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Characterization of conantokin *Rl*-A: molecular phylogeny as structure/function study

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A multidisciplinary strategy for discovery of new *Conus* venom peptides combines molecular genetics and phylogenetics with peptide chemistry and neuropharmacology. Here we describe application of this approach to the conantokin family of conopeptides targeting NMDA receptors. A new conantokin from *Conus rolani*, Con*Rl*-A, was identified using molecular phylogeny and subsequently synthesized and functionally characterized. Con*Rl*-A is a 24-residue peptide containing three γ-carboxyglutamic acid residues with a number of unique sequence features compared to conantokins previously characterized. The HPLC elution of Con*Rl*-A suggested that this peptide exists as two distinct, slowly exchanging conformers. Con*Rl*-A is predominantly helical (estimated helicity of 50%), both in the presence and absence of Ca^{++}. The order of potency for blocking the four NMDA receptor subtypes by Con*Rl*-A was NR2B>NR2D>NR2A>NR2C. This peptide has a greater discrimination between NR2B and NR2C than any other ligand reported so far. In summary, Con*Rl*-A is a new member of the conantokin family that expands our understanding of structure/function of this group of peptidic ligands targeted to NMDA receptors. Thus, incorporating phylogeny in the discovery of novel ligands for the given family of ion channels or receptors is an efficient means of exploring the megadiverse group of peptides from the genus *Conus*.

Keywords: conantokin; molecular phylogeny; conformational interconversion; helical peptide; electrophysiology

Introduction

The conantokins are a family of peptides found in *Conus* venoms that are antagonists of NMDA receptors [1–3]. Nine conantokin peptides from seven *Conus* species have been documented in the literature: Conantokin-*G* (Con*G*) from *Conus geographus* [4], Con*T* from *Conus tulipa* [5], Con*R* from *Conus radiatus* [6], Con*L* from *Conus lynceus* [7], Con*Pr*-A, *Pr*-B, *Pr*-C from *Conus parius* [8], Con*P* from *Conus purpurascens* [9], and Con*Br* from *Conus brettinghami* [10]. These peptides comprise the only group of peptidic ligands from animal venoms presently known to target NMDA receptors.

We have recently used a multidisciplinary strategy for discovery from *Conus* venoms; the general approach combines peptide chemistry (or recombinant expression), molecular genetics, and phylogenetics [11,12]. This approach has been used successfully for developing ligands targeted to various nicotinic receptors, resulting in the availability of selective pharmacological agents for diverse molecular isoforms of the nicotinic receptor family [13]. This general approach has not previously been systematically applied to the conantokin family. In this report, we have initiated the concerted discovery strategy for identifying novel conantokin peptides that can more efficiently allow us to assess structure/function relationships in these peptides.

A recently characterized conantokin peptide, Con*Br* from *C. brettinghami*, had a number of divergent characteristics compared to previously described conantokins [10]. We used a phylogenetic approach to find related natural peptides; our goal was a broad assessment of the sequence elements in Con*Br* that confer its particular selectivity profile. Because *Conus rolani*, a species not previously investigated, is a member of the same clade as *C. brettinghami,* we obtained the sequence of a conantokin peptide from *C. rolani* with homology to Con*Br*, which we designate Con*Rl*-A. The characterization of Con*Rl*-A and a comparison to Con*Br* are detailed below. These studies illustrate that molecular phylogeny can be used to quickly identify *Conus* species that have evolved conantokin peptides useful for targeted structure/function insights. We also describe an unexpected and novel conformational property of Con*Rl*-A that emerged from its characterization.

Experimental Procedures

Phylogenetic Analysis

Phylogenetic analysis was performed using combined sequences of 12S, 16S, and COI mitochondrial gene regions for each species, as previously described [14]. The lengths of the sequences used for analysis were 585–590, 546–548, and 594–709 base pairs for 12S, 16S, and COI, respectively. The sequences were aligned and neighbor-joining trees generated using ClustalX version 2.0.9 [15] with standard default options.

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Abbreviations used: *ConRl-A, conantokinRl-A; Gla, γ, γ-carboxyglutamate; NMDA, N-methyl-D-aspartate.*

Preparation of Genomic DNA and Characterization of Clones Encoding Con*RI*-A

Genomic DNA was prepared from 20 mg *C. rolani* tissue using the Gentra PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's standard protocol. A 10-ng *C. rolani* genomic DNA sample was used as a template for polymerase chain reaction (PCR) with oligonucleotides corresponding to conserved regions of the signal sequence (5′ GCG ATG CAA CTG TAC ACG TAT CTG) and 3′ UTR sequence (5′ AAT AAA CAT GAA AGA TTT GGG GAA) of conantokin prepropeptides. The resulting PCR product was purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's suggested protocol. The eluted DNA fragment was annealed to pNEB206A vector using the USER Friendly Cloning kit (New England BioLabs, Inc., Bever1y, MA, USA) following manufacturer's suggested protocol and the resulting product transformed into DH5a competent cells. The nucleic acid sequences of the resulting conantokin toxin-encoding clones were determined according to the standard protocol for automated sequencing.

