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Identification of a Novel Class of Nicotinic Receptor Antagonists

*DIMERIC CONOTOXINS VxXIIA, VxXIIB, and VxXIIC FROM CONUS VEXILLUM**

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The venoms of predatory marine snails (*Conus* spp.) contain diverse mixtures of peptide toxins with high potency and selectivity for a variety of voltage-gated and ligand-gated ion channels. Here we describe the chemical and functional characterization of three novel conotoxins, αD-VxXIIA, αD-VxXIIB, and αD-VxXIIC, purified from the venom of *Conus vexillum*. Each toxin was observed as an ~11-kDa protein by LC/MS, size exclusion chromatography, and SDS-PAGE. After reduction, the peptide sequences were determined by Edman degradation chemistry and tandem MS. Combining the sequence data together with LC/MS and NMR data revealed that in solution these toxins are pseudo-homodimers of paired 47–50-residue peptides. The toxin subunits exhibited a novel arrangement of 10 conserved cystine residues, and additional post-translational modifications contributed heterogeneity to the proteins. Binding assays and two-electrode voltage clamp analyses showed that αD-VxXIIA, αD-VxXIIB, and αD-VxXIIC are potent inhibitors of nicotinic acetylcholine receptors (nAChRs) with selectivity for α7 and β2 containing neuronal nAChR subtypes. These dimeric conotoxins represent a fifth and highly divergent structural class of conotoxins targeting nAChRs.

Nicotinic acetylcholine receptors (nAChRs)[4] belong to the Cys-loop superfamily of pentameric ligand-gated ion channels. Neuronal nAChRs are generally formed from a combination of α and β subunits (α2–α10 and β2–β4) that can assemble into a diversity of nAChR subtypes with different pharmacological and functional properties (1). Given their physiological importance, nAChRs are often targeted by venom peptides (2).

Our current knowledge of the structure and function of nAChRs owes much to studies using snake toxins as biochemical or pharmacological tools to isolate and characterize this receptor (3). The "bungarotoxin" family of proteins in the venom of elapid and hydrophid snakes includes the κ- and α-neurotoxins, muscarinic toxins, cytotoxins, cardiotoxins, fasciculins, calciseptins, and mambins (4, 5). The structurally related short-chain and long-chain α-neurotoxins and the "weak non-conventional" snake toxins are comprised of 60–74 amino acids, including 8 or 10 cystine residues and their structures have a three-fingered fold. The κ- and α-neurotoxins both inhibit nAChRs but differ in their specificity and binding kinetics (4).

Conotoxins are small disulfide-rich peptide toxins found in the venom of predatory marine snails from the genus *Conus.* These venom peptides generally target a variety of voltage-gated and ligand-gated ion channels (6, 7). Conotoxins acting at nAChRs include the α-conotoxin, αA-conotoxin, ψ-conotoxin, and αS-conotoxin families. The α-conotoxins are a large family of well characterized competitive nAChR antagonists with diverse subtype selectivities that allow the pharmacological dissection of nAChR subtypes (1, 8). The structurally different αA-conotoxins are competitive antagonists with specificity for muscle nAChRs but lack the selectivity for the α1/δ interface that is exhibited by the 3/5 subfamily of α-conotoxins (7), whereas the ψ-conotoxins are non-competitive antagonists at the muscle nAChR subtype (7). The recently described αS-conotoxin RVIIIA is a nAChR antagonist with broad subtype activity and a preference for the muscle subtype (9). Despite the importance of these toxins for prey capture, not all species of cone snails possess detectable levels of all four of these classes of nAChR antagonists in their venom. For example, no toxins from these families have previously been described from the venom of *Conus vexillum*. It has been suggested that different clades of *Conus* species might utilize unique conotoxin families for particular purposes (10–12).

Post-translational modifications contribute to the rich diversity and heterogeneity of conotoxins (13, 14) and may confer unique structural and functional traits. Examples of post-translational modifications in conotoxins include proline hydroxy-

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[4] The abbreviations used are: nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine binding protein; α-BgTx, α-bungarotoxin; ESI-MS, electrospray ionization mass spectrometry; LC/MS, liquid chromatography mass spectrometry; MS, mass spectrometry; MS/MS, tandem mass spectrometry; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reversed-phase high performance liquid chromatography; TOCSY, total correlation spectroscopy.

lation and glutamic acid γ-carboxylation as in GID (15), tyrosine sulfation as in EpI (16), and disulfide bonds and COOH-terminal amidation in most conotoxins. The only multimeric protein described so far from *Conus* venoms is a phospholipase (17). In contrast, there are numerous examples of heterodimers among venom toxins from several other genera including ants, spiders, scorpions, and snakes (17–23). Additionally, κ-neurotoxins from snake venom can form homodimers in solution at physiological concentrations, raising implications for both the mechanism of polypeptide chain folding during biosynthesis of the toxins and for their interaction with nAChRs (4).

Neuronal nAChRs are of increasing interest in human medicine as therapeutic targets for the treatment of chronic pain and neurological disorders such as Alzheimer and Parkinson diseases (24, 25). To decipher the functions and locations of the different nAChR subtypes, additional subtype-specific ligands are required. Cone snail venoms have been a particularly rich source of new inhibitors of nAChRs. This study describes the discovery and characterization of three novel post-translationally modified conotoxins, VxXIIA, VxXIIB, and VxXIIC, which occur as dimers and produce a slowly reversing block of α7 and α3β2 nAChRs. These αD-conotoxins contribute to our growing knowledge of ligands interacting at nAChRs.

EXPERIMENTAL PROCEDURES

Crude Venom Extraction—Specimens of *C. vexillum* (8), *Conus mustelinus* (7), *Conus miles* (9), and *Conus capitaneus* (10) were collected from the Great Barrier Reef, Australia. Crude extracts were prepared from venom duct material using 30% acetonitrile/water acidified with 0.1% trifluoroacetic acid. Soluble material was lyophilized and stored at −20 °C prior to use. The crude venom samples were tested for their ability to inhibit agonist-evoked currents of α7 and α3β2 nAChRs expressed in *Xenopus laevis* oocytes (see below).

Crude Venom Enrichment—A portion of each crude venom extract (10 mg) was fractionated by size-exclusion chromatography (Superdex Peptide, HR 10/30, Amersham Biosciences) to generate two fractions containing peptides and proteins in the size ranges 5–15 and 0.5–5 kDa. The column was eluted with 30% acetonitrile, 0.048% trifluoroacetic acid, aqueous at a flow rate of 0.5 ml/min, with detection at 214 nm. Only the 5–15-kDa fraction of each species inhibited oocyte-expressed α7 nAChRs.

Purification of Proteins—To isolate the 5–15-kDa components active at the nAChR, a portion of the crude venom extract (10 mg) was fractionated by semi-preparative RP-HPLC (10 μm C18, Vydac) eluted at 3 ml/min with a linear gradient of 0–90% solvent B over 80 min, using a Waters 600 solvent delivery system (A, 0.1% aqueous trifluoroacetic acid; B, 90% acetonitrile/0.09% aqueous trifluoroacetic acid). Each species contained ~11-kDa proteins that inhibited α7 and α3β2 nAChRs. The active fractions from *C. vexillum* were further purified by analytical C18 RP-HPLC using 5-μm 4.6 × 250-mm Jupiter and Vydac columns eluted at 1 ml/min, or a 3.5-μm 300 SB-C18 2.1 × 150-mm Zorbax column eluted at 0.18 ml/min using 0.5 or 1% linear gradients from 0 to 50% B over 45 min at 65 or 23 °C. The activity of each of the three ~11-kDa proteins isolated from *C. vexillum* venom was confirmed by retesting the purified material on oocyte-expressed nAChRs and an AChBP binding assay.

