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Mass Spectral Identification of Vc1.1 and Differential Distribution of Conopeptides in the Venom Duct of *Conus victoriae*. Effect of Post-Translational Modifications and Disulfide Isomerisation on Bioactivity

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Abstract Molluscs of the genus *Conus* (cone shells) are carnivorous, feeding on marine worms, small fish and other marine molluscs. They capture their prey by injecting venom containing hundreds of neurally active peptide components. These peptides are classed as conotoxins and consist of small disulfide-bonded peptides exhibiting a high degree of post-translational modifications (PTMs). The functional roles of these modifications remain largely unknown. Two of the most frequently observed modifications are γ-carboxylation of glutamate and hydroxylation of proline (Buczek et al. Cell Mol Life Sci 62:3067, 2005). Vc1.1 is an α-conotoxin from *Conus victoriae* (Sandall et al. Biochemistry 42(22):6904–6911, 2003) and the only form of this peptide which has been detected in the venom is the γ-glutamate and hydroxyproline (Vc1.1.P6O:E14-Gla) version of the molecule (Jakubowski et al. Toxicon 47(6):688–699, 2006). In order to investigate the role of PTMs, we did mass spectral profiling of the venom duct of *C. victoriae* looking at changes in mass and the number of peptides detected. We synthesised a number of predicted Vc1.1-PTM peptides together with the three possible disulfide isoforms of Vc1.1 and assessed the possible functional role of the PTM conopeptides by measuring the in vitro activity at the cognate neuronal nicotinic acetylcholine receptors (nAChRs). In addition we looked for their presence Vc1.1 venom by mass spectrometry and by this approach we were able to detect unmodified Vc1.1 in *C. victoriae* venom for the first time.

Keywords Conotoxins · Post-translational modification · PTM · Disulfide isoforms · Vc1.1 · Neuronal nicotinic acetylcholine receptor

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Introduction

Predatory Conus owe their evolutionary success to their ability to rapidly immobilize prey. Feeding on marine worms, small fish or marine molluscs, including other conus species, their venom, which is neurally active, causes paralysis, analgesia and death by targeting neurotransmitter receptors and ion channels. Among the biologically active components of the venom are the conotoxins, small disulfide-bonded peptides exhibiting a high degree of post-translational modification (PTM). The functional role of most of the modified residues in conotoxins remains largely unknown. Two of the most frequently observed modifications are γ-carboxylation of glutamate and hydroxylation of proline (Buczek et al. 2005). γ-Carboxy glutamate (symbolised by Gla) has been shown to promote calcium-assisted oxidative folding of Cys-rich conotoxins (see Bulaj and Olivera 2008). The role of 4-*trans*-hydroxyprolines (Hyp or O) in the in vitro oxidative folding and biological activity of conotoxins has only recently been studied (Lopez-Vera et al. 2008). For μ-conotoxin GIIIA

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which contains three Hyp residues, hydroxylation impaired its ability to block $Nav_{1.4}$ sodium channels but did not affect folding. In contrast, the presence of Hyp in omega-conotoxin MVIIC improved the oxidative folding but did not affect the biological activity. For α-conotoxin ImI, of the 4/3 loop subfamily, and α-conotoxin GI of the 3/5 loop subfamily, hydroxylation of the conserved Pro^6 residue improved their folding but impaired their activities against cognate target receptors. Similar effects of hydroxylation of conserved Pro^6 on activity were reported for a 4/7 loop α-conotoxin PnIB (Quiram et al. 2000) which targets neuronal nicotinic acetylcholine receptors (nAChRs). In view of these observations it was of interest to determine the effect of PTMs on the folding and biological activity of the 4/7 loop α-conotoxin Vc1.1 from *Conus victoriae* (Sandall et al. 2003), that has been shown to have significant analgesic activity in three animal models of neuropathic pain (Satkunanathan et al. 2005). In an earlier study, we showed by MS analysis that the venom of *C. victoriae* contained predominantly the post-translationally modified form of Vc1.1, Vc1.1[P6Hyp:E14Gla], termed vc1a (Jakobowski et al. 2004). However, the effect of these PTMs on bioactivity was not assessed. In the present study we have chemically synthesised a range of PTM forms of conotoxin Vc1.1 in order to positively identify the native forms present in the venom by mass spectral profiling and to assess the role of these PTMs on their in vitro activity at the cognate nAChR.