Peptide Synthesis

Con*RI*-A was synthesized using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) protected amino acids on ABI model 430A peptide synthesizer at the DNA/Peptide Core Synthesis Facility, University of Utah. The side chains of Asp, Glu, and Thr residues were protected with *t*-Bu (*tert*-butyl) group (Novabiochem, EMD Chemicals Inc., Gibbstown, NJ, USA). The side chains of Lys, Gln, and Arg residues were protected with Boc (*t*-butoxycarbonyl), Trt (trityl), and Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) groups (Novabiochem, EMD Chemicals Inc., Gibbstown, NJ, USA), respectively. The side chains of Gla were protected with tBu groups (Chem-Impex International, Inc. Wood Dale, IL, USA). The peptide was constructed on proline-containing preloaded resin, H-Pro-2-ClTrt resin (Novabiochem, EMD Chemicals Inc., Gibbstown, NJ, USA), using standard DCC/HOBt chemistry. The peptide was cleaved from the resin and simultaneous deprotection of side chains was achieved by agitating 20 mg of the resin in 1 ml of reagent K [TFA/phenol/thioanisole/water/ethanedithiol (82.5 : 5 : 5 : 5 : 2.5)] at room temperature. The mixture was filtered, precipitated using methyl-*tert*-butyl ether (MTBE) and spun by centrifugation. The pellet was repeatedly washed with cold MTBE and purified over a Vydac C_{18} column (10 mm $\times$ 250 mm, 5 µm particle size) using ACN/H_2O/TFA solvent system. The flow rate was maintained at 3 ml min^{-1} following a linear gradient of 10–40% ACN over 40 min and the fractions were detected at 220 nm. The purity of Con*RI*-A was confirmed using mass spectrometry. Mass spectra were obtained using a Micromass Quattro II mass spectrometer at the Mass Spectrometry and Proteomics Core Facility of the University of Utah.

Peptide Conformation Analysis

Conformational analysis of the peptide was achieved using C_{18} analytical RP-HPLC over the range of temperature from 4 to 45 °C. The desired temperature of the column was achieved by keeping it in a water bath over a period of time. Peptide (5 nmol) was dissolved in 50% ACN containing 0.01% TFA and incubated at room temperature for 5 min before injecting into HPLC. The peptide was eluted using a linear gradient of 20–50% Buffer B (90% ACN containing 0.1% TFA) over 35 min. Early- and late-eluting fractions were separated and incubated at 4 °C prior to the reapplication to column. Distinct fractions were separately run again under identical chromatographic conditions. Area under early- and late-eluting conformers was obtained by integrating the chromatograms using data analysis software provided by the manufacturer (Waters Corporation, Franklin, MA, USA).

Circular Dichrosim Spectroscopy

Far-UV CD spectra were recorded with an AVIV Model 62D spectropolarimeter, using a bandwidth of 1 nm, a step size of 1 nm, and an average time of 0.5 s. Peptide was dissolved in 10 mM HEPES buffer, pH 7.0, with or without 2 mM $CaCl_2$. All measurements were taken at room temperature, over 250–190 nm wavelength range, using cell of 0.1 cm path length. Peptide concentration was 100 µM, as determined by the absorbance at 280 nm. Five independent spectra were collected and averaged for each sample. The contribution of buffer to the CD signal was eliminated by subtracting the peptide CD signal from that of the buffer alone CD signal. All spectral intensities were expressed as mean residue ellipticities using the equation reported elsewhere [8]. Molar ellipticity of −35 086.66 degrees cm^2 $dmol^{-1}$ was estimated to be a perfect α-helix (100% α-helix). The percent helical conformation was calculated by assuming a linear relationship in comparison with 100% α-helix.