LC/MS Analyses—LC/MS analysis of crude venom extract or a partly purified mixture of dimeric toxins was undertaken using an Applied Biosystems/MDS SCIEX QSTAR Pulsar electrospray ionization quadrupole time-of-flight (ESI QqTOF) mass spectrometer equipped with an electrospray ionization source and linked to an upstream Agilent 1100 Series HPLC system. LC was performed with a Zorbax C3, 2.1 × 150-mm 5-μm RP-HPLC column eluted with 0–60% B for 60 min (A 0.1% formic acid, B 90% acetonitrile, 0.09% formic acid), at 180 μl/min and a temperature of 23 or 65 °C. Time of flight-MS scans were run in positive ion mode over a mass range of 500–2200 atomic mass units with an ion spray voltage of 5300 V. A positive mode "hi/lo" declustering LC/MS experiment as described previously (26) was conducted for additional verification of γ-carboxyglutamic acid residues. Data processing of LC/MS data were performed using the software package Bioanalyst (PE-Sciex, Canada). Apex mass is defined as the mass of the isotope distribution at maximum intensity as identified by the mass reconstruction tool in the Bioanalyst software.

Electrophoresis—Samples were solubilized in 8 M urea, 4% CHAPS, and loaded onto IPG strips (Immobilin Dry Strips pH 6–11, 7 cm, Amersham Biosciences) using passive hydration. SDS-PAGE was run with either 16% polyacrylamide or Novex precast 10–20% Tricine mini-gels under standard non-reducing conditions. Gels were either stained with colloidal Coomassie Blue G-250 or proteins were transferred to polyvinylidene difluoride using SDS/glycine buffers and then stained with Coomassie Blue.

Reduction and Alkylation of Cystine Residues—The purified ~11-kDa proteins (~20 pmol) were reduced in the presence of 10 mM TCEP, 50 mM ammonium acetate (pH 4.5), 10% acetonitrile (37 °C for 1 h) and subsequently alkylated in the added presence of 20 mM maleimide (37 °C for 1 h). The alkylated peptides were repurified by RP-HPLC.

Proteolytic Digestion—Samples of reduced and alkylated peptides from *C. vexillum* were subjected to digestion with the proteolytic enzymes pepsin, trypsin (Sigma), or endoproteinase Arg-C (sequencing grade, Roche). Briefly, for pepsin digestion, ~100 μg of reduced and alkylated peptide was suspended in 100 μl of a solution containing 50 mM formic and 50 mM acetic acid to which 2 μl of a 1 mg/ml stock solution of pepsin was added, and the reaction mixture was incubated for 3 h at 37 °C. Digestion was terminated by storage at −20 °C. For trypsin digestion, ~100 μg of reduced and alkylated peptide was suspended in 100 μl of 100 mM ammonium bicarbonate (pH 7.3) to which 2 μl of a 1 mg/ml stock solution of trypsin was added, and the reaction mixture incubated for 3 h at 37 °C. Digestion was terminated by addition of an equal volume of 0.1% trifluoroacetic acid (aqueous) and storage at −20 °C. Endoproteinase Arg-C digestion was achieved by dissolving ~100 μg of reduced and alkylated peptide in an incubation buffer of 100 mM Tris-HCl, 10 mM $CaCl_2$ (pH 7.6) to which 5 μl of an 0.1 mg/ml enzyme solution and 10 μl of activation solution (50 mM dithiothreitol, 5 mM EDTA) was added and allowed to incubate for 3 h at 37 °C. The resulting fragments from proteolytic diges-

tions were purified by analytical RP-HPLC and analyzed by Edman sequence chemistry as well as MS and MS/MS methods as described below.

Sequencing by Edman Chemistry—Edman NH_2-terminal sequence analysis was undertaken on purified material with disulfide bonds intact or with cysteine residues alkylated with maleimide after reduction with TCEP, as described above. Fragments generated by proteolytic digest reactions were similarly sequenced with an Applied Biosystems Procise Protein Sequencer (HT or 492cLC models).

Sequence Determination by MS/MS Analyses of Peptide Fragments—Tandem MS (MS/MS) experiments were conducted using the QSTAR. Samples in 20–50% aqueous acetonitrile, 0.1% formic acid were continuously infused into the ion source at 40 μl/min in a carrier of 70% eluent B (90% acetonitrile, 0.05% formic acid). Data were acquired over 400–2000 atomic mass units for 5–10 min with an ion spray voltage of ~9500 V and collision energy of 10–60 V, as required.

Additional MS Experiments—An ES-MS pH titration experiment was conducted using solvents containing ~60% acetonitrile and either 1% trifluoroacetic acid, 0.05% formic acid, 0.05% acetic acid, 20 mM ammonium acetate, or 20 mM ammonium bicarbonate to cover the pH range 1–9 in one pH unit increments. Samples of the ~11-kDa proteins in these solutions were introduced into the QSTAR by infusion at low flow rates and analysis performed as described above. A voltage step experiment was conducted to assess stability of the dimer association by application of stepwise increases in sample cone voltage (10–70 V) in parallel with extraction cone voltage (4–25 V) and a capillary voltage of 3500 V using an Micromass LCT ESI-TOF instrument.

Protein Quantitation—Crude venom extract was quantitated by BCA protein assay. VxXIIA was quantified initially by triplicate amino acid analysis, and used as an internal standard for quantitation of the other proteins. An extinction coefficient adjustment factor was determined for each protein (27) and used to adjust HPLC peak area to quantify VxXIIB and VxXIIC.

^1H NMR Spectroscopy—All NMR experiments were recorded on a Bruker Avance 600 spectrometer equipped with an *x,y,z* gradient unit. Protein concentration was ~1 mM. Native VxXIIA was examined in 90% H_2O, 10% D_2O (pH 3.0), at temperatures ranging from 280 to 313 K and in 100% D_2O (298 K). ^1H NMR experiments recorded were NOESY (28, 29), with mixing time of 300 ms, TOCSY (30) with a mixing time of 80 ms, and TOCSY and NOESY in 100% D_2O (31). All spectra were run over 7184 Hz (600 MHz) with 4 K data points, 256–512 free induction decays, 8–80 scans, and a recycle delay of 2 s. The solvent was suppressed using WATERGATE (32). Slowly exchanging amides were detected by dissolving protein in D_2O and recording a series of one-dimensional and TOCSY spectra at 298 K. Amide protons remaining after 24 h were classified as slowly exchanging. Spectra were processed using XWIN NMR and Aurelia, and subtraction of background was used to minimize T_1 noise. Chemical shift values of VxXIIA were referenced internally to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0.00 ppm. The protein was assigned using the "sequential walk" (33). Secondary Hα shifts were measured and compared with random coil shift values (34).

Expression of nAChRs in Xenopus Oocytes—RNA preparation, oocyte preparation, and expression of nAChRs in *Xenopus* oocytes were performed as described previously (15, 26). Briefly, plasmids containing cDNA encoding rat α3, α4, α7, β2, and β4 nAChR subunits (kindly provided by J. Patrick, Baylor College of Medicine, Houston, TX) were subcloned into the oocyte expression vector pNKS2 (35). cDNAs for the rat α1, β1, γ, and δ subunits of the muscle nAChR were provided by Dr. V. Witzemann (Max-Planck Institute for Medical Research). Oocytes were injected with 50 nl of cRNA (5–50 ng/μl), and kept at 19 °C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES at pH 7.4) supplemented with 50 mg/liter of gentamycin (Sigma).