Materials and Methods

Mass Spectral Analysis of Venom

Duct venom was extracted following dissection of the *C. victoriae* specimens. *C. victoriae* is a mollusc-hunting *Conus* species (Röckel et al. 1995) inhabiting the Indo-Pacific, specifically the North-western Australian coast. The specimens used in this study were obtained from Broome, western Australia, and maintained in captivity in the Department of Zoology salt-water aquarium facility (University of Melbourne). Venom from three animals was pooled. From another animal the duct was cut in transverse sections and the venom collected separately from each section. Air-dried venom was then suspended (1.0 mg/ml milliQ H_2O) and sonicated. The insoluble material was removed by centrifugation for 5 min at 1,800 rpm, and the supernatant stored at −20°C for subsequent LC/MS analysis. LC/ESI TOF MS analyses were carried out using a positive electrospray interface on an Agilent LC/MSD TOF Mass Spectrometer, coupled to an Agilent 1100 LC system (Agilent, USA). Chromatographic separation was performed using a Chromolith SpeedROD RP C18 column, 50 mm × 4.6 mm, 2 μm particle size (Merck, Germany). A 22 min linear gradient was employed. Buffer A: 0.1% formic acid/H_2O, buffer B: 95% acetonitrile/H_2O (0.1% formic acid). Flow: 0.5 ml/min. All data was acquired and reference mass corrected via a dual-spray ESI source. MS conditions: ionisation mode ESI, positive mode; drying gas flow 12 l/min; nebuliser 45 psi; drying gas temp 350°C; Vcap 4,000 V; fragmentor 250 V; skimmer 60 V; OCT RFV 250 V; scan range acquired m/z 100–2,500.

Synthetic Peptides

Synthetic α-conotoxin Vc1.1, Vc1.1-COOH, Vc1.1[P6Hyp], Vc1.1[E14Gla] and Vc1.1[P6Hyp:E14Gla] were synthesised by manual Fmoc solid-phase peptide chemistry, cleaved by treatment with a solution of TFA/water/triisopropylsilane/dithiothreitol (90/2.5/2.5/5), diethyl ether precipitated and lyophilised. The linear peptides containing four free sulfhydryls were purified by RP-HPLC on a Phenomenex C18 5um, 50 × 22 mm semiprep column and analysed by ESI–TOF mass spectrometry (direct injection at a flow of 0.25 ml/min). Other instrument parameters were as described above. Disulfide bonds were formed by air oxidation in 25 mM ammonium bicarbonate (peptide concentration 1 mg/ml) and the oxidation reaction followed by mass spectrometry (molecular weight decreased by 4 Da in the refolded material reflecting the formation of two disulfide bonds). The oxidised peptides were then purified in the same manner as the linear peptides on semi-preparative RP HPLC. Pure fractions were pooled and lyophilised.

In general, air oxidation of the Vc1.1 peptide analogues resulted in the formation of predominantly the native conformation of the peptide with lesser amounts of the other possible disulfide isomers (as evidenced by the HPLC traces in Fig. 4a and b). Selective synthesis of the three possible disulfide isomers of Vc1.1 was performed by orthogonal protection of the two cysteine pairs. In each case one cysteine pair was protected with the trityl group and the other pair by the acetamidomethyl (Acm) group. The linear peptide was, deprotected and purified as previously described to yield a peptide with two free sulfhydryl groups and two Acm protected cysteines. To form the two disulfide bonds was a one pot, two step process. First the linear peptide was dissolved in 80% acetic acid/water (1 mg/ml) and treated with two equivalents of iodine (from a stock solution of 50 mM in methanol) for 20 min. The reaction was followed by mass spectrometry where a decrease in mass of 2 Da was detected signifying the formation of the first disulfide bond (without affecting the Acm protection of the other cysteine pair). Next, the solution was treated with 20 equivalents of iodine (from the same stock solution of 50 mM in methanol) and the oxidation was allowed to proceed for two hours. Mass

spectral analysis after this time showed the loss of the two Acm groups (a decrease of 142 Da) and the formation of the second disulfide bond. Excess iodine was removed by treatment of the reaction solution with Dowex 4 X 8 mixed bed ion exchange resin (the solution colour changes from brown to clear). The solution was filtered and the oxidised peptide with two disulfide bonds was purified by RP HPLC. The three disulfide isomers of Vc1.1 had identical molecular mass (MH^+ 1807.6 Da), but each had a discrete retention time on reversed-phase HPLC. To demonstrate this a mixture of the disulfide isomers was analysed by reversed-phase HPLC and the mixture showed three discrete, separable peaks (Fig. 6). The isomers were also stable, showing no evidence of disulfide scrambling.

