A New Cysteine Framework in Sodium Channel Blocking Conotoxins†

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

Department of Cell and Animal Biology, Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel, Graduate School Neurosciences Amsterdam, Institute of Neuroscience, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands, Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446, and Bletterman Laboratory for Macromolecular Research, Interdepartment Equipment Unit, Faculty of Medicine, Hebrew University of Jerusalem, 91120 Jerusalem, Israel

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ABSTRACT: Two novel sodium channel blocking peptides from the venom of the molluscivorous snail *Conus pennaceus*, μPnIVA and μPnIVB, are described. Elucidation of their amino acid sequences was complicated by a previously undescribed anomalous product of reduction and pyridylethylation, which occurs on N-terminal cysteine residues and gives a PTH derivative eluting at the same position as PTH-Trp in reverse-phase chromatography. The amino acid sequences of the toxins were determined by a combination of Edman degradation and mass-spectrometric techniques as CCKYGWTCLLGCSPCGC (PnIVA) and CCKYGWTCWLGCSPCGC (PnIVB). These toxins block sodium channels in molluscan neurons, but have no effect on sodium currents in bovine chromaffin cells or in rat brain synaptosomes. Although there is only one amino acid difference in the two sequences, PnIVB is approximately 6 times more potent than PnIVA in blockade of the sodium current in *Lymnaea* neurons. The PnIV sequences reveal a new cysteine residue framework for conotoxins (CC-----C---C--C-C). Strikingly, the only charged residue in PnIVA/B is Lys3. Iodination reaction experiments on the adjacent Tyr4 suggest that this region of the peptide must be solvent exposed and essential for activity. These structurally novel μ-conotoxins target a sodium channel subtype with low affinity for tetrodotoxin and therefore provide new probes for functional studies on sodium channels.

Peptide toxins derived from *Conus* snail venoms have proved to be among the most versatile tools for the study of excitable systems. These small peptides are subdivided into pharmacological/chemical categories on the basis of their activities on target receptors and various aspects of their primary stuctures (Olivera et al., 1991). The most definitive aspect of the latter has been found to be invariant arrangements of cysteine residues in toxins of each category, commonly termed "cysteine frameworks" of conotoxins. Nearly all of the conotoxins described to date belong to one of three cysteine frameworks. These are the four Cys/two loop framework of the α-conotoxins, the six Cys/three loop framework of the μ-conotoxins, and the six Cys/four loop framework of the ω- and δ-conotoxins and related peptides (Olivera et al., 1990, 1991). It has been suggested that these conserved cysteine frameworks serve as lattices for generation of hypervariability in the residues comprising the intercysteine loops, thus enabling exquisite specialization of conotoxins to subtypes of receptors or ion channels (Olivera et al., 1991).

Subspecialization is clearly exemplified in the conotoxins that target sodium channels. Thus the channel blocker μ-conotoxins are effective only on vertebrate skeletal muscle (Gray et al., 1988), and although they compete with tetrodotoxin (TTX)[1] and saxitoxin on binding, recent mutagenesis studies on the microI skeletal muscle channel suggest that they have distinct attachment sites (Stephan et al., 1994). The recently described δ-conotoxins, which inhibit channel inactivation (Hasson et al., 1993), discriminate in a unique manner between mollusc neuronal sodium channels and those of rat brain (Fainzilber et al., 1994a). Although such high specificities are useful for probing microvariability in sodium channel structures, they can be frustrating for neurophysiologists who would like to have toxins that are consistently useful in different systems. This is a pressing problem when classical blockers are not effective. Thus for example TTX, which blocks vertebrate neuronal sodium channels at nanomolar concentrations, completely blocks *Aplysia* neuronal sodium currents only at 100−200 μM (Hasson et al., 1995).

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* Correspondence to this author at his current address: M. Fainzilber, Laboratory of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Doktorsringen 12A, BZ-Lab, Karolinska Institute, S-17177 Stockholm, Sweden. Fax: +46-8-341 960.

‡ Silberman Institute of Life Science, Hebrew University of Jerusalem.

§ Vrije Universiteit.

|| University of California, San Francisco.

⊥ Faculty of Medicine, Hebrew University of Jerusalem.

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[1] Abbreviations: CDC, caudodorsal neurons; CID, collision-induced dissociation; EC_{50}, effective concentration 50%; ESI/MS, electrospray ionization mass spectrometry; FMOC, [(9-fluorenylmethyl)oxy]carbonyl; GIIIA, μ-conotoxin-GIIIA from *Conus geographus*; GS, μ-conotoxin-GS from *Conus geographus*; HPLC, high-pressure liquid chromatography; LSI/MS, liquid matrix secondary ion mass spectometry; PD_{50}, paralytic dose 50%; PnIVA, μ-conotoxin-PnIVA from *Conus pennaceus*; PnIVB, μ-conotoxin-PnIVB from *Conus pennaceus*; PTH, phenylthiohydantoin; RPE, reduced and pyridylethylated; TFA, trifluoroacetic acid; STX, saxitoxin; TTX, tetrodotoxin; TxVIA, δ-conotoxin-TxVIA from *Conus textile.*

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Previous electrophysiological studies on the venom of *Conus pennaceus* revealed a potent block of the neuronal action potential in *Aplysia*. This potent action potential blockade was extremely interesting in comparison to the weak effects of TTX in molluscan neurons. Hasson et al. (1995) performed a detailed electrophysiological characterization of one purified action potential blocking toxin from *Conus pennaceus* venom, conotoxin-PnIVB. Voltage clamp experiments revealed that PnIVB is a selective and potent blocker of sodium currents in *Aplysia* neurons, with an effective concentration range below 0.1 μM. In this report we describe the chemical characterization of two sequence variants of these toxins, PnIVA and PnIVB, and examine their effects on sodium currents in *Lymnaea* neurons, bovine chromaffin cells, and rat brain synaptosomes. The amino acid sequences of PnIVA and PnIVB represent a new cysteine framework for conotoxins. The bioassay and electrophysiological data suggest that these two toxins selectively target a TTX-resistant sodium channel subtype, thus providing a structurally novel selective probe for sodium channels.