Two-electrode Voltage Clamp Recordings—Two-electrode voltage clamp recordings at a holding potential of −70 mV were performed in oocytes 1–10 days after cRNA injection. Assays were conducted using a custom-made manifold for rapid solution exchange (<300 ms) and a 50-μl funnel-shaped oocyte chamber as described previously (15) or an automated OpusXpress™ 6000A work station (Axon Instruments Inc., Union City, CA). 100 μM ACh (Sigma) was used to activate α3β2, α3β4, α4β4, and α4β2 combinations, 1 μM ACh was used to activate α1β1γδ nAChRs and 100 μM nicotine (Sigma) was used to activate the α7 nAChRs. Agonist pulses were applied in 2-s pulses at 4- or 5-min intervals until consistent control responses were obtained. A 5.5-μl aliquot of a 10-fold concentrated toxin solution was added directly into the static bath, to conserve material and avoid potential losses of the toxin to tubing surfaces. Each concentration was tested on at least three different oocytes from two separate batches. Estimates of potency were obtained by fitting concentration-response curves to the data by the equation: % response = $100/\{1 + ([\text{toxin}]/IC_{50})^{n_H}\}$ using Prism software (GraphPad version 3.0, San Diego, CA). The IC_{50} values were considered significantly different if the 95% confidence intervals did not overlap.

AChBP Binding Assay—Purified recombinant AChBP with a histidine tag was kindly provided by Titia Sixma and Angus Smit (Netherlands Cancer Institute and Vrije University, respectively, The Netherlands). An AChBP binding assay was performed according to the procedure of Smit *et al.* (36). Briefly, toxins were dried and resuspended in 85 μl of binding buffer (phosphate-buffered saline, 0.5 mg/ml bovine serum albumin, 0.1% Tween 20 (pH 7.4), plus 10 μl of AChBP (0.2 ng/μl) (7), 10 μl of ^{125}I-bungarotoxin (^{125}I-BgTx), (Amersham Biosciences), and 20 μl TALON metal affinity resin (Clontech). Assays were incubated for 1 h at 23 °C and filtered through GFB filters using a Millipore manifold and a wash buffer of 20 mM HEPES and 125 mM NaCl (pH 7.2). Retained radioactivity on the filters was measured using a γ-counter (Wallac). Specific binding was determined by subtraction of nonspecific binding determined in the presence of 80 μM tubocurarine (Sigma). (Similar results were obtained with 3.2 μM α-BgTx (Sigma).) A saturation binding experiment was conducted by measuring specific ^{125}I-BgTx binding in the absence or presence of toxin at a concentration of twice the toxin IC_{50}. ^{125}I-

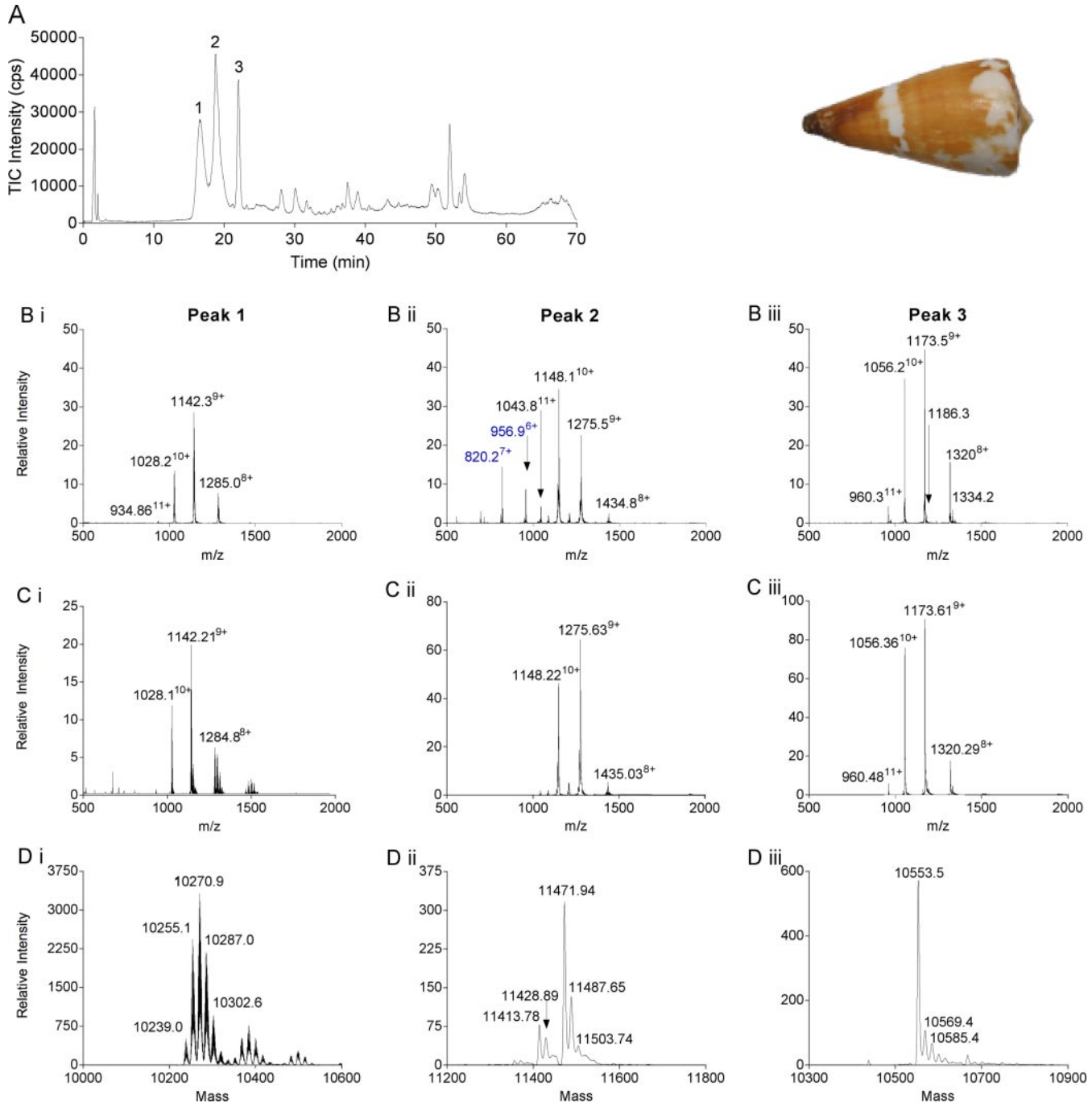

FIGURE 1. **LC/MS of *C. vexillum* crude venom.** *A*, the total ion current (*TIC*) shows native VxXIIA, VxXIIB, and VxXIIC marked *1*, *2*, and *3*, respectively. A *C. vexillum* shell is shown in the *inset*. *B*, *i–iii*, ion spectra from the three peptides in the crude venom are shown. *C*, *i–iii*, ion spectra from the three semi-purified peptides are shown. *D*, *i–iii*, deconvoluted/reconstructed mass spectra for the three peptides are shown with maximum ions and mass values labeled. The average mass values for components in the crude venom were ~10,270 Da for VxXIIA, ~11,472 Da for VxXIIB, and ~10,553 Da for VxXIIC. The VxXIIB spectra included ions consistent with a monomer mass at ~5,735 Da.

BgTx was added over the concentration range 0.5–75 nM. Nonlinear regressions were fitted to each experiment with Prism software (GraphPad).

