

**STRUCTURE AND ACTIVITY OF α-CONOTOXIN PeIA AT NICOTINIC ACETYLCHOLINE RECEPTOR SUBTYPES AND GABA<sub>B</sub> RECEPTOR-COUPLED N-TYPE CALCIUM CHANNELS**

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α-Conotoxins are peptides from cone snails that target the nicotinic acetylcholine receptor (nAChR). RgIA and Vc1.1 have analgesic activity in animal pain models. Both peptides target the α9 α10 nAChR and inhibit N-type calcium channels via GABA<sub>B</sub> receptor activation, but the mechanism of action of analgesic activity is unknown. PeIA has previously been shown to inhibit the α2α2 nAChRs. In this study, we have determined the structure of PeIA and shown that it is also a potent inhibitor of N-type calcium channels via GABA<sub>B</sub> receptor activation. The characteristic α-conotoxin fold is present in PeIA, but it has a different distribution of surface-exposed hydrophobic and charged residues to Vc1.1. Thus, the surface residue distribution, rather than the overall fold, appears to be responsible for the 50-fold increase in selectivity at the α2α2 nAChR by PeIA relative to Vc1.1. The equipotent activity of PeIA and Vc1.1 at the GABA<sub>B</sub> receptor, in contrast to their difference in potency at the nAChR, suggests that the GABA<sub>B</sub> receptor is more tolerant to changes in surface residues than is the nAChR. The conserved Asp-Pro-Arg motif of Vc1.1 and RgIA, which is crucial for potency at the α9 α10 nAChR, is not required for activity at GABA<sub>B</sub> receptor/N-type calcium channels, since PeIA has a His-Pro-Ala motif in the equivalent position. This study shows that different structure-activity relationships are associated with the targeting of the GABA<sub>B</sub> receptor versus nAChRs. Furthermore, there is probably a much more diverse range of conotoxins that target the GABA<sub>B</sub> receptor than currently realised.

α-Conotoxins are peptides from cone snail venoms that antagonize nicotinic acetylcholine receptors (nAChRs) (1-3). They typically comprise 12-19 amino acids and are characterized by two disulfide bonds that have a Cys<sub>1</sub>-Cys<sub>III</sub> and Cys<sub>II</sub>-Cys<sub>IV</sub> connectivity. Their three-dimensional structures incorporate an α-helix centered around Cys<sub>III</sub> (4) and they are regarded as rigid molecular frameworks based on their well defined secondary structure and disulfide-bracing (5). α-Conotoxins have a range of potential therapeutic applications, including the treatment of pain and disease states such as Parkinson’s disease (6,7).

nAChRs comprise five subunits and a range of different receptor subtypes exist depending on the subunit composition of the pentamer (8,9). Specific subtypes have different expression patterns and pharmacological and biophysical profiles. Targeting individual subtypes can potentially result in drug leads with minimal side effects (10,11). For example, the α9α10 nAChR subtype has been proposed as a potential target for pain relief (12,13). So far, the only α-conotoxins that have been reported to be potent antagonists of the α9α10 nAChR subtype are PeIA (14), RgIA (15) and Vc1.1 (16). PeIA and Vc1.1 belong to the same structural subclass of α-conotoxins in that they both contain four and seven residues respectively in their two inter-cysteine loops. In contrast, RgIA has four and three residues, respectively, in the two loops. The sequences of the three peptides are shown in Figure 1, which highlights that RgIA and Vc1.1 have identical sequences in loop 1 but very different sequences in loop 2. PeIA and Vc1.1 have similar sequences in loop 2 but significantly different charge distributions in loop 1.

RgIA and Vc1.1 exhibit analgesic activity in animal pain models when delivered via injection (16-18) and a cyclic form of Vc1.1 has been shown recently to be orally active, an exciting finding that augers well for its development as a therapeutic agent (19). However, the mechanism by which the analgesic activity of these peptides is exerted is...
unclear. Electrophysiological studies have shown that RgIA and Vc1.1 inhibit N-type calcium (Ca\(^{2+}\)) channel currents in dorsal root ganglia (DRG) neurons and Ca\(_{\alpha 2.2}\) channels expressed in Xenopus oocytes via activation of the G protein-coupled GABA\(_{B}\) receptor (13,20). The fact that the post-translationally modified version of Vc1.1 (vc1a), which has Pro6 hydroxylated and Glu14 in loop 2 converted to a \(\gamma\)-carboxy glutamic acid, is a potent inhibitor of the \(\alpha 9\alpha 10\) nAChR subtype but does not have analgesic activity (7,21) suggests that GABA\(_{B}\) activation is responsible for the analgesic activity. Furthermore, Vc1.1 reversal of mechanical allodynia was antagonized by pre-treatment with the orally active GABA\(_{B}\) receptor antagonist, SCH50911 (22). However, RgIA and Vc1.1 do not potently displace \(^3H\)CGP54626 binding in (22). However, RgIA and Vc1.1 do not potent inhibitor of the suggests that GABA\(_{B}\) activation is responsible for the analgesic activity. Furthermore, Vc1.1 reversal of mechanical allodynia was antagonized by pre-treatment with the orally active GABA\(_{B}\) receptor antagonist, SCH50911 (22). However, RgIA and Vc1.1 do not potently displace \(^3H\)CGP54626 binding in HEK293T cells transiently transfected with GABA\(_{B}\)(1b) and GABA\(_{B}\)(2) subunits nor activate human GABA\(_{B}\) receptors coupled to G protein-activated inwardly rectifying K\(^+\) (GIRK1/4) channels expressed in Xenopus oocytes (12). Thus, further studies are required to resolve the precise mechanism of action of RgIA and Vc1.1 that underlies their analgesic activity.

