Identification of a Novel Class of Nicotinic Receptor Antagonists

DIMERIC CONOTOXINS VxXIIA, VxXIIIB, and VxXIIIC FROM CONUS VEXILLUM

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-VxXIIIB, and αD-VxXIIIC, 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-VxXIIIB, and αD-VxXIIIC 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) 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, αА-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/β 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 hydroxylation mass spectrometry; TOCSY, total correlation spectroscopy.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 34, pp. 24745–24755, August 25, 2006
© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

M ARION LOUGHNAN,* ANNETTE NICKE,‡ ALUN JONES,‡ CHRISTINA I. SCHROEDER, SIMON T. NEVIN,§ DAVID J. ADAMS,* PAUL F. ALEWOOD,* AND RICHARD J. LEWIS,*‡

From the Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia, the School of Biomedical Sciences, University of Queensland, Brisbane, Queensland 4072, Australia, and the Max Planck Institute for Brain Research, Deutschordenstrasse 46, 60528 Frankfurt/Main, Germany

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; NCE/MS, tandem mass spectrometry; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reversed-phase high performance liquid chromatography; TOCSY, total correlation spectroscopy.

* This work was supported in part by Australian Research Council Discovery Grant DP0208295. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by an Australian Postgraduate Research Scholarship.

‡ Supported by Research Fellowship NI 592/2-1 of the Deutsche Forschungsgemeinschaft. Present address: Max-Planck Institute for Brain Research, Deutschordenstrasse 46, 60528 Frankfurt/Main, Germany.

§ To whom correspondence should be addressed. Tel.: 61-7-3346-2984; Fax: 61-7-3346-2101; E-mail: r.lewis@imb.uq.edu.au.

¶ 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; NCE/MS, tandem mass spectrometry; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reversed-phase high performance liquid chromatography; TOCSY, total correlation spectroscopy.

This article has been refereed.
Novel 11-kDa αD-conotoxins

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, VxXIIIB, and VxXIIIC, 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 capitanus (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. 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 −30 °C.

Crude Venom Enrichment—Specimens of C. vexillum (7), Conus capitaneus (17), and C. mustelinus (7) were further purified by anion-exchange chromatography (Superdex Peptide, HR 10/30, Amersham Biosciences) to enrich for peptides and proteins in the size range 5–15 kDa. An Agilent 1100 Series HPLC system was used, with a Superdex Peptide, HR 10/30, Amersham Biosciences column eluted with 0.1–90% 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 was performed using the software package Bioanalytist (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 Bioanalytist 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 acid 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 a solution containing 50 mM formic acid and 50 mM acetic acid to which 2 μl of a 1 mg/ml stock solution of trypsin was added, and the reaction mixture was 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 CaCl2 (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.

**Sequence by Edman Chemistry**—Edman NH₂-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 Precise Protein Sequencer (HT or 492C LC 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 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 VxXIIIB and VxXIIIC.

**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% H₂O, 10% D₂O (pH 3.0), at temperatures ranging from 280 to 313 K and in 100% D₂O (298 K).

**H 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% D₂O (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 D₂O 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 T₂ 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).
Novel 11-kDa αD-conotoxins

 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 α7 and α3β2 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 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).
Novel 11-kDa αD-conotoxins

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 NH₂-terminal sequences of several ~11-kDa nAChR antagonists from C. mustelinus, C. miles, and C. capitaneus gave a consensus sequence DXXXXQQXTGO-SKWRCC (X indicates sequence variation) that corresponded to the NH₂ 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. 2A) gave rise to two closely eluting peaks present in a 1:1 ratio (Fig. 2B). The ion spectrum (m/z) for the first eluting peak of reduced VxXIIA had a monoisotopic 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. 2A, inset). These data are
Novel 11-kDa αD-conotoxins

