Articles

γ-Conotoxin-PnVIIA, A γ-Carboxyglutamate-Containing Peptide Agonist of Neuronal Pacemaker Cation Currents†

Michael Fainzilber,*,‡ Takemichi Nakamura,§,|| Johannes C. Lodder,⊥ Eliahu Zlotkin,# Karel S. Kits,⊥ and Alma L. Burlingame§

Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel, Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California, Graduate School Neurosciences Amsterdam, Institute of Neuroscience Vrije Universiteit, Faculty of Biology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands, and Department of Cell & Animal Biology, Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel

Received June 30, 1997; Revised Manuscript Received October 22, 1997

ABSTRACT: A novel γ-carboxyglutamate-containing peptide, designated γ-conotoxin-PnVIIA, is described from the venom of the molluscivorous snail *Conus pennaceus*. γPnVIIA triggers depolarization and firing of action potential bursts in the caudodorsal neurons of *Lymnaea*. This effect is due to activation or enhancement of a slow inward cation current that may underly endogenous bursting activity of these neurons. The amino acid sequence of γPnVIIA was determined as DCTSWFGRCTVNSγCCSNSCDQTYCγ-LYAFOS (where γ is γ-carboxyglutamate, O is *trans*-4-hydroxyproline), thus γPnVIIA belongs to the six cysteine four loop structural family of conotoxins, and is most homologous to the previously described excitatory conotoxin-TxVIIA. Interestingly, TxVIIA did not induce action potentials in *Lymnaea* caudodorsal neurons. γPnVIIA is the prototype of a new class of γ-conotoxins that will provide tools for the study of voltage-gated pacemaker channels, which underly bursting processes in excitable systems.

Ion channels are integral plasma membrane proteins responsible for electrical activity in excitable tissues and, as such, are specifically targeted by many neurotoxins. One of the most successful groups of venomous predators, the marine *Conus* snails, have elaborated a wide array of peptide neurotoxins generally termed conotoxins. Conotoxins are typically disulfide-rich peptides of 12−35 amino acids, targeted against various subtypes of voltage or ligand-gated ion channels (*1, 2*). Piscivorous *Conus* venoms have been found to contain also another class of peptides, lacking disulfides but rich in γ-carboxyglutamate (γGlu[1]), termed conantokins (*3, 4*). Both classes of peptides, conotoxins, and conantokins are able to exhibit high receptor specificity due to constrained 3D structures, not generally found in peptides of this size (e.g., refs (*5*−*8*). These structures are imposed and stabilized by the density of disulfide bonds (conotoxins) or by helical interactions stabilized by γGlu-Ca^{2+} chelates (conantokins).

In contrast to the structural role of γ-carboxyglutamate in conantokins, a few reports have appeared describing *Conus* venom peptides with extensive disulfide bridging and also a small number of γGlu residues with putative functional roles. One of these is conotoxin-TxVIIA (previously designated TxIIA), a 27 amino acid residue peptide with six cysteines and two γGlus (*9, 10*). Initial electrophysiological observations using isolated *Aplysia* neurons revealed a strong excitatory effect of TxVIIA in causing spontaneous and repetitive firing of action potentials. Furthermore, bioactivity of the peptide is much reduced after partial decarboxylation upon prolonged storage (*9, 10*), suggesting a functional role of γGlu residues in TxVIIA. TxVIIA has so far revealed a very restricted specificity to marine molluscs and, in fact, has no observable effect in other molluscan neuronal systems such as the caudodorsal neurons of *Lymnaea* (K.S.K., and J.C.L. unpublished results). We therefore initiated a screen of *Conus* venoms for related conopeptides that might provide probes for a wider range of systems and to provide a background for structure−function analysis in these peptides.

† Financial support was provided by the National Center for Research Resources, NIH, Grant RR 01614 (to A.L.B.), the National Science Foundation, Grant DIR8700766 (to A.L.B.), and the European Neuroscience Program (to M.F.). T.N. was supported by funds from Sankyo Ltd.

* Correspondence to Department of Biological Chemistry, Ullman Building, Weizmann Institute of Science, Rehovot 76100, Israel. Fax: +972-8-9344112. E-mail: bmfainz@weizmann.weizmann.ac.il.

‡ Weizmann Institute of Science.

§ University of California.

|| Present address: Analytical and Metabolic Research Laboratories, Sankyo Co. Ltd., 2-58 Hiromachi I-chome, Shinagawa-ku, Tokyo 140, Japan.

⊥ Institute of Neuroscience Vrije Universiteit

Hebrew University of Jerusalem.

[1] Abbreviations: γGlu, γ-carboxyglutamate; CID, collision-induced-dissociation; ESI, electrospray ionization; HBS, HEPES-buffered saline; LC, liquid chromatography; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization.

S0006-2960(97)01571-7 CCC: $15.00
Published on Web 01/23/1998

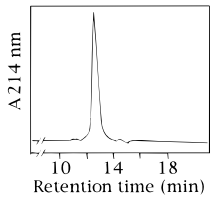

FIGURE 1: Phenyl column purification of of PnVIIA. The semi-preparative C18 column fraction was separated on a wide bore Vydac phenyl analytical column (250 × 4.6 mm, 5 μm particle size), equilibrated and eluted at a flow rate of 0.5 mL/min with 23% acetonitrile in 0.1% aqeous TFA.

In this study, we report the purification and characterization of a γGlu-containing conotoxin, PnVIIA, from the venom of the molluscivorous species, *Conus pennaceus*. PnVIIA triggers action potential bursting in isolated caudodorsal neurons from *Lymnaea* in a manner similar to the effects of TxVIIA in *Aplysia*. These effects are due to activation of an inward cation current underlying afterdischarge firing in these cells, thus PnVIIA represents the prototype of a conotoxin class targeted to such "pacemaker" currents and designated γ-conotoxins.

