Novel ω-Conotoxins from Conus catus Discriminate among Neuronal Calcium Channel Subtypes*

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ω-Conotoxins selective for N-type calcium channels are useful in the management of severe pain. In an attempt to expand the therapeutic potential of this class, four new ω-conotoxins (CVIA–D) have been discovered in the venom of the piscivorous cone snail, Conus catus, using assay-guided fractionation and gene cloning. Compared with other ω-conotoxins, CVIA has a novel loop 4 sequence and the highest selectivity for N-type over P/Q-type calcium channels in radioligand binding assays. CVIA–D also inhibited contractions of electrically stimulated rat vas deferens. In electrophysiological studies, ω-conotoxins CVIA and MVIIA had similar potencies to inhibit current through central (i.e. intrathecal) stimulated rat vas deferens. In electrophysiological studies, ω-conotoxins CVIA and MVIIA had similar potencies to inhibit current through central (α1B-d) and peripheral (α1B,b) splice variants of the rat N-type calcium channels when coexpressed with rat β₃ in Xenopus oocytes. However, the potency of CVID and MVIIA increased when α1B,d and α1B,b were expressed in the absence of rat β₃, an effect more pronounced for CVID at α1B,d (up to 540-fold) and least pronounced for MVIIA at α1B,d (3-fold). The novel selectivity of CVID may have therapeutic implications. 1H NMR studies reveal that CVID possesses a combination of unique structural features, including two hydrogen bonds that stabilize loop 2 and place loop 2 proximal to loop 4, creating a globular surface that is rigid and well defined.
purified by size exclusion HPLC (Superoxel HR10/30, Amersham Pharmacia Biotech) eluted with 30% acetonitrile, 0.1% trifluoroacetic acid at 0.5 ml/min. Active fractions were finally rechromatographed by analytical RP-HPLC (5 μM C18, 0.46 × 25 cm, Vydac) eluted at 1 ml/min with a linear gradient of 0–50% solvent B over 45 or 90 min. Peaks detected at 214 nm were collected and sequenced. Screening of the venom of other Australian cone snails, including the piscivorous Conus striatus, Conus tulipa, Conus geographus, and Conus magus, only identified sequences corresponding to known venom peptides.

Sequencing of Native Peptides—The purified peptides (~100 pmol) were reduced in the presence of TCEP, 50 mM ammonium acetate at pH 8.0 and then alkylated in the presence of maleimide (37 °C, 1 h). The alkylated peptides were purified by RP-HPLC, applied to a Biobrene-treated glass fiber filter, and analyzed by Edman chemistry using an Applied Biosystems, Inc., model 470A protein sequencer. Alkylation of peptides with maleimide allowed their cysteine residues to be observed as phenylthiohydantoin-Cys-maleimide doublets (diastereomers), and Ser was confirmed by the presence of dehydroserine. The high number of serine, threonine, and cysteine residues present in each conotoxin resulted in a relatively rapid decrease in the amino acid yields in successive cycles; however, complete sequences were obtained in each case. The low serie signals were accompanied by the presence of large dehydroserine peak.

Gene Isolation and Characterization—Ducts from two specimens of C. catus were emulsified, and poly(A)⁺-tailed mRNA was extracted using the QuickPrep mRNA purification system (Amersham Pharmacia Biotech). Strand-1 cDNA was 5' end-synthesized from the C. catus poly(A)⁺ mRNA templates using a Nor-d(T), bifunctional primer (5'-AACGTGAGAAATGGCGCCCGAGGATCA) (Amersham Pharmacia Biotech) extended with Superscript II reverse transcriptase (Life Technologies, Inc.). The resultant cDNA templates were used to manufacture double-stranded cDNA using a RNase H/DNA polymerase procedure (cDNA Timesaver system, Amersham Pharmacia Biotech). Marathon adaptors (CLONTECH) were added to the 5'-untranslated sequence (lowercase).

TABLE I

| Name      | Sequence | Selectivity | Ref.  |
|-----------|----------|-------------|-------|
| CVIA      | CKTGAGGACTGTSYDCTGGRS-R-GC* | N            | Present study |
| CVIB      | CKGGGAGCKTGYDCCTGCSSGRS-R-GC* | N            | Present study |
| CVIC      | CKGGGAGCYYDCCTGCSSRGRS-R-GC* | N            | Present study |
| CVID      | CKGGGAGCYYDCCTGCSSRGRS-R-GC* | N            | Present study |
| GVIA      | CKGGGAGCYYDCCTGCCGSGRRS-R-GC* | N            | Present study |
| GVIB      | CKGGGAGCYYDCCT GCCGSGRRS-R-GC* | N            | Present study |
| MVIA      | CKGGGAGCYYDCCTGCSSGRRS-R-GC* | N            | Present study |
| MVIC      | CKGGGAGCYYDCCTGCSSGRRS-R-GC* | N            | Present study |

*ω-Conotoxins from the venom of piscivorous C. catus (C), C. geographus (G), C. tulipa (T), C. magus (M), and C. striatus (S) are shown.

Selectivity determined from relative potencies to displace 125I-GVIA (N-type) and 125I-MVIIA (P/Q-type) binding to rat brain membrane.

Nonlinear regressions were fitted to each experiment (n = 3 data points per experiment) with Prism software (GraphPad, San Diego, CA).

