Characterization of contryphans from Conus loroisii and Conus amadis that target calcium channels

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1. Introduction

The venom of predatory cone snails consist of a diverse library of peptides, with multiply disulfide bonded conotoxins being the best-studied constituents [18,20–24,33]. The contryphans are 7–12 residue peptides, which contain only a single disulfide bond [7–13,17]. This class of conopeptides is characterized by a high degree of post-translational modification, which includes C-terminal amidation, epimerization of Trp and Leu (DTrp and DLeu), hydroxylation of Pro (Hyp, O), bromination of Trp and γ-carboxylation of Glu [1,7,9,11,12]. Contryphan sequences appear to show less sequence variability than the conotoxins. Leu-contryphans are characterized by DLeu within the disulfide loop, instead of DTrp, while glacontryphan possesses γ-carboxy glutamic acid (Gla, E) residues [7,9,13]. An interesting feature, characteristic of all the DTrp containing contryphans is the detection of multiple conformational states under reverse-phase chromatographic conditions [7–13,17]. Relatively few reported studies describe the nature of the receptors/channels that are targeted by the contryphans. The distinct effects of two closely related contryphans have been demonstrated on voltage-activated Ca2+ channels. The peptides Lo959 and Am975 were isolated from Conus loroisii, a vermiculous marine snail and Conus amadis, a molluscivore, respectively. The sequences of Lo959 and Am975 were deduced by mass spectrometric sequencing (MALDI-MS/MS) and confirmed by chemical synthesis. The sequences of Lo959, GCPDWDPWC-NH2 and Am975, GCODWDPWC-NH2 (O: 4-trans-hydroxyproline: Hyp), differ only at residue 3; Pro in Lo959, Hyp in Am975, which is identical to contryphan-P, previously isolated from Conus purpurascens, a piscivore; while Lo959 is a novel peptide. Both Lo959 and Am975 undergo slow conformational interconversion under reverse-phase chromatographic conditions, a characteristic feature of all contryphans reported thus far. Electrophysiological studies performed using dorsal root ganglion neurons reveal that both peptides target high voltage-activated Ca2+ channels. While Lo959 increases the Ca2+ current, Am975 causes inhibition. The results establish that subtle sequence effects, which accompany post-translational modifications in Conus peptides, can have dramatic effects on target ion channels.

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contraryphans, isolated from Conus ventricosus modulates voltage-gated and Ca\(^{2+}\)-dependent K\(^+\) channels [16] and the glacontrynchans, isolated from Conus marmoreus, blocks the L-type voltage-gated calcium channel in a calcium dependent manner [7].

In this report, we describe the sequences of two contryphans, Lo959 and Am975 isolated from the venom of Conus lorisii and Conus amadis, found off the southeast coast of India. The sequences have been derived by mass spectrometry and confirmed by chemical synthesis. The sequence of Am975 is shown to be identical to that of contrynch-P, isolated from Conus purpurascens. Lo959 differs from Am975 by the replacement of Hyp 3 by Pro 3. Electrophysiological studies using dorsal root ganglion neurons demonstrate that Am975 inhibits voltage-activated Ca\(^{2+}\) channels, while Lo959 enhances the magnitude of the Ca\(^{2+}\) current.

2. Materials and methods

2.1. Purification of peptides

The venom ducts of C. lorisii and C. amadis were dissected and stored in distilled ethanol. After concentrating in vacuo, the crude ethanol extracts were subjected to reverse-phase chromatographic purification. The fractionation of crude extracts was achieved on a Phenomenex C\(_{18}\) column (10 mm × 250 mm, 4 µm particle size, 90 Å pore size) using acetonitrile/water/0.1% trifluoroacetic acid (ACN/H\(_2\)O/0.1%TFA). The flow-rate was maintained at 1 ml min\(^{-1}\) and the fractions were detected at 226 nm. The fractions collected in the first chromatographic run were subjected to further purification on a Zorbax C\(_{18}\) (4.6 mm × 250 mm, 5 µm particle size, 300 Å pore size). The flow-rate was maintained at 0.5 ml min\(^{-1}\) and the fractions were detected at 226 nm.