Neuronal Nicotinic Receptor Assay

Adrenal chromaffin cells were isolated from adult bovine adrenal glands as described by Livett et al. (1987). Isolated cells were plated out on collagen coated 24-well plates at a density of 2.8×10^5 cells/cm^2. Fresh bovine adrenal glands were provided by Wagstaff Abattoirs (Cranbourne, Melbourne, Australia). Culture media and penicillin–streptomycin were from GIBCO BRL (USA). Foetal calf serum from CSL (Australia). Antimitotics, nystatin and nicotine from Sigma (USA). Collagenase Type I from Worthington Biochemical (USA), Percoll from GE Healthcare (UK). Cell culture plates from Sarstedt (USA). Collagen Type I (Rat Tail) from BD Biosciences (USA). BSA from Bovogen (Australia). Four-day-old cultured chromaffin cells were allowed to equilibrate to room temperature for 5 min. The incubation media was removed by two consecutive washes in Locke's buffer (154 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 10 mM D-glucose, 1.18 mM $MgSO_4.7H_2O$, 2.2 mM $CaCl_2 \cdot 2H_2O$, 0.5% BSA, pH 7.4) for 5 min. Cells were then incubated for 5 min with 0.1-20 µM of α-conotoxin Vc1.1 analogues before stimulation with 4.0 µM nicotine for a further 5 min. The incubation mixture was separated from the cells and acidified with 2.0 M perchloric acid, to give a final concentration of 0.4 M perchloric acid. The catecholamines remaining in the cells were released by lysing the cells with 0.01 M perchloric acid, and then acidified by addition of an equal volume of 0.8 M perchloric acid. Precipitated proteins were removed by centrifugation at 16,000 rcf for 10 min. To measure basal release of catecholamines, a control well contained Locke's buffer only, with no nicotine or Vc1.1 analogue. To determine the maximal release of catecholamines, a second control well contained nicotine but no Vc1.1 analogue. Catecholamines present in each sample were separated by RP-HPLC using a Waters HPLC system and C18 150 mm × 4.6 mm column, 5 µm particle size (Bio-Rad, USA), with 10% methanol in the mobile phase (70 mM KH_2PO_4, 0.1 mM NaEDTA, 0.2% heptane sulfonic acid). An isocratic elution was employed. Catecholamines eluting from the column were identified by their retention time and quantified by electrochemical detection (650 mV BAS model LC-3A). Known standards of adrenaline and noradrenaline were used to calculate the amount of catecholamines in each sample, expressed as a percentage of the total cell content. Concentration-response data was generated using GraphPad Prism Version 4.0 for Windows.

Results and Discussion

Duct Venom Analysis

The results in Fig. 1 show the LC/MS profile of duct venom from *C. victoriae* in the region where we identified vc1a (Vc1.1[P6Hyp:E14Gla], MH^+ 1866.6 Da) based on the previous LC/MS characterisation by Jakubowski et al. (2006). Two peaks are observed in the venom trace, one at 7.55 min and the other at 7.85 min. Synthetic Vc1.1[P6Hyp:E14Gla] was used to spike the venom, resulting in an increase in intensity of the peak at 7.55 min. This technique enabled us to confirm that the native duct venom peptide vc1a is identical to synthetic Vc1.1[P6Hyp:E14Gla], which allowed us to then use the same approach, in a separate experiment, using synthetic Vc1.1 (MH^+ 1807.6 Da) to confirm its presence in the venom.

Figure 2 shows the LC/MS profile of the duct venom from *C. victoriae* in the region where synthetic Vc1.1 elutes when spiked into the venom (8.75 min). Also at 8.75 min a low intensity peak was apparent in the unspiked venom. The mass of this component (MH^+ 1807.6 Da) corresponded to the mass of synthetic Vc1.1. This observation provides the first positive identification of Vc1.1 as a natural component of the venom, albeit in low abundance. We observed no other modified forms of Vc1.1, other than a trace of Vc1.1[P6Hyp] (MH^+ 1823.6 Da) co-eluting with the vc1a peak. As commented by Jakubowski et al. (2006) it is possible this mass may be formed by the loss of the Gla^{14} modification from vc1a during MS ionisation. Without the unmodified Vc1.1 co-elution experiment, the low intensity Vc1.1 signal would not have been easily identified within the complex venom.

The observation of unmodified Vc1.1 in the venom is of interest as it is known that γ-carboxyglutamation of conopeptides is brought about by enzymic modification of the pre- pro- region of the immature peptide (Brown et al. 2005). The presence of the unmodified, mature Vc1.1 peptide indicates that some of the pre- pro- peptide is cleaved prior to post-translational modification. This

