NMR Solution Structures of δ-Conotoxin EVIA from Conus ermineus That Selectively Acts on Vertebrate Neuronal Na⁺ Channels

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δ-Conotoxin EVIA, from Conus ermineus, is a 32-residue polypeptide cross-linked by three disulfide bonds forming a four-loop framework. δ-Conotoxin EVIA is the first conotoxin known to inhibit sodium channel inactivation in neuronal membranes from amphibians and mammals (subtypes rNa₁.2a, rNa₁.3, and rNa₁.6), without affecting rat skeletal muscle (subtype rNa₁.4) and human cardiac muscle (subtype hNa₁.5) sodium channel (Barbier, J., Lamthanht, H., Le Gall, F., Favreau, P., Benoit, E., Chen, H., Gilles, N., Ilan, N., Heinemann, S. F., Gordon, D., Ménez, A., and Molgò, J. (2004) J. Biol. Chem. 279, 4680–4685). Its structure was solved by NMR and is characterized by a 1:1 cis/trans isomerism of the Leu₁²-Pro₁³ peptide bond in slow exchange on the NMR time scale. The structure of both cis and trans isomers could be calculated separately. The isomerism occurs within a specific long disordered loop 2, including residues 11–19. These contribute to an important hydrophobic patch on the surface of the toxin. The rest of the structure matches the “inhibitor cystine-knot motif” of conotoxins from the “O superfamily” with a high structural order. To probe a possible functional role of the Leu₁²-Pro₁³ cis/trans isomerism, a Pro → Ala δ-conotoxin EVIA was synthesized and shown to exist only as a trans isomer. P13A δ-conotoxin EVIA was estimated only two times less active than the wild-type EVIA in binding competition to rat brain synaptosomes and when injected intracebroventricularly into mice.

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The atomic coordinates and structure factors (code 1G1P and 1G1Z) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

2 The on-line version of this article (available at http://www.jbc.org) contains one table of the H² chemical shifts of cis and trans isomers of δ-EVIA and of P13A δ-EVIA; one figure representing the H²-aliphatic region of the TOCSY spectra of δ-EVIA and P13A δ-EVIA; one figure representing the chemical differences between cis and trans isomers of δ-EVIA.

3 The on-line version of this article (available at http://www.jbc.org) contains on the cover and title page of this article an additional figure, Table S1, and Table S2.

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The new δ-conotoxin EVIA (δ-EVIA),1 a 32-amino acid conopeptide isolated from the venom of Conus ermineus, is the first conotoxin demonstrated to inhibit sodium channel inactivation in neuronal membranes from amphibians and mammals (subtypes rNa₁.2a, rNa₁.3, and rNa₁.6) without affecting rat skeletal muscle (subtype rNa₁.4) and human cardiac muscle (subtype hNa₁.5) sodium channel subtypes (1). This important recent discovery makes δ-EVIA a unique tool to study the modulation mechanisms of neuronal Na⁺ channels. As a consequence, δ-EVIA may also serve as a new lead molecule for the design of new drugs to treat neurological diseases characterized by defective nerve conduction, especially those causing an axonal demyelination (2, 3). Nerve conduction could be facilitated by specific inhibition of Na⁺ channel inactivation. The knowledge of the detailed three-dimensional structure is therefore the first step necessary to understand the structure-activity relationships of this new lead conotoxin.

Despite a low sequence identity with the κ-, ω-, and δ-conotoxins, δ-EVIA clearly belongs to the four-loop family of conotoxins characterized by a similar cysteine pairing giving a conserved 3-disulfide framework as shown in Fig. 1. Until now, the three-dimensional structure of 10 conotoxins belonging to this family was already determined as follows: the conotoxin κ-PVIIA (20) targeting potassium channels; conotoxins ω-MVIIA (21), ω-MVIIIC (22), ω-MVIIID (23), and ω-GVIA (24), targeting skeletal muscle sodium channels; the new δ-EVIA, targeting calcium channels (the μ-GS (27) targeting skeletal muscle sodium channels; conotoxin TVIIA (28); and the most recent conotoxin δ-TxVIA (12). The latter δ-TxVIA belongs to a specific class of conotoxins that affect Na⁺ channel inactivation exclusively in mollusks but exhibits high affinity to rat brain synaptosomes (15). In rat brain synaptosomes, δ-EVIA competes with δ-TxVIA for the same site 6 of voltage-dependent Na⁺ channels (see Ref. 1 and this work).