2.2. Reduction-alkylation

The purified peptides were reduced using dithiothreitol (DTT) at pH ~8 (0.1–0.2 M NH\(_4\)HCO\(_3\)) and incubated at 37 °C for about 3 h. The resulting mixture was treated with iodoacetamide (IAM), kept in the dark at room temperature for about 1 h, and the progress of the reaction analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometry.

2.3. Mass spectrometry

The MALDI-MS and MS/MS data were acquired on a Ultraflex TOF/TOF (Bruker Daltonics) spectrometer, equipped with a nitrogen laser (λ = 337 nm), operated in reflectron, positive ion mode. α-cyano-4-hydroxy-cinnamic acid and 2,5-dihydroxy benzoic acid were used as the matrices.

2.4. Peptide synthesis

Chemical synthesis was performed by standard solid phase peptide synthesis protocols on a LKB-Biolynx 4175, semi-automatic peptide synthesizer, using 9-fluorenylmethyloxy-carbonyl (Fmoc) chemistry [32]. The coupling reactions were mediated with pentafluorophenyl (Opfp) esters of the Fmoc protected amino acids. The C-terminal amino acid was linked to the amino functional group of Rink amide AM resin (200–400 mesh, Nova Biochem) by formation of an amide linkage to obtain C-terminal amidated peptide. The synthesis was performed on a target scale of 0.189 mequiv., corresponding to 300 mg of resin (loading capacity 0.63 mequiv/g). The side chains of Hyp and Asp were protected with tertiary butyl groups, while the side chain of Cys was protected with a trityl group. Hyp was coupled as the symmetric anhydride, prepared immediately before the coupling reaction. The Fmoc deprotections were achieved with 20% piperidine in dimethylformamide. Subsequent to complete synthesis, the peptide was simultaneously cleaved off the resin and deprotected using TFA/anisole/ethanediithiol (95:4:1). After complete deprotection, the resin was filtered, TFA removed by evaporation in vacuo and the peptide precipitated with ether. The precipitate was repeatedly washed with ether and purified over a C\(_{18}\) column (9.4 mm × 250 mm, 5–10 µm particle size) using ACN/H\(_2\)O/TFA solvent system. The flow-rate was maintained at 1.3 ml min\(^{-1}\) following a linear gradient of 10–95% ACN over 45 min and the fractions were detected at 226 nm.

The purified synthetic peptide was subjected to air oxidation in dilute solution (0.5 mM) to minimize intramolecular disulfide bond formation. Oxidation was carried out by stirring the peptide at 25 °C for 15 h at pH 8.0 NH\(_4\)HCO\(_3\) (0.1 M) solution, containing 1% (v/v) DMSO. The oxidation was monitored using mass spectrometry and the resulting mixtures lyophilized and further purified on a C\(_{18}\) column (9.4 mm × 250 mm, 5–10 µm particle size) using ACN/H\(_2\)O/TFA solvent system. The flow-rate was maintained at 1.3 ml min\(^{-1}\) following a linear gradient of 10–95% ACN over 45 min and the fractions were detected at 226 nm. The synthetic contrynchans were characterized using mass spectrometry.

(Monoisotopic masses: synthetic Lo959; [M + H]\(^{+}\)\(_{\text{red}}\) = 962.4 (calc.), 962.3 (obs.); [M + H]\(^{+}\)\(_{\text{ox}}\) = 960.4 (calc.), 960.2 (obs.). Synthetic Am975; [M + H]\(^{+}\)\(_{\text{red}}\) = 978.4 (calc.), 978.5 (obs.); [M + H]\(^{+}\)\(_{\text{ox}}\) = 976.4 (calc.), 976.5 (obs.).)

2.5. DRG neuron preparation and electrophysiology

The dorsal root ganglion (DRG) neurons were prepared as described earlier [32] with some minor modifications. The dorsal root ganglia were picked from the thoracic region of the spinal cord, from postnatal (P5) wistar rats. The ganglia were minced, treated with trypsin followed by a wash in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. Isolated medium sized DRG neurons (33–38 µm) were selected for electrophysiological studies [30]. The neuronal soma of the DRG neurons without processes was voltage clamped using the whole-cell patch clamp technique described previously [32].