EXPERIMENTAL PROCEDURES

Toxins and Bioassays. Venom of *C. pennaceus* was obtained from specimens collected in the northern Red Sea. Conotoxin-TxVIIA was from venom-purified aliquots (*9*). Assays for paralysis in limpet snails (*Patella caerulea*), bivalves (*Mytilus edulis*), and fish (*Gambusia affinis*) were performed as previously described (*11*).

Column Chromatography. *C. pennaceus* venom was extracted and fractionated on Sephadex G-50 (Pharmacia) and semipreparative C18 (Vydac) columns as previously described (*12*). Final purification of the active peptides was on wide pore reverse phase phenyl (Vydac, 25 × 0.46 cm, 0.5 μm particle size) as described in Figure 1, with on-line spectral analysis of peak purity utilizing a Hewlett-Packard 1040A diode array detector coupled with HP 300 Chemstation software.

Amino Acid Analysis. Analysis of amino acid composition after acid hydrolysis and 9-fluorenyl-methyl-oxycabonyl-chloride (FMOC) derivatization was performed on a Merck-Hitachi reverse-phase HPLC system. The system was calibrated prior to each analysis with FMOC-amino acid standards.

Reduction and Alkylation. Dried purified peptides were dissolved in 50 μL of 0.1 M NH_4HCO_3 (pH 8) containing 6 M guanidine-HCl and 10 μM EDTA, and reduced with 200 μg of DTT at 37 °C for 2 h under argon. 4-Vinylpyridine, iodoacetic acid, or iodoacetamide were added, and the mixture was incubated at 37 °C for 1.5 h under argon. The alkylated peptide sample was purified on reversed-phase HPLC column immediately after derivatization.

Edman Degradation Analyses. Reverse-phase purified peptides were applied to PVDF or glass fiber filters, and sequenced by automated Edman degradation on an Applied Biosystems 475A gas-phase protein sequencing system.

Proteolytic Digest. HPLC-purified sample of reduced and alkylated peptide was digested with TPCK-trypsin (Pierce, Rockford, IL) for 20 h at 37 °C. A portion of the digest was directly analyzed by LC/ESI/MS, and the remainder purified by reverse-phase HPLC. pH of the digest was adjusted to 3.0 prior to loading on the HPLC to minimize the possibility of γ-carboxyglutamate decomposition in extremely acidic conditions. Purified C-terminal peptide fragments were further digested by Endoproteinase Asp-N (Boehringer Mannheim, Indianapolis, IN) for 20 h at 37 °C, and immediately purified on reverse-phase HPLC. A portion of the purified Asp-N peptide was then methylated for LSI CID mass spectrometry (*13*).

Mass Spectrometry. Microbore LC/ESI/MS experiments were carried out on a VG/Fisons (Manchester, U.K.) Platform mass spectrometer using a C18 column (microsphere C18, 5 μm particle size, 1 × 250 mm, Alltech, Deerfield, IL) with a linear gradient of 2 to 62% acetonitrile in 0.1% TFA in 60 min. A postcolumn addition of make-up solvent, 2-propanol/2-methoxyethanol (1:1), was used to optimize spraying and ionization performance (*14*). High-energy CID mass spectra were obtained with a Kratos (Manchester, U.K.) Concept IIHH tandem mass spectrometer equipped with a continuous flow liquid secondary ionization (LSI) source (*13*) and a scanning charge-coupled device array detector (*15*).

Electrophysiology. Isolated *Lymnaea* caudodorsal neurons were kept in Petri dishes (Costar) and bathed in Hepes buffered saline (in millimolar): 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 pipet solutions used to selectively record specific currents were as follows (in millimolar). 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 0.1, 4-aminopyridine 1, pH 7.8 set with NaOH. Pipet saline (I_{Ca} and I_{Na}): CsCl 29, $CaCl_2$ 2.3, HEPES 10, EGTA 11, ATPMg 2, GTPtris 0.1, pH 7.4 adjusted with CsOH. Pipet saline (nonselective): KCl 29, $CaCl_2$ 2.3, HEPES 10, EGTA 11, ATPMg 2, GTPtris 0.1, pH 7.4, adjusted with KOH. Na^+/Ca^{2+} selective conditions were obtained by using standard HBS in the bath in combination with the above-described Cs^+ containing pipet saline. Toxin was administered by means of a laboratory-built pressure ejection system through a small glass pipet (tip diameter 20 μM) placed at ~100 μM from the recorded cell. This enabled rapid application of toxins, which were applied continuously during voltage ramps or 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. Pipets (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, to allow equilibration with the pipet solution. Data acquisition was controlled by a CED AD/DA converter

Table 1: Edman Degradation of PnVIIA

cycle	assigned residue	yield (pmol)	cycle	assigned residue	yield (pmol)
1	Asp	185	17	Ser	18
2	Cys	170	18	Asn	35
3	Thr	180	19	Ser	15
4	Ser	190	20	Cys	11
5	Trp	170	21	Asp	23
6	Phe	140	22	Gln	26
7	Gly	210	23	Thr	22
8	Arg	85	24	Tyr	17
9	Cys	93	25	Cys	14
10	Thr	150	26	Glu	3
11	Val	170	27	Leu	17
12	Asn	85	28	Tyr	14
13	Ser	110	29	Ala	11
14	Glu	9	30	Phe	13
15	Cys	50	31	Hyp	8
16	Cys	56	32	Ser	9

(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) 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.