Heterologous Expression of NMDA Receptors in Xenopus Oocytes

The rat NMDA receptor clones used were NR2A, NR2B, NR2C, NR2D, and NR1-2b; GenBank numbers were AF001423, U11419, U08259, U08260, and U08264, respectively. We previously used the NR1-3b splice variant to express recombinant NMDA receptors [8–10]; in this study, we have used NR1-2b splice variant due to its robust expression in the central nervous system [16]. To confirm this splice variant difference does not affect the potency or order of conantokin selectivity toward NMDA receptor complexes, we also assayed conantokins for potency on differing combinations of NR2(A–D) and NR1-3b and found no difference between splice variants (data not shown). All of the expression clones were driven by a T7 promoter and were used to make capped RNA (cRNA) for injection into the oocytes of *Xenopus laevis* frogs. cRNA was prepared *in vitro* using Ambion RNA transcription kits (Ambion, Inc., Austin, TX, USA) according to manufacturer's protocols. To express NMDA receptors, 2–5 ng of cRNA for each subunit was injected per oocyte. Oocytes were maintained at 18 °C in ND96 solution (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.2–7.5) containing antibiotics (septra, amikacin, pen/strep). All voltage-clamp electrophysiology was done using oocytes 1–6 days postinjection.

Two-Electrode Voltage-Clamp Electrophysiology

All oocytes were voltage clamped at −70 mV at room temperature. Oocytes were gravity perfused with Mg^{2+}-free ND96 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.2–7.5). Mg^{2+} was not included in the ND96 buffer because Mg^{2+} blocks NMDA receptors at the voltage potential used to clamp oocytes (−70 mV). To reduce nonspecific absorption of peptide, bovine serum albumin was added to ND96 buffer at a final concentration of 0.1 mg/ml. To elicit current from oocytes expressing NMDA

receptors, 1-s pulses of gravity-perfused agonist solution were administered at intervals of 60, 90, or 120 s, depending on the rate of receptor recovery from desensitization. Agonist solution comprised of glutamate and co-agonist glycine suspended in Mg^{2+}-free ND96 buffer at final concentrations of 200 and 20 μM, respectively. Buffer was perfused continuously over the oocytes between agonist pulses, except during equilibration periods. During equilibration period, buffer flow was halted for 5 min to create a static bath for application of either peptide (suspended in ND96 buffer at various concentrations) or control solution (ND96 buffer alone). The length of equilibration period was equal to or greater than the time necessary to achieve maximal current inhibition at a given concentration. The effect of a peptide on NMDA receptor-mediated current was determined by measuring the amplitude of the first agonist-elicited current pulse immediately following the equilibration period as a percentage of the amplitude of the baseline current (agonist-elicited current immediately preceding equilibration period). Data acquisition was automated by a virtual instrument made by Doju Yoshikami of the University of Utah. Concentration–response curves were generated using Prism software (GraphPad Software, Inc., San Diego, CA, USA), using the following equation:

$$\%\ \text{Response} = 100/\{1 + [(\text{peptide})/IC_{50}]^{n_H}\}$$

where n_H is the Hill coefficient and IC_{50} is the concentration of peptide causing half-maximal block.

Results

Phylogenetic Analysis

C. brettinghami is a member of the so-called *Conus sulcatus* complex, a set of deepwater *Conus* species all believed to belong to the *Asprella* clade, with *C. sulcatus* as the type species (*Asprella* is regarded as a subgenus of *Conus* in most, but not all, taxonomic works). One species in this branch of *Conus* is *C. rolani*; since we had collected this species, genomic DNA clones from *C. rolani* were analyzed. *C. rolani* is part of a group of deepwater *Conus* (known as the *Asprella* clade) from the tropical Western Pacific. There is little known regarding the biology of these species; they are typically collected at depths of 100–300 m by fishermen who use deepwater tangle nets. The presence of conantokin peptides that target NMDA receptors in the venom of *C. rolani* and other species in the *Asprella* clade [10] is suggestive (but does not establish) that these species are likely to envenomate fish. The phylogenetic relationship of *C. rolani* to *C. brettinghami*, the source of Con*Br*, was determined by the analysis of the combined sequences of 12S, 16S, and COI mitochondrial gene regions and is shown in Figure 1; all *Conus* species that have yielded other conantokin sequences are shown in the figure.

Analysis of a Clone Encoding Con*Rl*-A from *C. rolani*

Prior work on α-conopeptides targeted at nicotinic acetylcholine receptors revealed that the analysis of *Conus* species closely related to each other has potential for identifying homologous peptides with a sufficient level of amino acid sequence divergence to gain structure/function insights. Con*Br*, a conantokin peptide from *C. brettinghami*, had some intriguing functional features. To assess structure/function relationships for Con*Br*, we wanted to identify a homologous peptide with significant amino acid sequence differences.