The Ca\(^{2+}\) currents in the cell bodies of DRG neurons were isolated using the following solution compositions [15]. The bath solution contained choline chloride (125 mM), MgCl\(_2\)-6H\(_2\)O (1 mM), CaCl\(_2\) (5 mM), glucose (22.5 mM), tetrodotoxin (TTX) (0.002 mM), tetraethyl ammonium chloride (10 mM), 4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid (HEPES) (10 mM), pH 7.4. The pipette solution contained CsCl (100 mM), tetraethyl ammonium chloride (20 mM), HEPES (30 mM), and tetraethylammonium chloride (30 mM).
(10 mM), ethylene glycol tetraacetic acid (EGTA) (10 mM), CaCl₂/CH₂O (1 mM), MgATP (2 mM), cAMP (0.050 mM), pH 7.2. The pH was adjusted with 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris). The solution composition ensured that the main permeant cation was calcium.

Outward calcium-activated K⁺ currents were recorded with an extracellular solution containing choline chloride (130 mM), KCl (3 mM), CaCl₂-2H₂O (2.5 mM), MgCl₂-6H₂O (0.6 mM), HEPES (10 mM), NaHCO₃ (1.2 mM), and glucose (10 mM), pH 7.4 with Tris. To remove the contribution of Ca²⁺-activated K⁺ current in the recordings, 0.1 mM CdCl₂ was included in the bath solution. The internal solution contained KCl (140 mM), CaCl₂-2H₂O (1 mM), MgCl₂-6H₂O (2 mM), EGTA (11 mM), and HEPES (10 mM), pH 7.2 with Tris. The currents were capacity and leak subtracted using a P/4 subtraction protocol. A 50% Rs compensation was applied in all the recordings.

The currents were filtered at 3 kHz, and sampled at 20 kHz. The temperature of the bath was maintained at 20 °C. The toxins were dissolved in water, and applied as a bolus to achieve the final bath concentration of 100 nM. The effects of contryphans on ionic currents were seen in 3–5 cells.

3. Results

3.1. Mass spectrometric sequencing

The cone snails, C. loroisii and C. amadis were collected off the southeast coast of India. C. loroisii is a worm-hunting species (vermicorous), whereas C. amadis feeds on mollusks (moluscivorous). Fig. 1 shows the chromatograms of the crude venom extracts of C. loroisii and C. amadis. The insets in Fig. 1 show the MALDI-MS spectra of the marked peaks in the chromatograms. The fractions corresponding to the marked peaks are hereafter designated as Lo959 (from C. loroisii, Fig. 1(A) and Am975 (from C. amadis, Fig. 1(B). Reduction-alkylation of the peptide fractions, achieved by DTT/IAM, resulted in increment of their molecular mass by 116 Da (2 × 58 Da), establishing the presence of a single disulfide bond in both peptides. Fig. 2 shows the MALDI-MS/MS spectra of the reduced-alkylated Lo959 (Lo959*, precursor ion m/z 1076.6, Fig. 2(A) and Am975 (Am975*, precursor ion m/z 1092.2, Fig. 2(B). The presence of peaks at m/z 283.9, 284.3; m/z 460.9, 461.6; m/z 576.1, 576.8 in both cases indicates that Lo959 and Am975 share significant similarities in their sequences. The sequences are readily derived from the observed fragments and the assignments of the observed bₙ and yₙ ions are indicated in Fig. 2. The most intense peaks correspond to cleavages at X-Pro bonds. Notably, fragments resulting from the cleavage of ‘Asp-Pro’ are the most intense, consistent with the reported lability of this bond under MALDI mass spectrometric conditions [34].