Despite the differences in the loop length between cysteine residues (especially for the loops 2–4), their three-dimensional solution structures reveal a common scaffold consisting of a small β-hairpin structure and several types of tight turns. All conotoxins of this group exhibit a rather well defined backbone conformation, stabilized by a number of hydrogen bonds located in the different secondary structures, and by the three disulfide bridges. The scaffold forms the classical “cysteine-knot” motif known for toxic and inhibitory polypeptides (29). A
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Fig. 1. Sequence comparison of conotoxins. Identical residues are shown in boldface type. The standard one-letter code for amino acid residues is used except for the post-translationally modified 4-trans-1-hydroxyproline (O) and γ-carboxy-glutamic acid (X). Spacers (–) are inserted to show maximum homology based on the conserved Cys framework. ω, hydrophobicity calculated according to Fauchère et al. (87), without the six cysteines. Asterisks indicate an amidated C terminus. a, HWTX-I, Huwentoxin-I. b, NAR, nicotinic acetylcholine receptor. c, μ-Aga-I, μ-Agatoxin-Ω, d, an additional disulfide bridge occurs between Cys24 and Cys30. e, δ-ACTX-Hv1, δ-Atoxicon-Hv1 (versutoxin). f, an additional disulfide bridge occurs between Cys30 and Cys21. g, Analysis of peak C yielded a significant increase in the Cys (S-carboxymethyl) signal at cycles 10 and 25, due to the open disulfide bridge Cys10, Cys25 in addition to the disulfide bridge Cys3, Cys21. h, The mixture of δ-EVIA-containing fractions (~100 ml) was diluted with deionized water (final volume ~500 ml) containing the redox couple cysteine/cystine, 0.25 mmol. The volume was adjusted to 1 liter with degassed buffer (0.1 m NH4SO4, 0.1 m ammonium acetate, 1 mM EDTA, pH 8.5), and the solution was adjusted to pH 8.5 with NH4OH and reneutralized by incubation (48 h at 4 °C, followed by 12 h at 22 °C). The mixture of oxidized peptides was adjusted to pH 4 with trifluoroacetic acid, and loaded on a reverse phase preparative column (Vydac 218TP, 250 × 25 mm), and eluted with the acetonitrile gradient described previously. Based on HPLC co-elution experiments and electrospray ionization mass spectrometry, δ-EVIA was identified as a minor product under the oxidation conditions used. The peptide was further purified with a semi-preparative column (Zorbax SB C18, 250 × 9.4 mm). Disulfide Pairing Assignment—δ-EVIA disulfide pairing pattern was determined after partial reduction with tris(2-chloroethyl)phosphate (40 °C, 5 min), as reported previously (32). The reverse phase chromatographic profile of the mixture is shown in Fig. 2A. The fingerprint pattern displayed a huge peak of nonreduced δ-EVIA and three intermediates. After the intermediates in peaks A–C were alkylated with a large excess of iodoacetamide and purified by reverse phase HPLC, the amino acid sequences of their Cys (S-carboxamidomethyl) derivatives were determined (Fig. 2B). Sequencing the Cys (S-cam) derivative in peak A indicated SS bridging of Cysγ and Cysγ. Sequencing the Cys (S-cam) derivative in peak B revealed an increased signal for Cys (S-cam) at cycles 3, 10, 21, and 25. Thus, peak B has the disulfide bridge Cysγ-Cysγ5 in addition to the disulfide bridge Cysγ, Cysγ. Analysis of peak C yielded a significant increase in the Cys (S-cam) signal at cycles 10 and 25, due to the open disulfide bridge Cysγ, Cysγ. Therefore, we deduce SS bridging between Cysγ, Cysγ5 in addition to the detected Cysγ, Cysγ, and Cysγ, Cysγ5 disulfide bonds. The cystine framework of δ-EVIA was summarized as Cysγ, Cysγ, Cysγ, Cysγ, and Cysγ5, Cysγ. Radioiodination and Binding Assays—δ-Conotoxin TxAII was radiiodinated by using 1 nmol of toxin, 0.5 mCi of carrier-free Na125I in a potassium phosphate buffer, pH 7.25, containing H2O2 (10 μl of 1:50,000 solution) and lactoperoxidase (0.7 unit, EC 1.11.1.7 from bovine milk) for a 2-min incubation time. The moniodotoxin was purified.
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on a Vydac C18 column. Rat brain synaptosomes were prepared from adult Sprague-Dawley rats (300 g), according to the method described by Kanner (33). Equilibrium competition assays were performed using increasing concentrations of unlabeled toxins in the presence of a constant low concentration of the radioactive toxin. Competition binding experiments were analyzed by the program Kaleidagraph (Synergy Software) by using a non-linear Hill equation (for IC\textsubscript{50} determination).

The K\textsubscript{d} values of EVIA were calculated by the equation K\textsubscript{d} = (L\textsubscript{t} + L\textsubscript{i}/K\textsubscript{c}) where L\textsubscript{t} is the concentration of the hot δ-TxVIA, and K\textsubscript{c} is its dissociation constant (34). Standard binding medium composition was (in mM) as follows: choline Cl 130, CaCl\textsubscript{2} 1.8, KCl 5, MgSO\textsubscript{4} 0.8, HEPES 50, glucose 10, and 2 mg/ml bovine serum albumin. Following incubation for the designated times, the reaction was terminated by dilution with 5 ml ice-cold wash buffer of the following composition (in mM): choline Cl 140, CaCl\textsubscript{2} 1.8, KCl 5.4, MgSO\textsubscript{4} 0.8, HEPES 50, pH 7.2, 5 mg/ml bovine serum albumin. Separation of free from bound toxin was achieved by rapid filtration under vacuum using Whatman GF/C filters preincubated with 0.5% polyethyleneimine. The filter discs were then rapidly washed twice with 2 ml of buffer. Nonspecific toxin binding was determined in the presence of a high concentration of the unlabeled toxin.

Biochemical Activity—To quantify the biochemical activity of native and synthetic conotoxins, Swiss-Webster mice (15 g) were injected intracerebroventricularly with a stereotaxic system (Harvard/ASI Apparatus, UK). The ED\textsubscript{50} value was defined as the dose that produces hyperactivity in 50% of the tested animals within 12 h postinjection. The Ki values in 50% of the tested animals within 12 h postinjection.

Structure Calculations—Models were calculated following a protocol described previously (49) using the X-PLOR software version 3.851 (50). Structures were analyzed using the program check-nmr (51) and promotif (52) and displayed using Molmol version 2.4 (53) and Molscript (54).