A *C. rolani* clone encoding a peptide precursor with a high degree of homology to previously identified conantokin precursors [9] was identified, as described under experimental procedures, and designated Con*Rl*-A. The sequences of the predicted corresponding open reading frames of Con*Rl*-A and Con*Br* precursors are shown in Figure 2. The striking similarity of the sequences encoding Con*Rl*-A and Con*Br* establishes that Con*Rl*-A is a member of the conantokin superfamily. A notable feature of Con*Rl*-A is that the predicted amino acid at position 1 of the mature peptide (alanine) differs from that of the canonical *N*-terminal glycine (or aspartate) at position 1 in all previously identified conantokins.

A comparison of the predicted mature sequences of Con*Rl*-A and Con*Br* after the predicted posttranslational modification is shown in Figure 3. In a similar manner to Con*Br*, the glutamate residues that are known to be γ-carboxylated in members of the conantokin family purified from venom (i.e. glutamates at positions 3–4 and each Glu that occurs in a spacing of every 3–4 amino acids thereafter, such that they align on a single side of the helical peptide) are predicted to be posttranslationally modified to γ-carboxyglutamate in Con*Rl*-A. However, the glutamate at position 11, which immediately follows the carboxyglutamate predicted at position 10, is not predicted to undergo posttranslational modification.

Characterization of Con*Rl*-A

In order to characterize the deduced conantokin from *C. rolani*, chemical synthesis of the predicted sequence as shown in Figure 3 was carried out as described in the Experimental Procedures section. The observed mass of the peptide, as revealed from ESI-MS, is 3042.5, 2997.2, 2953.9, and 2909.7. Occurrence of multiple peaks in ESI-MS corresponds to the loss of carboxyl groups, a phenomenon previously observed in Gla containing *Conus* peptides [9,17].

The elution profile of Con*Rl*-A is unusual with two peaks separated by a broad trough. This apparent chromatographic heterogeneity was assessed by eluting the peptide from the HPLC column at different temperatures. Figure 4(a) shows the effect of temperature on chromatography of the peptide. At low (4 °C) temperature, Con*Rl*-A yields two well-resolved peaks; as the temperature increases (22 °C), the trough between two peaks increases and at high temperature (45 °C), a single homogeneous peak is found. These observations clearly suggest that Con*Rl*-A exists as two distinct, slow-exchanging conformers in solution. The broad trough in the chromatogram at 22 °C can be attributed to interconversion of the two conformers; at higher temperature, conformational interconversion may be sufficiently rapid that the C_{18} column cannot resolve the two conformers resulting in a single peak in the elution profile. Under these conditions, the conformational interconversion is presumably faster than the chromatographic resolution.

The conformational equilibrium between two conformers in Con*Rl*-A was probed at 4 °C. Figure 4(b) shows elution profiles of the fractions isolated from the chromatogram of Con*Rl*-A. The early-eluting hydrophilic component and late-eluting hydrophobic fraction of Con*Rl*-A were separately collected and reinjected into the column at 4 °C. Both components were incubated at 4 °C for 20 min prior to reinjection. Reinjection of either of the fraction results in reappearance of both the HPLC peaks, and a ratio between two peaks of ~2.6:1 was obtained in both cases. This observation is consistent with the presence of

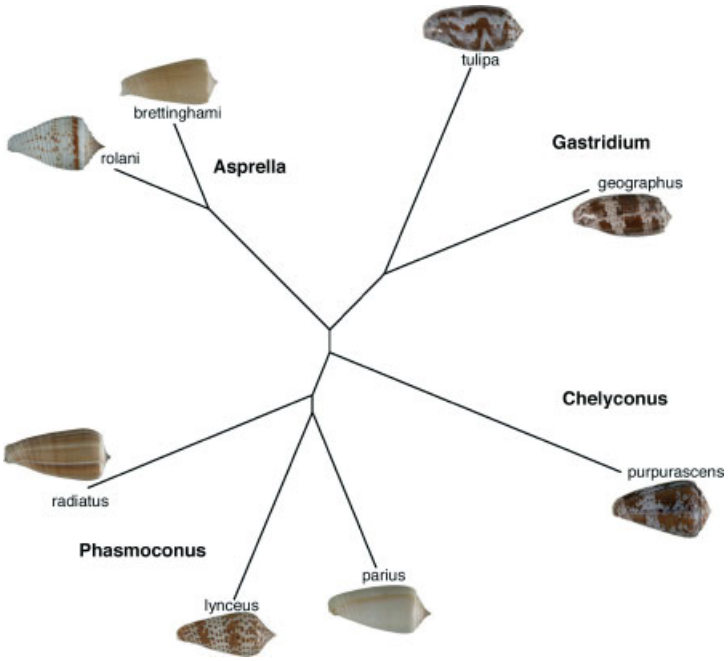

Figure 1. Phylogenetic analysis using combined 12S, 16S and COI sequences showing all *Conus* species from which conantokin peptides have been identified. *Conus rolani* was collected from the Philippines coast using tangle nets.

two interconvertible conformers of Con*Rl*-A in solution, which can be separated upon the appropriate chromatographic conditions at 4 °C. Since the ratio of major and minor conformers of Con*Rl*-A is independent of peptide concentration, it is likely that the chromatographic heterogeneity is due to intramolecular changes (and not, for instance, dimerization). For example, proline-containing peptides exhibiting peak splitting in an RP-HPLC column have been documented in the literature [18–23].