The two sequences are identical with the sole exception of residue 3, which is Pro in Lo959 and Hyp in Am975. Hydroxylation of Pro is a common post-translational modification in peptides derived from cone snail venoms. Am975 from C. amadis (a molluscivorous snail) is identical to contryphan-P, isolated previously from the piscivorous snail, Conus purpurascens. Confirmation of the sequences, in particular the ‘D’ configuration of W (4), has been achieved by comparison of synthetic peptides with the natural contryphans. Epimerization at residue 3 in the disulfide loop of contryphans has been established earlier [7,9–13,17]. Fig. 3 illustrates the HPLC profiles of natural and synthetic

Fig. 1 – Purification of peptides: (A) chromatogram of crude ethanol extract of the venom duct of Conus loroisii. (B) Chromatogram of crude ethanol extract of the venom duct of Conus amadis (inset) MALDI-MS spectra of the fractions corresponding to the marked peaks: (A) Lo959 and (B) Am975.
Am975. Likewise, the identity of HPLC profiles of the natural Lo959 and its synthetic counterpart was obtained and reported elsewhere [8]. Such unusual chromatographic behavior of purified contryphans has been previously demonstrated by Olivera and co-workers [9–13]. The two distinct HPLC fractions correspond to two slowly interconverting conformations. This is readily demonstrated by the fact that isolation and reinjection of a single component (reinjection of peaks marked P.1 and P.2) results in the reappearance of both the HPLC peaks (Fig. 3A inset). This slow conformational interconversion has been attributed to enhanced activation barriers of rotation about X-Pro bonds, resulting from the constraints of disulfide bridge [2,5,6,25–27,31].

Fig. 2 – (A) MALDI-MS/MS spectrum of [M + H]+ of Lo959*, precursor ion m/z 1076.6. (B) MALDI-MS/MS spectrum of [M + H]+ of Am975*, precursor ion m/z 1092.2. Note: the fragment ion masses are calculated using monoisotopic masses of amino acid residues (O: 4-trans-hydroxyproline; C*: carboxamidomethyl cysteine).

Fig. 3 – Comparison of chromatographic traces of (A) natural Am975 with that of (B) synthetic Am975 (inset) chromatographic traces obtained by reinjection of the fractions corresponding to P.1 and P.2 (column: Zorbax C18, 4.6 mm × 250 mm, 5 μm particle size, 300 Å pore size).
3.2. Ion channel activity studies

An earlier study by Massilia et al. reported the use of dorsal unpaired median neuron cells from cockroach nerve cord to demonstrate that contryphan-Vn from Conus ventricosus blocks the Ca\[^{2+}\]-dependent K\(^+\) current \[16\]. These authors also demonstrated the effect of the peptide on the global outward K\(^+\) current in the rat fetal chromaffin cells \[16\]. The effect of the purified natural Am975 that was available first was checked on the Ca\[^{2+}\]-activated K\(^+\) currents in medium sized DRG neurons. In the absence of external Cd\[^{2+}\], which is known to block certain subtypes of high voltage-gated Ca\[^{2+}\] channels \[3\], natural Am975 showed appreciable decrease in the Ca\[^{2+}\]-activated K\(^+\) currents (Fig. 4A). However, in experimental conditions where Cd\[^{2+}\] (0.1 mM) was present in the bath solution, the decrease in the Ca\[^{2+}\]-activated K\(^+\) currents with natural Am975 were much smaller (Fig. 4B), suggesting that the attenuation of the Ca\[^{2+}\]-activated K\(^+\) currents could be a secondary effect due to block of the voltage-activated Ca\[^{2+}\] currents by natural Am975. Subsequently, experiments were conducted to check the effect of Am975 on isolated voltage-gated Ca\[^{2+}\] currents. Both natural and synthetic Am975 decreased the whole-cell Ca\[^{2+}\] current amplitudes (Fig. 4C). In order to distinguish whether the action of Am975 was on the low or high threshold voltage-gated Ca\[^{2+}\] currents, the effects of Am975 were checked on isolated Ca\[^{2+}\] current recorded in response to a ramp stimulation from \(-100\) mV to \(+60\) mV; duration, 300 ms \[14\]. A decrease in the high threshold current component was evident from these experiments (data not shown). The effects were further confirmed using two-pulse protocols, shown in Fig. 5, to observe both the low voltage-activated (LVA) T-type Ca\[^{2+}\] current and the high voltage-activated (HVA) Ca\[^{2+}\] current in relative isolation \[30\]. The membrane potential was held at \(-100\) mV. A 150 ms pre-pulse to \(-40\) mV activated the LVA/T-type Ca\[^{2+}\] current, which inactivated with time and remained inactivated for another 165 ms that was confirmed by an identical test pulse.