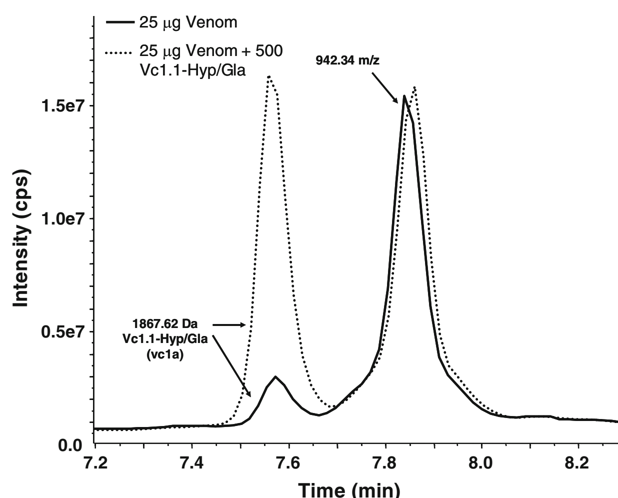

Fig. 1 Overlay of the LC/MS traces of unspiked venom and Vc1.1[P6O:E14Gla] spiked venom. A 22 min linear gradient was employed for RP C-18 HPLC. Buffer A: 0.1% formic acid; buffer B: 95% acetonitrile in water. The TIC is shown as detected by online-TOF MS. Fragmentor: 250 V; Skimmer: 60 V; OCT RFV: 250 V. The *solid line* represents the trace from ~25 µg of crude venom (by weight). The *broken line* represents the trace of ~25 µg crude venom mixed with 500 ng of purified Vc1.1[P6O:E14Gla]. Absolute retention times varied from experiment to experiment, but in each trace in the area of interest spanning 7.0–9.0 min retention time, a characteristic peak (which was termed a flagpole peak) corresponding to non-α conotoxin with m/z 942 was present. In the synthetic Vc1.1[P6O:E14Gla] spiking experiment traces from separate experiments were overlaid with reference to this peak at 7.85 min

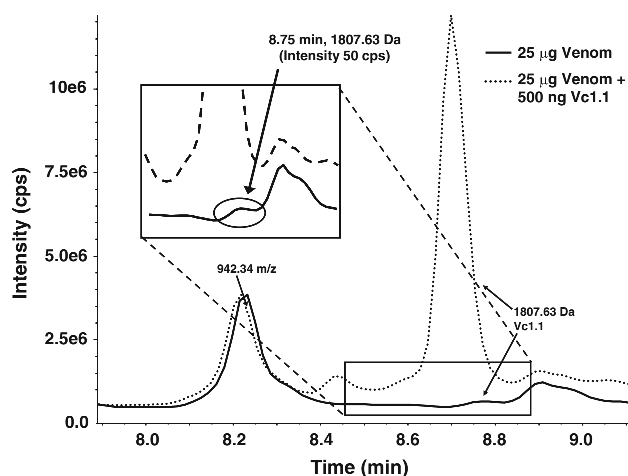

Fig. 2 Overlay of the LC/MS traces of unspiked venom and Vc1.1 spiked venom. A 22 min gradient was employed for RP C-18 HPLC. Buffer A: 0.1% formic acid; buffer B: 95% acetonitrile in water. The TIC is shown as detected by online-TOF MS. Fragmentor: 250 V; Skimmer: 60 V; OCT RFV: 250 V. The *solid line* represents the trace from ~25 µg of crude venom (by weight). The *broken line* represents the trace of ~25 µg crude venom mixed with 500 ng of purified Vc1.1. In the synthetic Vc1.1 spiking experiment traces are overlaid with reference to the flagpole peak with the m/z of 942 (see Fig. 1 legend) and the retention time of 8.25 min

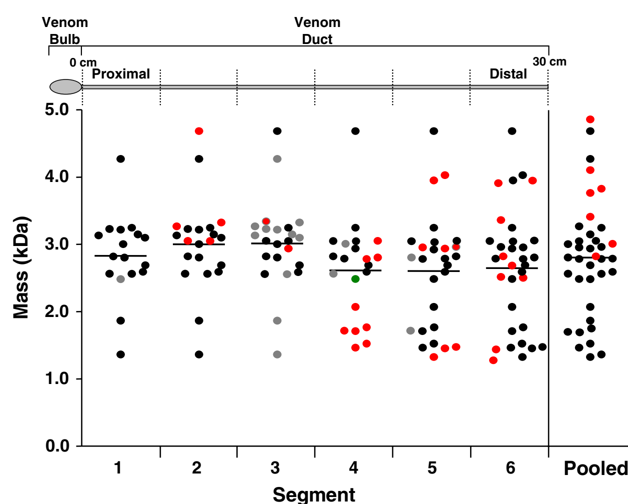

Fig. 3 Dominant masses in each section of the venom duct. A venom duct from one animal was transversely divided into six segments, and venom collected separately from each segment. Each point represents a distinct mass, as calculated by Mass Hunter (applied biosystems), with an intensity cutoff of 2.0×10^6 cps. Masses that were not present in previous sections are coloured *red*. Masses that are not present in subsequent sections are coloured *grey*. The average mass is indicated by a *horizontal line*. Pooled refers to total duct venom obtained from three animals and pooled

implies that cleavage is independent of post-translational modification.

To determine where post-translational modifications of the venom take place, we examined the LC/MS mass profile of venom obtained from segments of the venom duct, from the bulb to the proboscis. Figure 3 shows the mass distribution of components along the venom duct. The plot shows the predominant masses in each segment as determined by Mass Hunter (applied biosystems), using an abundance cut-off of 2×10^6 cps as a selectivity criterion.