RESULTS

Protein Identification—Initial analyses of the crude venom from several vermivorous cone snail species indicated the presence of a number of ~11-kDa proteins with inhibitory activity at $\alpha7$ and $\alpha3\beta2$ nAChRs (data not shown). Comparing the MS of intact and reduced material revealed that these proteins in *C. mustelinus*, *C. miles*, *C. capitaneus*, and *C. vexillum* occurred as dimers of two peptide subunits. The crude venom of *C. vexillum* was the least complex, containing three dominant and apparently homogeneous proteins of 10,300–11,400 Da (Fig. 1) that we named VxXIIA, VxXIIB, and VxXIIC. Each protein was purified to a single peak by RP-HPLC, however, varying levels of micro-heterogeneity were revealed by LC/MS analysis of crude venom (Fig. 1). The reduced subunits were half the size of the

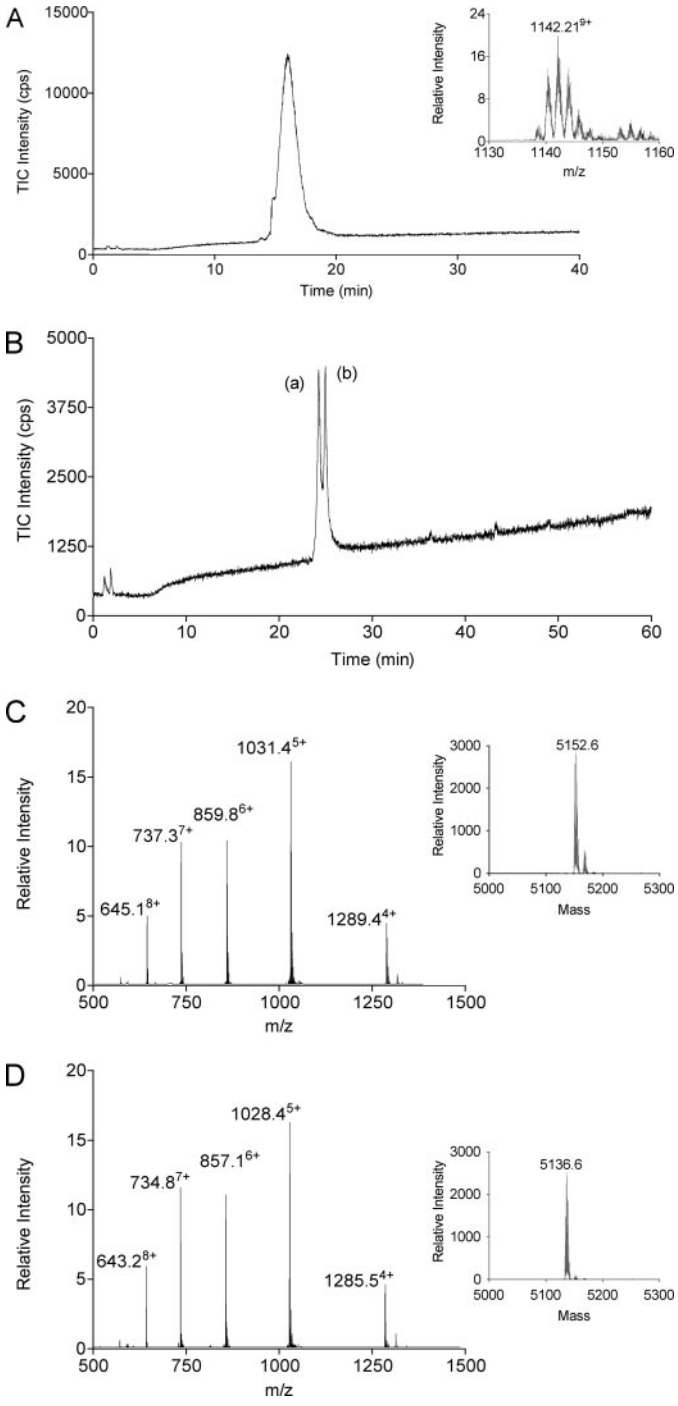

FIGURE 2. **LC/MS of VxXIIA.** *A*, the total ion current (*TIC*) for intact VxXIIA is shown with the corresponding ion spectra shown in the *inset*. *B*, the total ion current for reduced VxXIIA shows two components. *C*, the ion spectra from peak 1 of reduced VxXIIA (mass 5150 Da, monoisotopic value), and the deconvoluted mass spectrum, *inset*, are shown. *D*, ion spectra from peak 2 of reduced VxXIIA (mass 5134 Da, monoisotopic value) and the deconvoluted mass spectrum, *inset*, are shown.

~11-kDa native toxins and for *C. vexillum* ranged in monoisotopic mass from 5135–5741 Da (Fig. 2, Table 1). SDS-PAGE of the native proteins indicated an apparent size of 10–12 kDa (data not shown), supporting the conclusion from SE-HPLC and MS data that the proteins occur as dimers. The LC/MS analysis of crude venom also detected ions for minor monomer forms of VxXIIB, whereas VxXIIA and VxXIIC occurred exclusively as dimers (Fig. 1).

MS analysis at raised voltages and LC/MS at raised temperature (65 °C) failed to disrupt the dimers (data not shown). The higher temperature improved HPLC separation of components from the heterogeneous mixtures but promoted decarboxylation of putative γ-carboxyglutamic acid residues in VxXIIB, as indicated by a mass shift of −44 Da (data not shown). ESI-MS of native VxXIIB over pH 1–9 revealed no discernible shift from dimer to monomer at conditions up to pH 5, but beyond that pH neither the dimer nor monomer could be detected. MS analyses showed that the dimer remained intact following denaturation treatments with urea, guanidine HCl, or 50% trifluoroethanol (data not shown).

Determination of Primary Sequences—Sequence information obtained directly from Edman N-terminal sequence analyses of the reduced and alkylated peptides was limited, due in part to rapid losses in successive cycles of Edman chemistry despite adequate initial yields. As a result, an unambiguous sequence assignment of the full-length peptide could be made for only the first 15–20 cycles. Proteolytic digests with trypsin and pepsin, or endoproteinase Arg-C and pepsin, were used to generate nested sets of overlapping peptide fragments from each precursor peptide. Sequence information deduced from peptide fragments by MS/MS was used to complete the sequences suggested by Edman N-terminal sequencing data in some cases, and for *de novo* sequence determination in other cases. The resulting peptide sequences (Table 1) had ~43% homology, sharing 21 of the 47–50 residues including a conserved WGRCC motif. The determined monoisotopic molecular masses of the reduced peptides, together with the sequence data, suggested that the three peptides VxXIIA, VxXIIB, and VxXIIC all had a free carboxyl terminus (Table 1).

The Edman NH_2-terminal sequences of several ~11-kDa nAChR antagonists from *C. mustelinus*, *C. miles*, and *C. capitaneus* gave a consensus sequence D*XXX*CQ*XX*TOGSKWGRCC (*X* indicates sequence variation) that corresponded to the NH_2 terminus of VxXIIA, indicating these species also produce αD-conotoxins. The observed apex mass values of the 11-kDa proteins from these species ranged from 10,840 to 10,940 Da for *C. mustelinus*, ~10,280 Da for *C. miles*, and 10,990 to 11,000 Da for *C. capitaneus*. As seen for *C. vexillum*, upon reduction each protein separated into peptides of half the size of the native protein.

Post-translational Modifications—The ion spectrum for native VxXIIA revealed apex mass values of ~10,255, ~10,271, and 10,287 Da (Fig. 1). This ion series reflected differences of 16 mass units that could be attributable to hydroxyproline/proline heterogeneity or perhaps to partial methionine oxidation. A minor ion series with differences of 112 mass units probably reflected the presence of trifluoroacetic acid adducts. Upon reduction, intact VxXIIA (Fig. 2*A*) gave rise to two closely eluting peaks present in a 1:1 ratio (Fig. 2*B*). The ion spectrum (*m*/*z*) for the first eluting peak of reduced VxXIIA had a monisotopic mass of 5150 Da, whereas the second peak had a monoisotopic mass of 5134 Da (Fig. 2). An expanded view of the 9^+ ion for the intact protein of VxXIIA revealed that the three co-eluting components exist in a 1:2:1 ratio (Fig. 2*A*, *inset*). These data are

TABLE 1

Comparison of deduced sequences for *C. vexillum* components VxXIIA, VxXIIB, and VxXIIC

The sequences were derived from intact material by Edman chemistry, and from fragments generated by enzyme digestions, by both Edman chemistry and MS/MS. Residues shown underlined were determined by MS/MS, either by revision from Edman data or by *de novo* sequencing. Other residues were determined by Edman sequencing and confirmed by MS and MS/MS. J denotes residues likely to be γ-carboxyglutamic acid, O denotes hydroxyproline, and gaps are for sequence alignment. Sites of potential Pro/Hyp substitution between variants of a peptide are shadowed. Cystine residues are highlighted in bold. Residues or motifs conserved between all three peptides are indicated. The mass values are consistent with all three peptides having a free carboxyl terminus.