**Rat Vas Deferens—**Male Wistar rats (250–350 g) were killed by an overdose of pentobarbital (50 mg/kg) and then decapitated. The vas deferens was dissected free of surrounding connective tissue. It was then placed in cold physiological saline solution (PSS) and washed with PSS to remove any blood. The tissue was then equilibrated at room temperature for 1 h.

### Ducts from two specimens of C. catus

| Channels | Sequence | Selectivity | Ref.  |
|----------|----------|-------------|-------|
| N/P/Q    |          |             |       |
| P/Q      |          |             |       |
| N/P      |          |             |       |
| N        |          |             |       |
| N        |          |             |       |
| N        |          |             |       |
| N/P/Q    |          |             |       |
| N/P      |          |             |       |

**Nonlinear regressions were fitted to each experiment (n = 3 data points per experiment) with Prism software (GraphPad, San Diego, CA).**

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cervical dislocation and exsanguinated. Prostatic halves of each vas deferens were mounted under 0.5 × g tension in 5-ml organ baths containing physiological solution containing the following (mM): 119 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.18 KH₂PO₄, 25.0 NaHCO₃, 5.5 glucose, 2.5 CaCl₂, 0.026 EDTA, at 37 °C and bubbled with 5% CO₂ in O₂ (pH 7.4). For the 4.5 min of electrical field stimulation every 3 min (single 55 V, 0.1-ms duration pulses generated by a Grass S44 stimulator) were applied via two platinum-stimulating electrodes straddling the tissue. Isometric contractions were measured using a force transducer (F-60, Narco Bio-System) and recorded digitally on a Power Macintosh computer using Chart version 3.5.6 sw software and a MacLab data acquisition system (ADInstruments, Australia) at a sampling frequency of 200 Hz. Evoked contractions were abolished by tetrodotoxin (0.1 μM), indicating that they were of neurogenic origin. Conotoxins were added cumulatively at 18-min intervals, with allowance made for a small (0.16%/min) deterioration of the evoked response. CVID effect on ATP (3 × 10⁻³ μM, Sigma) and norepinephrine (10⁻³ μM, Sigma) responses in the bisected epididymal portion of the rat vas deferens was also determined. Dose-response data (means ± S.E.; n = 5) were analyzed using Prism software.

Oocyte Injection and Recording—Oocytes were surgically removed from mature Xenopus laevis frogs anesthetized by submersion in 0.1% ethyl m-aminobenzoate (MS 222). Stage V–VI oocytes were prepared for injection by dissociation in Ca²⁺-free solution containing the following (mM): 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES (pH 7.4), plus 5 μg/ml gentamicin (pH 7.4), were injected with 25–50 ng of total cRNA using a precision injector (Drummond “Nanojet”) and incubated at 18 °C for 3–5 days. Injected cRNA obtained using an Ambion mMessage mMachine kit comprised rat brain peripheral (α₁₈₈₈) or central (α₂₉₉₉) subunits of the N-type VSCC expressed in the absence or presence (1:1 ratio) of the rat β₃ subunit provided by D. Lipscombe (12). Recordings were made in a nominally Ca²⁺-free solution containing (mM) 85 tetraethylammonium, 5 BaCl₂, 5 KCl, 5 HEPES, adjusted to pH 7.4 with methanesulfonic acid per fused at a rate of ~10 μl/min. Depolarization-activated Ba²⁺ currents were recorded using a GeneClamp 500B two-electrode voltage-clamp amplifier (Axon Instruments Inc., Foster City, CA) at room temperature (21–23 °C). Voltage and current electrodes were filled with 3 M KCl and had final resistances of 0.2–1.0 MΩ. VSCC currents in oocytes were evoked by a step depolarization to 0 mV from a holding potential of −80 mV using pClamp programs (Axon Instruments Inc). The linear membrane capacitative and leak currents were subtracted using a P/4 pulse protocol. Membrane currents were sampled at 10 kHz and filtered at 2 kHz. Oocytes showing <15% change in peak current amplitude over a 10-min incubation period were used in these studies to avoid effects associated with current run down. Oocytes that exhibited a slowly developing Cl⁻ current were discarded, and in control experiments on the remaining oocytes, BAPTA injection did not affect the Ba²⁺ current, indicating that this current was not contaminated by Ca²⁺-activated Cl⁻ current. Perfusion was stopped, and cumulative additions of α-conotoxins were added to a Teflon chamber (0.8 ml volume) to achieve rapid mixing. Inhibition of peak Ba²⁺ current amplitude was measured when the inhibitory effects of each concentration of MVIIA and CVID approached steady state values, typically requiring a 5–7-min incubation to achieve ~2% change in peak current amplitude per min. Data (means ± S.E.; n = 3–5) were fitted with nonlinear regressions using Origin software.

²H NMR and Three-dimensional Structure Calculations—All NMR experiments were recorded on a Bruker ARX 500 spectrometer equipped with a z-gradient unit, or a Bruker DMX 750 spectrometer equipped with an x,y,z-gradient unit. The Ho chemical shifts were obtained from spectra of CVIA–D in 95% H₂O, 5% D₂O (pH 2.5–3.5) at 293 K, and restraints for three-dimensional structure calculations of CVID were obtained from spectra recorded at 280 and 293 K at pH 3 and 5.5 (18). Structures were calculated using the torsion angle dynamics/stimulated annealing protocol in XPLOR version 3.8 (22–25), as described previously (20).