RESULTS

Purification of Conotoxin-PnVIIA. *C. pennaceus* venom was fractionated as described in the Experimental Procedures, and fractions were assayed for γGlu content by dot-blotting and staining with 4-diazobenzenesulfonic acid (DBS) (*16*). The fraction indicated as PnVII in Figure 1B of Fainzilber et al. (*12*) stained positively (data not shown) and was verified for γGlu content by a comparison of positive versus negative ion mode MALDI mass spectrometry (*10*). The fraction was then repurified by reverse-phase phenyl chromatography (Figure 1), and the major component obtained was designated conotoxin-PnVIIA. On-line spectral analyses of the final chromatographic step suggested homogeneity of the purified toxin (data not shown). ESI/MS measurements of the purified peptide revealed a single mass of 3718.4, further confirming homogeneity of PnVIIA.

Chemical Characterization. Automated Edman sequencing of PnVIIA after alkylation with 4-vinylpyridine revealed a 32 amino acid sequence, allowing assignments of 30 residues (Table 1). The extremely low yields of Glu at steps 14 and 26, taken together with the positive DBS stain and MALDI signature of the peptide (see above), suggested the presence of γ-carboxyglutamate residues at these positions. Amino acid composition analysis was consistent with the proposed sequence (Table 2), and measurement of the chemical average molecular weight of the natural toxin by ESI/MS yielded a value (measured 3718.4, calculated 3719.0) supporting the Edman sequence above, assuming two γ-carboxyglutamate residues, three disulfide bridges, and a free carboxy-terminus. To establish the γ-carboxyglutamate residues and free C-terminus unambiguously, the peptide was further characterized by mass spectrometric identification of suitable chemical derivatives and enzymatic digests. A tryptic digest of reduced and carboxymethylated PnVIIA gave two peptides, T1 and T2, whose average molecular masses (*17*) by ESI/MS were 1029.0 and 3062.6, respectively. These mass values confirm those anticipated for both PnVIIA tryptic peptides, namely 1029.1 for the sequence DXTSWFGR (where X is carboxymethylCys), and 3062.2 for the sequence XTVNSγXXSNSXDQTYXγLYAFOS (where γ is γ-carboxyglutamate, O is 4-transhydroxyproline). Asp-N digest of the C-terminal tryptic peptide T2 gave two products, AN1 and AN2. ESI/MS average mass for AN1 was 1525.4, fitting the predicted mass of the Asp-N fragment XTVNSγXXSNSX (predicted 1525.6). The measured monoisotopic LSI/MS value (*17*) for the C-terminal fragment AN2 was 1553.7, in agreement with the presence of a C-terminal carboxyl function. An attempt to further confirm the C-terminal sequence of PnVIIA by LSI tandem MS failed, perhaps due to poor ionization efficiency of AN2. Therefore, PnVIIA was reduced and alkylated with iodoacetamide, a procedure expected to generate derivatives with better CID spectra than carboxymethylated peptides. After trypsin followed by Asp-N digests, the C-terminal carbamoylmethylation peptide AN2u was isolated. Methylation with HCl/MeOH gave a tetra ester, with monoisotopic LSI/MS mass of 1608.9. This mass fits a peptide with incorporation of four methyl groups, one at the side chain of Asp, two at the carboxyl groups of the γ-carboxyglutamate, and the fourth at the presumed C-terminal free carboxyl (predicted monoisotopic mass 1608.7). The protonated tetra-methylated AN2u was further analyzed by CID mass spectrometry, yielding a spectrum that independently established the sequence and structural nature of this C-terminal peptide (Figure 2). The location and identity of γGlu as residue 6 is determined by the values of the b5 and b6 sequence ions, and the occurrence of the γGlu residue is established by the unique immonium ion at *m*/*z* 174. The y2 ion confirms a C-terminal structure of −Hyp-Ser-OMe, derived from the free carboxy-terminal of PnVIIA. Thus, the structure of this C-terminally free peptide including the identity and location of both covalent modifications was established by tandem mass spectrometry independently of both Edman and amino acid analysis data.

Table 2: Amino Acid Composition Analysis of Conotoxin-PnVIIA[a]

amino acid	mole ratio
Asx	3.9 (4)
Ser	4.7 (5)
Glx	3.0 (3)
Cys	5.2 (6)
Thr	2.8 (3)
Gly	1.1 (1)
Arg	1.0 (1)
Hyp	0.8 (1)
Ala	1.2 (1)
Tyr	2.0 (2)
Val	1.2 (1)
Phe	2.0 (2)
Leu	1.2 (1)
Trp	nd (1)

[a] Molar ratios of amino acids determined after acid hydrolysis and FMOC derivatization. Values in brackets are those predicted from the amino acid sequence.

PnVIIA belongs to the large group of conotoxins with the cysteine framework of ω and δ conotoxins, however the sequence is most similar to conotoxin-TxVIIA (Table 3). These homologies comprise approximately 48% amino acid identity and 63% similarity, including positioning of most