Name	Sequence	Mass (Da) Monomer (reduced)[1] Observed Mass	Monomer (reduced)[1] Predicted Mass	Dimer (intact)[2] Observed Mass	Dimer (intact)[2] Predicted Mass
	Cysteine framework and conserved residues 10| 20| 30| 40| 50| -----C----------CC----C---CC----C-C---------C-C D S WGR G P Y RG S				
VxXIIA	DVQD-**C**QVST**O**GSKWGR**CC**LNRV**C**GP**M**C**C**P**A**SH**C**Y**C**VYHRGRGHG**C**S**C**#	5134.6	5134.09	10255	10255.76
VxXIIB	DDJSJ**C**IINTRDS**P**WGR**CC**RTRM**C**GSM**CC**P**R**NG**C**T**C**VYHWRRGHG**C**S**C**P**G**#	5741.4	5741.26	11472	11470.88
VxXIIC	DLRQ-**C**TRNA**P**GSTWGR**CC**LN**P**M**C**GNF**CC**P**R**SG**C**T**C**AYNWRRGIY**C**S**C**#	5282.4	5282.19	10554	10552.18

[1] Mass values given for the reduced monomers are monoisotopic values.
[2] Mass values for native dimers are given as observed maximum or apex mass values, and predicted (calculated) average values because the monoisotopic values could not be determined with confidence for components in that mass range.

consistent with dimers composed of two subunits containing either a proline or hydroxyproline in a 1:1 ratio. MS also revealed the presence of additional minor variants consistent with both more and less frequent hydroxyproline substitution (data not shown).

The sequence obtained for VxXIIA (Table 1) was consistent with the observed monoisotopic mass value of 5134.6 Da for one of the two reduced subunits (Fig. 2, *Peak 2*). An equally abundant variant, [Hyp^{29}]VxXIIA, was identified by sequencing and explained the second observed monoisotopic value of 5150.6 Da seen for the first eluting peak (Fig. 2). Additionally the minor variants [Hyp^{25},Hyp^{29}]VxXIIA and [Pro^{10}]VxXIIA were identified. Thus the observed mass values for the three major components of the native VxXIIA complex, ~10,255, ~10,271, and ~10,287 Da (apex values), corresponded to a homodimer of VxXIIA, a pseudo-homodimer of both VxXIIA and [Hyp^{29}]VxXIIA, and a homodimer of [Hyp^{29}]VxXIIA. These three components occurred in a 1:2:1 ratio consistent with the random association of two different subunits of equivalent abundance as suggested above. Their predicted average mass values are 10,255.76, 10,271.76, and 10,287.76 Da, respectively.

VxXIIB also purified to a single peak by RP-HPLC but again LC/MS analysis revealed microheterogeneity. The major form had a mass of ~11,472 Da and there was another component with a mass deficit of ~58 Da. Additional minor variants, consistent with hydroxyproline/proline substitution or methionine oxidation, were also observed. Further heterogeneity was attributable to decarboxylation of γ-carboxyglutamic acid residues. It is not known if the differently decarboxylated forms are natural variants or arose from sample degradation. Chromatography of VxXIIB after reduction revealed one dominant peak, in contrast to the two peaks observed for VxXIIA. The observed monoisotopic mass of the major form of reduced VxXIIB was 5,741 Da.

A single dominant VxXIIB sequence was identified (Table 1). A minor [*des*-Gly^{50}]VxXIIB variant with a likely amidated COOH terminus was determined by MS/MS. MS of NH_2-terminal peptic fragments of VxXIIB indicated the presence of a further minor variant [Hyp^{14}]VxXIIB (data not shown). These variants were consistent with mass data from the native VxXIIB sample, with observed apex mass values of ~11,472, 11,414, and 11,488 Da (Fig. 1). The corresponding predicted average mass values were 11,470.88, 11,412.88, and 11,486.88 Da. Standard Edman chemistry analysis of intact VxXIIB showed low levels of glutamate in positions three and five, indicative of γ-carboxyglutamic acid residues at those locations. However, the results of multiple sequence analyses were ambiguous, with varying levels of glutamate in those cycles. MS analysis of the intact peptide and NH_2-terminal fragments showed mass values that fitted a sequence containing two γ-carboxyglutamic acid residues (data not shown). It also showed the presence of components with mass values −44 and −88, indicating partial decarboxylation of γ-carboxyglutamic acid, typically observed for conotoxins containing this residue. The second of the two putative γ-carboxyglutamic acid residues did not readily decarboxylate in MS, even at elevated voltage settings (data not shown). Heat treatments (65 °C for 1 h prior to LC/MS, or LC/MS at 65 °C) predictably resulted in increased γ-carboxyglutamic acid decarboxylation, with decarboxylated VxXIIB eluting earlier than the fully carboxylated form. Tandem MS of an NH_2-terminal pepsin-cleaved fragment 1-23 (mass 3121), also showed a loss of 88 Da, again consistent with the presence of two γ-carboxyglutamic acid residues in the sequence.

VxXIIC was also purified to a sharp single peak by RP-HPLC analysis and LC/MS analysis showed little evidence of heterogeneity, except for a minor form with a mass consistent with either a proline/hydroxyproline substitution or methionine oxidation. The major form had a mass of ~10,553 Da. Chromatography of VxXIIC after reduction revealed one dominant

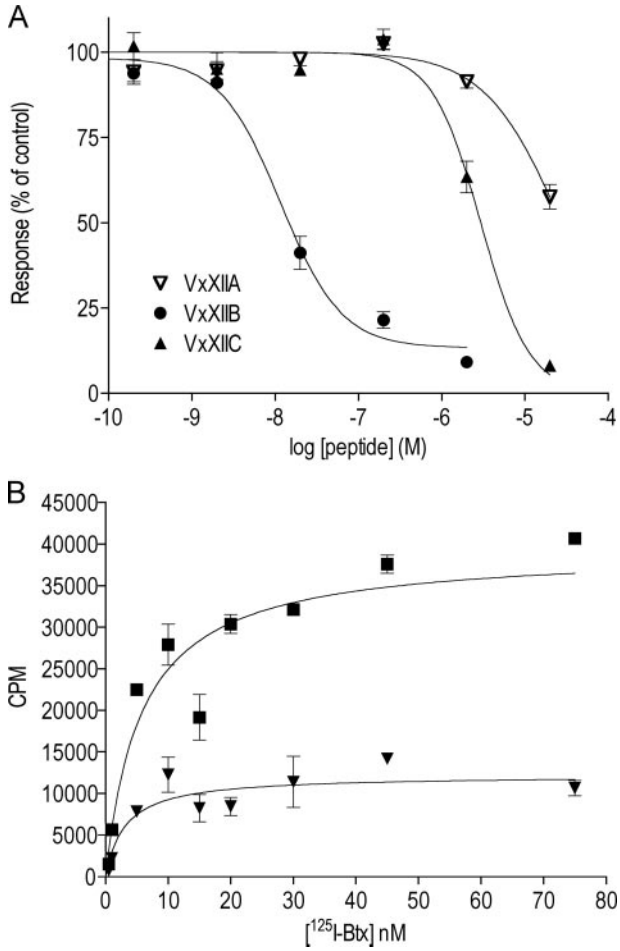

FIGURE 3. **Native VxXIIA, VxXIIB, and VxXIIC binding to AChBP.** *A*, displacement of ^{125}I-BgTx from AChBP by VxXIIA, VxXIIB, and VxXIIC ($n = 6$ for each data point). *B*, saturation binding of ^{125}I-BgTx to AChBP in the absence (■; K_d 5.8 ± 1.6 nM, B_{max} 39,300 ± 2,800 cpm) or presence of VxXIIB (22 nM) (▼; K_d 3.2 ± 1.5 nM, B_{max} 12,200 ± 1,200 cpm), after correction for nonspecific binding, $n = 2$–3 for each data point.