RESULTS
Isolation and Synthesis of α-Conotoxins from C. catus—The four α-conotoxins, CVIA–D, were isolated from the crude venom of C. catus using activity-directed fractionation. Each comprised <1% of total venom peptide as determined by HPLC (214 nm) or MS detection (m/z 500–2000). The amino acid sequences of CVIA and CVIB were cosequenced at a 3:2 ratio, whereas CVIC and CVID were obtained from separate fractions. Synthetic C-terminally amidated CVIA, CVIC, and CVID gave retention times and masses that were indistinguishable from the corresponding native peptides (Fig. 1), thus CVIA, CVIC, and CVID were determined to be C-terminally amidated. For each peptide, the oxidized form eluted earlier than the reduced form.

Gene Isolation and Characterization—PCR of the C. catus venom duct cDNA templates produced a DNA product of approximately 380–500 base pairs (data not shown). A relatively broad DNA band indicated the presence of a composite PCR product. Subsequent sequence analysis of more than 100 clones derived from the PCR product libraries confirmed this observation. The PCR products contained peptides of the ω- and δ-conotoxin types, as well as sequences from at least two other conotoxin families yet to be assigned. Within the ω-conotoxin sequences isolated were two that translated to putative mature peptides identical to the CVIA and CVID sequences. Despite an extensive screening of the two C. catus PCR product libraries, clones for the conotoxins CVIB and CVIC were not located, and the CVID clone was located in only one specimen. The complete nucleotide and predicted amino acid sequences derived for CVIA and CVID are shown in Fig. 2 and are aligned with the sequences for MVIIA and MVIA. Homology screening of public nucleotide and amino acid data bases with the CVIA and CVID sequences indicated that both sequences were unique.

Radioligand Binding—Synthetic CVIA–D, GVIA, MVIA, and MVIC each fully displaced [¹²⁵]I-GVIA (defining N-type VSCCs) and [¹²⁵]I-MVIIIC (defining P/Q-type VSCCs) binding to crude rat brain membrane (Fig. 3; Table II). GVIA, MVIA, and

![Fig. 1. HPLC/MS of native and synthetic α-conotoxins from C. catus.](http://www.jbc.org/)
CVID had similarly high affinity for the N-type VSCC (pIC$_{50}$ S.E. values of 10.4 ± 0.05, 10.3 ± 0.03, and 10.2 ± 0.03 M, respectively). In comparison, CVIA was a moderate inhibitor, and CVIB and CVIC were relatively poor inhibitors at the N-type VSCC. GVIA, MVIIA, and CVID also fully displaced $^{125}$I-MVIIA, with potencies (IC$_{50}$ values of 49, 29, and 50 pM, respectively) similar to those obtained for displacement of $^{125}$I-GVIA. At the P/Q-type VSCC MVIIIC had highest affinity (pIC$_{50}$ 9.2 ± 0.04 M); CVIB and CVIC were moderate inhibitors; MVIIA, CVIA, and MVIIA were poor inhibitors; and CVID was an exceptionally weak inhibitor (IC$_{50}$ 55 μM) (Fig. 3B and Table II).

Saturation binding studies indicated that $^{125}$I-CVID and $^{125}$I-MVIIA (67 ± 11 and 37 ± 12 pM, respectively) had potencies similar to those obtained for displacement of $^{125}$I-GVIA. At the P/Q-type VSCC MVIIIC had highest affinity (pIC$_{50}$ 9.2 ± 0.04 M); CVIB and CVIC were moderate inhibitors; MVIIA, CVIA, and MVIIA were poor inhibitors; and CVID was an exceptionally weak inhibitor (IC$_{50}$ 55 μM) (Fig. 3B and Table II).

Saturation binding studies indicated that $^{125}$I-CVID and $^{125}$I-MVIIA (67 ± 11 and 37 ± 12 pM, respectively) had poten-
Novel ω-Conotoxins Inhibit Neuronal Ca\(^{2+}\) Channels

The two-dimensional 1H NMR spectra of ω-conotoxins we have examined (18, 20, 30), peaks were well dispersed, and there was little overlap. CVIA–D secondary shifts closely paralleled those of MVIIA (Fig. 6, Table III). The voltage dependences of activation of these VSCC conductances were unaffected by CVID or MVIIA (data not shown). CVID and MVIIA had similar potencies to inhibit depolarization-activated inward Ba\(^{2+}\) current arising from \(\alpha_{1B,4}\) or \(\alpha_{1B,4}^{ex}\) coexpressed with rat \(\beta_3\) (Fig. 6, C and D). At the \(\alpha_{1B,4}\), the potency of both MVIIA and CVID increased 10-fold in the absence of rat \(\beta_3\). At the \(\alpha_{1B,4}^{ex}\), in contrast, CVID potency increased up to 540-fold, whereas MVIIA potency was only slightly increased in the absence of excess rat \(\beta_3\). Inhibition of the inward Ba\(^{2+}\) current by CVID was slowly reversed upon washout, and neither conopeptide inhibited the small endogenous calcium channel current (<5 nA) seen in oocytes that were not injected with cRNA (data not shown). For the \(\alpha_{1D/B}\) subunit combinations tested, a relatively small residual N-type current (~10% of maximum peak current) remained in the presence of saturating concentrations of CVID or MVIIA (Fig. 6, C and D). The residual component had reversal potentials and inward current maxima typical of N-type VSCCs and were completely blocked by bath application of 100 \(\mu\)M Cd\(^{2+}\) (data not shown), suggesting that both peptides are unable to occlude completely current through open N-type VSCCs. Further studies are required to confirm that CVID and MVIIA are allosteric inhibitors of the N-type VSCC, as suggested for GVIA from single channel activity (28).