EXPERIMENTAL PROCEDURES

Toxic Substances. Venom of *Conus pennaceus* was obtained from specimens collected in the northern Red Sea. Tetrodotoxin (TTX) and μ-conotoxin-GIIIA (μGIIIA) were from Sigma.

Experimental Animals and Bioassays. Bivalve mussels (*Mytilus edulis*) were collected from the Mediterranean coast of Israel and maintained in laboratory aquaria. These animals were used as a new bioassay for flaccid paralysis in molluscs. Up to 10 μL aliquots were injected through the byssal cleft into mussels of 300 mg body weight. Paralysis was determined 15 min post-injection. A positive response was defined as an obvious relaxation of the two valves, resulting in a loss of the animal's ability to hold its shell closed. Bioassays on fish (*Gambusia*) and blowfly larvae (*Sarcophaga*) were carried out as previously described (Fainzilber & Zlotkin, 1992; Reed & Muench, 1938).

Column Chromatography. Conus pennaceus venom was extracted and fractionated on Sephadex G-50 (Pharmacia) and semipreperative C18 (Vydac) columns as previously described (Fainzilber et al., 1994b). 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. Purified peptides were quantified by amino acid analysis.

Amino Acid Analysis. Analysis of amino acid composition after acid hydrolysis and [(9-fluorenylmethyl)oxy]cabonyl (FMOC) chloride derivatization was performed on a Merck-Hitachi reverse-phase HPLC system, according to Betner and Foldi (1988). 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 mM EDTA and were reduced with 200 μg of dithiothreitol at 37 °C for 2 h under argon. To the solution was added 600 μg of the appropriate alkylation agent, i.e., 4-vinylpyridine, iodoacetic acid, or iodoacetamide, and the mixture was kept at 37 °C for 2 h under argon. The alkylated peptides were purified by a reversed-phase HPLC immediately after the reaction.

Thermolytic Digestion. A dried sample of reduced and carbamoylmethylated PnIVA was dissolved in 20 μL of 20% 2-PrOH in 0.1 M NH_4HCO_3 containing 0.5 mM $CaCl_2$ (Welinder, 1988) and digested with thermolysin (2 μg, Sigma) for 22 h at 37 °C. A portion of the digest was directly analyzed by LC/MS, and the remainder was purified by reverse-phase HPLC.

Edman Degradation Analyses. Reverse-phase purified peptides were applied to PVDF or glass fiber filters and sequenced by automated Edman degradation on Applied Biosystems 470A or 475A protein sequencing systems.

Mass Spectrometry. Liquid secondary ion (LSI) mass spectra were obtained with a Kratos MS-50S mass spectrometer (Aberth et al., 1982; Falick et al., 1986). Microbore HPLC/ESI/MS experiments were carried out on a Fisons/VG Platform mass spectrometer with an ethanol/propanol/water/formic acid solvent system (Medzihradszky et al., 1994). High-energy CID mass spectra were obtained with a Kratos Concept IIHH tandem mass spectrometer equipped with a continuous flow liquid secondary ionization source and a scanning charge-coupled device array detector (Burlingame, 1994; Burlingame et al., 1990; Walls et al., 1993).

^{22}Na Flux Assays. ^{22}Na influx assays in rat brain synaptosomes were carried out as previously described (Tamkun & Catterall, 1981; Fainzilber et al., 1994a). Briefly, rat brain synaptosomes (0.3−0.5 mg of membrane protein per reaction vial) were preincubated for 10 min with toxins at twice their final concentrations in 100 μL of Na^+-free preincubation buffer at 37 °C. Flux was initiated by adding 100 μL of influx buffer containing 2.66 mM NaCl and 1.5 μCi/mL ^{22}-NaCl. After 30 s at 37 °C flux was terminated by addition of 2 mL of ice-cold wash buffer and rapid filtration through BA85 nitrocellulose membrane filters under vacuum. Background ^{22}Na influx was determined in the presence of 2 μM TTX and subtracted from all data points (background was always less than 20% of total flux after subtraction of blank filters). All flux experiments were done in quadruplicate and repeated at least three times.

Electrophysiology. (a) Lymnaea. Whole-cell voltage clamp recordings were taken from dissociated caudodorsal neurons (CDC) from laboratory-bred adult *Lymnaea stagnalis*. The cells were isolated by mechanical dissociation after a 30-min incubation in 0.2% trypsin (type III, Sigma) in HEPES buffered saline (HBS) at 37 °C, as previously described (Dreijer & Kits, 1995). Cells were used within 7 h of isolation. For recording of sodium currents HBS was washed out and replaced under constant perfusion with a solution containing NaCl (40 mM), $CaCl_2$ (4 mM), $CdCl_2$ (0.5 mM), HEPES (10), and 4-aminopyridine (4-AP) (2 mM), pH 7.8, adjusted with NaOH. The internal pipette solution was composed of CsCl (29 mM), $CaCl_2$ (2.3 mM), HEPES (10 mM), EGTA (11 mM), MgATP (2 mM), and Tris−GTP (0.1 mM), pH 7.4, adjusted with CsOH; the calculated free calcium concentration was 10^{-8} M. Conotoxins were applied by means of a picospritzer system that allows rapid application of drugs. Toxin applications commenced 2 s before the test pulse and ended immediately after it. Whole-cell voltage clamp experiments were performed and data was analyzed as previously described (Dreijer & Kits, 1995), except that for CDCs an Axopatch 2A amplifier (Axon