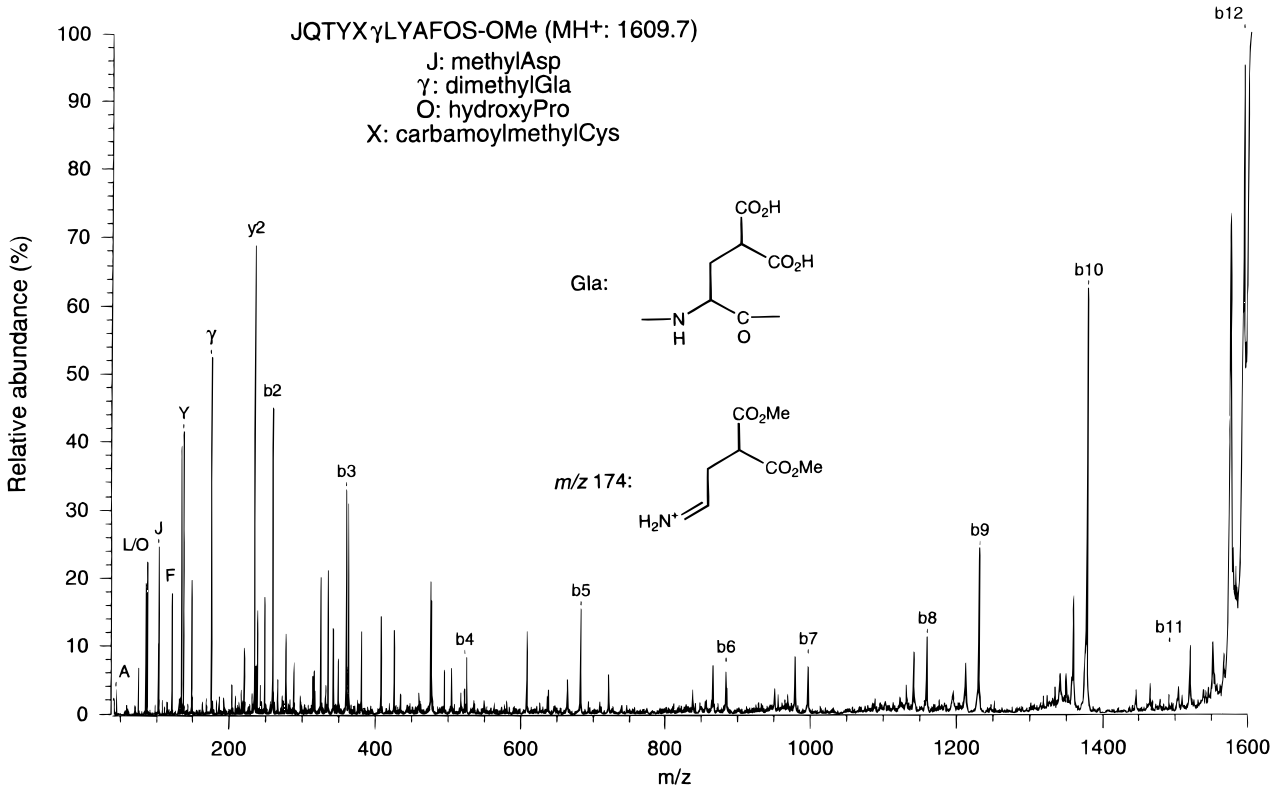

FIGURE 2: High-energy CID spectrum of the monoisotopic ^{12}C isobar of the protonated C-terminal Asp-N product of reduced and carbamoylmethylated PnVIIA (tetramethylated AN2′), *m*/*z* 1609.7, generated by LSI-MS. The b-series ions by Biemann's nomenclature (*37*) and the immonium ions of several residues (represented by one-letter code) are annotated on the spectrum. The inset shows a proposed structure of the immonium ion generated from the *O*,*O*′-methylated γ-carboxyglutamate residue at *m*/*z* 174, which is annotated as γ.

Table 3: Amino Acid Sequence of Conotoxin-PnVIIA Compared with TxVIIA[a]

PnVIIA	D	**C**	TS	**W**	FGR	**C**	T	**V**	N	**SγCCS**					**N**	S	**C**	DQ	**T**	**YC**	γ	**L**	**Y**	AFOS-COOH
TxVIIA		**C**	GG	**Y**	STY	**C**	γ	**V**	D	**SγCCS**	D	**N**					**C**	VR	**S**	**YC**	T	**L**	**F**	-NH2

[a] Sequence identities are boxed, and similarities are in bold type. Spaces inserted to maximize homologies.

hydrophobic and some charged residues, as well as one of the γ-carboxyglutamates.

Paralytic Activity of PnVIIA. Initial injections of PnVIIA to limpet snails (*Patella*) did not reveal the contractile paralysis previously observed for TxVIIA and other conotoxins in this bioassay (*9*); however, at doses above 50 pmol/100 mg of body weight, some flaccidity of the foot musculature could be observed. Flaccid or relaxation paralytic effects are more easily observed in bioassays on bivalve molluscs; hence, toxicity of PnVIIA was quantified in bioassays in freshwater mussels (*Mytilus*), as previously done for conotoxins PnIVA and PnIVB (*11*). The ED_{50} for *Mytilus* paralysis was 63.2 pmol/100 mg of body weight. No toxic or other effects could be observed upon injection of 1 nmol of PnVIIA (15-fold higher than the *Mytilus* ED50) per 100 mg of body weight in *Gambusia* fish or blowfly (*Sarcophaga*) larvae.

Electrophysiological Effects of PnVIIA on Lymnaea Neuroendocrine Cells. Effects of PnVIIA were first screened in a number of mollusc or vertebrate electrophysiological preparations. Consistent effects were observed only on caudodorsal neurons from the snail *Lymnaea stagnalis*, and this system was therefore used for detailed investigations on toxin activity. The caudodorsal neurons are typical rythmic bursting cells responsible for production of egg laying hormone, and their ionic currents have been characterized exhaustively (*18*−*20*). In the first series of experiments, we applied PnVIIA to caudodorsal neurons recorded under current clamp and investigated the effects on membrane potential and action potential firing. As shown in Figure 3A, PnVIIA enhances the excitability of these cells in a dose-dependent way. Cells that were silent responded to low doses (≤ 1 μM) of the toxin by depolarization, while doses of 10 μM or more induced trains of action potentials. The number of action potentials increased with increasing doses. The duration of PnVIIA application also markedly influenced the response. In silent cells, responding with a burst of action potentials, the number of action potentials and the duration of the burst increased with increasing duration of the PnVIIA pulse (Figure 3B). Cells that were spontaneously active responded by a temporary increase in firing frequency, followed by an afterburst hyperpolarization during which the cell stops firing for a short period. Increasing the duration of PnVIIA application under these circumstances led to an increase in the duration of the burst, but even more so in the duration of the afterburst silent refractory period (Figure 3C). The latter effect is possibly indirect, as a natural