Circular Dichroism Spectroscophy

Specific bands in the far-UV CD spectra have been widely used for rapid determination of backbone conformation of proteins or peptides. Secondary structural information derived from CD spectra can represent qualitatively the overall fold of molecule and such studies have been extensively employed in the characterization of conantokins. Figure 5 shows the CD spectra of Con*Rl*-A. Con*Rl*-A is predominantly alpha helical and the estimated helicity is 50%, which is essentially same in the presence and absence of the divalent cation calcium. Molecular modeling of homologous Con*Br* indicates the presence of helical structure between 2 and 12 residues of the molecule [10]. Con*Rl*-A contains eight identical residues compared to that of Con*Br* among structured 2–12 peptide segment. The observed percentage of helicity for Con*Rl*-A and its homology to Con*Br* strongly suggest that both the peptides may adopt a similar helical conformation. Surprisingly, Con*Br* results in a single homogenous peak in RP-HPLC elution profile at room temperature, whereas Con*Rl*-A exhibits pronounced asymmetry.

Functional Characterization of Con*Rl*-A

Due to the high degree of homology of Con*Rl*-A with Con*Br* (a known antagonist of the NR2B and NR2D NMDA receptor subtypes), [10], we assessed the functional antagonism of Con*Rl*-A on the four NMDA receptor subtypes with different NR2 subunits (NR2A–NR2D). These were heterologously expressed in *Xenopus* oocytes in combination with the NR1-2b splice variant. Using two-electrode voltage-clamp electrophysiology, we determined the function of Con*Rl*-A by applying the peptide to oocytes and allowing for a 5-min equilibration period in a static bath. The effect of the peptide was measured by normalizing the agonist-elicited response following equilibration period to baseline response in the absence of peptide. Concentration–response curves for Con*Rl*-A on each of the four NMDA receptor subtypes are shown in Figure 6. The order of potency for Con*Rl*-A was NR2B>NR2D>NR2A>NR2C. Interestingly, although nine amino acids differ between the corresponding positions of Con*Rl*-A and Con*Br* (Figure 3(b)), the order of NR2 subunit selectivity was identical for the two peptides, and the degree of potency on each subunit was similar (Table 1).

Table 1. Comparison of approximate IC_{50} (μM) values of Con*Rl*-A and Con*Br*

Peptide	NMDA receptor subtypes NR2(A–D)			
	NR2A IC_{50}	NR2B IC_{50}	NR2C IC_{50}	NR2D IC_{50}
Con*Br*	0.68	0.14	4.9	0.31
Con*Rl*-A	2.1	0.11	6.1	0.48

Discussion

We have characterized a novel conantokin, Con*Rl*-A from *C. rolani*, a *Conus* species that belongs to the *Asprella* clade. We previously characterized Con*Br* from *C. brettinghami*, another species in *Asprella*; Con*Br* had a pharmacological profile for the different NMDA receptor subtypes distinct from previously characterized members of the conantokin family. The rationale for focusing on species in the same clade of *Conus* as *C. brettinghami* was to gain structure/function insights using a phylogenetic approach

Figure 2. Precursor sequences of *ConRl-A* and *ConBr*. The arrows indicate the signal sequence/propeptide and propeptide/mature conantokin boundaries.

(a)

Conantokin	Nucleotide and Predicted Amino Acid Sequences
Con*Rl*-A	A D E E Y L K F I E E Q R K Q G K L D P T K F P GCCGATGAAGAATATTTAAAGTTTATAGAGGAACAACGCAAACAAGGAAAACTGGATCCGACAAAATTCCCG
Con*Br*	G D E E Y S K F I E R E R E A G R L D L S K F P GGCGATGAAGAATATTCAAAGTTTATAGAGAGAGAACGCGAAGCAGGAAGACTGGATTTGTCAAAATTCCCG

(b)

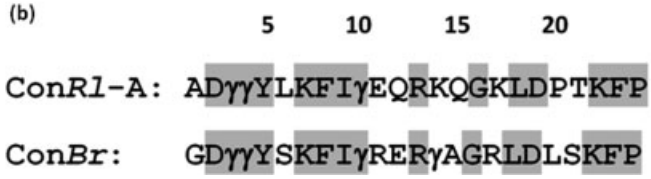

Figure 3. cDNA sequences encoding conantokins. (a) Nucleotide and predicted amino acid sequences for Con*Rl*-A and Con*Br*. (b) Predicted mature peptides. Posttranslational modifications (γ: Gla) were assumed based on consistent occurrence of Gla in naturally isolated conantokins and amino acid spacing between Gla residues.