It is apparent from Fig. 3 that there is an overall decrease in average mass towards the distal region with the

Table 1 Modification of venom peptides within the venom duct

Section of duct[1]	Total masses[2]	Masses gained[3]	Masses lost[4]
1 (posterior)	17	NA	NA
2	21	5	1
3	23	2	0
4	21	10	10
5	27	8	2
6 (anterior)	33	9	2
Total venom[5]	37	7	NA

[1] Venom duct was divided into six segments (see Fig. 3)

[2] Number of distinct masses identified in each section

[3] Number of masses not present in the previous sections

[4] Number of masses not present in subsequent sections

[5] Number of masses identified in the total venom duct

change occurring half way along the duct (at Segment 3–4). As shown in Table 1, the largest number of peptides exhibiting a change in mass occurred in the last three segments. The number of masses gained exceeds the number of masses lost. This is consistent with a previous report (Marshall et al. 2002) that the proximal half of the venom duct is specialised for synthesis and the distal half for active transport, as the venom migrates to the distal region of the duct ready for envenomation.

Our result showing the appearance of larger molecular weight species in the distal segments is consistent with reports by Marshall et al. (2002)and Dr. Jon-Paul Bingham (see Nelson 2004) of an increased number of higher molecular weight peptides observed in the distal region of the venom duct, as well as within the radulae and in milked venom for envenomation. The observation of functionally relevant conotoxin dimers (Loughnan et al. 2004), specifically in *C. victoriae* venom (J. A. Jakubowski, unpublished data) suggests that some higher molecular weight peptides (>4,000 Da) seen in proximal and pooled venom are formed by aggregation of smaller peptides. The reduction in average mass in distal segments may be due to cleavage of pre- pro- peptides. The disappearance of some masses in distal segments may be due to their MS signal being suppressed by other major species, or they may fail to migrate to the end of the duct (see Bingham et al. 1996). Alternatively, they may aggregate or gain PTMs, as one peptide present in Segment 5 was observed to gain the mass shift of a bromotryptophan modification (+78 Da) in Segment 6 and potential mass additions due to Hyp and Gla were also observed (results not shown). It would be of interest to use LC/MS coupled with MS/MS to analyse venom in the radulae (Marshall et al. 2002) of *C. victoriae* to potentially confirm these processing events and gain further understanding of the processing of duct venom (Bingham et al. 1996).

Table 2 Synthetic PTM analogues of Vc1.1

Analogue	Sequence	Mass (Da)
Vc1.1-COOH	NH_2–GCCSDPRCNYDHPEIC–COOH	1807.6
Vc1.1	NH_2–GCCSDPRCNYDHPEIC–$CONH_2$	1806.6
Vc1.1-Gla	NH_2–GCCSDPRCNYDHP**X**IC–$CONH_2$	1849.6
Vc1.1-Hyp	NH_2–GCCSD**O**RCNYDHPEIC–$CONH_2$	1822.6
Vc1.1-Hyp/Gla	NH_2–GCCSD**O**RCNYDHP**X**IC–$CONH_2$	1866.6

X = Gla^{14} and **O** = Hyp^{6}

Synthetic Analogues of Vc1.1

In order to determine the effect of C-terminal amidation, γ-carboxyglutamation, and hydroxyprolination of Vc1.1 on biological activity, a range of PTM analogues of Vc1.1 were synthesised (Table 2).

Figure 4a and b show the LC traces of non-purified, air-oxidised synthetic Vc1.1, and Vc1.1-COOH, respectively. The LC trace of synthetic Vc1.1 shows one distinct peak shown by MS to be 1,806.6 Da, whereas Vc1.1-COOH exhibits three distinct peaks, all subsequently shown by MS to be of the same mass, 1,807.6 Da. These three peaks represent the three disulfide isomers that formed spontaneously.

The α-conotoxins, having two disulfide bonds, are capable of forming three distinct disulfide isomers, characterised as the 'globular', 'ribbon', and 'beads-on-a-string' conformers (Gehrmann et al. 1998). These three isomers of Vc1.1 have 2–8; 3–16 (globular), 2–16; 3–8 (ribbon), or 2–3; 8–16 connectivity (beads) as illustrated in Fig. 5.

These three disulfide isomers of Vc1.1 were individually synthesised and purified using an orthogonal cysteine protection strategy (Fletcher and Hughes 2004). Figure 6 shows the HPLC trace following co-injection of the three purified isomers (with 2–8; 3–16, 2–16; 3–8, and 2–3; 8–16 connectivities). The isomers in the synthetic mixture were identified based upon their individual retention times. The globular isomer corresponded to the front eluting peak, the ribbon isomer the middle peak and the bead isomer the last eluting peak. This is in accordance with a report by Clark et al. 2006.