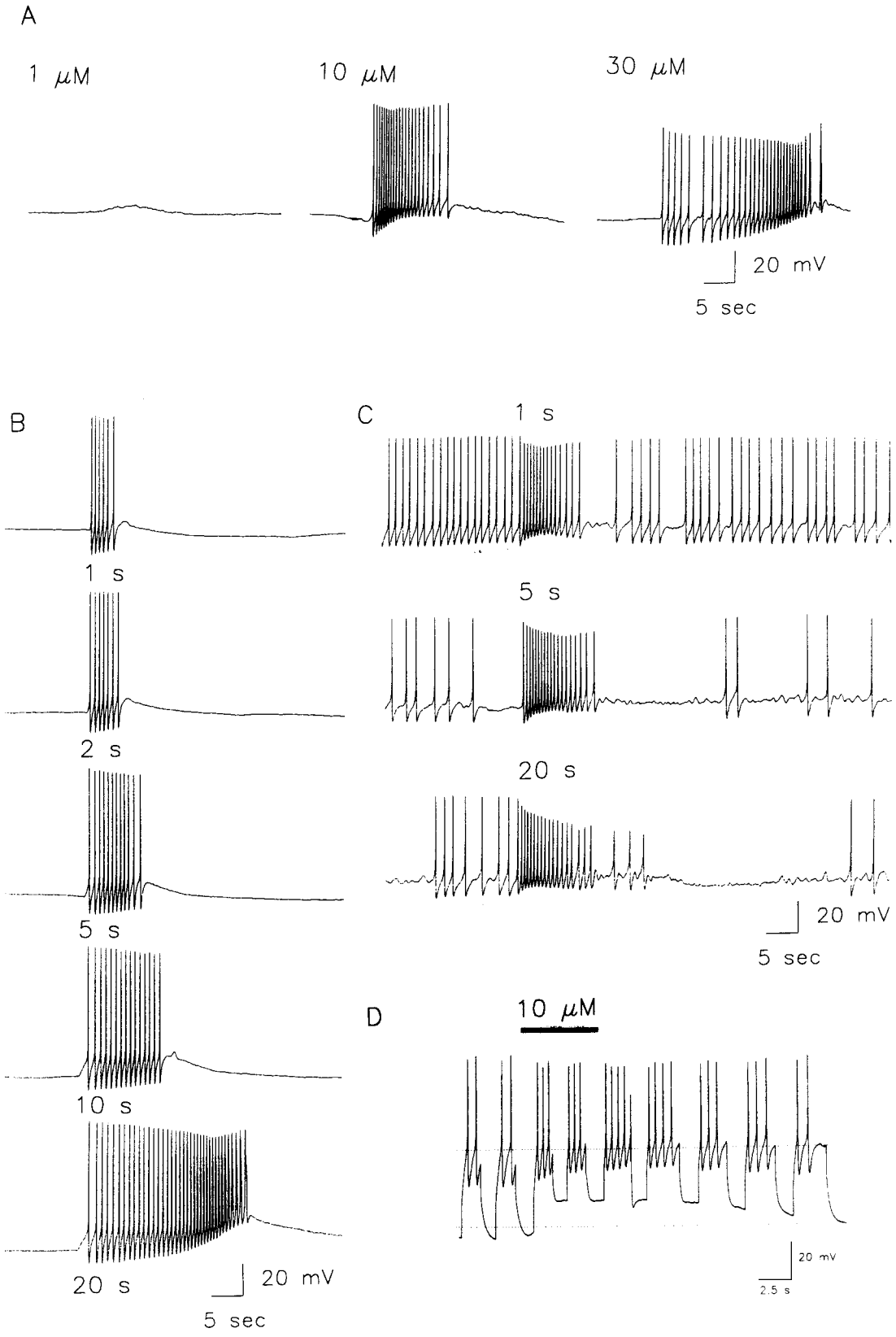

FIGURE 3: Effects of PnVIIA on caudodorsal neurons recorded under current clamp conditions ($n = 5$). (A) Dose-dependent increase in excitability of caudodorsal neurons, inducing depolarization and repetitive spiking upon application of micromolar doses of PnVIIA. (B) Time dependence of the excitatory effect of PnVIIA in silent caudodorsal neurons, showing increased duration of spiking with increased duration of application. (C) Time dependence of the excitatory effect of PnVIIA in spontaneously active caudodorsal neurons, showing that not only spiking increases but also the duration of the silent period after the afterburst increases with longer applications. (D) Effect of PnVIIA on the membrane resistance of the caudodorsal neurons. Pulses of hyperpolarizing current were injected into the cells, giving rise to hyperpolarizations of the membrane potential. While the injected current is constant, the hyperpolarizing response decreases upon application of PnVIIA, showing that the membrane resistance decreases.

consequence from the increased firing frequency induced by the toxin.

We then investigated whether the effect was due to closure (blockade) or opening (activation) of ion channels, by measuring input resistance of the cell membrane upon injection of hyperpolarizing current pulses (30 μA). The amplitude of the resulting hyperpolarization is a direct measure of the membrane resistance. Figure 3D clearly shows that during PnVIIA application hyperpolarization amplitude is strongly decreased (~50% attenuation), thus revealing a marked decrease in membrane resistance. Thus, PnVIIA induces an increase in conduction, i.e., leads to the opening of ion channels, and therefore acts primarily as a channel agonist or activator, rather then as a channel blocker.