FIG. 3. ω-Conotoxin potency at N-type and P/Q-type VSCCs in rat brain. A, potency at N-type VSCCs measured by displacement of \(^{125}\)I-GVIA binding. B, potency at P/Q-type VSCCs measured by displacement of \(^{125}\)I-MVIIA binding. Displacement data was best fit to a single-site competition model (shown). C, selectivity for N-type versus P/Q-type VSCCs in rat brain. Shown are the means ± S.E. for \(pIC_{50}\) (N-type) and \(pIC_{50}\) (P/Q-type).

A (rat brain N-type VSCC)  
| Conotoxin | \(^{125}\)I-GVIA Binding (%) |
|-----------|----------------|
| CVIA      | 100            |
| GVIA      | 80             |
| CVID      | 60             |
| MVIIA     | 40             |
| VCIA      | 20             |
| MVIC      | 10             |

B (rat brain P/Q-type VSCC)  
| Conotoxin | \(^{125}\)I-MVIIA Binding (%) |
|-----------|----------------|
| CVIA      | 100            |
| GVIA      | 90             |
| CVID      | 80             |
| MVIIA     | 70             |
| VCIA      | 60             |
| MVIC      | 50             |

C (N-type vs P/Q-type VSCC selectivity)  
| Conotoxin | \(\Delta pIC_{50}\) |
|-----------|----------------|
| CVIA      | 6              |
| GVIA      | 4              |
| CVID      | 2              |
| MVIIA     | 0              |
| VCIA      | -2             |
| MVIC      | -4             |

\(\Delta pIC_{50}\) was calculated from kinetic data (23 and 7 pM, respectively) were used in the displacement assays (20% of its \(K_d\) concentration). Displacement of higher concentrations of \(^{125}\)I-MVIIA by MVIIA, GVIA, CVID, or \(^{125}\)I-CVID also did not identify a significant resistant component, possibly due to the reduction in the signal to noise of the assay with increased labeled ligand concentrations (data not shown). The difference in \(B_{max}\) did not arise from differences in binding kinetics; \(^{125}\)I-CVID and \(^{125}\)I-MVIIA had similar \(k_{on}\) values (0.024 and 0.08 min\(^{-1}\) pmol\(^{-1}\) after adjusting for \(k_{off}\) and \(k_{on}\) values (0.56 and 0.53 min\(^{-1}\), respectively). The \(K_d\) values for \(^{125}\)I-CVID and \(^{125}\)I-MVIIA calculated from kinetic data (23 and 7 pM, respectively) were similar to the estimates obtained from saturation binding studies (67 and 37 pM, respectively).

Rat Vas Deferens—CVIA–D inhibited electrically stimulated rat vas deferens contractions (Fig. 5A). The rank order of potency was GVIA > CVID > MVIIA > CVIC > GVIC > CVID (Fig. 5B and Table II). Inhibition of the nerve-evoked responses in rat vas deferens was positively correlated with displacement of \(^{125}\)I-GVIA binding across the ω-conotoxins tested (log(rat vas deferens IC\(_{50}\)) = 0.78(log(\(^{125}\)I-GVIA IC\(_{50}\)) + 0.1) (\(r^2 = 0.92\)). CVID and MVIIA gave Hill slopes significantly greater than unity (2.9 and 3.3, respectively), whereas CVIA-C and GVIA gave Hill slopes that were not significantly different from unity. The origin of these differences is unclear; however, differences in N-type versus P/Q-type VSCC selectivity do not appear to be a contributing factor. The nonlinear relationship between calcium influx and transmitter release (26) is expected to give rise to a Hill slope greater than unity. Base-line tension was not affected by this series of peptides, and CVID (3 × 10\(^{-7}\) M) had no significant effect on the size of ATP and norepinephrine-induced contractions in rat vas deferens (data not shown, n = 3). A residual component of rat vas deferens contractile activity remained at saturating concentrations of CVID, MVIIA, and GVIA (14.8% (95% CI, 12–18%), 10.5% (7–14%), and 10% (6–14%), respectively) (Fig. 5B).