Fig. 4 – Block of voltage-gated Ca\[^{2+}\] channels attenuates the decrease in the Ca\[^{2+}\]-activated K\(^+\) currents by Am975 (natural). Family of Ca\[^{2+}\]-activated K\(^+\) currents recorded: (A) in the absence of external Cd\[^{2+}\] (i) control; (ii) in the presence of 100 nM Am975 (natural) in the bath. (B) In the presence of external Cd\[^{2+}\] (i) control; (ii) in the presence of 100 nM Am975 (natural) in the bath. Holding potential, \(-80\) mV; test pulses were applied in 5 mV steps from \(-50\) to \(+50\) mV. (C) Am975 blocks isolated voltage-gated Ca\[^{2+}\] currents. (i) effect of Am975 (natural) (100 nM) (control, grey trace) (ii) effect of Am975 (synthetic) (100 nM) (control, grey trace) Holding potential, \(-100\) mV; test potential, \(-10\) mV. Note: (A); (B); (C) (i) and (ii) are recordings from different cells.
depolarization to \(-40\) mV, 10 ms after the pre-pulse (data not shown). A recovery time of 2 s was allowed between the successive two-pulse protocols. However, when a similar two-pulse protocol was applied but with the test pulse depolarization to \(-10\) mV, most of the T-type Ca\(_{2+}\) current remained inactivated, but evoked a high voltage-activated Ca\(_{2+}\) current in relative isolation. Both the natural and synthetic Am975 decreased the amplitude of high voltage-activated Ca\(_{2+}\) current with negligible effect on the low voltage-activated Ca\(_{2+}\) current and the result of only the synthetic Am975 effects is shown in Fig. 5D. However, both the natural and synthetic Lo959 increased the amplitude of the high threshold Ca\(_{2+}\) current (Fig. 5A and C). Observation of overlapping high voltage-activated Ca\(_{2+}\) currents with similar amplitudes even after 10–15 min following the onset of whole-cell recording (Fig. 5B), ruled out the possibility that the changes in the high voltage-activated Ca\(_{2+}\) current result from a wash out effect of intracellular biochemical messenger molecules.

A similarity in the biological effects seen with the natural and synthetic contryphans on voltage-activated Ca\(_{2+}\) channels along with the similarity in the HPLC profiles confirms the homogeneity of the isolated contryphans and their sequences.

4. Discussion

The number of well-characterized contryphan sequences from diverse Conus species is steadily growing. Table 1 lists the available contryphan sequences and summarizes the observed biological activity. There have been relatively few studies, which have directly focused on the molecular targets of the contryphans. While the contryphan-Vn has been reported to modulate both voltage-gated and Ca\(_{2+}\)-activated K\(^+\) channels [16], the glacontryphan-M has been shown to block L-type Ca\(_{2+}\) channels [7]. In the present study, Lo959 and Am975 have been shown to target voltage-activated Ca\(_{2+}\) channels, albeit with distinctly different consequences. Inspection of the aligned contryphan sequences in Table 1, suggests that three broad classes may be identified. In the first group, which contains seven sequences, the residue 2 in the disulfide loop is either Pro or Hyp, while residue 4 can be Glu, Asp or Gln. In the second group, residue 4 in the disulfide loop is a positively charged basic residue, Lys or His. This group is also characterized by extensions of the sequence at the N-terminus, which harbor negatively charged residues like Asp or Gla (E). The third group differs from the other two in that 2Leu replaces 2Trp in the disulfide loop and the group is referred to as ‘Leu-contryphans’. Also, unlike the other two groups, where the residue 2 in the