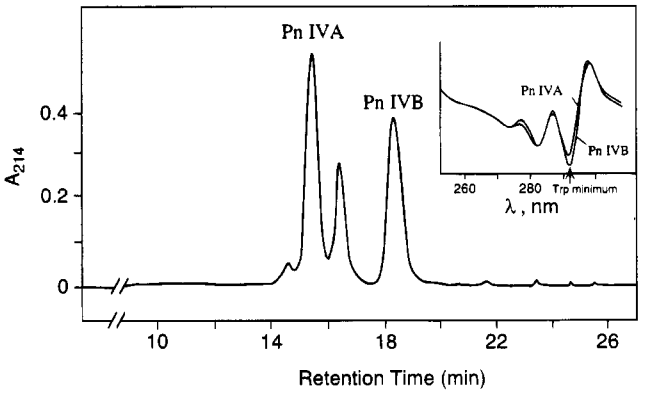

FIGURE 1: Phenyl column purification of PnIVA and PnIVB. The bioactive fraction from a semipreparative C18 column was separated on a wide-bore Vydac phenyl analytical column (250 × 4.6 mm, 5 μm particle size), equilibrated and eluted at a flow rate of 0.5 mL/min with 23% acetonitrile in 0.1% aqeous TFA. The inset shows comparative second-order derivative spectra (Grego et al., 1986) from the PnIVA and PnIVB peaks.

Instruments) was used. Current recordings were filtered at 2 kHz and sampled at >5 kHz.

(b) Chromaffin Cells. Bovine chromaffin cells were isolated as described by Greenberg and Zinder (1982) and cultured according to the methods of Artalejo et al. (1991). Cells were used up to four days after isolation. Whole-cell sodium currents were measured using patch-clamp techniques (Hamil et al., 1981), utilizing an EPC-7 amplifier (List, Darmstadt). Media compositions were as follows. Pipette solution: CsCl (135 mM), $MgCl_2$ (2 mM), $CaCl_2$ (1 mM), HEPES (10 mM), EGTA (11 mM), MgATP (2 mM), Tris−GTP (0.1 mM), pH 7.4, adjusted with CsOH. Bath solution: NaCl (130 mM), $BaCl_2$ (13 mM), (+)-D-glucose (10 mM), $MgCl_2$ (2 mM), $CdCl_2$ (0.5 mM), and HEPES (10 mM), pH 7.4, adjusted with TEAOH.

RESULTS

Purification and Chemical Characterization of Conotoxins PnIVA and PnIVB. Conus pennaceus venom was extracted and fractionated as previously described (Fainzilber et al., 1994b). Fractions were assayed as previously described (Hasson et al., 1995), and the major active peak was found to be that eluting at approximately 50% acetonitrile at the second fractionation step. Refractionation of this peak on analytical C18 columns did not give homogenous products (data not shown); however, spectral analysis indicated the presence of closely related peptides differing in the aromatic regions of their UV spectra. Therefore, final purification was performed using aromatic interactions on a Vydac phenyl column. The fraction was separated into three peaks on Vydac phenyl (Figure 1), the first and third of which contained action potential blocking activity. Second-order derivative UV spectra (Grego et al., 1986) of these peaks suggested that they represent two very similar peptides, differing in their Trp content (Figure 1, inset).

The amino acid sequences of both toxins were first determined by automated Edman sequencing after reduction and pyridylethylation. Unambiguous sequences of 16 and 17 amino acid residues were obtained (Table 1), with a single clear Trp signal in the first cycle for both peptides. However, in an Edman degradation run of non-alkylated PnIVB no signal was observed in the first cycle, although further

Table 1: Initial Edman Degradation Analyses of PnIVA/B

	RPE-PnIVA		RPE-PnIVB		non-alkylated PnIVB	
cycle	assigned residue	yield (pmol)	assigned residue	yield (pmol)	assigned residue	yield (pmol)
1	Trp	225	Trp	300		
2	Cys	205	Cys	250		
3	Lys	270	Lys	480	Lys	450
4	Tyr	220	Tyr	550	Tyr	435
5	Gly	214	Gly	260	Gly	240
6	Trp	135	Trp	175	Trp	70
7	Thr	120	Thr	240	Thr	160
8	Cys	81	Cys	200		
9	Leu	100	Trp	84	Trp	20
10	Leu	80	Leu	225	Leu	57
11	Gly	42	Gly	185	Gly	60
12	Cys	28	Cys	120		
13	Ser	16	Ser	165	Ser	45
14	Pro	15	Pro	120	Pro	26
15	Cys	11	Cys	65		
16	Gly	7	Gly	41	Gly	10
17			Cys	15		

Table 2: Amino Acid Composition Analyses of PnIVA/B

	mole ratio	
amino acid	PnIVA	PnIVB
Ser	0.9 (1)	1.0 (1)
Thr	1.0 (1)	1.0 (1)
Gly	2.8 (3)	3.1 (3)
Tyr	1.0 (1)	0.9 (1)
Pro	1.2 (1)	1.1 (1)
Leu	2.0 (1)	2.2 (2)
Lys	0.9 (1)	0.9 (1)

assignments were consistent with those of the pyridylethylated samples (Table 1). Amino acid composition analyses of both peptides were in good agreement with the Edman data (Table 2), but this technique does not enable accurate quantification of cysteines, or determination of tryptophan. We therefore examined whether the mass spectrometrically determined molecular weights were consistent with the amino acid composition and Edman degradation data in hand.

Subsequent measurement of the molecular weight of PnIVA using liquid secondary ion mass spectrometry (LSIMS) was 1789.5 (based on the ^{12}C mass scale), which did not in fact agree with the proposed Edman degradation sequence in Table 1 (predicted monoisotopic molecular weight, 1771.7, assuming two disulfide bridges). In addition, the value obtained for PnIVB was 1862.8, again in conflict with that predicted from the Edman sequence (1947.7, assuming two disulfide bridges). Reduction and pyridylethylation (RPE) of the native peptides should give a 106-Da (H + 4-vinylpyridine) mass increment per each half-cystine residue. However, the LSI MS observed molecular mass for RPE-PnIVA was 2332.9 Da (monoisotopic), giving a mass increment of 543.4 Da, which cannot be explained by normal reaction products. The molecular mass of RPE-PnIVB was found to be 2406.0 Da, and the unexpected mass increment of ca. 543 Da was observed again. These facts suggested the occurence of an unexpected reaction that gave rise to a partly incorrect Edman sequencing result. Therefore, the structures of the peptides were analyzed in detail by mass spectrometric techniques.