Analysis of the structure-activity relationships of new peptides that target \(\alpha 9\alpha 10\) or GABA\(_{B}\) receptors might shed light on the mechanism of action of analgesic \(\alpha\)-conotoxins. In this study, we have determined the activity of PeIA on GABA\(_{B}\) receptor modulation of N-type Ca\(^{2+}\) channels for comparison with its activity at \(\alpha 9\alpha 10\) nAChR. We also determined the three-dimensional structure of PeIA. The study reveals that an \(\alpha\)-conotoxin with a distinctive loop 1 compared to Vc1.1 and RgIA is also a potent inhibitor of N-type Ca\(^{2+}\) channels via GABA\(_{B}\) receptor activation.

**Experimental Procedures**

*Peptide synthesis*—PeIA was synthesized using methods described previously for Vc1.1 (23). Oxidation of the disulfide bonds was achieved by stirring overnight in a 0.1 M ammonium bicarbonate buffer.

*NMR spectroscopy*—Spectra were recorded at 600 MHz (Bruker Avance NMR spectrometer) on a sample containing 1 mM PeIA in 10% D\(_2\)O/90% H\(_2\)O. The two-dimensional spectra were recorded and three-dimensional structures calculated as previously described (24). Distance restraints were obtained from a NOESY spectrum recorded with a 200 ms mixing time at 290 K.

*Oocyte electrophysiology*—RNA preparation, oocyte preparation and expression of nAChR subunits in oocytes obtained from Xenopus laevis were carried out as described previously (21). Plasmids with cDNA encoding the rat \(\alpha 9\) and \(\alpha 10\) nAChR subunits were kindly provided by Dr. A.B. Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina) and all other nAChR subunit clones were kindly provided by Dr. J. Boulter (UCLA, Los Angeles, CA). All oocytes were injected with 5 ng of cRNA and then kept at 18°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\) and 5 mM HEPES at pH 7.4) supplemented with 50 mg/L gentamycin, 5 mM pyruvic acid and 5% horse serum 2-5 days before recording.

Membrane currents were recorded from Xenopus oocytes using a two-electrode voltage clamp amplifier (OC-725C Oocyte Clamp; Warner Instruments, Hamden, CT). Recording electrodes were pulled from borosilicate glass (GC150T-15, Harvard Apparatus Ltd., Edenbridge, UK) and had resistances of 0.3-1.5 M\(\Omega\) when filled with 3M KCl. All recordings were conducted at room temperature (21-23°C) using a bath solution of ND96 as described above. During recordings, the oocytes were voltage clamped in a small recording chamber (≤40 µl) at a holding potential of −80 mV and superfused continuously with ND96 via gravity feed tubes at 0.1 - 0.2 ml/min, with 5 min incubation times for the bath applied conotoxins. Acetylcholine was applied via gravity feed tubes switched until peak currents amplitude was obtained (1 - 3 s), with 1 - 2 min washout periods between applications. Data were sampled at 500 Hz and filtered at 200 Hz. Peak current amplitude was measured before and following incubation of the peptide.

All data were pooled (n = 3-7 for each data point) and represent arithmetic mean ± S.E.M. Concentration-response curves for antagonists were fitted by unweighted non-linear regression to the logistic equation: \[ E_x = E_{max} X^n / (X^n + IC_{0.5}^n) \]

where \(E_x\) is the response; \(X\) the antagonist concentration; \(E_{max}\) the maximal response; \(n\) is the slope factor; and \(IC_{0.5}\), the antagonist concentration producing half-maximal inhibition of the agonist response. Computation was done using SigmaPlot 8.0 (Jandel Corporation, San Rafael, CA).