TABLE 1
Comparison of deduced sequences for C. vexillum components VxXIIA, VxXIIB, and VxXIIC

| Name       | Sequence | Cysteine framework and conserved residues | Monomer (reduced) | Dimer (intact) |
|------------|----------|------------------------------------------|-------------------|----------------|
| VxXIIA     | DVQD—CQVSTQGSKGRCCINRVCNHPCCPAHRYCVYHRGRFHCSC# | 10|20|30|40|50 | Observed Mass | Predicted Mass | Observed Mass | Predicted Mass |
| VxXIIB     | DDJSJCIINTRDPWGRCCRTNCGCMNCNPAPGCTCVYHRGRFHCSCPG# | 10|20|30|40|50 | 5741.4 | 5741.26 | 11472 | 11470.88 |
| VxXIIC     | DLRQ—CTRNAPGSTWGRCCINPHCMFPCCPRSGCTCAAYRHRGHCSC# | 10|20|30|40|50 | 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, [Hyp29]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 [Hyp29,Hyp29]VxXIIA and [Pro10]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 [Hyp29]VxXIIA, and a homodimer of [Hyp29]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-Gly88]VxXIIB variant with a likely amidated COOH terminus was identified by MS/MS. MS of NH2-terminal peptic fragments of VxXIIB indicated the presence of a further minor variant [Hyp14]VxXIIB (data not shown). These variants were consistent with mass data from the native VxXIIB sample, with observed mass values consistent with hydroxyproline/proline substitution or methionine oxidation. The major form had a mass 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 NH2-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 NH2-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
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 NH2-terminal sequence obtained using Edman chemistry data. A minor variant [Hyp10]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 nAChRs, and muscle (3 μM peptide) nAChR subunit combinations. B, concentration-response analyses for VxXIIB at oocyte-expressed nAChR subunit combinations. IC50 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.
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 $\text{B} > \text{C} > \text{A}$ with IC$_{50}$ values of 27 $\mu$m for VxXIIA (Hill slope $-0.94$), 11 $\mu$m (Hill slope of $-1.19$) for VxXIIB, and 3 $\mu$m for VxXIIC (Hill slope $-1.32$) (Fig. 3A). Saturation binding experiments with $^{125}$I-BgTx in the presence of 22 nm VxXIIB revealed a significant, non-saturable reduction of $^{125}$I-BgTx binding to AChBP by VxXIIB (Fig. 3B).

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. 4A). All three $\alpha$D-conotoxins showed selectivity for $\alpha_7$ and $\alpha_3\beta_2$ nAChRs, with VxXIIB being the most potent toxin on all subunit combinations. The rank orders of potency were $\text{B} > \text{A} > \text{C}$ at $\alpha_7$ and $\alpha_3\beta_2$ subtypes and $\text{B} > \text{C} > \text{A}$ at $\alpha_4\beta_2$ and muscle subtypes.

For a more detailed functional characterization of the subtype selectivity of VxXIIB, concentration-response relationships were determined at oocyte-expressed $\alpha_3\beta_2$, $\alpha_7$, $\alpha_4\beta_2$, and muscle subunit combinations (Table 2, Fig. 4B). VxXIIB had low nanomolar potency at $\alpha_3\beta_2$ nAChRs (IC$_{50}$ 8.4 nm) and $\alpha_7$ receptors (IC$_{50}$ 0.4 nm), modest affinity at the $\alpha_4\beta_2$ (IC$_{50}$ 0.23 $\mu$m) and muscle subtypes (IC$_{50}$ 3.5 $\mu$m), and little activity at $\beta 4$ containing receptors at concentrations up to 3 $\mu$m (Table 2, Fig. 4B). The concentration-response analysis of VxXIIA at $\alpha_3\beta_2$ nAChRs gave an IC$_{50}$ of 370 nm. To start to identify where these $\sim 11$-kDa proteins acted on the nAChR, the activity of VxXIIB was investigated at two mutated nAChRs, $\alpha_3\beta_2$ L119Q and $\alpha_3\beta_2$ F117A, which affect small $\alpha$-conotoxin binding (37). VxXIIB off-rates were slower at the mutant nAChR $\alpha_3\beta_2$ L119Q compared with $\alpha_3\beta_2$ nAChRs (data not shown).

$\alpha$D-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 $\delta$ chemical shifts indicated $\beta$-sheet formation across residues 14–26, with the rest of the protein approaching a random coil structure (Fig. 5A). 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 Glu$^{6}$.
TABLE 3
Sequence comparisons with examples of five classes of conotoxins that act at nAChRs or the 5HT3 receptors (GVIIIA)