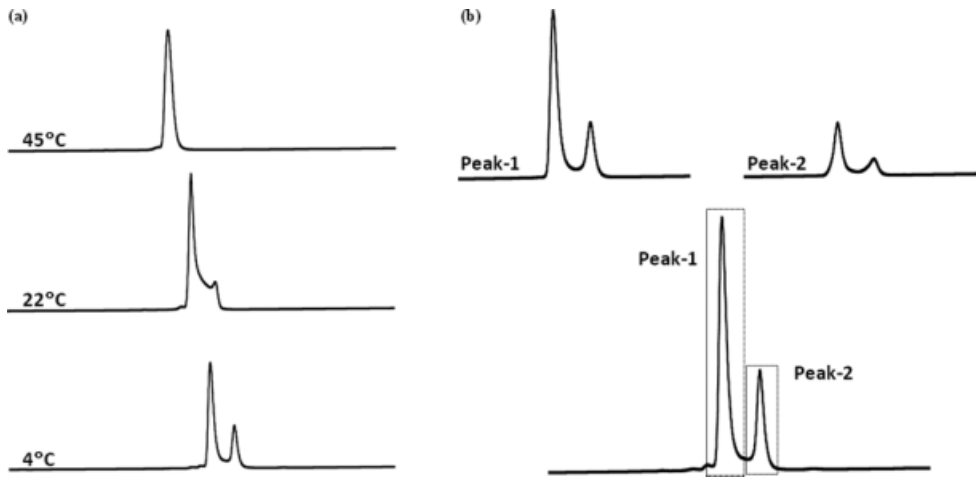

Figure 4. Conformational analysis of Con*Rl*-A using RP-HPLC. (a) Effect of temperature on chromatography of the peptide at indicated temperature. The temperature of the C_{18} analytical column was maintained either by incubating in ice (or) water bath of the desired temperature. (b) Elution profiles obtained by reinjection of the fractions corresponding to Peak-1 and Peak-2 of the peptide at 4 °C. Both major and minor conformers are represented from each peak.

(instead of, for example, using the standard 'alanine walk'). Analysis of a *C. rolani* library revealed a conantokin peptide that differed in 9 out of 24 amino acids in its primary sequence from Con*Br*. The conserved sequence features included the Tyr 5 residue previously shown to confer a relatively high affinity for the NR2D subtype [10].

The precursor sequences of Con*Br* and Con*Rl*-A are typical in the pattern of divergence seen in other conopeptide superfamilies. Thus, the mature toxin sequences are both 24 AA, but the initially translated open reading frame is 103 amino acids. There is a signal sequence of 21 AA that is completely identical in the two predicted precursors. A relatively large propeptide region (58 AA) is found between the signal sequence and the mature conantokin peptide, with a typical proteolytic cleavage site at the *C*-terminus indicated by the arrow in Figure 2. There are only two divergent AA positions in the 58 amino acid propeptide region (divergence =

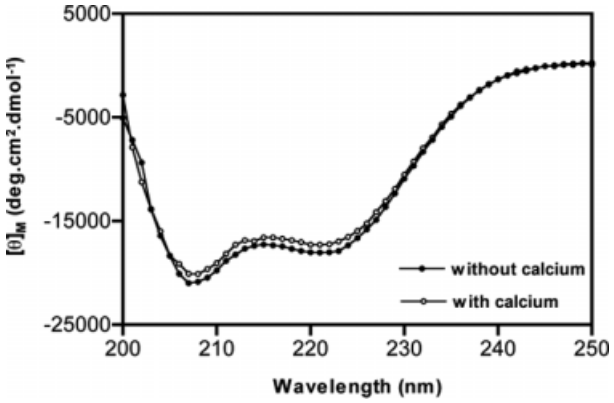

Figure 5. Circular dichroism spectra of Con*Rl*-A. Spectra were recorded by dissolving peptide in 10 mM HEPES buffer at pH 7.0, containing either 2 mM $CaCl_2$ (or) absence of $CaCl_2$ ($n = 5$ scans per condition). Percentage of helicity of peptide in the presence (or) absence of calcium was found to be 50%.