*Electrophysiological recording from rat DRG neurons*—Dorsal root ganglion (DRG) neurons...
were enzymatically dissociated from ganglia of 7-14 day old Wistar rats according to standard protocols. Briefly, rats were killed by cervical dislocation in accordance with the University of Queensland and RMIT University Animal Ethics Committees, the spinal column was semi-segmented and the spinal cord removed. Ganglia were removed and rinsed in cold Hanks’ balanced salt solution (HBSS; MultiCel), minced and incubated in 1 mg/ml collagenase (Type 2; 405U/mg, Worthington Biochemical Corp., Lakewood NJ) in HBSS at 37°C for approximately 30 mins. Following incubation, ganglia were rinsed three times with warm (37°C) Dulbecco’s modified Eagle media (DMEM; GIBCO) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin and gently triturated with a fire-polished Pasteur pipette. Cells were plated on glass cover slips, incubated at 37°C in 95% O₂:5% CO₂ and used within 4-36 hours.

The external recording solution for rat DRG neurons contained (in mM): 150 TEACl, 2 BaCl₂, 10 d-glucose, 10 HEPES, pH 7.3-7.4. Recording electrodes were filled with an internal solution containing (in mM): 140 CsCl, 1 MgCl₂, 5 MgATP, 0.1 NaGTP, 5 BAPTA-Cs₂, 10 HEPES, pH 7.3 with CsOH and had resistances of 1.0-2.5 MΩ. Membrane currents were recorded using the whole cell configuration of the patch clamp technique with an Axopatch 200B amplifier (Molecular Devices Corp., Sunnyvale, CA). A voltage protocol using step depolarisations from ~80 mV to ~10 mV was used when examining high voltage-activated (HVA) Ca²⁺ channel currents. Test potentials of 150 ms duration were applied every 20 s. CGP55845 hydrochloride was obtained from Tocris Bioscience (Bristol, UK) and ω-conotoxin CVID, 100 nM and 300 nM) also antagonized the effects of PeIA (n = 3, Figure 4B). Bath application of PeIA was synthesised using solid phase chemistry and one major disulfide isomer was formed during oxidation. We confirmed that this isomer contained the native disulfide connectivity using an analysis of NMR chemical shifts compared with Vc1.1, as shown in Figure 2A. The three-dimensional structure of PeIA was determined using simulated annealing based on NMR data and the ensemble of the 20-lowest energy structures is shown in Figure 2B. The major element of secondary structure is an α-helix spanning residues 6-10. In addition, type I β-turns are present at residues 1-4, 2-5 and 9-12. The secondary structure present in PeIA is similar to that determined for Vc1.1 (24).

The activity of the synthesized α-conotoxin PeIA was assessed on ACh-evoked currents in Xenopus oocytes expressing different nAChR subunit combinations. Concentration-response curves revealed an inhibition of α9α10 and αββ2 nAChR currents with IC₅₀’s and Hill slopes of 54.9 ± 9.0 nM (n ≥ 4) and nH = 0.6 and 97.5 ± 10.9 nM (n ≥ 4) and nH = 0.8, respectively (Figure 3A, B). In contrast, no significant inhibition of ACh-evoked currents was observed at αββ2, human α7 and muscle αβγδ nAChRs in the presence of 1 µM PeIA.

Depolarization-activated whole-cell Ba²⁺ currents elicited by voltage steps from a holding potential of ~80 mV to ~10 mV were inhibited by PeIA in a concentration-dependent manner. The concentration-response relationship obtained for inhibition of HVA Ca²⁺ channel currents by PeIA gave an IC₅₀ of 1.1 nM (Figure 4A,B). In the presence of the selective N-type Ca²⁺ channel inhibitor, ω-conotoxin CVID, 100 nM PeIA failed to further inhibit the inward Ba²⁺ current. CVID (200 nM) alone reduced the Ca²⁺ channel current amplitude to 57.2 ± 10.8% of control (n = 8, Figure 4A). The expression of the GABAB₁ and GABAB₂ receptor subunits in rat DRG neurons has been confirmed at both the mRNA and protein level (26). Furthermore, inhibition of N-type Ca²⁺ channel currents in rat DRG neurons by the GABAB agonist baclofen has been reported previously (27-29). The inhibition of HVA Ca²⁺ channel currents by PeIA (100 nM) was antagonized in the presence of the selective GABAB receptor antagonist CGP55845A (1 µM) to 87.9 ± 6.1% of control (n = 8, Figure 4C). Lower concentrations of CGP55845 (100 nM and 300 nM) also antagonized the effects of PeIA (n = 3, Figure 4B). Bath application of
1 μM CGP 55845A alone, does not effect HVA Ca$^{2+}$ channel current amplitude (96.4 ± 5.1% of control). In preliminary experiments, PeIA (100 nM) inhibited the peak amplitude of depolarization-activated Ca$^{2+}$ channel currents to 63.3 ± 4.4% (n = 3) of control in baclofen-sensitive Xenopus oocytes expressing recombinant Ca$^{2+}$-sensitive αβ3 (α2β3 + α2δ + β3) (13). Taken together, these data suggest that PeIA inhibits α-conotoxin-sensitive, N-type Ca$^{2+}$ channels via the activation of the G protein-coupled GABA$\textsubscript{B}$ receptor in DRG neurons.