An initial attempt to sequence the intact peptide by high energy collision induced dissociation (CID) mass spectrometry failed, probably due to the multiple disulfide bonds in

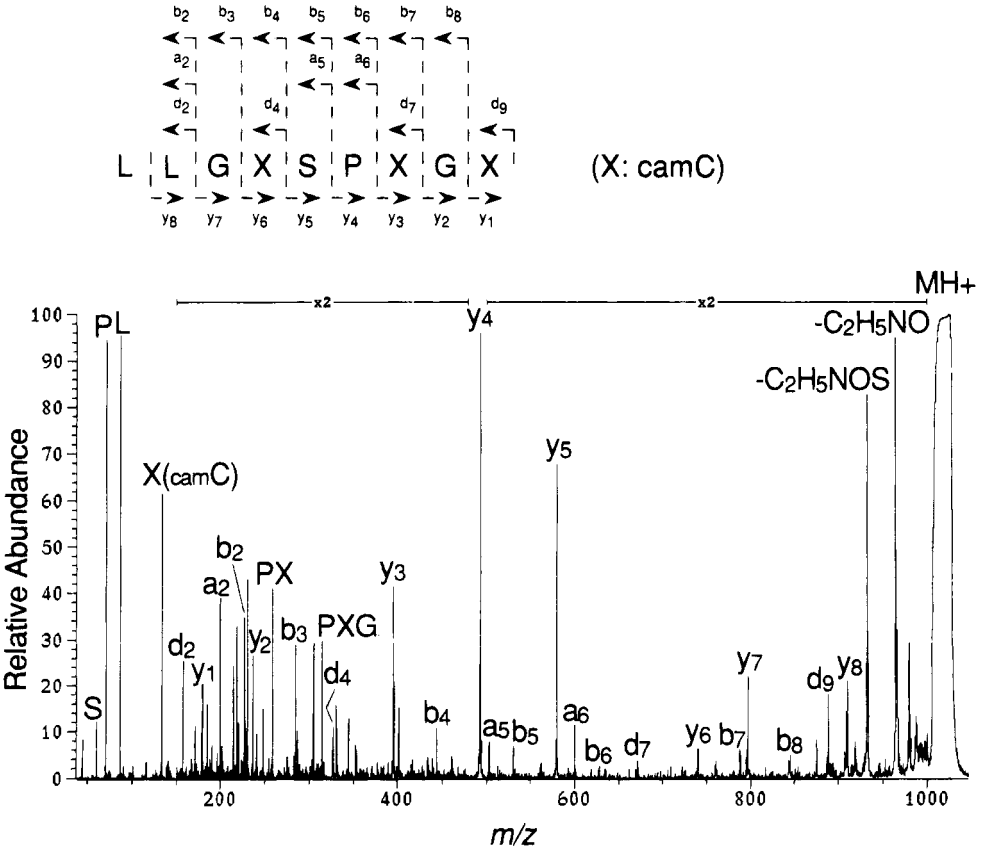

FIGURE 2: High-energy CID spectrum of the monoisotopic ^{12}C isobar of the protonated peptide TH2 (C-terminal thermolytic digest product of PnIVA), *m/z* 1023.4, which was generated by LSI/MS. The observed sequence ions are annotated on the spectrum with Biemann's nomenclature (Biemann, 1988). S, P, L, and X represent immonium ions of serine, proline, leucine, and *S*-carbamoylmethylcysteine (camC), respectively. PX and PXG represent internal fragment ions corresponding to the internal sequences from proline onward.

the chain. Only a few small internal fragment ions were observed in the spectra. Since the multiple disulfide bonds were resistant to in situ reduction with β-mercaptoethanol, conventional reduction and alkylation were carried out to generate fragmentable peptides. Among several alkylating methods, carbamoylmethylation was chosen because of its preferable fragmentation in high-energy CID analysis of cysteine-rich peptides in this class, which was demonstrated by a study of the high-energy CID spectra of various derivatives of a model peptide, α-conotoxin SI (data not shown). In the CID spectrum of the pyridylethyl derivative, a loss of 106 u (C7H8N: pyridylethyl radical) and formation of m/z 106 were predominant and only a few sequence ions were observed. Although the carboxymethyl derivative and the carbamoylmethyl derivative gave spectra of similar appearances with higher relative abundance of sequence ions, the latter gave a spectrum with a better signal to noise ratio. The immonium ion of carbamoylmethylated Cys was clearly observed at m/z 133.

Reduction and carbamoylmethylation of PnIVA gave an alkylated peptide with a monoisotopic molecular mass of 2137.9 Da (determined by LSI MS). The 348.4 unit mass increment suggested the presence of six Cys residues [6 × 58 unit (CH_2CONH_2) mass increment per each half cystine residue]. The reduced and carbamoylmethylated peptide was enzymatically digested because the peptide was too large for direct CID analysis. While neither basic nor acidic residues were indicated in the middle of the peptide chain by the Edman sequence, the presence of two leucine residues near the center of the chain was observed (Table 1). Therefore, a thermolytic digest was carried out under specificity restricting conditions (Welinder, 1988) for cleaving the peptide at these positions (N-terminal of Leu). The digest mixture was first analyzed by liquid chromatography electrospray ionization mass spectrometry (LC/ESI/MS) to monitor the extent of digestion and then purified by reverse-phase HPLC. The digest gave a pair of peptides, TH1 and TH2, which correspond to the N- and C-terminal halves of the original peptide, respectively. The observed average molecular masses by LC/ESI/MS for TH1 and TH2 were 1134.2 and 1022.9 Da, respectively.