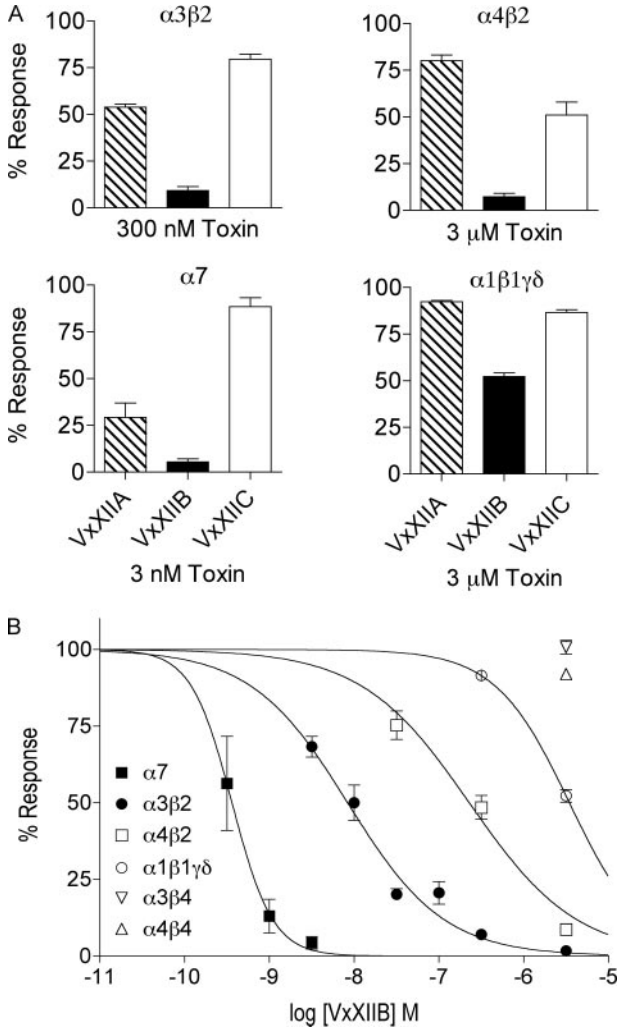

FIGURE 4. **Two-electrode voltage clamp analysis of the selectivity and potency of native VxXIIA, VxXIIB, and VxXIIC at oocyte-expressed rat nAChR subunit combinations.** *Xenopus* oocytes were clamped at −70 mV and responses to 2-s pulses of 100 μM ACh were recorded after a 3-min preincubation with the indicated toxin. *A*, comparison of the activity of VxXIIA, VxXIIB, and VxXIIC at α3β2 (300 nM peptide), α7 (3 nM peptide), α4β2 (3 μM peptide) nAChRs, and muscle (3 μM peptide) nAChR subunit combinations. *B*, concentration-response analyses for VxXIIB at oocyte-expressed nAChR subunit combinations. IC_{50} values and Hill slopes are given in Table 2. Each mean represents the average of measurements from at least three different oocytes. *Error bars* represent S.E.

peak in contrast to the two peaks observed for VxXIIA. The observed monoisotopic mass of the major form of reduced VxXIIC was 5,282 Da. The sequence of VxXIIC (Table 1) was determined largely from MS/MS of fragments generated from trypsin and pepsin digests, in addition to the partial NH_2-terminal sequence obtained using Edman chemistry data. A minor variant [Hyp^{10}]VxXIIC was determined by MS/MS. The observed apex mass values 10,553, 10,569.4, and 10,585.4 Da corresponded to the predicted average mass values (assuming an additional hydroxyproline substitution in one subunit variant) of 10,552.18, 10,568.18, and 10,584.18 Da, respectively.

The αD-conotoxins showed marked increases in hydrophobicity after reduction, reflected by 4–9-min delays in elution time on RP-HPLC (data not shown). This was particularly apparent for VxXIIB and suggests that substantial shielding of hydrophobic residues occurs in the native toxins. Two-dimensional gel electrophoresis of VxXIIA showed that the protein had a pI of ~10 (data not shown) consistent with VxXIIA, VxXIIB, and VxXIIC containing five to seven basic residues (see Table 1). Interestingly, there was no MS evidence of heterodimers with mass values corresponding to mixtures of VxXIIA and VxXIIB, VxXIIA, and VxXIIC, or VxXIIB and VxXIIC.

Activity Assay of Crude Venom—C. vexillum crude venom was screened for its ability to inhibit agonist-evoked currents of different combinations of rat neuronal nAChR subunits (α3β2, α3β4, α4β2, α4β4, and α7) heterologously expressed in *Xenopus* oocytes. Crude venom (50 μg of protein/ml) caused 100% block of both the α3β2 and α7 nAChR subtypes, and 69% block of the α4β2 subtype (data not shown). No significant activity was detected at the α4β4 or α3β4 subtypes. Assay at the α3β2 nAChR of fractions generated by size-exclusion chromatography of the crude venom indicated the presence of active components in the 5–15-kDa fraction (data not shown). Surprisingly, no activity was detected in the small peptide fraction where α-conotoxins typically elute. The other species investi-

gated in this study showed a similar activity profile (see "Experimental Procedures").

Characterization of Activity of Purified Proteins—Analysis of the inhibition of ^{125}I-BgTx binding to AChBP by VxXII proteins indicated a rank order of potency of B > C > A with IC_{50} values of 27 μM for VxXIIA (Hill slope −0.94), 11 nM (Hill slope of −1.19) for VxXIIB, and 3 μM for VxXIIC (Hill slope −1.32) (Fig. 3*A*). Saturation binding experiments with ^{125}I-BgTx in the presence of 22 nM VxXIIB revealed a significant, non-surmountable reduction of ^{125}I-BgTx binding to AChBP by VxXIIB (Fig. 3*B*).

TABLE 2

IC_{50} values (nM), 95% confidence intervals (CI), and Hill slopes (n_H) for inhibition of agonist-evoked current by VxXIIB at mammalian nAChR subunit combinations expressed in *Xenopus* oocytes

nAChR	IC_{50} (95% CI)	n_H
α7	0.4 (0.2–0.6)	−1.80
α3β2	8.4 (6.6–10.6)	−0.76
α4β2	228 (144–360)	−0.68
Muscle	3460 (2800–4300)	−0.99

To establish nAChR subtype selectivity, VxXIIA, -B, and -C were tested for inhibition of agonist-evoked responses on four oocyte-expressed receptor subunit combinations (Fig. 4*A*). All three αD-conotoxins showed selectivity for α7 and α3β2 nAChRs, with VxXIIB being the most potent toxin on all subunit combinations. The rank orders of potency were B > A > C at α7 and α3β2 subtypes and B > C > A at α4β2 and muscle subtypes.

For a more detailed functional characterization of the subtype selectivity of VxXIIB, concentration-response relationships were determined at oocyte-expressed α3β2, α7, α4β2, and muscle subunit combinations (Table 2, Fig. 4*B*). VxXIIB had low nanomolar potency at α3β2 nAChRs (IC_{50} 8.4 nM) and α7 receptors (IC_{50} 0.4 nM), modest affinity at the α4β2 (IC_{50} 0.23 μM) and muscle subtypes (IC_{50} 3.5 μM), and little activity at β4 containing receptors at concentrations up to 3 μM (Table 2, Fig. 4*B*). The concentration-response analysis of VxXIIA at α3β2 nAChRs gave an IC_{50} of 370 nM. To start to identify where these ~11-kDa proteins acted on the nAChR, the activity of VxXIIB was investigated at two mutated nAChRs, α3β2 L119Q and α3β2 F117A, which affect small α-conotoxin binding (37). VxXIIB off-rates were slower at the mutant nAChR α3β2 L119Q compared with α3β2 nAChRs (data not shown).