In a further series of experiments, we examined the identity of the channel(s) activated by PnVIIA. To this end, whole cell voltage clamp experiments were performed on caudodorsal neurons; however, no significant effects of the toxin could be observed on fast voltage gated sodium ($n = 3$) or calcium currents ($n = 10$) activated in a standard voltage step protocol (data not shown). We then applied a slow ramp protocol (Figure 4) to investigate possible effects on slow voltage-gated currents (also designated as pacemaker currents) that are believed to underly spontaneous firing. Figure 4A shows the current response in standard HBS to a voltage ramp protocol, indicating that a noninactivating inward current is activated at voltages above −30 mV. In the presence of 1 mM Cd^{2+} or 1 mM Ni^{2+} this inward current was 85 or 61% blocked, respectively (means of three independent experiments for each, SEM = 6 or 8%). Both sodium-free and calcium-free salines affected the amplitude and voltage dependence of the slow inward current, indicating that both ions contribute to this current. These properties are strongly reminiscent of pacemaker currents characterized in other *Lymnaea* neurons (*21*).

In the presence of 10 μM PnVIIA, we observed a dual effect on the current response to a ramp protocol in standard HBS ($n = 8$). First, the activation range of the slow inward current shifted by approximately 10 mV to a more negative potential, thus, accounting for the enhanced excitability of the cells. Second, we saw an increase in noninactivating outward current at potentials above 0 mV (Figure 4A). Whether the latter is a direct effect of PnVIIA or an indirect effect due to the increased inward current remains to be determined. It is however in line with the previously observed prolongation of afterburst hyperpolarization under current clamp conditions. Since the primary effect in current clamp recordings was an increase in excitability accompanied by an increase in membrane conductance, we next focused on the slow inward current activated by PnVIIA. A series of experiments under Na^+/Ca^{2+} selective conditions, (potassium currents blocked by replacing K^+ for Cs^+ in the pipet medium) confirmed that PnVIIA activates an inward current carried by Na^+ and/or Ca^{2+} ions (Figure 4B). PnVIIA enhanced the maximal slow inward current by 35% ($n = 11$; SEM = 7%), with the current averaging 474 pA in control conditions versus 625 pA in the presence of the toxin. All subsequent experiments utilized Cs^+ instead of K^+ in the pipet medium. Under these conditions in Ca^{2+} selective saline, PnVIIA increased the slow inward current by 26% ($n = 6$; SEM = 6%), further indicating that the channel activated by PnVIIA is permeable to calcium ions.

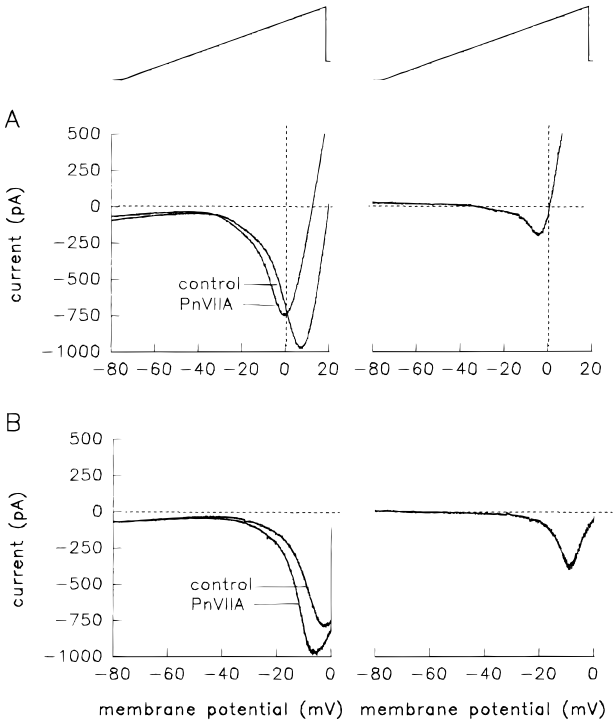

FIGURE 4: Effects of PnVIIA on caudodorsal neurons recorded under voltage clamp conditions. *Pulse protocol:* a voltage ramp is applied during which the membrane potentials goes from −80 to +20 mV at a rate of 4 mV/s. This protocol will only reveal slow, voltage gated currents, as fast currents will inactivate during the slow voltage ramp. (A) Left panel: current responses in standard HBS to the voltage ramp in the absence or presence of PnVIIA, as indicated. Under control conditions, an inward current is activated at −30 mV and above. Most likely this represents a pacemaker current. With 10 μM PnVIIA the voltage dependence shifts to the left (i.e. the current is activated at more hyperpolarized potentials). Furthermore, an increase in outward current at >0 mV occurs. Right panel: the current induced by 10 μM PnVIIA, obtained by subtracting the control response from the response in the presence of the toxin. A slow inward current is activated at <0 mV, while at >0 mV, the response is outward. (B) Like panel A, but now under conditions selective for Na^+/Ca^{2+}-currents (K^+ currents blocked by replacing K^+ with Cs^+ ions in the pipet saline). Under these conditions, the inward current activated by PnVIIA is more apparent (note the larger amplitude of the PnVIIA activated current) probably as it is no longer obscured by outward currents). Right panel: current responses in the absence or presence of PnVIIA; left panel: isolated PnVIIA activated current.

To obtain an initial pharmacological characterization of the PnVIIA activated current, we probed the effect of PnVIIA in the presence of 1 mM Ni^{2+} or 1 mM Cd^{2+}. While Ni^{2+} ions blocked the control slow inward current by approximately 60%, the response to PnVIIA was not affected. Thus, PnVIIA application increased the residual current by 97% ($n = 3$; SEM = 7%). In the presence of Ni^{2+}, control currents averaged 135 pA versus an average current of 261 pA after PnVIIA application. In the presence of 1 mM Cd^{2+}, we no longer observed an effect of PnVIIA ($n = 3$), showing that Cd^{2+} completely blocks the PnVIIA-activated current.