To compare the efficacy of the three disulfide isomers, their ability to antagonise the nicotinic receptor response was assessed in vitro in primary monolayer cultures of bovine adrenal chromaffin cells (Livett et al. 1987). Vc1.1 is a known competitive antagonist in this model system of neuronal nicotinic receptor function (Sandall et al. 2003). Figure 7 shows that only the (2–8; 3–16) globular isomer produced a dose-dependent inhibition of the nAChR

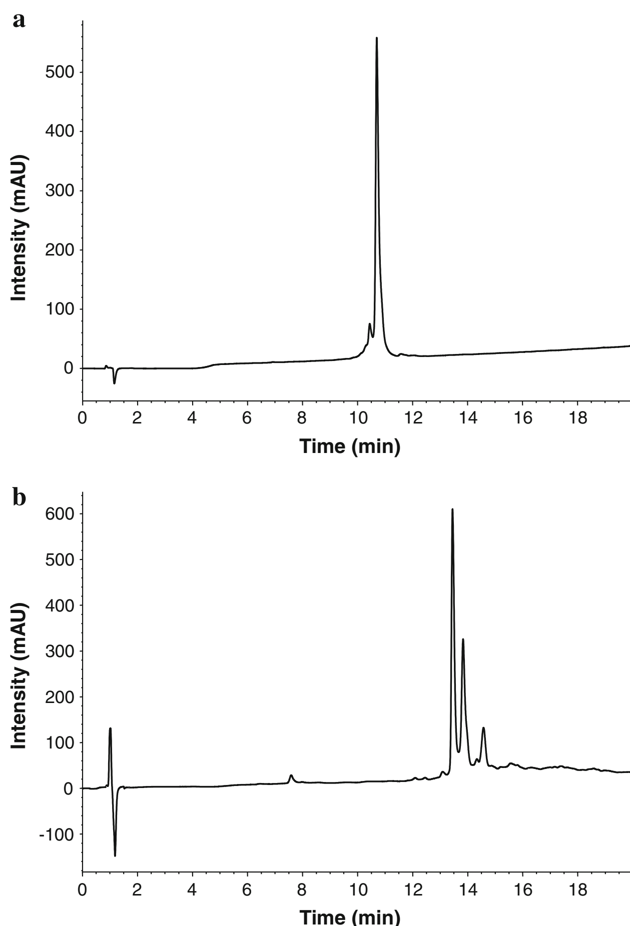

Fig. 4 **a** Analytical RP HPLC trace of crude, air oxidised Vc1.1. The linear peptide is oxidised in 25 mM ammonium bicarbonate buffer (concentration 1 mg/ml) and the product is a single peptide conformation with two disulfide bonds having a mass of 1807 Da, by ESI–TOF mass spectrometry. **b** Analytical RP HPLC trace of crude, oxidised Vc1.1-COOH. The linear peptide is oxidised in 25 mM ammonium bicarbonate buffer (concentration 1 mg/ml) and the product is a mixture of three peptides containing two disulfide bonds. The three peptides were isolated by semi-preparative HPLC and each peptide had a mass of 1,808 Da, by ESI–TOF mass spectrometry representing the three possible disulfide combinations. Only the early eluting peak was active in the nAChR assay and in fact was equipotent with Vc1.1 in this assay

Isomer	Disulfide Connectivities
Globular 2-8:3-16	NH_2-G-C-C-S-D-P-R-C-N-Y-D-H-P-E-I-C-$CONH_2$
Ribbon 2-16:3-8	NH_2-G-C-C-S-D-P-R-C-N-Y-D-H-P-E-I-C-$CONH_2$
Beads 2-3:8-16	NH_2-G-C-C-S-D-P-R-C-N-Y-D-H-P-E-I-C-$CONH_2$

Fig. 5 Schematic diagram of the disulfide connectivities of the three possible Vc1.1 isomers, namely, the globular, ribbon and bead forms of the peptide

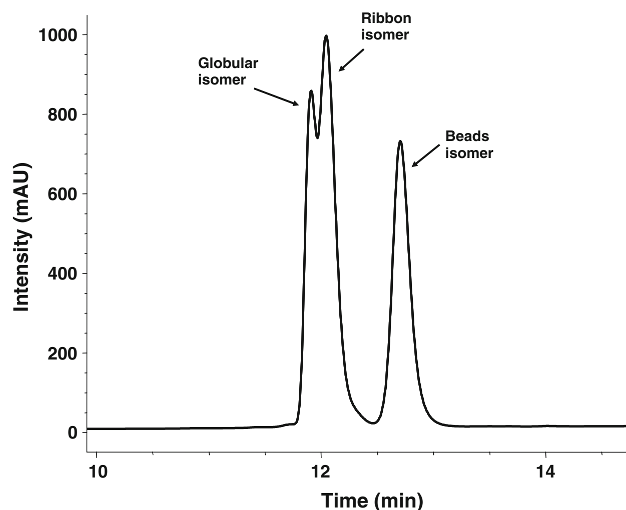

Fig. 6 The three possible disulfide isomers of Vc1.1 were synthesised by orthogonal cysteine protection as described in the ''Materials and Methods''. The three peptide isomers were found to have different retention times on analytical RP HPLC. The elution times were Vc1.1[2,8–3,16] 11.7 min, Vc1.1[3,8–2,16] 12.1 min and Vc1.1[2,3–8,16] 12.7 min. The early eluting peak (11.7 min) had the normal disulfide bonding connectivity as other known 4,7 α-conotoxins and was the only peptide with activity in the nAChR assay