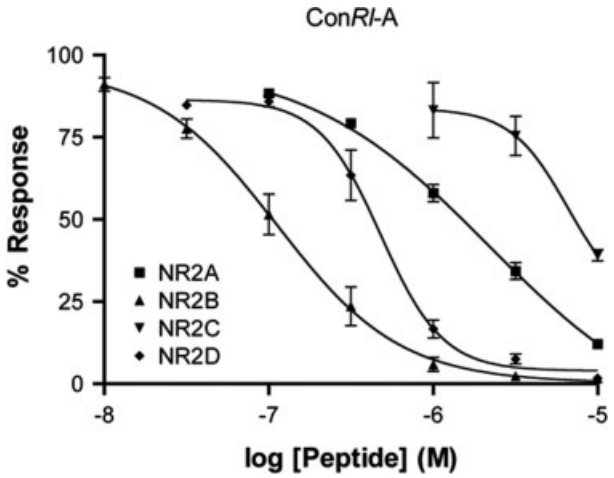

Figure 6. Concentration–response curve of Con*Rl*-A on the four different NR2 NMDA receptor subunits separately co-expressed with NR1-2b in *Xenopus* oocytes [$n = 3$ tests for each data point; error bars represent standard error of the mean (SEM)]. Curve fitting was performed as described in experimental procedures.

3.4%). On the other hand, the very considerable divergence in the mature peptide region (9/24 nonidentical AA; 37.5% divergence) is a characteristic result of the focal hypermutation of conopeptides that has been repeatedly noted [24–26]. These mature peptide regions are among the most rapidly evolving gene sequences known.

The precursors of γ-carboxylated peptides isolated from *Conus* contain a propeptide sequence that serves as the γ-carboxylase recognition sequence (γ-CRS) for the *Conus* γ-glutamyl carboxylase. In the case of Con*G* from *C. geographus,* Bandyopadhyay *et al*. [27] demonstrated that the γ-CRS was within the -20 to -1 region of the propeptide sequences within which there are two divergent positions between Con*Rl*-A and Con*Br*. γ-CRS has also been identified in the propeptide regions of other γ-carboxylated peptides, e.g. in εTxIX(tx5a) [28,29] belonging to the T-superfamily and Tx9a [30] in the P-superfamily. The γ-CRS in Gla-TxX and Gla-TxXI is however present as a 'post-peptide'.

Since the γ-CRS in peptides belonging to different superfamilies is highly divergent it has not been possible to assign any common sequence features. Czerwiec *et al*. [31] pointed out the presence of conserved basic residues and α-helicity of the propeptide. A structure/function study of εTxIX [32] suggested the importance of hydrophobic residues. Hydrophobic residues are also important in the mammalian γ-CRS [31]. In the case of the γ-CRS from Con*G*, substitution of the basic residues by alanine did not have any significant effect [33]. However, the roles of hydrophobic residues in the γ-CRS from conantokins have not been investigated. The putative γ-CRS region of Con*Br* and Con*Rl*-A is highly similar to that of Con*G*, and all of the large hydrophobic residues are conserved. Thus, we do not expect the amino acid changes between Con*Br* and Con*Rl*-A, nor between these peptide precursors and the Con*G* precursors to affect the function of the γ-CRS.

The characterization of Con*Rl*-A resulted in the unexpected discovery that Con*Rl*-A equilibrates between two conformational states. If the temperature is lowered, the equilibration slows down sufficiently to separate the two conformational forms using standard HPLC conditions. Discrete conformational states have not been previously detected for any conantokin peptide. Most *Conus* peptides appear to have a single solution conformation with some notable exceptions [34–39]. The contryphan family of peptides, that have a single disulfide bond as well as a D-amino acid, has two interconverting conformational states, dependent on the presence of the D-amino acid [40]. Another peptide with more than one conformational state in solution is α-conotoxin MI; this peptide is equilibrated between the two alternative conformational states relatively slowly [41].

In all these prior cases, the conformational equilibrium was in the context of a small peptide with disulfide bonds. What is novel about the discovery of the conformational equilibration between two states in Con*Rl*-A is that this peptide has no disulfide cross-links at all. Given its sequence homology to Con*Br*, the presence of two conformers was rather unexpected, since the latter peptide elutes from an HPLC column as a single homogenous peak. At present, the molecular basis of unique chromatographic behavior of Con*Rl*-A is unclear.