We conclude from these results that PnVIIA activates a slow inward current, carried by Na^+/Ca^{2+} permeable channel(s) sensitive to Cd^{2+} but not Ni^{2+}. This is based on the following lines of evidence: (a) PnVIIA excites caudodorsal neurons and induces action potentials by eliciting a response that involves a decrease in membrane resistance and therefore an increase in conductance; (b) PnVIIA activates a slow or

sustained inward current, which response is not affected after block of K^+ channels by replacing K^+ ions with impermeant Cs^+ ions; (c) partial responses are still present in Ca^{2+} selective saline; and (d) the slow inward current is blocked by Cd^{2+} ions.

DISCUSSION

The amino acid sequence of PnVIIA conserves the six-cysteine-four loop framework C···C···CC···C···C typical of ω- and δ-conotoxins and as shown in Table 3 is most similar to the sequence of conotoxin-TxVIIA, an excitatory toxin from *Conus textile* venom (*10*). Although both toxins conserve the cysteine framework typical of ω- and δ-conotoxins, the half-cystine pairing for PnVIIA and TxVIIA has not yet been determined, thus, it is not yet apparent if they share the same fold. Nonetheless, both these toxins have an identical acidic net charge (−5) and are similar in their surface hydrophobic/hydrophilic interaction properties, as evidenced by comparable elution properties in reverse phase chromatography (compare Figure 1B in refs *9*, and *12*). Furthermore the gross effects of these toxins in the respective sensitive systems (*Aplysia* versus *Lymnaea* neurons) are very similar, comprising an enhancement of excitability, decreased membrane resistance, and increased repetitive firing (Figure 3 and ref *9*). PnVIIA and TxVIIA may therefore represent closely related members of the same family, or convergent evolution to closely related receptor/channel targets. Thus, the selectivity in their targeting to *Aplysia* or *Lymnaea* receptors is intriguing. It should be noted that this selectivity could also be due to differences in ionic strength and composition between the systems (see, for example, ref *22*), as the ionic strength of physiological *Aplysia* saline is 10-fold higher than that of *Lymnaea*, reflecting the differences between their respective marine and freshwater habitats.

The primary event mediating the excitatory effects of PnVIIA on *Lymnaea* caudodorsal neurons is an enhancement of a slow, voltage activated inward cation current (Figures 3 and 4). Changes in the slow inward currents carried by such nonspecific cation channels may play a crucial role in bursting and pacemaker activities in a variety of excitable systems, ranging from mammalian heart muscle to molluscan neurons (reviewed in ref *23*). Indeed, the mechanism of action of PnVIIA is reminiscent of that recently reported by Wilson et al. (*24*) for crude *C. textile* venom extracts on isolated bag cell neurons of *Aplysia*. The bag cells in *Aplysia* have an analogous role to the caudodorsal neurons in *Lymnaea*, and both cell types have similar overall electrophysiological characteristics, including the characteristic bursting afterdischarge. This is a stereotyped firing pattern, lasting about 30 min in *Lymnaea*, and consisting of synchronized action potential firing in all cells of the network (*25*). Crude *C. textile* venom applied to isolated bag cells, as well as excised patch-clamped membranes, revealed a slow inward cation current resulting from an increase in the opening probability of a Ca^{2+}-sensitive nonspecific cation channel (*24*). Since the venom was observed to increase cation channel activity also in patch configurations without direct access to the extracellular side, Wilson et al. (*24*) suggested that channel activation occurs indirectly via activation of intracellular messengers. Although various intracellular messengers are well-established modulators of nonspecific cation channels (*26*, *27*), this interpretation is ambiguous since crude venom extracts such as that used by Wilson et al. (*24*) may contain various intracellular messenger type molecules. Specifically, in the case of *C. textile*, high levels of arachidonic acid have been reported in the venom (*28*). Furthermore, the cocktail of enzymatic and cytolytic activities (*29*−*31*) and multitude of neurotoxins in crude venom extracts, clearly allow for numerous mechanisms when using whole venoms in sensitive pharmacological or physiological assays. Taking into account the preliminary observations on TxVIIA excitatory effects on *Aplysia* neurons (*9*), it would clearly be of interest to test if TxVIIA might be the component responsible for the effects reported by Wilson et al. (*24*) on the bag cell cation current.

Wilson et al. (*24*) further suggested that the effects of *C. textile* venom on bag cells might be mediated by binding to the receptor for the neuropeptide that normally triggers afterdischarge. Such a mechanism seems unlikely in the present case, since Brussaard et al. (*25*) have previously shown that activation of afterdischarge in silent caudodorsal neurons by endogenous neuropeptides requires a specific combination of four different caudodorsal peptides. Thus, the simplest explanation for the effects of PnVIIA is direct action on a voltage-gated, nonspecific cation channel. The recent discovery of a directly neuropeptide-gated sodium channel (*32*) is to some extent reminiscent of the proposed mechanism. Thus, we consider PnVIIA as the prototype of a novel pharmacological class of conotoxins, designated γ-conotoxins. γPnVIIA provides the first chemically defined selective tool for stimulation and study of cation pacemaker channels. Previous discovery of the δ-conotoxins as a novel pharmacological class specific for molluscan sodium channels (*22*) swiftly lead to identification of related piscivorous venom toxins active on mammalian channels (*33*, *34*). As slow inward cation channels in vertebrates may be involved in syndromes of clinical relevance, such as epileleptic activity in hippocampus (*35*) and pacemaker potentials in heart muscle (*36*), piscivorous *Conus* toxins related to γPnVIIA are awaited with much interest.

ACKNOWLEDGMENT

We thank Dr. Ariel Gaathon (Bletterman Sequencing Laboratory, Hebrew University) for initial Edman sequencing.