Xenopus Oocytes—Two-electrode voltage clamp studies revealed that CVID and MVIIA were potent inhibitors of ionic currents through peripheral (\(\alpha_{1B,4}\)) and central (\(\alpha_{1B,4}^{ex}\)) splice variants of the rat N-type calcium channels expressed in Xenopus oocytes in the presence or absence of rat \(\beta_3\) (Fig. 6, Table III). The voltage dependences of activation of these VSCC conductances were unaffected by CVID or MVIIA (data not shown). CVID and MVIIA had similar potencies to inhibit depolarization-activated inward Ba\(^{2+}\) current arising from \(\alpha_{1B,4}^{ex}\) or \(\alpha_{1B,4}^{ex}\) coexpressed with rat \(\beta_3\) (Fig. 6, C and D). At the \(\alpha_{1B,4}\), the potency of both MVIIA and CVID increased 10-fold in the absence of rat \(\beta_3\). At the \(\alpha_{1B,4}^{ex}\), in contrast, CVID potency increased up to 540-fold, whereas MVIIA potency was only slightly increased in the absence of excess rat \(\beta_3\). Inhibition of the inward Ba\(^{2+}\) current by CVID was slowly reversed upon washout, and neither conopeptide inhibited the small endogenous calcium channel current (<5 nA) seen in oocytes that were not injected with cRNA (data not shown). For the \(\alpha_{1D/B}\) subunit combinations tested, a relatively small residual N-type current (~10% of maximum peak current) remained in the presence of saturating concentrations of CVID or MVIIA (Fig. 6, C and D). The residual component had reversal potentials and inward current maxima typical of N-type VSCCs and were completely blocked by bath application of 100 \(\mu\)M Cd\(^{2+}\) (data not shown), suggesting that both peptides are unable to occlude completely current through open N-type VSCCs. Further studies are required to confirm that CVID and MVIIA are allosteric inhibitors of the N-type VSCC, as suggested for GVIA from single channel data (27). VSCC chimera studies indicate that GVIA may occlude the pore of the N-type VSCC (28).

\(^{1}H\) NMR Studies—The two-dimensional \(^{1}H\) NMR spectra of ω-conotoxins CVIA–D were each assigned using standard protocols (29). As with other ω-conotoxins we have examined (18, 20, 30), peaks were well dispersed, and there was little overlap. CVIA–D secondary shifts closely paralleled those of MVIIA (Fig. 7), whose structure is well defined (30), indicating that the overall fold and disulfide connectivity of these peptides are the
Novel \(\omega\)-Conotoxins Inhibit Neuronal \(Ca^{2+}\) Channels

### Table II

| Conotoxin | Displacement of \(^{125}\text{I}-\text{GVIA}\), IC\(_{50}\) (95% CI) | Inhibition of vas defereens, IC\(_{50}\) (95% CI) | Displacement of \(^{125}\text{I}-\text{MVIIC}\), IC\(_{50}\) (95% CI) |
|-----------|-------------------------------------------------|---------------------------------|---------------------------------|
| CVIA      | 0.56 (0.44–0.70)                                | 205 (170–250)                   | 850 (700–1020)                  |
| CVIB      | 7.7 (7.1–8.5)                                   | 630 (480–830)                   | 11 (9.0–13)                     |
| CVIC      | 7.6 (7.0–8.3)                                   | 410 (290–590)                   | 31 (26–37)                      |
| CVID      | 0.070 (0.058–0.077)                             | 18.4 (16–21)                    | 55,000 (49,000–62,000)          |
| MVIIA     | 0.055 (0.047–0.066)                             | 18.2 (15–22)                    | 440 (380–520)                   |
| GVIA      | 0.038 (0.030–0.047)                             | 4.9 (4.0–6.0)                   | 1050 (830–1320)                |
| MVIIC     | 7.0 (5.3–9.8)                                   | 0.60 (0.49–0.72)                |                                 |

**FIG. 4.** Saturation binding of \(^{125}\text{I}-\text{CVID}\) and \(^{125}\text{I}-\text{MVIIA}\) to rat brain membrane. Specific binding (open symbols) was best fitted by rectangular hyperbola describing a single binding site. The corresponding solid symbols represent nonspecific binding determined in the presence of an excess of the corresponding non-iodinated peptide.

**FIG. 5.** \(\omega\)-Conotoxin inhibition of electrically stimulated rat vas deferens. A, contractile response of guinea pig vas deferens to increasing concentrations of CVID, followed by washout. The inset compares single twitch responses recorded before (1), during (2), and after washout (3) of CVID, as indicated. B, concentration-dependent inhibition of rat vas deferens twitches by CVIA–D, MVIIA, and GVIA. Note the steep Hill slopes for CVID and MVIIA, and the incomplete inhibition caused by CVID, GVIA, and MVIIA.

The greatest difference in \(\Delta\) secondary shifts compared with MVIIA was seen in loops 2 and 4 of CVID. Although differences in loop 4 are not surprising given that CVID has two additional residues, the differences in loop 2 are remarkable given the similarities between MVIIA and CVID at loop 2. The secondary shifts of residues 9–14 in loop 2 of CVID follow the same pattern of those in MVIIA but are of greater magnitude, indicating that the structure of loop 2 in CVID may be more stabilized. This could stem from a long range interaction with loop 4, where the major differences in primary structure are located. Loop 2 has previously been the least defined region of \(\omega\)-conotoxin structures, with residues of this loop characterized by relatively broad peaks in the \(^1H\) NMR spectra, indicative of conformational exchange (30, 33). This lack of structure definition has hindered attempts to understand the crucial role loop 2 plays in activity, function, and selectivity of \(\omega\)-conotoxins, particularly that of the principal binding determinant Tyr\(^{13}\), as well as residues of secondary importance such as Leu\(^{11}\) and Arg\(^{10}\) in MVIIA (34). Because no line broadening is observed in loop 2 or elsewhere in CVID but is prominent in loop 2 of MVIIA, CVID may provide a significantly improved structural template for pharmacophore development. Given that the significant differences in secondary \(\Delta\) shifts for residues in loops 2 and 4 in CVID preclude accurate modeling of CVID from existing \(\omega\)-conotoxin structures, and given its enhanced N-type selectivity and structural stability, we determined the three-dimensional structure of CVID using \(^1\)H NMR spectroscopy, as described below.