The structure was generated by the hybrid distance geometry dynamically simulated annealing method (55, 56). In a first stage, the substructures generated using metric matrix distance geometry algorithms were regularized and refined by a high temperature simulated annealing procedure with the radial bias pro force field of X-PLOR. The non-bonded van der Waals interactions were represented by a simple pulsed quadratic term (55, 57). The experimental distance restraints were represented as a soft asymptotic potential, and electrostatic interactions were ignored. The force constant associated with the distance restraints was kept to 50 kcalmol\textsuperscript{-1}Å\textsuperscript{-2} throughout the protocol. One cycle of simulated annealing refinement consisted of 1,500 steps of 3 fs at 1,000 K followed by 3,000 cooling steps of 1 fs from 1,000 to 300 K. At the end, each structure was subjected to 1,500 steps of conjugate gradient energy minimization.

In the second stage of the calculation, structures with good experimental and geometric energies were further refined using the full CHARMM22 force field of X-PLOR. In this stage of the calculation, the non-bonded interactions such as electrostatic interactions and van der Waals’ interactions (described by the Lennard-Jones empirical energy function) were taken into account. An approximate solvent electrostatic screening effect was introduced by using a distance-dependent dielectric constant and by reducing the electric charges of the formally charged amino acid side chain (Asp and Lys) to 20% of its nominal charges defined in the CHARMM22 force field. The force constant used for the NOE potential was reduced to 25 kcalmol\textsuperscript{-1}Å\textsuperscript{-2}. After 1,500 steps of conjugate gradient energy minimization, the dynamic was initiated at 750 K. The system was equilibrated for 0.5 ps with an integration step of 1 fs and then coupled to a heat bath at 750 K, and the simulation was allowed to evolve for 10 ps before the recycle process to re-energize the system was performed for a period of 5.4 ps and allowed to evolve again for 15 ps. Finally, the structures were energy-minimized by 1,500 steps of the conjugate gradient algorithm.

RESULTS
δ-EVIA was synthesized using Fmoc chemistry, based on its amino acid sequence. The N\textsuperscript{-}Fmoc linear/denatured and reduced peptide was obtained in high yield (151/350 mg of calculated Fmoc peptide). Its folding/renaturation was the limiting step in the synthesis of bioactive δ-EVIA. Typically, the folding procedure yielded only 5–10 mg of synthetic and bioactive δ-EVIA (3.3–6.6% yield, based on 151 mg of Fmoc linear peptide). The HPLC pattern of the preparation obtained after the folding procedure indicated the presence of a large quantity of monomer isomass peptides (data not shown), probably because of misfolding of the linear, reduced peptide. Synthetic δ-EVIA co-eluted with native δ-EVIA (Fig. 2C), and the predicted MW of synthetic δ-EVIA was confirmed by ESI-MS (observed MH\textsuperscript{+} 3288.1; calculated MH\textsuperscript{+} 3288.4).

Sequence Resonance Assignments of the Spin Systems—From the DQF-COSY and TOCSY spectra, we first noticed the presence of 53 amide protons (HN), instead of the 30 expected. The DQF-COSY and TOCSY spectra, we first noticed the presence of 53 amide protons (HN), instead of the 30 expected. The DQF-COSY and TOCSY spectra recorded at 0.5, 1, 3, 19, 36, and 54 h. For the P13A conotoxin—EVIA was confirmed by ESI-MS (observed MH\textsuperscript{+} 3288.1; calculated MH\textsuperscript{+} 3288.4).

Sequence Resonance Assignments of the Spin Systems—From the DQF-COSY and TOCSY spectra, we first noticed the presence of 53 amide protons (HN), instead of the 30 expected. The sequence-specific resonance assignment (46) was undertaken and revealed two distinct NOE sets of resonances with only Asp\textsuperscript{1}, Asp\textsuperscript{2}, Lys\textsuperscript{6}, Hyp\textsuperscript{8}, Gly\textsuperscript{9}, Cys\textsuperscript{11}, and Leu\textsuperscript{22} common to the two systems. The d\textsubscript{\textsuperscript{14}N} connectivities proceeded unambiguously for the two sets of resonances except for Cys\textsuperscript{3}/Ser\textsuperscript{2}/. Tyr\textsuperscript{7}, Phe\textsuperscript{9}, Pro\textsuperscript{13}, Ala\textsuperscript{14}, and Ala\textsuperscript{20} spin systems were used as starting points for the sequential assignment due to their specific pattern. In one of the two systems, the Leu\textsuperscript{12}/Pro\textsuperscript{13} peptide bond was unambiguously assigned to the cis peptide conformation due to the observation of a strong d\textsuperscript{2} and a weak d\textsuperscript{2} in NOE spectra. For the second system, the peptide bond conformation was unambiguously assigned to the trans conformation due to the observation of a strong d\textsuperscript{2} with no d\textsuperscript{2}. Then the two systems
clearly resulted from a Leu$^{12}$-Pro$^{13}$ cis/trans mixture of δ-EVIA in slow exchange on the time scale of the NMR chemical shift. No exchange peaks between the two conformers were detected either in the TOCSY or the NOESY experiments (Fig. 3). The chemical shift differences (where $\Delta \delta^{\text{cis-trans}}$ indicates difference of the chemical shifts between cis and trans conformers) observed in the NMR spectra between δ-EVIA 12–13cis and 12–13trans are the most important for the H$^N$ proton compared with the C$^\beta$H protons and very small for all the side chain protons but four of the nine residues in loop 2 (Ser$^{11}$, Leu$^{12}$, Pro$^{13}$, and Asn$^{17}$) (see "Discussion"). By taking into account the experimental uncertainties and using the relative intensities of the TOCSY cross-peak intensities of the same proton pair in the two different conformers (the spin system of Cys$^{10}$, Ser$^{22}$, Gly$^{27}$, and Cys$^{29}$ were the best resolved), a 1:1 ratio of each population was estimated. This ratio is not temperature-dependent between 283 and 313 K.