One of the HPLC-purified thermolytic peptides, TH2, was analyzed by high-energy CID mass spectrometry and provided the complete C-terminal sequence of the cysteine-rich peptide, PnIVA, as shown in Figure 2. The CID spectrum thus confirms the Edman determination for this region of the peptide, with the addition of a C-terminal cysteine that was not determined previously. It was not possible to obtain clear CID spectra for TH1, which was therefore subjected to automated Edman sequencing, giving an N-terminal sequence of CCKYG---. This confirmed the presence of a sixth cysteine residue at the N-terminus, which was erroneously identified as Trp in the initial Edman degradation of reduced and pyridylethylated peptide. Consequently, the final confirmed amino acid sequence of PnIVA was determined as CCKYGWTCLLGCSPCGC. The molecular mass calculated from this final corrected sequence, 1789.6 Da (monoisotopic, assuming three disulfide bridges), correlates well with the observed value of 1789.5 Da.

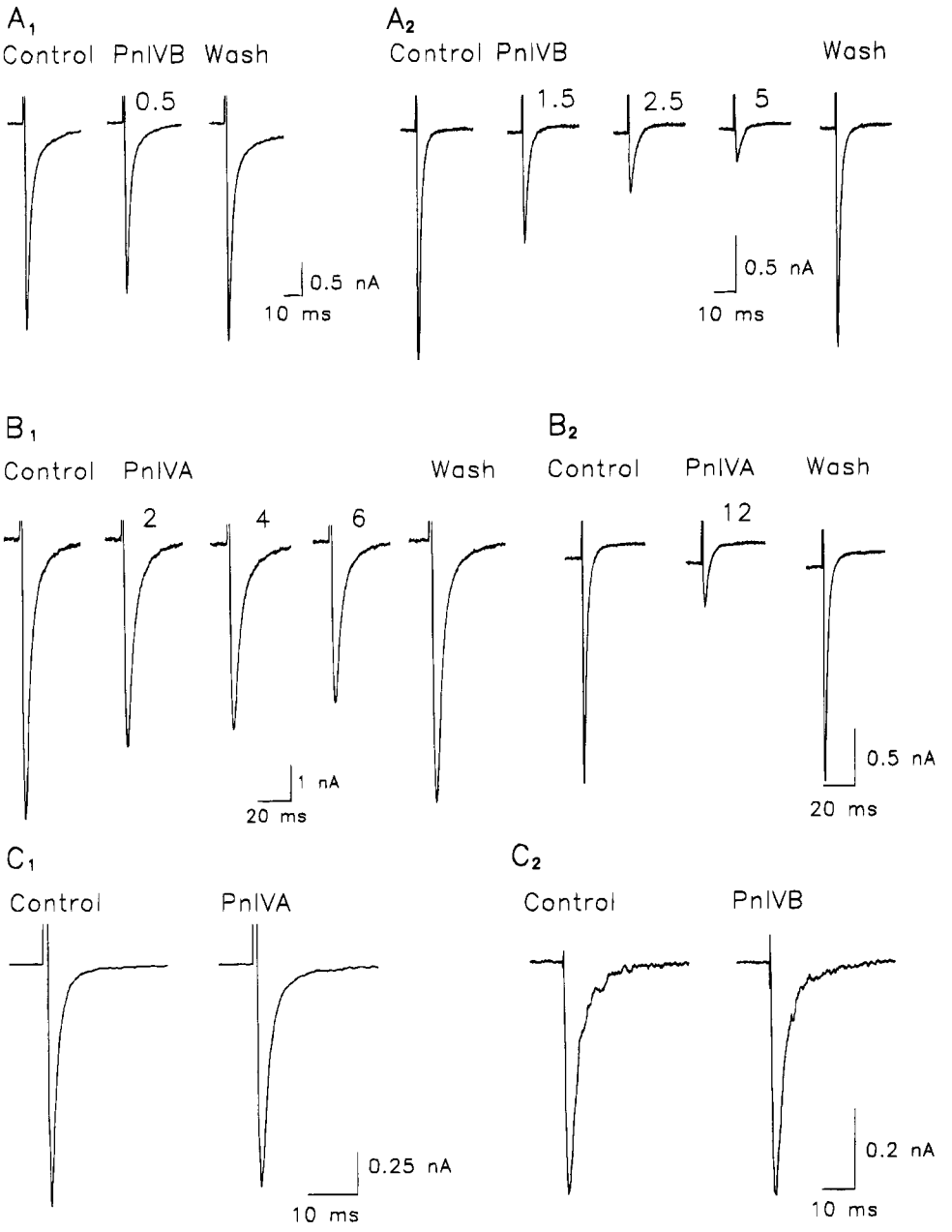

FIGURE 3: Effects of PnIVA and PnIVB on voltage-dependent sodium currents in *Lymnaea* CDC neurons and bovine chromaffin cells. Sodium currents at 10 mV, from a holding potential of −80 mV, were recorded from caudodorsal neurons of the snail *Lymnaea stagnalis*, in the whole-cell voltage clamp mode (A, B). Each panel shows a representative experiment on a single cell. The concentration of toxin applied (μM) is indicated above each trace. (A_1) Partial block of CDC sodium current by 0.5 μM PnIVB. (A_2) A series of doses, 1.5, 2.5, and 5 μM PnIVB, tested on the same cell. A 1-min wash between toxin applications sufficed for return of the current to control values (shown only for the beginning and end of the experiment). Panels B_1 and B_2 show a similar series with PnIVA; note the higher concentrations required. Sodium currents in bovine chromaffin cells were recorded at −10 mV, from a holding potential of −80 mV (C). Panel C_1 shows that application of 12 μM PnIVA was without a significant effect on the current; a similar result is shown in panel C_2 with 5 μM PnIVB.