αD-conotoxins are stable for at least several months when stored dry or as a 30% ACN, 0.05% trifluoroacetic acid stock. However, some dilute aqueous solutions had reduced stability and therefore toxin dilutions were freshly prepared prior to testing.

NMR Analysis—The NOESY spectra of VxXIIA showed well dispersed NH protons allowing relatively straightforward assignment of the protein. The Hα chemical shifts indicated β-sheet formation across residues 14–26, with the rest of the protein approaching a random coil structure (Fig. 5*A*). Slowly exchanging amides were located mainly in the 14–26-residue region, with Trp[14], Arg[16], Cys[17], Leu[19], Asp[20], Arg[21], Cys[23], Met[26] as well as Gln[6],

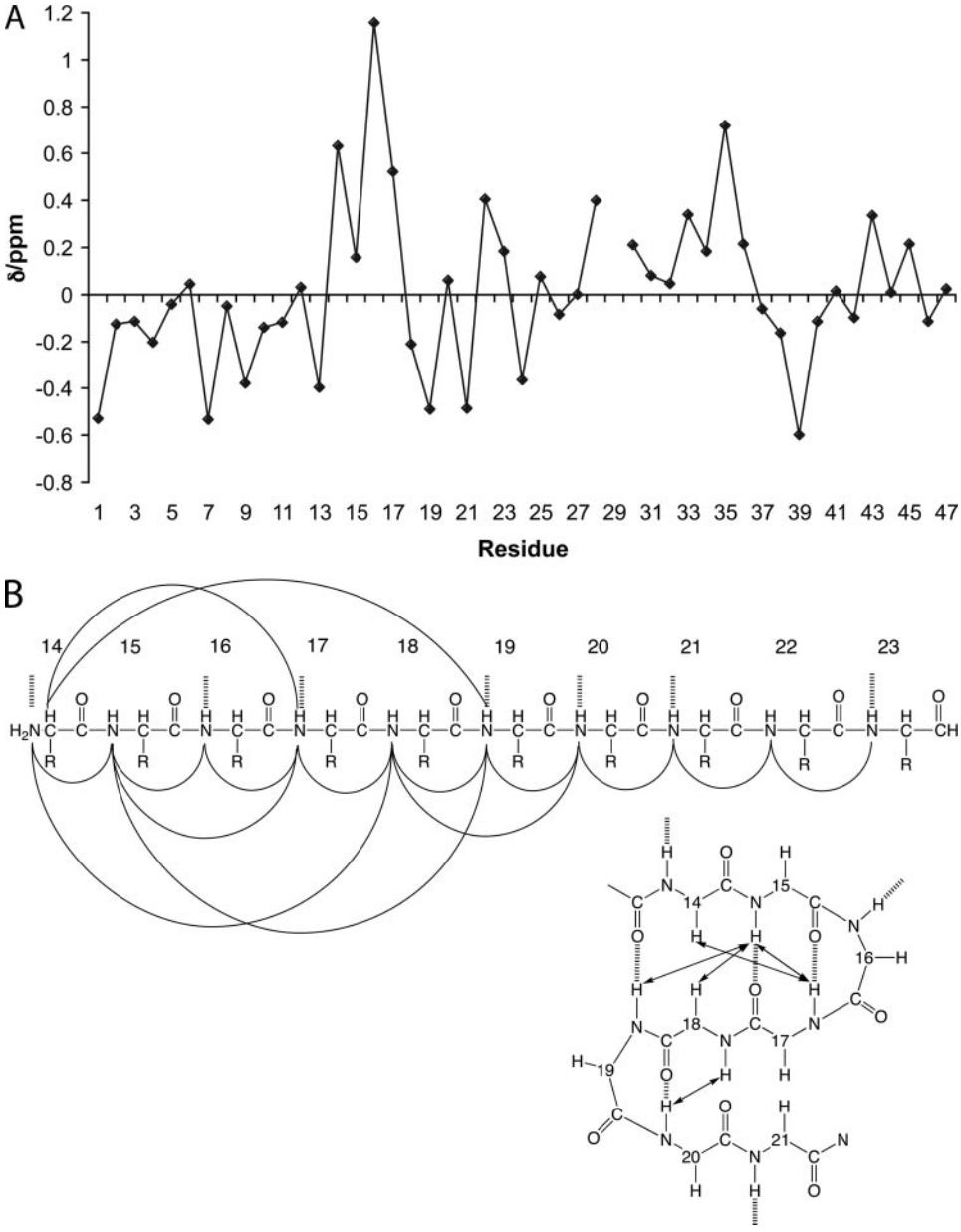

FIGURE 5. **NMR studies of VxXIIA.** *A*, secondary Hα chemical shift data for VxXIIA compared with random coil chemical shift values. *B*, the β-sheet network of VxXIIA is shown with *double-headed arrows* represent the observed NOEs, and *dashed lines* represent H-bonds.

TABLE 3
Sequence comparisons with examples of five classes of conotoxins that act at nAChRs or the $5HT_3$ receptors (GVIIIA)

Peptide and family[a]	Cystine residues	Mass	Superfamily	Sequence and cystine framework	Antagonism	Ref.
		Da				
α-GID	4	2184	A	IRDγ**CC**SNPA**C**RVNNOHV**C**[b]	Competitive	15
αA-EIVA	6	3094	A	G**CC**GPYONAA**C**HO**C**G**C**KVGROOY**C**DROSGG[c]	Competitive	68
ψ-PIIIF	6	2664	M	GOO**CC**LYGS**C**ROFOG**C**YNAL**CC**RK[c]	Non-competitive	69
αS-RVIIIA	10	5167	S	K**C**NFDK**C**KGTGVYN**C**Gγ**S**C**S**C**γ**GLHS**C**R**C**TYNIGSMKSG**C**A **C**I**C**TYY[b]	Undefined	9
α-GVIIIA	10	4189	S	G**C**TRT**C**GGOK**C**TGT**C**T**C**TNSSK**C**G**C**RYNVHPSGBG**C**G**C**A**C**S[c]	$5HT_3$	70
αD-VxXIIA	10	5134	D	DVQD**C**QVSTOGSKWGR**CC**LNRV**C**GPM**CC**PASH**C**Y**C**VYHR GRGHG**C**S**C**[b]	Non-competitive	This study

[a] Names include framework identifiers: I/II for α-conotoxins, IV for αA-conotoxins, VIII for αS-conotoxins, and II for ψ-conotoxins. The conotoxins GID, EIVA, PIIIF, RVIIIA, GVIIIA, and VxXIIA (reduced form) are from *C. geographus, C. ermineus, C. purpurascens, C. radiatus, C. geographus,* and *C. vexillum*, respectively.
[b] Free carboxyl terminus (COOH), γ,γ-carboxyglutamic acid (Gla), *O*-hydroxyproline (Hyp), and B, Br-Trp.
[c] Amidated COOH terminus (NH_2).

Tyr[34], and Ser[46] were present after 24 h, presumably as a result of stabilizing H-bond interactions. Gly[15] exchanged at an intermediate rate and disappeared after 4.5 h. An antiparallel β-sheet structure comprising residues 13–20 (Fig. 5*B*) was proposed after comparison of shielded amide protons with long range NOEs. NMR analysis of VxXIIB was unproductive with extensive heterogeneity being evident. The NMR spectrum from VxXIIC was well dispersed and further NMR studies are in progress.