The assignment of the Hyp$^6$ was straightforward with strong $d_\alpha$ and no $d_\beta$ between Lys$^5$ and Hyp$^6$, indicating that the peptide bond is in a trans conformation. However, the observation of $d_\alpha$ between Lys$^5$ and Hyp$^6$ could not be observed due to the superposition of the strong intra-residual NOE C$^\beta$H-C$^\alpha$H of Gly$^{23}$. A number of cross-peaks were not found in the H$^N$-aliphatic region of the DQF-COSY spectrum. It was the case for Lys$^5$, Lys$^{16}$, Cys$^{20}$, Cys$^{21}$, and Ala$^{30}$. The two sequence-specific resonance assignments of δ-EVIA are given as Supplemental Material.

Secondary Structure and Molecular Topology—On the basis of the NMR data (Fig. 4 and Fig. 5), we identified the presence of a small β-sheet composed of three short antiparallel strands, involving Gly$^8$-Cys$^{10}$ (strand 1), Gly$^{23}$-Val$^{26}$ (strand 2), and Val$^{26}$-Asp$^{31}$ (strand 3). The double-stranded anti-parallel β-sheet comprising strands 2 and 3 are connected by a typical γ-turn Val$^{26}$-Val$^{28}$ and forms a β-hairpin structure. Finally, two β-turns including amino acids Lys$^5$-Gly$^8$ and Cys$^{20}$-Gly$^{23}$ were identified according to the standard criteria (58).

Structure Calculations—The input data for the structural calculation consisted of 206 distance and 24 dihedral restraints. The 206 distance restraints include 56 intraresidual, 68 sequential ($i$, $i+1$), 27 medium range ($i$, $i$ + 2 to $i$, $i$ + 4), and 55 long range (>4, $i$ + 4) restraints (Fig. 5A) for the trans conformer. The number of distance restraints is a little different for the cis conformer with 134 and 56 distance restraints for...
the sequential and medium range restraints, respectively. Sixteen slowly exchanging amide protons were assigned from the D2O exchange experiment, and 11 of them were used to define 22 hydrogen-bond restraints (see under “Experimental Procedures”). These 11 with the 5 remaining slowly exchanging amide protons will be discussed below. Dihedral angle restraints included 15 $\chi_1$ angles and 9 $\phi$ angles.

Both cis and trans Pro13 were modeled separately with the appropriate $\phi$ angle restraints in the simulated annealing calculations using paralhdg.pro force field. Thirty initial structures were calculated, leading to 16 and 18 final structures for the trans and the cis conformer, respectively. None of these models have an NOE violation exceeding 0.15 Å or a dihedral angle violation exceeding $5^\circ$. These structures were refined with the CHARMM22 force field and show a few distance violations lower than 0.25 Å and no dihedral angle violations exceeding $5^\circ$. The geometric and energetic statistics of these final structures, given in Table I, satisfy both the force fields and the experimental restraints. Side-by-side views of the final structures (Fig. 6A) show that residues 3–10 and 20–30 (corresponding to loops 1, 3, and 4) have well defined backbone dihedral angles (Fig. 7, B, C, and E) and low r.m.s.d. for the backbone atoms (Fig. 7D), whereas loop 2 (residues 11–19) appears completely disordered. The two residues of the N- and C-terminal parts also exhibit higher disorder. This correlates with the small number of medium and long range NOEs for these parts of the molecule (Fig. 7A).

Analysis of the Hydrogen Bond Network—The hydrogen-exchange rates in D2O identified 16 slowly exchanging amide protons in the structure. It concerned the Ile4, Lys5, Tyr7, Gly8, Phe9, Cys10, Ile14, Leu15, Cys21, Ser22, Gly23, Val26, Val28, Cys29, and Asp30 amide protons. Eleven of them were used as restraints in the structure calculation from which 7 were involved in the triple-stranded antiparallel $\beta$-sheet (Fig. 4). Two amide protons, Gly8 and Gly23, were involved in the formation of $\beta$-turns. The last two exchanging amide protons, Ile4 and Cys21, restrained as hydrogen-bonded with Leu19 (O) and Ile4 (O), respectively, characterize an additional $\beta$-bridge between residues Ile4 and Cys20, providing an additional stabilization to the N terminus. Such hydrogen bonds were also found for the $\omega$-GVIA (24), $\mu$-GS (27), $\kappa$-PVIIA (20), and $\delta$-TxVIA (12) conotoxins. Furthermore, three other hydrogen bonds, which were not experimentally imposed, participate in the stability of these two regions together with the C-terminal part in the models. These hydrogen bonds involve the $\delta$-hydroxyl proton (H9) of Hyp6 with the hydroxyl oxygen (O6) of Ser22, the Ser22 (H9) with Asp30 (O), and the Ser22 (HO) (corresponding to a slowly exchanging amide proton) with Hyp6 (O6). Each of them was found in 12, 16, and 7 of the 16 refined structures, respectively.