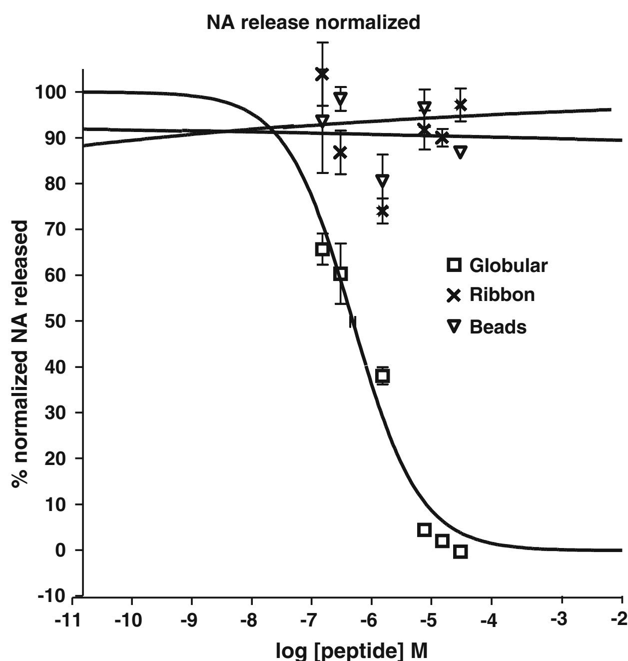

Fig. 7 Dose-dependent antagonism of nAChRs by the forced-synthesis disulfide isomers of Vc1.1. Four-day-old cultures of bovine adrenal chromaffin cells where pre-incubated with 0.1–20 μM of each isomer, then agonised to release noradrenaline using 4.0 μM nicotine. Relative noradrenaline release was assessed by electrochemical detection of the cell supernatant and cell lysate. The % release of noradrenaline is calculated by normalising with untreated cells as 0% and cells treated only with nicotine as 100%

response. The other two isomers failed to inhibit the response.

The globular conformation of the α-conotoxins has the least conformational heterogeneity by NMR (Gehrmann et al. 1998; Dutton et al. 2002) and is considered to be the most energetically favoured and stable conformation (Zhang and Snyder 1991). In this case the globular form is the only active form and therefore we would expect it to be the only expressed form in the venom. This is not always the case since for some conotoxins, such as AuIB, it has been observed that the ribbon conformer has the greatest activity (Nicke et al. 2004). Likewise, the χ- or λ-conotoxins exhibit the ribbon isomer as their native form (Balaji et al. 2000; McIntosh et al. 2000; Sharpe et al. 2001). For α-conotoxin GI the beads conformation has been shown to be the least stable and least globular in shape (Gehrmann et al. 1998).

In Fig. 8 the dose-dependency of a range of PTM analogues of Vc1.1 (listed in Table 2) is shown. The IC_{50}s for these analogues are listed in Table 3. It is observed that the greatest loss of activity occurs with the P6Hyp modification, which results in a 75-fold loss of activity. Modification of a carboxy to an amide C-terminus produced a three-fold loss of activity. The E14Gla modification had a similarly small effect which resulted in a 5-fold loss of activity. This finding is consistent with studies indicating the importance of the highly conserved Pro^6 for receptor binding by 4/7 loop α-conotoxins (Clark et al. 2006). Recent studies by Lopez-Vera et al. (2008) suggest that the P6Hyp modification greatly improves stability of the native conopeptide. They suggest that the gain in stability counterbalances the associated loss of activity.

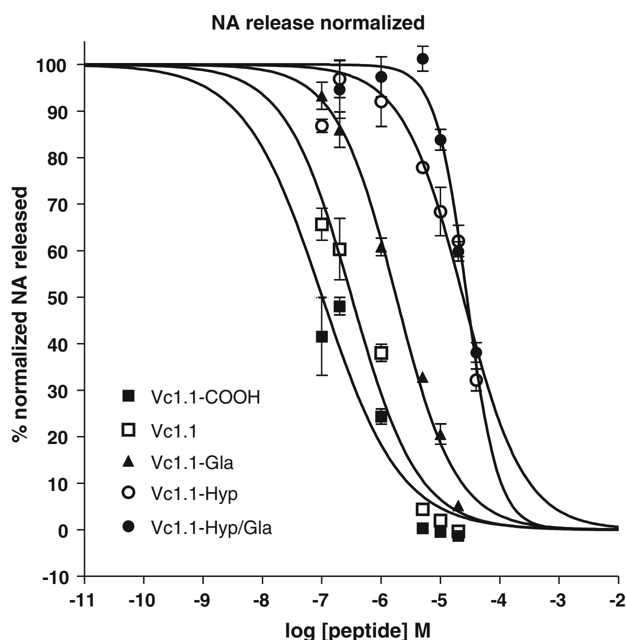

Fig. 8 Dose-dependent antagonism of nAChRs by a range of PTM analogues of Vc1.1. Four-day-old cultures of bovine adrenal chromaffin cells where pre-incubated with 0.1–40 μM of each analogue, then agonised to release noradrenaline using 4 μM nicotine. Relative noradrenaline release was assessed by electrochemical detection of the cell supernatant and cell lysate. The % release of noradrenaline is calculated by normalising with untreated cells as 0% and cells treated only with nicotine as 100%