A tryptic digestion of RPE-PnIVB gave a C-terminal peptide, YGWTCWLGCSPCGC (data not shown), that corresponds to the original Edman sequence. As the N-terminal Edman degradation of native PnIVB suggests an N-terminus of CCKY (Table 1), the sequence of PnIVB was also corrected as CCKYGWTCWLGCSPCGC. The calculated molecular mass of the corrected sequence, 1862.6 Da (monoisotopic, assuming three disulfide bridges), was again in good agreement with the observed value of 1862.8 Da. The elucidated sequences show a highly Cys-rich structural feature of the peptides, six Cys residues in each heptade-capeptide, and a new Cys framework for conotoxins, CC-----C---C--C-C (Figure 4).

PnIVA	**C**C**K**YGW**T**C**L**L**G**C**S**P**C**G**C**
PnIVB	**C**C**K**YGW**T**C**W**L**G**C**S**P**C**G**C**
GIIIA	RD**CC**TOOKK**C**KDRQ**C**KOQR**CC**A-$_{NH2}$
GS	A**C**SGRGSR**C**OOQ**CC**MGLR**C**GRGNPQK**C**IGAH*E*DV

FIGURE 4: Three cysteine frameworks in sodium channel blocking conotoxins. The amino acid sequences of PnIVA, PnIVB, GIIIA and GS are shown. The standard one-letter code is used, except O = trans-4-hydroxyproline and *E* = carboxyglutamate. Cysteine residues are shown in boldface type. The single amino acid difference between PnIVA and PnIVB is underlined.

Paralytic Activity in Vivo of PnIVA/B. The paralytic activity of PnIVA and PnIVB was examined in bioassays in bivalve molluscs (*Mytilus*), mosquito fish (*Gambusia*), and fly larvae (*Sarcophaga*). ED_{50} values for *Mytilus* paralysis were 33.5 pmol/100 mg of body weight for PnIVA and 21.9 pmol/100 mg for PnIVB. Both toxins were completely inactive when injected into *Gambusia* fish or *Sarcophaga* blowfly larvae, at doses 10−15 times the ED_{50} values determined on *Mytilus*. The phylogenic specificity of these *Conus pennaceus* toxins contrasts with that of μGIIIA, which was inactive on *Mytilus* and revealed an ED_{50} for paralysis in *Gambusia* fish of 9.6 pmol/100 mg.

Electrophysiology and Sodium Flux Assays. PnIVB was previously shown to be a potent blocker of the voltage-dependent sodium current in *Aplysia* neurons, with an EC_{50} of 16 nM (Hasson et al., 1995). In order to test the subtype and/or phyletic selectivity of PnIVA and PnIVB, we examined their effects on isolated sodium currents in *Lymnaea* CDC and bovine chromaffin cells, and also on veratridine-stimulated sodium flux in rat brain synaptosomes. In our initial experiments on *Lymnaea* CDC, PnIVB at 0.5 μM blocked approximately 20% of the sodium current (Figure $3A_1$), although this concentration is sufficient for a complete block in *Aplysia* neurons (Hasson et al., 1995). A series of further experiments ($N = 5$) show that PnIVB completely blocks the CDC sodium current at concentrations of 5 μM and above, with an EC_{50} of 1.2 μM. An example of an increasing dose series on the same cell is shown in Figure $3A_2$. Similar experiments with PnIVA revealed a surprising difference in the efficacies of these almost identical peptides. The EC_{50} of PnIVA for blockade of *Lymnaea* CDC sodium current was 7.5 μM; that is approximately 6 times less potent than PnIVB (Figure 3B). The blockade caused by both toxins was reversible within a 1-min wash at all concentrations tested.

Both toxins had no observable effect in the two mammalian systems we tested. Applications of 12 μM PnIVA or 5 μM PnIVB to bovine chromaffin cells were without effect on the isolated sodium currents (Figure 3C). Furthermore, PnIVB at 2 μM had no effect on the veratridine-induced sodium influx in rat brain synaptosomes, whereas TTX at the same concentration blocked more than 80% of the measured uptake (data not shown).

Iodination Blocks Toxicity of the PnIV Toxins. In order to enable a direct study on the binding characteristics of these new toxins, we attempted to radiodinate both PnIV toxins using either lactoperoxidase or iodogen protocols designed for modification of Tyr4 with iodine. Incorporation of ^{125}I into the peptide was very rapid in both cases; thus for example a reaction of 0.5 nmole PnIVA with 1 mCi carrier-free $Na^{125}I$ in the presence of 2 nmol of iodogen (Pierce) for 3 min on ice resulted in incorporation of over 30% of the available ^{125}I. Unfortunately no specific binding of the purified radiodinated derivatives could be observed using a number of experimental protocols. Therefore PnIVA was incubated with lactoperoxidase with or without NaI. The incubation conditions alone did not markedly affect its toxicity as measured in the *Mytilus* bioassay, but the toxin incubated in the presence of iodine totally lost its activity (data not shown). It therefore seems that the lack of specific binding of radiodinated PnIVA is not a result of loss of activity due to the reaction conditions, but rather a direct consequence of iodine modification of the tyrosine residue. The rapidity of the iodination suggests that the single tyrosine residue in these peptides is solvent exposed on the outer surface of the molecule.

DISCUSSION

In the present study we have characterized two new conotoxins from the venom of the molluscivorous species *Conus pennaceus*. As will be detailed below, these toxins represent a new cysteine framework for conotoxins, and they apparently target a different pharmacological subtype of sodium channels than previously described ligands.

Elucidation of the amino acid sequences of PnIVA/B was complicated by a previously undescribed anomalous product of reduction and pyridylethylation (Table 1). The extremely limited amounts of native toxins available did not enable a detailed characterization of this unexpected chemistry; however, analyses of other conotoxin sequences under these reaction conditions reveal the following (T. Nakamura and A. L. Burlingame, unpublished data): (1) The anomalous reaction is reagent-derived and may originate with unknown impurities in certain commercial batches of 4-vinylpyridine; (2) The reaction appears to occur only on N-terminal cysteine residues. (3) The PTH derivative of this uncharacterized cysteine modification elutes at precisely the same position as PTH-Trp in reverse-phase chromatography systems. The series of experiments leading to the final sequence elucidation for PnIVA/B clearly emphasizes the importance of mass spectrometric techniques in such studies.