The four remaining slow exchanging amide protons (Lys5, Phe9, Ile14, and Leu15) are more rapidly exchanged than the preceding ones, except for Lys5 as shown in Fig. 5. The two first amide protons corresponding to the second and the last residues of the first loop are also slowly exchanged in $\omega$-MVIIA and $\omega$-SVIB conotoxins (25), but a posteriori no justification was given. The slow exchange of Ile14 and Leu15 amide protons, located in the disordered loop, could be due to the high hydrophobicity in this region (see “Discussion” below).

Description of the Three-dimensional Structure—$\delta$-EVIA is

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**FIG. 4.** Schematic representation of the consensus hydrogen bond network for the two models of $\delta$-EVIA with Leu14-Pro13 cis and trans conformation. Only the central portion of the molecule is shown. All depicted hydrogen bonds were restricted except between Ser22 (H9) and Ala30 (O) (see text). Observed interstrand NOEs are indicated by bars. Some canonical inter-strand NOEs, such as Cys29-Cys29, were not observed due to the spectral degeneracy.

**FIG. 5.** Summary of the sequential NOE connectivities, $^2J_{HN,HN}$ coupling constants, slowly exchanging backbone NH protons, and side chain rotamers for the Leu14-Pro13 trans conformation of $\delta$-EVIA. The sequential NOEs, $d_{NN}$, $d_{NN}$, $d_{NN}(i, i + 2)$, and $d_{NN}(i, i + 2)$ are indicated by bars between two residues and classified from strong to very weak according to the height of the filled bars. Open and filled circles indicate backbone NH protons that did not exchange with D2O in a TOCSY spectrum recorded after 36 h and in a TOCSY spectrum recorded after 54 h, respectively; open and filled squares indicate NH protons that are still observed in a NOESY spectrum recorded after 36 h and in a TOCSY spectrum recorded after 54 h, respectively. The side chain rotamers were determined from coupling constant, and NOE data are also indicated: $g^t$, $g^t$, and $t$ correspond to $g^t$ ($60^\circ$), $g^t$ ($60^\circ$), and $t$ ($180^\circ$), respectively.
energy function, and $F_{\text{b}}$ solution structure to the geometric average. The structural topology of Molscript (54) scheme of the backbone polypeptide folding of the closest neighbor with 3 hydrogen bonds for strand 1 and 3 are hydrogen-bonded from Ala 24 (HN) to Val 26 (O) and from Val28 (HN) to Ala30 (O), respectively (i.e. 2 residues). When calculated on the heavy atoms of the Cys 3–Cys 10 plus Cys 20–Asp 30 segments, the r.m.s.d. with respect to the mean coordinate positions is only equal to 0.59 Å because of the restriction of 14 $\chi_1$ angles (Fig. 5). The $\chi_1$ angles of all the Cys residues were restricted and thus contribute to stabilize the core of the molecule. As observed for the majority of the conotoxins, the Cys 10–Cys 20 disulfide bridge is a right-handed spiral motif, whereas the Cys 20–Cys 29 disulfide bridge is a left-handed spiral motif (52). The third disulfide bridge Cys 3–Cys 21 is less well defined due to the proximity of the undefined peripheral disulfide bond between Cys 3 and Cys 20 (Fig. 6 – SVIB (25) conotoxins).

In addition, one $\gamma$- and two $\beta$-turns were characterized in the models. Based on the analysis of the $\phi$ and $\psi$ angles (60), the models describe a type II $\beta$ 5–8 and a type I $\beta$ 20–23 turn (Fig. 7, B and C). Also, the hydrogen bond between Val 28 (H N) and Val 26 (O) defined a classic $\gamma$ 26–28 turn ($\gamma$-turn).

The buried side chains correspond to residues Ile 6, Hyp 6, Ser 11, Ser 22, Ala 24, Val 26, Val 28, and Ala 30 and the six cystines. When calculated on the heavy atoms of the Cys 8–Cys 10 plus Cys 20–Asp 30 segments, the r.m.s.d. with respect to the mean coordinate positions is only equal to 0.59 Å because of the restriction of 14 $\chi_1$ angles (Fig. 5). The $\chi_1$ angles of all the Cys residues were restricted and thus contribute to stabilize the core of the molecule. As observed for the majority of the conotoxins, the Cys 10–Cys 20 disulfide bridge is a right-handed spiral motif, whereas the Cys 20–Cys 29 disulfide bridge is a left-handed spiral motif (52). The third disulfide bridge Cys 3–Cys 21 is less well defined due to the proximity of the undefined N-terminal part. It appears to be represented by two conformations, with 7 structures displaying a right-handed hook conformation and 9 structures having a non-standard conformation. The ordering of the three disulfide bridges with the three-stranded antiparallel $\beta$-sheet form the so-called "inhibitor cystine-knot motif" described by Pallaghy et al. (29).

**NMR Analysis of the Pro**$^{13}$ → Ala $\delta$-EVIA—The P13A mutation restores a unique NMR spin system (given as Supplemental Material), and the sequential NOEs are characteristic of a trans peptide bond between Leu 12 and Ala 13. The chemical shifts are very close in frequency to the trans wild-type toxin; this observation indicates a conserved global solution structure. The chemical shift similarity between the Leu$^{12}$–Ala$^{13}$ peptide bond in P13A mutant and the Leu$^{12}$–Pro$^{13}$ trans conformer in wild-type conotoxin is particularly noteworthy for the H$^3$ proton of Ile$^4$, which has the highest difference with the cis isomer (|$\Delta$$\delta^H$| = 0.73 ppm). Most of the observed chemical shift

![Fig. 6. Stereoview of the NMR structures of conotoxin $\delta$-EVIA](image)

A, stereoview 16-structure superimposed for a minimum r.m.s.d. to the (C, Ca, and N) atoms from residues Cys$^8$ to Cys$^{10}$ and Cys$^{20}$ to Asp$^{30}$; B, Molscript (54) scheme of the backbone polypeptide folding of the closest solution structure to the geometric average. The structural topology of the triple-stranded antiparallel $\beta$-sheet is shown with the three disulfide bridges and the Pro$^{13}$ residue. N-term and C-term indicate N-terminal and C-terminal positions.