Table 3 Inhibition of noradrenaline release by PTM analogues of Vc1.1

Analogue	IC_{50} (μM)
Vc1.1-COOH	0.10
Vc1.1	0.32
Vc1.1-Gla	1.80
Vc1.1-Hyp	24.04
Vc1.1-Hyp/Gla	27.93

NMR studies of Vc1.1, Vc1.1[P6Hyp], and Vc1.1 [P6Hyp:E14Gla] (Clark et al. 2006) showed the structure of Vc1.1 to be typical of 4/X α-conotoxins, with a central α-helical region spanning residues Pro^6 to Asp^{11}. This structure was unaltered by the PTMs indicating that the loss of activity of the PTM analogues is not due to a structural change but to differences in the nature of the modified side chains. The 4/7 α-conotoxins, which include Vc1.1, typically have a patch of hydrophobic residues on one face that are thought to mediate receptor binding and specificity for different nAChR subtypes (Shon et al. 1997; Hu et al. 1998; Hill et al. 1998). The surface properties of Vc1.1 (Clark et al. 2006) present fewer hydrophobic residues than other members of this class, which may account for it being the most potent α-conotoxin in tests for analgesia (Satkunanathan et al. 2005). The nAChR subunit specificity of Vc1.1 remains unknown, but has variously been reported to be $\alpha3\beta2$, $\alpha3\beta4$, $\alpha3\alpha5\beta2$ and $\alpha9\alpha10$, based on transgenic *Xenopus* oocyte studies (Clark et al. 2006; Ellison and Olivera 2007). However, no one combination has as yet been identified as the high-affinity target.

The PTMs of Vc1.1 expressed in the venom are seen here to lessen its specificity for mammalian (bovine) nAChRs. It is likely that the functional activity of the native venom is highly specialised towards molluscs, its prey species. This specialisation may be enhanced by the post-translational modification of venom peptides (Olivera et al. 1990; Bingham et al. 1996). Reports of fatal stinging by *Conus* species are restricted to fish-hunting (rather than mollusc- or worm- hunting) species (Flecker 1936; Rice and Halstead 1968). There are no reports of humans being envenomated by *C. victoriae*. Mammalian nAChRs exhibit greater similarity with those in vertebrate fish nAChRs than with those in invertebrate molluscs or worms (Le Novere et al. 2002). This may explain why 3/5 α-conotoxins targeted to fish has potency in mammals. Fish-hunting species

express a greater proportion of 3/5 α-conotoxins, which target muscle-type nAChRs to immobilise fast-moving prey (by paralysis of the pectoral fin). In mammals, these act on the diaphragm to cause death by asphyxiation.

Any effects of *C. victoriae* and related mollusc-hunting species may be due to a small proportion of the conopeptides being present as unmodified species. This is also suggested by our observation of the presence of unmodified Vc1.1 in venom. It is quite possible that a variety of PTMs of any one peptide exist in the venom with the major form being that which is required for the specific prey. This would enable rapid evolution of the venom, through alterations via post-translational modification to meet challenges brought about by changes in the snail's environment. Experiments to confirm this hypothesis could include assays of the efficacy of PTM forms in insect cell lines expressing nAChRs which have been developed for assay of agonistic insecticides (Towers and Sattelle 2002; Jepson et al. 2006). The insect nAChRs provide a closer model of the native activity of venom peptides in invertebrates.

Conclusions

We have shown by use of co-elution experiments that α-conotoxin Vc1.1 is expressed in the venom duct of *C. victoriae* not only as the modified vc1a, as previously reported (Jakubowski et al. 2006) but also as the unmodified Vc1.1. LC/MS analysis of masses in venom obtained from sectioned duct revealed that the venom peptides gained PTMs as they migrated to the distal region. Assay of the three forced-synthesis disulfide isomers of Vc1.1 showed that the globular isomer was the only active form and confirmed that it is also the natively expressed form. Assay of a range of PTM analogues of Vc1.1 found that the P6Hyp modification resulted in the most dramatic loss of activity, some 75-fold, while the other modifications displayed by vc1a had a minimal effect on activity. This confirms that the Pro^6 is essential for receptor interaction and that hydroxylation of Pro^6 reduces receptor specificity. We hypothesise that natively expressed venom components are modified in the venom duct to confer specificity to molluscan nicotinic receptors, coincidentally reducing specificity for mammalian receptors. It is crucial to the development of Vc1.1 as a therapeutic as the absence of PTMs consequently broadens the specificity of the conopeptide to include mammalian receptors.

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