| Peptide and family | Cystine residues | Mass | Superfamily | Sequence and cystine framework | Antagonism | Ref. |
|-------------------|-----------------|------|-------------|--------------------------------|------------|-----|
| α-GID             | 4               | 2184 | A           | IREDyyCCSNPACVRVGNHVW⁴         | Competitive| 15  |
| αA-EIVA           | 6               | 3094 | A           | GCCGPONAACHEOCCVRGROYCDROSG⁵   | Competitive| 68  |
| ψ-PIIF            | 6               | 2664 | M           | GGGCCLYGSCEPOCGCNALCRR⁹        | Non-competitive| 69 |
| αS-RVIIIA         | 10              | 5167 | S           | KCHDFKCGTVQNGYSGCGYLSHCRTCYNMGKSG⁶ | Undefined| 9   |
| α-GVIIIA          | 10              | 4189 | S           | GCTRTQCCGOKCTTGCTCTNSEEKCCCRVNYHPSGBCDGCA⁷ | 5HT₃ | 70  |
| αD-VxXIIIA        | 10              | 5134 | D           | DVQDVQSTOGKQGCRQCGQPEASGCYQVRHRGRQGGSCE⁸ | Non-competitive| This study |

⁴ 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, PIIF, RVIIIA, GVIIIA, and VxXIIIA (reduced form) are from C. geographus, C. ermineus, C. purpurascens, C. radiatus, C. geographus, and C. vexillum, respectively.
⁵ Free carboxyl terminus (COOH), γ-carboxyglutamic acid (Gla), O-hydroxyproline (Hyp), and B, Br-Trp.
⁶ Amidated COOH terminus (NH₃).

Novel 11-kDa αD-conotoxins

Tyr³⁴, and Ser⁴⁶ were present after 24 h, presumably as a result of stabilizing H-bond interactions. Gly¹⁵ exchanged at an intermediate rate and disappeared after 4.5 h. An antiparallel β-sheet structure comprising residues 13–20 (Fig. 5B) was proposed after comparison of shielded amide protons with long range NOEs. NMR analysis of VxXIIIB was unproductive with extensive heterogeneity being evident. The NMR spectrum from VxXIIIC 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, VxXIIIB, and VxXIIIC, from the venom of the vermiculous 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, VxXIIIB, and VxXIIIC 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 venom 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 α-GVIIIA and αS-RVIIIA in having 10 cystine residues, but their sequences and cystine spacing are substantially different (see Table 3). Like αS-RVIIIA, VxXIIIB has undergone a post-translational modification from a glutamate to γ-carboxyglutamic acid at two positions. VxXIIIA and a [Hyp²⁸]VxXIIIA occur in a 1:1 ratio, which gives rise to a distinctive pattern of heterogeneity of the dimers. VxXIIIB and VxXIIIC 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, α-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 denaturing 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, VxXIIIB, and VxXIIIC, 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 (Trp¹⁴,

AUGUST 25, 2006•VOLUME 281•NUMBER 34 JOURNAL OF BIOLOGICAL CHEMISTRY 24753
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 \kappa-neurotoxins, \beta-defensins, and defensin-related peptides. Defensin-like structural folds and antiparallel \beta-sheets are contained in some toxins from platypus and scorpion venoms (48, 49). Features such as hydrophobic interactions and characteristic \beta-sheet hydrogen bonds have been suggested to play an important role in formation and stability of these dimers (49–53). Similarly, features of the \alphaD-conotoxins such as the \beta-sheet identified in VxXIIB, 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 \alpha7 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 \alphaD-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 \alphaD-conotoxins, especially VxXIIB, are potent antagonists at nAChRs. Purified VxXIIB, VxXIIB, and Vx XIIIC had substantial activity at \alpha7- and \beta2-containing nAChRs, modest activity at the muscle subtype, and little activity at \beta4-containing receptors. The rank order of potency of VxXIIB, -B, and -C differed for \alpha7 and \alpha3/\beta2 subtypes (B > A > C) and AChBP (B > C > A).