* $F_{\text{bond}}$ is the bond length deviation energy; $F_{\text{angle}}$ is the valence angle deviation energy; $F_{\text{impr}}$ deviation energy for the improper angles used to maintain the planarity of certain groups of atoms; $F_{\text{elec}}$ is the coulombic energy contribution to $F_{\text{total}}$. $F_{\text{elec}}$ in the Lennard-Jones van der Waals energy function, and $F_{\text{NMR}}$ is the experimental NOE function calculated using a force constant of 50 and 25 kcal mol$^{-1}$ Å$^{-1}$ in the case of the allhdg.pro and the CHARMM22 force field, respectively.

* In the case of the allhdg.pro force field, only the repulsion term is given.
Fig. 7. Structural data of the 16 NMR models of δ-EVIA as function of the protein sequence. A, distribution of the distance restraints classified from black to light gray into intraresidual, sequential, medium range, and long range restraints. B and C, values of the ϕ (B) and ψ (C) backbone dihedral angles describing δ-EVIA. D, r.m.s.d. from the average structure for the backbone atoms. E, order parameter S of the ϕ (shaded bars) and ψ (solid bars) angles; 0 = randomly distributed, and 1 = perfectly defined.
Fig. 8. Competition curves for $^{125}$I-labeled $\delta$-TxVIA binding inhibition by $\delta$-conotoxin EVIA and its P13A variant. Synaptosomes (65.5 $\mu$g/ml) were incubated for 60 min at 22 °C with 180 pm $^{125}$I-labeled $\delta$-TxVIA and increasing concentration of $\delta$-EVIA toxins. Nonspecific binding, determined in the presence of 1 $\mu$M of $\delta$-TxVIA, was subtracted. The amount of $^{125}$I-labeled $\delta$-TxVIA bound is expressed as the percentage of the maximal specific binding without additional toxin. Competition curves are fitted by the Hill equation (with a Hill coefficient of 1) to determine the IC$_{50}$ values (see “Experimental Procedures”). The IC$_{50}$ values are (in nM, average of three experiments) as follows: 475 ± 75 nM (open circle) for $\delta$-EVIA and 1,100 ± 140 nM (black circle) for P13A $\delta$-EVIA.

differences with the wild-type conotoxin are essentially located in the region of the Leu$^{12}$-Ala$^{13}$ dipeptide (given as Supplementary Material).

Biological Activity Pro$^{13}$→Ala $\delta$-EVIA—Competition curves for $^{125}$I-labeled $\delta$-TxVIA binding inhibition by $\delta$-EVIA and its P13A variant on rat brain synaptosomes revealed that both conotoxins, in a dose-dependent manner, fully displaced the radiolabeled conotoxin from its receptor site (Fig. 8). The calculated $K_i$ values were about two times higher for the P13A variant (1,100 ± 140 nM, n = 3) than for $\delta$-EVIA (475 ± 75 nM, n = 3). These results indicate that the P13A variant binds to receptor site 6 of voltage-dependent Na$^+$ channels, like $\delta$-EVIA, but with lower affinity. Consistent with this finding, a 2-fold reduction in the ED$_{50}$ for the excitotoxic activity of the P13A variant was observed (following intracerebroventricular injection to mice), when compared with natural and synthetic $\delta$-EVIA toxins. Competition curves for $^{125}$I-labeled $\delta$-EVIA and its P13A variant (Fig. 8) indicate that the mutant toxin keeps its selectivity for neuronal excitability in muscle fibers (data not shown). These results suggest that the folding process increases the $\delta$-EVIA-accessible hydrophobic area and (ii) the hydrophobic character of $\delta$-EVIA likely contributes to the low yield folding process. The detergent-assisted oxidative folding process recently described by De la Cruz et al. (63) could therefore improve the $\delta$-EVIA folding yield. In this respect, we have observed a higher amount of folded P13A $\delta$-EVIA variant compared with that of the wild-type $\delta$-EVIA, suggesting a folding effect of Pro$^{13}$.

Comparison to Other Toxins—The three-dimensional structure of $\delta$-EVIA consists of a $\beta$-hairpin, involving residues Gly$^{23}$ to Asp$^{31}$ (Fig. 6B), and several turns. Such a conformation, stabilized by a number of disulfide bridges and hydrogen bonds, is adopted by several other small proteins (Fig. 1) coming from phylogenetically divergent species including spiders and cone shells but also fungi or plants (29). The r.m.s.d. of the three disulfide bridges with or without loops 1 and 3 was calculated for these different peptides relative to the $\delta$-EVIA (Table III). Despite the relatively low amino acid sequence homology (Fig. 1), it reveals a strong correlation between this four-loop family and the scaffold of the peptide backbone. In particular, the loop 1, stabilized by a $\beta$-turn, and the disulfide bridges appear very close. The loop 3 is constituted of a type I ($\kappa$-conotoxin PVIIA, huwentoxin-I, and $\delta$-EVIA), type I' ($\omega$-conotoxin GVIA, $\omega$-conotoxin MVIIA, and $\mu$-agatoxin) $\beta$-turn.