**Three-dimensional Structure of \(\omega\)-Conotoxin CVID—**CVID structures were calculated based on a total of 481 distance restraints derived from 159 intraresidue, 110 sequential, 184 medium and long range NOEs, 28 hydrogen bond restraints defining a total of 14 hydrogen bonds, and 23 \(\phi\) and 10 \(\chi^1\) dihedral angle restraints. A total of 47 of 50 structures converged to a consensus fold, with no NOE distance violation greater than 0.2 \(\AA\) and no dihedral violations greater than 3°. Of these, the 20 lowest energy structures were chosen to represent the structure of CVID. The structures are exceptionally well defined, with a backbone pairwise root mean squared difference of 0.35 \(\AA\) (calculated over all residues). The angular order parameters (S) for a backbone pairwise root mean squared structure of CVID from existing \(\omega\)-conotoxin structures, and given its enhanced N-type selectivity and structural stability, we determined the three-dimensional structure of CVID using \(^1\)H NMR spectroscopy, as described below.

The CVID structures are shown superimposed globally over the backbone atoms, together with the lowest energy structure displayed in secondary structure mode in the same orientation (Fig. 8A). The same structures are shown rotated 90°, to display side chains of residues in loops 2 and 4 which are important for binding (Fig. 8B). As for other \(\omega\)-conotoxins, the secondary structure of CVID is dominated by a triple-stranded \(\beta\)-sheet, in this case incorporating residues 7–9 and 25–27 as the two peripheral strands and residues 19–21 as the central \(\beta\)-strand. Other secondary structural features include a
\( \beta \)-bridge composed of residues 1–2 and 14–16 and several \( \beta \)-turns. The \( \beta \)-turns that link the \( \beta \)-sheet and \( \beta \)-bridge regions comprise residues 3–6 (type II), 9–12 (type I), 10–13 (type I), 15–18 (type I), and 21–24 (type IV). The \( \beta \)-bridge has not previously been reported explicitly for other \( \omega \)-conotoxins but is likely to exist in MVIIA, GVIA, and MVIIC and has been identified in \( \kappa \)-conotoxin PVIIA, which adopts the same structure as the \( \omega \)-conotoxins but targets the Shaker potassium channel (35). Structural features of CVID that have not been described for other \( \omega \)-conotoxins are the presence of two hydrogen bonds from the NH protons of Lys10 and Leu11 to the C\( \beta \)O oxygen atoms of Gly22 and Thr23, respectively. It is likely that these enhance the stability of loop 2 in CVID compared with other \( \omega \)-conotoxins. Importantly, the backbone of Tyr13 is stabilized in the \( \alpha \_L \) conformation, with the \( \chi_1 \) side chain torsion angle at \(-60^\circ\). NMR data confirming this conformation for Tyr13 in CVID include the presence of a strong intraresidue NH-HOEP NOE, together with a weaker H\( \alpha \)-\( \gamma \)-NH\( \beta \), and a \( ^3J_{\text{NH-HEP}} \) coupling constant of 7 Hz (36). Attempts to define the conformation of Tyr13 in other \( \omega \)-conotoxins have been ambig-
of an absence of any disulfide bond isomerization. The Hβ shifts differences of Cys8, Tyr13, and Asp14 are also significantly greater in CVID than in MVIIA. This trend is followed over the temperature range 280–298 and the pH range 3–6 (data not shown).

**DISCUSSION**

By using assay-directed fractionation, we have isolated four new ω-conotoxins from *C. catus*, named ω-conotoxins CVIA–D. These four conotoxins were chemically synthesized and folded to be indistinguishable from the native peptides. *C. catus* is the sixth piscivorous cone snail to evolve mammalian active ω-conotoxins for prey capture. Analogous to other ω-conotoxins identified to date, CVIA–D have three disulfide bonds, fall within the mass range 2500–3600 Da (4), and are relatively hydrophilic. Inspection of the sequences in Table I reveals a conserved cysteine framework and three conserved residues Gly5, Tyr13, and Ser19 (CVID numbering) among ω-conotoxins. Homology at loops 1–4 varies from 0 to 60% sequence identity among these ω-conotoxins, with the largest difference occurring in loop 4 of CVID. Cloning and sequencing of mRNA transcripts from the venom ducts of two specimens of *C. catus* confirmed CVIA and CVID are expressed in this tissue. Our inability to isolate mRNA transcripts encoding CVIB and CVIC was most likely associated with the relatively low abundance of these peptides. Variation in the levels of specific peptides between individual *C. catus* could also be involved.2 Isolation of cDNA templates for CVIB and CVIC may require the analysis of additional *C. catus* specimens or the use of additional “PCR markers” that are more specific for the mature peptide sequences.