A saturation binding study suggested that VxXIIB inhibited $^{125}$I-\alpha-BgTx to AChBP in a non-competitive manner, indicating their binding sites on AChBP do not overlap. Initial studies investigating \alphaD-conotoxins interactions at mammalian nAChRs also suggests they interact with nAChRs in a different manner to the \alpha-conotoxins. The slow recovery of the \alpha3/\beta2 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 \alpha-conotoxin MII. These results suggest that ACh and VxXIIB binding sites do not overlap and that both can bind simultaneously to the nAChR. Substitution of \beta2 subunit residues located in the cleft has been shown to influence the binding of \alpha-Conotoxins MII, PnIA, and GID, with the \beta2-LI19Q mutation strongly reducing affinity for all three \alpha-conotoxins as evidenced by a reduction in potency and a fast recovery from \alpha-conotoxin block (37). In contrast, this mutation slowed the recovery from VxXIIB block. These results suggest that the binding sites of \alpha-conotoxins and \alphaD-conotoxins partially overlap but that \alphaD-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 VxXIIB, -B, and -C are the first described members of a new family of conotoxins that target nAChRs. Phylogenetic analysis places C. veixillum in cone snail Clade XII (11, 12, 61) with C. mustelinus, C. miles, and C. capitaneus, which also possess \alphaD-conotoxins with masses of ~11 kDa. The \alphaD-conotoxins were dominant components in the venom of C. veixillum. Given their abundance in crude venom and variation across species, the \alphaD-conotoxins appear to have evolved as an alternative strategy for prey capture for species lacking \alpha-, \alphaA-, \alphaS-, and \psi-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 \alphaD-conotoxins and to establish the molecular basis of their binding interaction at the nAChR.

Acknowledgments—We thank John Holland for advice concerning two-dimensional gel electrophoresis, Sebastien Dutertre for assistance with the AChBP binding assays, and Roger Pearson (CSIRO Livestock Industries) for partial sequence analysis of peptides from C. muselii, C. miles, and C. capitaneus.

REFERENCES

1. Nicke, A., Wonnacott, S., and Lewis, R. J. (2004) Eur. J. Biochem. 271, 2305–2319
2. Lewis, R. J., and Garcia, M. L. (2003) Nat. Rev. Drug Discovery 2, 790–802
3. Nirthanan, S., and Gwue, M. C. (2004) J. Pharmacol. Sci. 94, 1–17
4. Grant, G. A. (1998) J. Toxicol. Toxins Res. 17, 239–260
5. Gawade, S. P. (2004) J. Toxicol. Toxins Res. 23, 37–96
6. Lewis, R. J. (2004) IIJMBM Life 56, 89–93
7. Terlau, H., and Olivera, B. M. (2004) Physiol. Rev. 84, 41–68
8. Arias, H. R., and Blanton, M. P. (2000) Int. J. Biochem. Cell Biol. 32, 1017–1028
9. Teichert, R. W., Jimenez, E. C., and Olivera, B. M. (2005) Biochemistry 44, 7897–7902
10. Teichert, R. W., Rivier, J., Dykert, J., Cervini, L., Gulyas, J., Bulaj, G., Ellison, M., and Olivera, B. M. (2004) Toxicon 44, 207–214
11. Espiritu, D. J., Watkins, M., Dia-Monje, V., Cartier, G. E., Cruz, L. J., and Olivera, B. M. (2001) Toxicon 39, 1899–1916
12. Olivera, B. M. (2002) Annu. Rev. Ecol. Syst. 33, 25–47
13. Craig, A. G., Bandypadhyay, P., and Olivera, B. M. (1999) Eur. J. Biochem. 264, 271–275
14. Loughnan, M. L., and Alewood, P. F. (2004) Eur. J. Biochem. 271, 2294–2304
15. Nicke, A., Loughnan, M., Millard, E., Alewood, P., Adams, D., Daly, N., Craik, D., and Lewis, R. (2003) J. Biol. Chem. 278, 3137–3144
16. Loughnan, M. L., Bond, T., Atkins, J., Ads, D. J., Broxton, N. M., Livett, B. G., Down, J. G., Jones, A. Alewood, P. F., and Lewis, R. J. (1998) J. Biol. Chem. 273, 15667–15674
17. McIntosh, J. M., Ghomashchi, F., Gelb, M. H., Dooley, D. L., Stoehr, S. L., Giordani, A. B., Naisbit, S. R., and Olivera, B. M. (1995) J. Biol. Chem. 270, 3518–3526
18. Davies, N. W., Wiese, M. D., and Browne, S. G. A. (2004) Toxicon 43, 173–183
19. Santos, A. D., McIntosh, J. M., Hillyard, D. R., Cruz, L. J., and Olivera, B. M. (2004) J. Biol. Chem. 279, 17596–17606
20. Valdez-Cruz, N. A., Batista, C. V., and Possani, L. D. (2004) Eur. J. Biochem. 271, 1453–1464
21. Corona, M., Valdez-Cruz, N. A., Merino, E., Zúñiga, M., and Possani, L. D. (2001) Toxicon 39, 1893–1898