REFERENCES

1. Myers, R. A., Cruz, L. J., Rivier, E., and Olivera, B. M. (1993) *Chem. Rev. 93*, 1923−1936.
2. Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., Mena, E. E., Woodward, S. R., Hillyard, D. R., and Cruz, L. J. (1990) *Science 249*, 257−263.
3. Haack, J. A., Rivier, J., Parks, T. N., Mena, E. E., Cruz, L. J., and Olivera, B. M. (1990) *J. Biol. Chem. 265*, 6025−6029.
4. Haack, J. A., Parks, T. N., and Olivera, B. M. (1993) *Neurosci. Lett. 163*, 63−66.
5. Prorok, M., Warder, S. E., Blandl, T., and Castellino, F. J. (1996) *Biochemistry 35*, 16528−16534.
6. Skjaerbaek, N., Nielsen, K. J., Lewis, R. J., Alewood, P., and Craik, D. J. (1997) *J. Biol. Chem. 272*, 2291−2299.
7. Nielsen, K. J., Thomas, L., Lewis, R. J., Alewood, P. F., and Craik, D. J. (1996) *J. Mol. Biol. 263*, 297−310.
8. Guddat, L. W., Martin, J. A., Shan, L., Edmundson, A. B., and Gray, W. R. (1996) *Biochemistry 35*, 11329−11335.

9. Fainzilber, M., Gordon, D., Hasson, A., Spira, M. E., and Zlotkin, E. (1991) *Eur. J. Biochem. 202*, 589−595.
10. Nakamura, T., Yu, Z., Fainzilber, M., and Burlingame, A. L. (1996) *Protein Sci. 5*, 524−30.
11. Fainzilber, M., Nakamura, T., Gaathon, A., Lodder, J. C., Kits, K. S., Burlingame, A. L., and Zlotkin, E. (1995) *Biochemistry 34*, 8649−56.
12. Fainzilber, M., Hasson, A., Oren, R., Burlingame, A. L., Gordon, D., Spira, M. E., and Zlotkin, E. (1994) *Biochemistry 33*, 9523−9529.
13. Hall, S. C., et al. (1996) in *Mass Spectrometry in the Biological Sciences* (Burlingame, A. L., and Carr, S. A., Eds.) pp 171−202, Humana Press, Totowa, NJ.
14. Medzirhadszky, K. F. (1994) *J. Am. Soc. Mass Spectrom. 5*, 350−358.
15. Burlingame, A. L. (1994) in *Biological Mass Spectrometry: Present and Future* (Matsuo, T., Caprioli, R. M., Gross, M. L., and Seyama, Y., Eds.) pp 147−164, John Wiley & Sons Ltd.
16. Nishimoto, S. K. (1990) *Anal. Biochem. 186*, 273−279.
17. Burlingame, A. L., and Carr, S. A. (1996) in *Mass Spectrometry in the Biological Sciences* (Burlingame, A. L., and Carr, S. A., Eds.) Appendix XI, Humana Press, Totowa, NJ.
18. Brussaard, A. B., Lodder, J. C., ter, M. A., de, V. T., and Kits, K. S. (1991) *J. Physiol. 441*, 385−404.
19. Dreijer, A. M., and Kits, K. S. (1995) *Neuroscience 64*, 787−800.
20. Kits, K. S., and Mansvelder, H. D. (1996) *Invert. Neurosci. 2*, 9−34.
21. van-Soest, P. F., and Kits, K. S. (1997) *J. Neurophysiol. 78*, 1384−1393.
22. Fainzilber, M., Kofman, O., Zlotkin, E., and Gordon, D. (1994) *J. Biol. Chem. 269*, 2574−80.
23. Partridge, L. D., Muller, T. H., and Swandulla, D. (1994) *Brain Res. Rev. 19*, 319−25.
24. Wilson, G. F., Richardson, F. C., Fisher, T. E., Olivera, B. M., and Kaczmarek, L. K. (1996) *J. Neurosci. 16*, 3661−3671.
25. Brussaard, A. B., Schluter, N. C., Ebberink, R. H., Kits, K. S., and Ter, M. A. (1990) *Neuroscience 39*, 479−91.
26. Sudlow, L. C., Huang, R. C., Green, D. J., and Gillette, R. (1993) *J. Neurosci. 13*, 5188−5193.
27. Wilson, G. F., and Kaczmarek, L. K. (1993) *Nature 366*, 433−438.
28. Nakamura, H., Kobayashi, J., Ohizumi, Y., and Hirata, Y. (1982) *Experientia 38*.
29. Kreger, A. S. (1989) *Toxicon 27*, 56−57.
30. Marsh, H. (1970) *Toxicon 8*, 271−277.
31. Marsh, H. (1971) *Toxicon 9*, 63−67.
32. Lingueglia, E., Champigny, G., Lazdunski, M., and Barbry, P. (1995) *Nature 378*, 730−733.
33. Fainzilber, M., Lodder, J. C., Kits, K. S., Kofman, O., Vinnitsky, I., Van, R. J., Zlotkin, E., and Gordon, D. (1995) *J. Biol. Chem. 270*, 1123−1129.
34. Shon, K. J., Grilley, M. M., Marsh, M., Yoshikami, D., Hall, A. R., Kurz, B., Gray, W. R., Imperial, J. S., Hillyard, D. R., and Olivera, B. M. (1995) *Biochemistry 34*, 4913−4918.
35. Hoehn, K., Watson, T. W., and MacVicar, B. A. (1993) *Neuron 10*, 543−552.
36. Reuter, H. (1984) *Annu. Rev. Physiol. 46*, 473−484.
37. Biemann, K. (1988) *Biomed. Environ. Mass Spectrom. 16*, 99−111.

BI971571F