CVIA–D each fully displaced 125I-GVIA and 125I-MVIIC binding to rat brain membrane. CVID, GVIA, and MVIIA were similarly potent at rat brain N-type VSCCs. However, CVID was 100-fold more selective than MVIIA for N-type over P/Q-type VSCCs, and 125I-CVID bound to 30% fewer sites in rat brain membrane than 125I-MVIIA. Similar differences in Bmax were not observed in saturation binding of 125I-CVID and 125I-MVIIA to human brain membrane, suggesting that pharmacological differences exist in the N-type VSCCs present in rat and human brain. Further studies are required to determine the origin of these differences. CVIA–D were reversible inhibitors of peripheral N-type VSCCs involved in neurally evoked transmitter release in rat vas deferens. Inhibition of neurotransmitter release was positively correlated with the displacement of 125I-GVIA binding, consistent with N-type VSCCs being the major source of Ca2+ influx contributing to neurotransmitter release in the rat vas deferens stimulated at low frequencies (37–40). A correlation between inhibition of Ca2+ influx and ligand displacement at the N-type VSCC was shown for a series of MVIIA and TVIA analogues (41) but not for a series of GVIA analogues (33). Potency estimates obtained using the vas deferens were lower than estimates obtained from radioligand displacement studies (Table II), consistent with previous observations (37, 42). The relatively high levels of Ca2+ in the physiological solution for the vas deferens compared with the binding assays is likely to contribute to this difference in potency, since Ca2+ is a non-competitive inhibitor of ω-conotoxin binding (13). CVID had no effect on contractions of the vas deferens induced by exogenously applied ATP or norepinephrine or on voltage-sensitive sodium channels in rat autonomic neurons.2

In *Xenopus* oocytes, CVID and MVIIA inhibited the inward current through central (αβδ,α) and peripheral (αβδ,α) splice variants of the rat N-type VSCC. In the presence of rat β3 subunit, chosen because it assembles with α1D (43) and is widely distributed in rat brain (44), MVIIA and CVID had

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**FIG. 9. Comparison of CVID and MVIIA (55) structures.** Shown in the same global orientation are the backbone superimposition of the 20 lowest energy structures of CVID (green) (A), and a set of 34 structures of MVIIA (gray backbone, Protein Data Bank code IDW5, see Ref. 55) comprising both χ3 families that were calculated with a force constant of 40 kcal/mol applied to the three disulfide bridge dihedral angles Cβ–Sγ1–Sγ2–Cβ (B). The disulfide bridge Cys8–Cys20 (purple), and the side chains of important binding residues Arg10 (Lys10 in CVID) (blue), Leu11 (yellow), and Tyr13 (orange) are identified. The position of loop 4 is indicated in A and B. C shows a comparison of differences in Hβ shifts for selected residues of CVID (green) and MVIIA (gray, 30). Note that CVID has greater separations of Hβ shifts, particularly at Cys8, Tyr13, Asp14, and Cys20.

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numous, and indeed Tyr13 may adopt an averaged conformation in other ω-conotoxins. The enhanced structural stability of CVID is further reinforced by a comparison of the Hβ shifts of CVID and MVIIA (Fig. 9C). The Hβ protons of Cys20 are degenerate in MVIIA indicative of disulfide bond isomerization, whereas the corresponding protons in CVID have disparate shifts indic-
similar potencies. However, the potency of MVIIA and CVID increased at $\alpha_{1B,4}$ and $\alpha_{1B,6}$ expressed in the absence of rat $\beta_3$. This increase was most pronounced for CVID at $\alpha_{1B,4}$ (up to 540-fold), intermediate for CVID and MVIIA at $\alpha_{1B,6}$ (~10-fold), and least pronounced for MVIIA at $\alpha_{1B,4}$ (3-fold). Previous studies have shown that the $\beta$ subunit can influence the affinity of local anesthetics (45) and spider toxins (46) for $\alpha_{1A}$ (PQ-type) calcium channels expressed in oocytes and mammalian cells. Oocytes express low levels of an endogenous $\beta$ subunit ($\beta_3\text{no}$) with high homology to rat $\beta_3$ that is essential for expression of functional channels, and overexpression of either of these $\beta_3$ subunits causes the same facilitation of VSCC current activation and inactivation (47). We propose that expression of excess $\beta_3$ can differentially modify $\omega$-conotoxin pharmacology at central and peripheral splice variants of the N-type VSCC, presumably by binding to an intracellular domain of $\alpha_{1B}$ to cause an extracellular conformational change that reduces $\omega$-conotoxin binding.