The major structural differences between these molecules result from the different sizes of the loops 2 and 4 (Fig. 1). The resulting secondary structure in loop 2, except for peptides with nine amino acids, is a type I $\beta$-turn in the majority of the cases. $\delta$-EVIA, which is the first structurally resolved conotoxin with a large second loop, displays a disordered loop from Ser 11 to C with 180 pm (Fig. 9), with a similar hydrogen bond network, constraining in this case the hairpin turn to a $\gamma$-turn.

**DISCUSSION**

**Chemical Synthesis and Folding**—As shown in Fig. 2A, the retention time ($R_t$) for natively folded and biologically active $\delta$-EVIA was significantly longer than that of the linear, fully reduced peptide, in contrast to the observed $R_t$ for the natively folded hydrophobic $\omega$- or $\kappa$-conotoxin (20, 61). This observation suggests (i) that the folding process increases the $\delta$-EVIA-accessible hydrophobic area and (ii) the hydrophobic character of $\delta$-EVIA likely contributes to the low yield folding process. The detergent-assisted oxidative folding process recently described by De la Cruz et al. (63) could therefore improve the $\delta$-EVIA folding yield. In this respect, we have observed a higher amount of folded P13A $\delta$-EVIA variant compared with that of the wild-type $\delta$-EVIA, suggesting a folding effect of Pro$^{13}$.

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**The cis/trans Isomerism of Pro$^{13}$ in $\delta$-EVIA**—Considering the NMR ensemble of $\delta$-EVIA, the great disorder of loop 2 could correlate with the Leu$^{12}$-Pro$^{13}$ peptide bond cis/trans isomerism. The absence of exchange peaks between the two conformers indicates, however, a very slow exchange rate relative to the NMR time scales. For instance, such a feature was already observed for the Lqh-8/6, a toxin from a scorpion venom (64), or for a cyclic peptide (65). Several statistical studies (66, 67) tentatively suggested possible correlations between the steric and electronic properties of the residues surrounding the proline and the stability of the cis isomer, by examination of peptide bonds from the Protein Data Bank (68). According to these studies, a cis peptide bond in a sequence Leu$^{12}$-Pro$^{13}$-
TABLE III

The closest structure to the geometric average was used for the calculation.

| Protein data bank entry | 6 Cys | | 6 Cys + loop 1, | | 6 Cys + loop 1 and 3, |
|-------------------------|-------| | Backbone^b | Heavy^d | Backbone^b | Backbone^b |
| µ-GS^d | 1AG7 | | 0.53 | 0.58 | 0.58 |
| δ-TxVIA^d | 11FU3 | | 0.25 | 0.31 | 0.35 | 0.43 |
| κ-PVIIA^d | 1KCP | | 0.30 | 0.30 | 0.30 | 0.32 |
| ω-GVIA^d | 10MC | | 0.36 | 0.39 | 0.37 |
| ω-MVIIA^d | 1KW5 | | 0.36 | 0.45 | 0.48 | 0.50 |
| ω-MVIIIC | 1OMN | | 0.45 | 0.49 | 0.50 | 0.56 |
| Huwentoxin I | 1QK6 | | 0.39 | 0.38 | 0.41 | 0.44 |
| ACTX Hv1 | 1AXH | | 0.72 | 0.82 | 0.72 |
| µ-agatoxin I | 1KCP | | 0.36 | 0.37 | 0.36 | 0.43 |

^a r.m.s.d. was calculated when loop 3 contain 3 residues, as for the conotoxin δ-EVIA.
^b r.m.s.d. are calculated for atoms (C, N, and Ca).
^c r.m.s.d. are calculated for atoms (C, N, Ca, Cβ, and Sγ).
^d Conotoxin µ-GS from Hill et al. (27).
^e Conotoxin δ-TxVIA from Kohn et al. (12).
^f Conotoxin κ-PVIIA from Savarin et al. (20).
^g Conotoxin ω-GVIA from Davis et al. (24).
^h Conotoxin ω-MVIIA from Atkinson et al. (88).
^i Conotoxin ω-MVIIIC from Farr-Jones et al. (22).
^j Conotoxin ω-SVIB from Nielsen et al. (25).
^k Neurotoxin huwentoxin-I from the venom of the spider Selenocosmia huwena (14).
^l Versutoxin (δ-tracotoxin-Hv1) from the venom of the Australian Blue Mountains funnel web spider, Hadronyche versuta (30).
^m µ-agatoxin-I from the venom of the American funnel web spider, Agelenopsis aperta (62).

Fig. 9. Solution structures of some four-loop conotoxins. A, GVIAM, B, MVIIA; C, GS; D, δ-EVIA. The structures are superimposed to the backbone (C, Ca, and N) atoms of the well defined regions encompassing residues 1–8 and 15–26 for GVIAM, 1–8 and 15–25 for MVIIA, 2–20 and 25–31 for GS, and 3–10 and 20–29 for δ-EVIA. The structures are shown with the same orientation. Nt, and Ct indicate N-terminal and C-terminal positions.