DISCUSSION

In the search for new inhibitors of nAChRs, we isolated and characterized three novel post-translationally modified conotoxins, VxXIIA, VxXIIB, and VxXIIC, from the venom of the vermivorous cone snail *C. vexillum*. The toxins were found to be novel, subtype-specific antagonists of the nAChR. Sequences of the reduced peptides were obtained using Edman chemistry combined with ESI-MS and tandem MS of both intact proteins and proteolysis fragments. VxXIIA, VxXIIB, and VxXIIC naturally occur as ~11-kDa pseudo-homodimers of paired 47–50-residue peptides (see Table 1). The peptide monomers have a novel arrangement of 10 cystine residues that defines a new conotoxin superfamily. These toxins contain one or two post-translationally modified amino acids, hydroxyproline and γ-carboxyglutamic acid, which confer additional heterogeneity. Comparison of VxXIIA-C sequences reveals conserved WGRCC and CSC motifs, which might be important in the biological activity and/or the structure of these proteins, but little homology to previously described conotoxins. Thus, the VxXII peptides represent a novel class of conotoxins with an unusual structure.

Native VxXIIA-C are unusually large conotoxins. Proteins from cone snail venom include some incompletely characterized larger toxins and a number of enzymes and proteases ranging in size from 13 to 130 kDa (7, 17, 38–44). However, the majority of conotoxins identified so far are 1–4 kDa (45). A small number of conotoxins of >40 residues (~4.4 kDa) have been described (see Ref. 9). These peptides contain 8 or 10 cystine residues and mostly belong to the I or S superfamilies. In contrast to the typically small conotoxins, the toxins from venoms of other animal genera are generally larger, including the 3–8-kDa spider toxins (46), the 4–9-kDa ant venom toxins (18), and the 6–9-kDa three-finger snake toxins (3).

VxXIIA-C resemble σ-GVIIIA and αS-RVIIIA in having 10 cystine residues, but their sequences and cystine spacing are substantially different (see Table 3). Like αS-RVIIIA, VxXIIB has undergone a post-translational modification from a glutamate to γ-carboxyglutamic acid at two positions. VxXIIA and a [Hyp[29]]VxXIIA occur in a 1:1 ratio, which gives rise to a distinctive pattern of heterogeneity of the dimers. VxXIIB and VxXIIC also have variants with proline to hydroxyproline substitutions for at least one site, but only as minor forms. These toxins are antagonists of ligand-gated ion channels as is αS-RVIIIA. Teichert *et al.* (9) have proposed that S-superfamily conotoxins predominantly target ligand-gated ion channels and that some piscivores rely on αS-conotoxin muscle nAChR antagonists. The two S-superfamily representatives described to date, σ-GVIIIA and αS-RVIIIA, are from fish-hunting species, whereas *C. vexillum* is a worm hunter. The most widespread peptide families targeting the nAChR, the α- and αA-conotoxins, belong to the A-superfamily. Because we detected no α-, αA-, αS-, and ψ-conotoxins in the venom of *C. vexillum*, it appears that this species utilizes a new superfamily of toxins to target the nAChR. Given these are dimeric toxins, we proposed that the superfamily be named the αD superfamily.

The protein dimers from *C. vexillum* remained intact in high voltage MS, size exclusion chromatography and SDS-PAGE. Their stability was further emphasized by their resistance to denaturating treatments and RP-HPLC at 65 °C. Exposure to reducing conditions separated the dimer components, indicating that disulfide bonds were necessary for the dimer structure of all three toxins. In contrast to work by Kashiwagi *et al.* (47) showing that heterodimeric yeast killer toxin SMKT subunits were non-covalently associated under acidic conditions but dissociated under neutral and basic conditions, pH-dependent dimer denaturation was not observed for the VxXII peptides. The Vx subunit combinations were discrete with no evidence of heterodimers formed from intermixes of VxXIIA, VxXIIB, and VxXIIC, indicating sequence-specific dimer formation.

The NMR analysis of VxXIIA revealed 47 resonances, in alignment with the peptide sequence data and consistent with the 11-kDa protein occurring as a symmetrical homodimer. Analysis of the Hα chemical shifts, NOEs, and slowly exchanging protons indicated that the structure contained an antiparallel β-sheet across residues 13–20, whereas the remainder of the protein had less well defined structure. Some residues identified as shielded from the solvent though H-bonds (Trp[14],

Arg[16], Cys[17], and Ser[46]) are in the conserved WGRCC and CSC motifs and may thus make an important contribution to the structure of these toxins.

Dimers have also been described from among the snake venom κ-neurotoxins, β-defensins, and defensin-related peptides. Defensin-like structural folds and antiparallel β-sheets are contained in some toxins from platypus and scorpion venoms (48, 49). Features such as hydrophobic interactions and characteristic β-sheet hydrogen bonds have been suggested to play an important role in formation and stability of these dimers (49–53). Similarly, features of the αD-conotoxins such as the β-sheet identified in VxXIIA, and conserved motifs, might be expected to contribute to dimer formation in these proteins. However, the specific residues involved in dimer formation, through either noncovalent and/or covalent interactions, have not yet been identified.

AChBPs are homologous to the extracellular ligand-binding domain of the nAChR, especially the α7 nAChR. These proteins provide useful structural templates for modeling the ligand-binding domain of mammalian nAChRs (54–57). Recent AChBP co-crystallization studies have provided a detailed insight into the molecular basis of ligand binding (58–60). Here we tested the potency and selectivity of αD-conotoxins in a binding assay using recombinant AChBP from *Lymnea stagnalis* and by two-electrode voltage clamp analysis at different oocyte-expressed mammalian nAChR subunit combinations. Both approaches revealed that αD-conotoxins, especially VxXIIB, are potent antagonists at nAChRs. Purified VxXIIA, VxXIIB, and VxXIIC had substantial activity at α7- and β2-containing nAChRs, modest activity at the muscle subtype, and little activity at β4-containing receptors. The rank order of potency of VxXIIA, -B, and -C differed for α7 and α3β2 subtypes (B > A > C) and AChBP (B > C > A).

A saturation binding study suggested that VxXIIB inhibited ^{125}I-α-BgTx to AChBP in a non-competitive manner, indicating their binding sites on AChBP do not overlap. Initial studies investigating αD-conotoxins interactions at mammalian nAChRs also suggests they interact with nAChRs in a different manner to the α-conotoxins. The slow recovery of the α3β2 nAChR from the VxXIIB block was enhanced when ACh pulses were increased from 2 to 30-s durations, whereas the prolonged presence of ACh did not influence the recovery of block from α-conotoxin MII.[5] These results suggest that ACh and VxXIIB binding sites do not overlap and that both can bind simultaneously to the nAChR. Substitution of β2 subunit residues located in the cleft has been shown to influence the binding of α-conotoxins MII, PnIA, and GID, with the β2-L119Q mutation strongly reducing affinity for all three α-conotoxins as evidenced by a reduction in potency and a fast recovery from α-conotoxin block (37). In contrast, this mutation slowed the recovery from VxXIIB block. These results suggest that the binding sites of α-conotoxins and αD-conotoxins partially overlap but that αD-conotoxin binding does not extend into the ACh binding pocket. Interestingly, it appears that nAChR inhibitors acting in the cleft do not need to act competitively with ACh, perhaps because they stabilize the cleft in a conformation that does not favor agonist binding.

The VxXIIA, -B, and -C are the first described members of a new family of conotoxins that target nAChRs. Phylogenetic analysis places *C. vexillum* in cone snail Clade XII (11, 12, 61) with *C. mustelinus*, *C. miles*, and *C. capitaneus*, which also possess αD-conotoxins with masses of ~11 kDa. The αD-conotoxins were dominant components in the venom of *C. vexillum*. Given their abundance in crude venom and variation across species, the αD-conotoxins appear to have evolved as an alternative strategy for prey capture for species lacking α-, αA-, αS-, and ψ-conotoxins. Whereas chemical synthesis of these proteins is expected to be challenging, recombinant production of other dimeric toxins of comparable size has been achieved (49, 62–67). Co-crystallization studies with AChBP are expected to more precisely define the structure of αD-conotoxins and to establish the molecular basis of their binding interaction at the nAChR.

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Identification of a Novel Class of Nicotinic Receptor Antagonists: DIMERIC CONOTOXINS VxXIIA, VxXIIB, and VxXIIC FROM CONUS VEXILLUM

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