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**Fig. 5** – Selective modulation of the HVA-type Ca\(_{2+}\) current by the contryphans in medium sized DRG neurons. (A) Lo959 (natural) enhances the HVA Ca\(_{2+}\) current elicited by the test pulse depolarization to \(-10\) mV, when recorded 5 min after application (current trace in black) without affecting the LVA Ca\(_{2+}\) current. The current trace is compared to that of control without contryphan in the bath (control, grey trace). (B) Control recordings of LVA and HVA Ca\(_{2+}\) currents using the two-pulse protocol, just after achieving the whole-cell recording mode (control, grey trace) and 15 min later (15 min, black trace). (C) (i) Lo959 (synthetic) enhances the HVA Ca\(_{2+}\) current selectively similar to that of Lo959 (natural) without affecting the LVA Ca\(_{2+}\) current (ii) Lo959 (synthetic) sensitive Ca\(_{2+}\) current obtained by digital subtraction of the control Ca\(_{2+}\) current trace (grey) from the Ca\(_{2+}\) current trace recorded in the presence of Lo959 (synthetic) (black) shown in (C) (i). (D) (i) Am975 (synthetic) attenuates the HVA Ca\(_{2+}\) current selectively. (ii) Am975 (synthetic) sensitive Ca\(_{2+}\) current obtained by subtracting the Ca\(_{2+}\) current trace recorded 5 min after bath application of Am975 (synthetic) (black) from the control (grey) Ca\(_{2+}\) current trace. **Note:** (A)–(D) are recordings from different cells.
disulfide loop is Hyp or Pro, the peptides of this group have Val at position 2 in the disulfide loop. Further, unlike the normal contryphans, Leu-contryphans are less effective in eliciting ‘stiff-tail’ syndrome in mice, however exhibit significant activity in fishes.

A distinctive feature of the contryphans is the high level of sequence conservation observed in the mature peptide sequences. In the 7-residue segment, which constitutes the disulfide loop, sequence variability is largely restricted to residues 2 and 4 in the disulfide loop. In most cases, variability at position 3 is generated by post-translational modification of the Pro residue by hydroxylation. The extent of conservation across species in the mature contryphan sequences is much greater than the multiply disulfide bonded conotoxins. The observation of multiple conformational states of contryphans suggests that three-dimensional structural variability may provide a mechanism for targeting diverse receptors. Together with post-translational modifications, like Pro hydroxylation and Trp bromination, predatory cone snails may have evolved mechanisms to enhance the diversity of natural contryphan libraries, permitting the targeting of a wide-range of receptors and channel subtypes.

An intriguing observation in the present study is the observed difference in the effects of the closely related peptides, Lo959 and Am975 on voltage-activated Ca\(^{2+}\) currents in DRG neurons. While the former enhances the Ca\(^{2+}\) current, the latter appears to diminish the magnitude of the whole-cell Ca\(^{2+}\) current. The sole structural difference between these two sequences is the residue 2 in the disulfide loop; Pro in Lo959 and Hyp in Am975. γ-Hydroxylation of Pro residues is a common modification found in many conopeptide families that target both voltage and ligand-gated ion channels. Investigations on the roles of the Hyp10 and Hyp21 hydroxyl groups in omega-conotoxin GVIA, studied by proline substitutions indicated that they contribute to structural changes as monitored by NMR. Small decreases in biological activities were also reported in assays that monitored N-type voltage-gated Ca\(^{2+}\) channel activity [4]. The protrusion of hydroxyproline into the solvent in conotoxin e-TxIX has been suggested to be important in Ca\(^{2+}\) channel binding, causing decrease in the presynaptic Ca\(^{2+}\) current in Aplysia neurons [28]. The structural origins of the differences in the activities of Lo959 and Am975 remain to be elucidated. The opposite effects on the high voltage-activated (HVA) Ca\(^{2+}\) currents of DRG neurons have to be interpreted with caution, since it cannot be concluded with certainty, whether the action is on the same calcium channel subtype. The DRG neurons have more than one HVA calcium channel subtype (N-, L-, P/Q- and R-type) [19,29]. The fraction of unblocked Ca\(^{2+}\) current shown in Fig. 5 may indicate the presence of other HVA current subtypes that were not explored further by using known blockers of HVA currents. While a search for the specific calcium channel molecular target requires considerable effort, the DRG neuron with its repertoire of multiple Ca\(^{2+}\) channel subtypes has however proved to be a useful biological assay system in this study, helping to identify the broad class of ion channels targeted by Lo959 and Am975.

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