The alternatively spliced $\alpha_{1B,4}$ and $\alpha_{1B,6}$ differ at extracellular loops I/IIIS-S4 (+SFMG and -SFMG, respectively) and IVS3-S4 (~ET and + ET, respectively), indicating that relatively small changes in the sequence of $\alpha_{1B}$ not only influence channel kinetics (insertion of ET in $\alpha_{1B,6}$ slows activation kinetics (48)), but modify $\omega$-conotoxin pharmacology. Since $\omega$-conotoxins bind preferentially to the inactivated state than the resting state of N-type calcium channels (49), the observed effect is not through excess $\beta$ subunit that would act to increase the proportion of channels in the inactivated state (47). This auxiliary subunit effect may occur through the action of excess $\beta$ subunit at a second site that is present at the C terminus of $\alpha_{1A,1}$, $\alpha_{1B,1}$, and $\alpha_{1E,1}$ (50), but a differential effect of rat and *Xenopus* $\beta_3$ on the pharmacology of $\omega$-conotoxins may also be involved. The potency of Aga IVA and funnel web spider toxin were reduced ~10-fold when $\alpha_{1A,1}$ was coexpressed in mammalian cells with $\beta_3$ or $\beta_2$, rather than with $\beta 1$B (46). The extent to which $\omega$-conotoxin pharmacology at central and peripheral splice variants of $\alpha_{1B}$ is influenced by different combinations of the five $\alpha 2/\beta$ subunits and the four $\beta$ subunits requires further investigation, given the heterogeneity of $\alpha_{1B,1}$/auxiliary subunit combinations in the brain (43). The differential effect of $\beta$ subunits on $\omega$-conotoxin pharmacology at central and peripheral splice variants of $\alpha_{1B}$ may lead to the development of $\omega$-conotoxins with selectivity for specific forms of the N-type VSCC present in different neural pathways. However, the precise location of specific combinations of $\alpha_{1B}$ splice variants and different $\beta$ subunits have not yet been determined.

The ability of CVID to discriminate among different forms of the N-type VSCC may also have therapeutic implications. In a Freund’s complete adjuvant model of inflammatory pain in rats, chronic intrathecal administration of CVID (AM336; 0.36 nmol/h) produced sustained antinociception with mild behavioral side effects (mean scores 0.5–1 on a scale of 7) over a 7-day infusion period. In comparison, MVIIA (Ziconotide; 0.1 for first 24 h and 0.13 nmol/h for next 6 days) produced similar initial levels of antinociception that returned to baseline by day 6, whereas levels of motor side effects (mean scores 3–4) were greater and maintained throughout the 7-day infusion period (52). These effects of MVIIA and CVID were completely reversed on ceasing conotoxin infusion. CVID has recently gained approval to enter clinical trials for the treatment of severe pain.

1H NMR studies reveal that CVID adopts a unique fold, with loop 4 curved toward loop 2 to create a globular surface. In contrast, loop 4 is oriented parallel to loop 2 in MVIIA and MVIII and away from loop 2 in GVIA (18, 30, 53, 54). The presence of two hydrogen bonds between loops 2 and 4 in CVID favor loop 4 in this orientation, whereas hydrogen bonds between loops 2 and 4 have not been reported previously for GVIA, MVIIA or MVIII. Early structural studies on MVIIA (30) raised the possibility of loop 2 instability, based on the observation of broadened lines in this region. This was supported by a recent study on the dynamics of MVIIA (55), where it was suggested that the motion of loop 2 was facilitated by isomerization of the disulfide bridge between Cys$^8$ and Cys$^{20}$.

In that study, the introduction of a force constant of 40 kcal mol$^{-1}$ into the empirical force field restrained the $\chi_3$ angle of this disulfide bridge to $\pm 90^\circ$ resulting in two closely related families of structures (55). No such isomerization is evident in CVID, where a corresponding force constant of only 10 kcal/mol yielded a conformation of $+90^\circ$ in all structures. In addition, the $\chi_1$ angles at Cys$^{20}$ for MVIIA ($+180^\circ$) and CVID ($-60^\circ$) differed significantly, as do the magnitude of $\Delta H_b/\Delta H_\beta$ shift differences of AMX residues (Fig. 9C). The greater $H_b$ shift differences of Cys$^8$, Cys$^{20}$, Tyr$^{13}$, and Asp$^{14}$ in CVID indicate that the side chains of these residues undergo less conformational exchange than MVIIA. The improved stability of this disulfide bridge in CVID together with the interloop hydrogen bonds may act to restrain loop 2 motion, providing an entropic advantage for binding. This overall stability of CVID is also likely to contribute to its selectivity, since loop 2/4 combinations contain many of the primary determinants for $\omega$-conotoxin selectivity between N-type and P/Q-type VSCCs (18).

CVID and two closely related families of MVIIA (55) structures are compared to highlight important differences in the orientation of loops 4 and 2 (Fig. 9). Although Tyr$^{13}$ occupies similar spatial positions, the positions of the side chains of the important binding residues 10 and 11 appear reversed in the two peptides. According to Atkinson et al. (55), motion in MVIIA results in time-averaged NOEs that ultimately produce flaws in the resulting structure. Such motion does not appear to be present in CVID, since no line broadening is observed in loop 2 or elsewhere in the peptide. This, together with the increased number of distance restraints, particularly in the loop 2 region, ensures that the structure of CVID is represented by a single, well defined conformation. Interestingly, CVID lacks the secondary binding residues present in loop 4 of MVIIA (Arg$^{21}$) (34) or GVIA (Lys$^{24}$ and Tyr$^{23}$) (33) but utilizes a new $\omega$-conotoxin/VSCC interaction through the relatively exposed Val$^{24}$. These structural features presumably act in concert to influence the selectivity of CVID, as well as providing a well defined template for pharmacophore development and the design of peptidomimetic inhibitors of N-type VSCCs.

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