There are several noteworthy features of Con*Rl*-A. In Con*G*, the first conantokin characterized, there were five γ-carboxyglutamate residues – a number of studies have established that these are important for giving the peptide its helical structure in solution [42]. As shown in Table 2, there have been a number of conantokin peptides characterized in which γ-carboxyglutamate residues are substituted with other amino acids. Most notably, the γ 7 in Con*G* is substituted with a lysine residue, in most of the conantokin peptides characterized so far (this is the case for both Con*Br* and Con*Rl*-A). The literature to date suggests that in conantokins such as Con*G* with a γ-carboxyglutamate at residue 7, Ca^{++} is required to maintain the helical conformation [43]; however, the presence of lysine at this position confers stability to the helical conformation, even in the absence of Ca^{++}, as we have demonstrated for Con*Rl*-A using CD. We note that Con*Rl*-A is the only conantokin peptide characterized so far in which two of the five Gla residues in Con*G* are substituted by Lys; the presence of Lys14 was only found in Con*Pr*B; however, in this peptide, position 7 has Gla rather than Lys.

Another unusual feature of Con*Rl*-A is that it is the first native conantokin sequence characterized with alanine at position 1. Previous mutational studies of Con*G*, Con*T*, and Con*R* indicated that an Ala substitution for glycine at position 1 resulted in an ~10-fold reduction in potency [44–46]. However, since the potency of Con*Rl*-A and Con*Br* does not differ significantly, it would appear that the Ala substitution for Gly in these peptides from *Conus* species in the *Asprella* clade is not as functionally significant as was

Table 2. Comparison of known conantokin sequences

Conus clade	Conantokin	Peptide sequence	Reference
Gastridium	Con*T*	GE$\gamma\gamma$ YQK MLγ NLRγ AEVK KNA[a]	5
	Con*G*	GE$\gamma\gamma$ LQγ NQγ LIRγ KSN[a]	4
Phasmoconus	Con*L*	GE$\gamma\gamma$ VAK MAAγ LARγ DAVN[a]	7
	Con*R*	GE$\gamma\gamma$ VAK MAAγ LARγ NIAK GCKVNCYP[b]	6
	Con*Pr*-A	GEDγ YAγ GIRγ YQLI HGK I[b]	8
	Con*Pr*-B	DEOγ YAγ AIRγ YQLK YGK I[b]	8
	Con*Pr*-C	GEOγ VAK WAγ GLRγ KAASN[a]	8
Chelyconus	Con*P*	GE$\gamma\gamma$ HSK YQγ CLRγ IRVNK VQQγC[b]	9
Asprella	Con*Br*	GD$\gamma\gamma$ YSK FIγ RERγ AGR LDLSKFP[b]	10
	Con*Rl*-A	AD$\gamma\gamma$ YLK FIγ EQRK QGK LDPTKFP[b]	This work

Con, conantokin; γ, γ-carboxyglutamate; O, 4-*trans*-hydroxyproline.
[a] *C*-terminal amidation.
[b] *C*-terminal free acid.

found for the other conantokin peptides, suggesting that there are sequence features of these peptides that allow a substitution at position 1 to be more readily tolerated with respect to its functional activity.

Although the mature Con*Br* and Con*Rl*-A sequences diverge significantly from each other (37.5% divergence), they are nevertheless much more similar to each other than either peptide is to any other conantokin sequences that have been identified so far (Table 2). Indeed, the sequence identity of peptides that come from *Conus* species in the same clade (43%) is generally greater than is found for two conantokin sequences from species that come from different clades (20–25% sequence identity is typical). It is for this reason that a systematic comparison of conantokin sequences from species within a clade is equivalent to a detailed mutational study of a particular peptide, since such a phylogenetically informed analysis has the potential to reveal which amino acid sequences are stringently conserved and which substitutions are acceptable in sets of peptides that may have homologous physiological functions.

The comparison of the sequences of Con*Br* and Con*Rl*-A, shown in Figure 3, reveals that 15 of the 24 amino acids are identical, but there are 9 substitutions. Three of these (Ala1, Lys17, and Thr21) are conservative substitutions with the other five nonconservative. Correlating the sequence changes with the functional characterization of Con*Rl*-A suggests that at these five positions, particular AA are not stringently required for either potency or selectivity because the functional activities of Con*Br* and Con*Rl*-A are similar (Table 1). It seems reasonable to regard them as functionally homologous venom components of two different *Conus* species, given the similarities in potency and selectivity for the four different subtypes of NMDA receptors. One of the conserved residues, Tyr 5, was previously shown to be critical for an unusual pharmacological characteristic of these peptides – compared to other conantokins, these peptides exhibit a relatively high affinity for the NR2D receptor subtype [10].

Although the shift in affinity from Con*Br* is modest, the increase in NR2B affinity and decrease in NR2C affinity give the peptide a 55-fold preference for NR2B over NR2C, the greatest discrimination yet found among conantokins for these two NMDA receptor subtypes. This makes Con*Rl*-A a potentially useful pharmacological tool for B/C differentiation.

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