Sequences of the two peptides are summarized in Figure 4 and compared with the previously described vertebrate muscle sodium channel blockers μGIIIA and μGS. It is immediately apparent that there is very little homology between the *Conus pennaceus* toxins and the piscivorous venom derived μ-conotoxins. There is also no discernable homology between μGIIIA and the six cysteine/four loop μGS, both of which target the same sodium channel macrosite (Yanagawa et al., 1988). PnIVA and PnIVB represent a new Cys framework (CC-----C---C--C-C), which has not been described so far for conotoxins. In accordance with the proposed rules for conotoxin nomenclature, the roman numeral in a toxin designation should represent its cysteine framework. The roman numeral IV is therefore designated for this new CC-----C---C--C-C framework. It is perhaps noteworthy that conotoxins exhibiting three different cysteine frameworks (GIIIA, GS, and PnIV) are all sodium channel blockers. This is perhaps somewhat anomalous for conopeptides, as for example the only unifying element in the large diversity of ω-conotoxin calcium channel blockers characterized to date is their invariant cysteine framework (Olivera et al., 1991; Adams & Olivera, 1994). In this context it is interesting to note that two additional conopeptides, MrVIA and MrVIB, affecting both sodium and calcium channels in *Lymnaea* were recently described (Fainzilber et al., 1995b).

A number of studies have shown that positively charged residues centering on Arg13 in the peptide sequence are crucial for sodium channel block by μGIIIA (Sato et al., 1991; Wakamatsu et al., 1992; Becker et al., 1992). Sato et al. (1991) further suggested that arginine might be a required residue for peptide blockers of sodium channels, on the basis of the notion that the guanidinium side chain of arginine is

analogous to the guanidino groups of small organic sodium channel blockers. It is therefore extremely interesting that the only positively charged residue in PnIVA/B is a lysine (Figure 4). Both *Conus pennaceus* toxins also differ from GIIIA and GS in their relatively high content of hydrophobic residues (Figure 4). Our iodination data suggest that at least one of these hydrophobic residues, Tyr4, is important for binding and activity of the toxin. It should however be noted that our data cannot discriminate at this stage between a direct effect on Tyr4 and a steric hindrance of the iodine modification of the Tyr on Lys3. Furthermore, the difference in potencies between PnIVB versus PnIVA when tested on *Lymnaea* CDCs (Figure 3) indicates that substitution of Trp for Leu at position 9 has functional significance. The importance of the single lysine and the aromatic hydrophobic residues for toxin activity should be one of the first priorities in structure−function analyses of these toxins.

In light of their different cysteine framework and unusual chemical composition, it is perhaps not surprising that PnIVA and PnIVB target a different subtype of sodium channels than the piscivorous venom derived GIII and GS conotoxins. Efficacy of the PnIV toxins for sodium channel blockade appears to be in inverse proportion to the tetrodotoxin sensitivity of the channel subtype. Thus in *Aplysia* neurons PnIVB causes a complete blockade at 80 nM concentration, versus a required 200 μM for TTX (Hasson et al., 1995); in *Lymnaea* CDCs the toxin concentrations required for complete blockade are 5 μM for PnIVB (Figure 3) versus 30 μM for TTX (Brussaard et al., 1991); and in the vertebrate systems no effects of PnIVA or PnIVB could be observed, whereas TTX effects on vertebrate sodium channels are observed at sub-micromolar concentrations even on the relatively resistant muscle subtypes (Strichartz et al., 1987). It is noteworthy that *Aplysia* and *Lymnaea* sodium channels do not seem to differ significantly in their sensitivity to δ-conotoxin-TxVIA (Fainzilber et al., 1995a), and eventual comparison of sequences of these channels may therefore shed light on residues important for interactions with PnIVA and PnIVB. However, it should be noted that differences in media composition may significantly affect toxin activity in electrophysiological systems. For example, binding of δTxVIA is calcium dependent, and a low Ca^{2+} concentration buffer reduces its affinity by approximately 2-fold (Fainzilber et al., 1994a). Effects of different ion compositions might account for part of the difference in activity of PnIVB on *Aplysia* versus *Lymnaea* channels. It should also be noted that we were not able to directly examine the effects of PnIVA/B on vertebrate skeletal or heart muscle sodium channels. Although these toxins' lack of toxicity in fish makes it unlikely that they have significant effects on these channel subtypes, the tetrodotoxin-"resistant" pharmacology of heart channels suggest it may be of interest to examine this possibility.

All of the categories of sodium channel blocking conotoxins seem to bind close to macrosite 1 of the sodium channel (Moczydlowski et al., 1986; Yanagawa et al., 1988; M. Fainzilber, unpublished data). This may suggest overall similarities in their three-dimensional structures enabling recognition of the channel pore, while specific differences target them to different channel subtypes. The set of GIII, GS, and PnIV should therefore enable high-resolution studies of functionally important structural changes in the pore region of the sodium channel. An *Aplysia* sodium channel clone has been reported in abstract form (Dyer et al., 1993). Comparison of the *Aplysia* sequence, when it is available, to those of the cloned rat brain and skeletal muscle subtypes may facilitate localization of residues important for binding and activity of these toxins. The mollusc neuronal sodium channel targeted by PnVIA/B may represent a separate pharmacological subtype of channel. This channel subtype is also characterized by its low affinity for TTX, its relatively weak response to sea anemone toxins, and its specific targeting by δTxVIA (Hasson et al., 1993; Fainzilber et al., 1994a). Three other pharmacological subtypes of sodium channels were previously defined on the basis of sensitivity to μ-conotoxins and TTX (Moczydlowski et al., 1986). On the basis of their unique cysteine framework and pharmacological specificity, the *Conus pennaceus* toxins described in this study should serve as novel pharmacological tools for functional studies on sodium channels.