Ile14 of δ-EVIA would not be frequent due to the presence of branched aliphatic residues preceding and following the proline (67). In δ-EVIA, amide protons of Ile14 and Leu15 slowly exchanged with D2O. Unfortunately, the H2 of Ile14 is degenerate in the trans conformer with the intra-residual CαH/H2 cross-peak of Gly23, so we cannot conclude about a similar exchange for this conformer (Fig. 4). Two additional weak NOEs, Leu12 (CαH)–Ile14 (H3), and Leu12 (CαH)–Ile14 (CαH), are found for the cis isomer relative to the trans isomer. Calculation of structures for the cis conformer induced the formation of a non-hydrogen-bonded type VIb-3 β-turn involving Ser11 to Ile14 (68, 67), and a significant decrease of the disorder for the φ and ψ angles of Leu15 (−122 ± 9 and 168 ± 8°) and Pro13 (−65 ± 8 and 157 ± 11°) (see Fig. 7, B and C, for comparison with the trans conformer). However, the presence of this β-turn is not sufficient to render ordered the entire loop 2. The slow exchange observed for the H2 of Ile14 and Leu15 would then originate in the high local hydrobobicity (Leu12–Pro13–Ile14–Leu15) rather than the formation of hydrogen bonds.

As a consequence of the cis/trans isomerism of Pro13, the Δ8α-δ is not uniformly distributed along the sequence (data given as Supplemental Material). Ile14 HN is the most affected as well as GN of Asn17, Gly38, and Leu19 located in front of Pro13 in the loop 2. In addition to the backbone protons, the Δ5α-δ of the side chain protons of Cys46, Ser11, Leu12, Pro13, and especially Asn17 residues are relatively high (0.10 to 0.30 ppm). The comparison of the structure of the two conformers in this region show more...
hydrogen bonds for the cis conformer involving the side chain of Asn
the systematic study of the Pro
13 would slow a folding step as observed recently in the case of
H9004
with Ser11 (O

Interaction with the Na
+ Channel—As for µO- and δ-conotoxins targeting sodium channels, δ-EVIA displays a high degree of hydrophobicity and a small number of charges (Figs. 1 and 10). This fact was recently discussed for the NMR structure of δ-TxVIA (12), a 27-mer peptide that competes with δ-EVIA for the same receptor site 6 on the Na
+ channels (1) (Fig. 8). In particular, a characteristic of δ-TxVIA is to bind strongly to the receptor site 6 of Na
+ channels of mammals without modifying the spontaneous inactivation period, while it delays significantly this period in mollusks (75–77). A hydrophobic cluster is revealed on the surface δ-TxVIA including, in particular, Met
6, Leu
11, Leu
12, Tyr
20, Val
23, Leu
24, and Val
25. Among these seven amino acids, five are homologously conserved in δ-EVIA (Phe
6, Pro
13, Ile
14, Val
23, and Val
25) (Fig. 1). The hydrophobic patch revealed on the surface δ-TxVIA is even extended by Ile
4, Tyr
1, and Leu
12 as shown in Fig. 10. The positively charged amino acids are located at the beginning of the loop 1 (Lys
5) and in the loop 2 (Lys
16), whereas the negatively charged amino acids are located at the opposite N and C termini (Asp
1, Asp
2, and Asp
31), making δ-EVIA quite amphipathic.

The apparent disorder observed in the loop 2 of δ-EVIA, which includes Leu
12, Pro
13, and Ile
14 participating in the hydrophobic patch, could be important for receptor binding as it was suggested for the αα-conotoxin PIVA (78), which has a flexible 3–11 loop and a rigid 14–23 loop. Such a flexible region has already been proposed to be stabilized upon interaction with the receptor (79–81). In particular, a cis/trans proline isomerization was supposed to occur in the free form of the human acidic fibroblast growth factor (80), but when complexed to the succrose octasulfate, the slow conformational motion corresponding to this isomerization is thwarted by this binding leading to a more rigid conformation. Also, conformational changes of small peptidic ligands upon binding have been observed in other systems (82–85). It can be presumed that the loop 2, which is quite flexible in solution, might allow structural rearrangements to accommodate its binding, whereas the rigid core of δ-EVIA serves as a delivery scaffold, with possible direct additional interactions with the receptor.

Due to the peculiar apparent low structural order and the hydrophobic character of the loop 2 from Leu
12 to Leu
15, and to the specific cis/trans isomerism of the Leu
12–Pro
13 peptide bond, another question arose about a possible biological and functional interest of such a structural singularity. The fact is that a trans, or a cis, amino-acetyl proline peptide bond does not offer at all the same local binding abilities. In the trans conformation, the peptide carbonyl (a potent hydrogen bond acceptor) of the preceding residue, is trans to the hydrophobic cyclic side chain of the proline amino acid (i.e. on the two opposite sides of the main peptide chain). In the cis conformer, the carbonyl of the peptide bond is cis to the hydrophobic proline side chain (i.e. on the same side of the main peptide chain). In terms of molecular interactions between the neurotoxin and the receptor, it could make strong and significant differences. Such differences have been demonstrated, for instance, in a study related to an opioid peptide (86) in which increasing the proportion of the cis conformation of a prolyl peptide bond affects its affinity for the receptor. If we hypothesize the requirement of a cis amino-acetyl proline peptide motif for a tight molecular contact within the neurotoxin-receptor complex, the small but significant change in activity of the P13A mutant δ-EVIA could be interpreted as a deficiency in presenting on the same side of the main peptide chain both a hydrophobic cyclic proline side chain and a peptide bond carbonyl, a potent acceptor of a hydrogen bond. Further explorations and probing of this hypothesis are currently in progress using peptido-mimics simulating either cis or trans amino-acetyl proline as well as using other single-point mutations in the regions expected to be important for the toxin-receptor interactions.

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NMR Structures of δ-Conotoxins EVIA

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NMR Solution Structures of δ-Conotoxin EVIA from Conus ermineus That Selectively Acts on Vertebrate Neuronal Na⁺ Channels
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