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REFERENCES

Aberth, W., Straub, K. M., & Burlingame, A. L. (1982) *Anal. Chem. 54*, 2029−2034.

Adams, M. E., & Olivera, B. M. (1994) *Trends Neurosci. 17*, 151−155.

Artalejo, C. R., Dahmer, M. K., Perlman, R. L., & Fox, A. P. (1991) *J. Physiol. 432*, 681−707.

Becker, S., Prusak-Sochaczewski, E., Zamponi, G., Beck-Sickinger, A. G., Gordon, R. D., & French, R. J. (1992) *Biochemistry 31*, 8229−8238.

Betner, I., & Foldi, P. (1986) *Chromatographia 22*, 381−387.

Biemann, K. (1988) *Biomed. Environ. Mass Spectrom. 16*, 99−111.

Brussaard, A. B., Lodder, J. C., Ter Maat, A., de Vlieger, T. A., & Kits, K. S. (1991) *J. Physiol. 441*, 385−404.

Burlingame, A. L., Walls, F. C., Falick, A. M., Evans, S., Riddoch, A., & Buchanan, R. (1990) *Rapid Commun. Mass Spectrom. 4*, 447−448.

Burlingame, A. L. (1994) in *Biological Mass Spectrometry, Present and Future* (Matsuo, T., Caprioli, R. M., Gross, M. L., & Seyama, Y., Eds.) pp 147−164, Wiley, Chichester.

Dreijer, A. M. C., & Kits, K. S. (1995) *Neuroscience 64*, 787−800.

Dyer, J. R., Johnston, W. L., & Dunn, R. J. (1993) *Neurobiology of Aplysia Conference Abstracts*, p 26, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Fainzilber, M., & Zlotkin, E. (1992) *Toxicon 30*, 465−469.

Fainzilber, M., Gordon, D., Hasson, A., Spira, M. E., & Zlotkin, E. (1991) *Eur. J. Biochem. 202*, 589−595.

Fainzilber, M., Kofman, O., Zlotkin, E., & Gordon, D. (1994a) *J. Biol. Chem. 269*, 2574−2580.

Fainzilber, M., Hasson, A., Oren, R., Burlingame, A. L., Gordon, D., Spira, M. E., & Zlotkin, E. (1994b) *Biochemistry 33*, 9523−9529.

Fainzilber, M., Lodder, J. C., Kits, K. S., Kofman, O., Vinnitsky, I., Van Rietschoten, J., Zlotkin, E., & Gordon, D. (1995a) *J. Biol. Chem. 270*, 1123−1129.

Fainzilber, M., van der Schors, R., Lodder, J. C., Li, K. W., Geraerts, W. P. M., & Kits, K. S. (1995b) *Biochemistry 34*, 5364−5371.

Falick, A. M., Wang, G. H., & Walls, F. C. (1986) *Anal. Chem. 58*, 1308−1311.

Gray, W. R., Olivera, B. M., & Cruz, L. J. (1988) *Annu. Rev. Biochem. 57*, 665−700.

Greenberg, A., & Zinder, O. (1982) *Cell Tissue Res. 226*, 655−665.
Grego, B., Nice, E., & Simpson, R. J. (1986) *J. Chromatogr. 352*, 359−368.
Hamill, O. P., Marty, A., Neher, E., Sakmann, B., & Sligworth, F. G. (1981) *Pflugers Arch. Eur. J. Physiol. 391*, 85−100.
Hasson, A., Fainzilber, M., Gordon, D., Zlotkin, E., & Spira, M. E. (1993) *Eur. J. Neurosci. 5*, 56−64.
Hasson, A., Fainzilber, M., Zlotkin, E., & Spira, M. E. (1995) *Eur. J. Neurosci. 7*, 815−818.
Medzihradszky, K. F., Maltby, D. A., Hall, S. C., Settineri, C. A., & Burlingame, A. L. (1994) *J. Am. Soc. Mass Spectrom. 5*, 350−358.
Moczydlowski, E., Olivera, B. M., Gray, W. R., & Strichartz, G. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5321−5325.
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., & Cruz, L. J. (1990) *Science 249*, 257−263.
Olivera, B. M., Rivier, J., Scott, J. C., Hillyard, D. R., & Cruz, L. J. (1991) *J. Biol. Chem. 266*, 22067−22070.
Reed, L. J., & Muench, S. (1938) *Am. J. Hyg. 27*, 493−497.
Sato, K., Ishida, Y., Wakamatsu, K., Kato, R., Honda, H., Ohizumi, Y., Nakamura, H., Ohya, M., Lancelin, J. M., Kohda, D., & Inagaki, F. (1991) *J. Biol. Chem. 266*, 16989−16991.
Stephan, M. M., Potts, J. F., & Agnew, W. S. (1994) *J. Membr. Biol. 137*, 1−8.
Tamkun, M. M., & Catterall, W. A. (1981) *Mol. Pharmacol. 19*, 78−86.
Wakamatsu, K., Kohda, D., Hatanaka, H., Lancelin, J. M., Ishida, Y., Oya, M., Nakamura, H., Inagaki, F., & Sato, K. (1992) *Biochemistry 31*, 12577−12584.
Walls, F. C., Hall, S. C., Medzihradszky, K. F., Yu, Z., Burlingame, A. L., Evans, S., Hoffman, A. D., Buchanan, R., & Glover, S. (1993) *Proc. ASMS Conf. 41*, 937a−937b.
Welinder, K. G. (1988) *Anal. Biochem. 174*, 54−64.
Yanagawa, Y., Abe, T., Satake, M., Odani, S., Suzuki, J., & Ishikawa, K. (1988) *Biochemistry 27*, 6256−6262.

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