**DISCUSSION**

We report here, for the first time, that PeIA is a new member of the select group of conotoxins known to target G protein-coupled GABA$\textsubscript{B}$ receptors as well as the α9α10 nAChR. The inhibition of N-type Ca$^{2+}$ channels via GABA$\textsubscript{B}$ receptor modulation in DRG neurons by PeIA is similar to that reported for Vc1.1 (1.1 nM for PeIA compared to 1.7 nM for Vc1.1 (13)). Our observations allow some deductions on structure-activity relationships to be made. As well as their shared Cys framework, PeIA and Vc1.1 share two residues in common in loop 1 (Ser4 and Pro6) and four residues in loop 2 (residues 12-14 are identical and residue 15 involves a conservative Leu to Ile substitution). Consequently, it appears that the residues which differ, ie, residues 5 and 7, and 9-11 are not critically important for activity at the GABA$\textsubscript{B}$ receptor based on the similar levels of activity for the two peptides. In particular, our results clearly show that there is not an absolute requirement for a negatively charged residue at position 5 and a positively charged residue at position 7 for GABA$\textsubscript{B}$ receptor activity, as might have been supposed from the conservation of these residues in Vc1.1 and RgIA. The results further suggest that there is probably a much more diverse range of conotoxins that target the GABA$\textsubscript{B}$ receptor than is currently realised, based on the substantial differences seen in loop 1 between PeIA on the one hand and Vc1.1 and RgIA on the other.

The activities of PeIA and Vc1.1 at the α9α10 nAChR are also similar but there are differences observed for other nAChR subtypes. For instance, PeIA is a potent antagonist of the α3β2 subtype, whereas Vc1.1 is >50-fold less active (14,30). A comparison of the structure of PeIA with Vc1.1 is shown in Figure 2B,C. The overall fold of PeIA is similar to that of Vc1.1 but there are differences in the surface characteristics of PeIA in terms of the distribution of charged and hydrophobic residues. These surface differences presumably account for the differences in activity at specific nAChR subtypes.

Mutagenesis studies on Vc1.1 (23) and RgIA (31) have shown that the Asp-Pro-Arg motif in loop 1 is crucial for activity at the α9α10 nAChR. However, in PeIA the equivalent residues are His-Pro-Ala, yet it remains a potent antagonist of the α9α10 nAChR. Interestingly, mutation of Arg7 in Vc1.1 to an alanine, the equivalent residue in PeIA, will also be critical for activity but this probably derives from a structural, rather than functional, role of this residue. Previous studies have shown that when this proline is mutated to an alanine in Vc1.1 significant perturbations in structure are observed (23). Furthermore, we recently showed that a naturally occurring α-conotoxin, L11a, which does not contain this conserved proline, does not have a well-defined structure in solution (32).

PeIA, Vc1.1 and RgIA all have different pi values, varying from 4.5 to 9, and this distinction is reflected in the charge distribution on the surfaces of these molecules. These differences appear more critical at the nAChR based on the different potencies observed at different subtypes. By contrast, the three peptides have similar potency at the GABA$\textsubscript{B}$/N-type Ca$^{2+}$ channel, indicating that electrostatic interactions are not crucial for activity at this receptor. A more complete understanding of the structure-activity relationships of α-conotoxins will require further mutational studies and the testing of naturally occurring α-conotoxins at a range of membrane receptors but the studies reported here provide a valuable starting point.

In summary, we have shown that PeIA has a well-defined three-dimensional structure in solution and report for the first time that it is a potent inhibitor of N-type Ca$^{2+}$ channels coupled to GABA$\textsubscript{B}$ receptors. The information reported here on the structure-activity relationships of α-conotoxins that have
applications as analgesic agents potentially should be useful in the design of novel drug leads for the treatment of pain.

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FOOTNOTES

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The abbreviations used are: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; DRG, dorsal root ganglion; high voltage-activated, HVA; NMR, nuclear magnetic resonance;

FIGURE LEGENDS

Figure 1: Sequences of α-conotoxins that target the α9α10 nAChR. The cysteine residues are connected in the native disulfide connectivity as indicated. The asterisk represents an amidated C-terminus.

Figure 2: NMR chemical shifts and structure of PeIA. (A) The secondary shifts of PeIA (red) and Vc1.1 (blue) were calculated by subtracting random coil α-H shifts (33) from the experimental shifts. The negative secondary shifts for residues 6-11 are indicative of helical structure. The similarities in secondary shifts between the two peptides indicate that the three-dimensional structures are similar. (B) Superposition of the 20 lowest energy structures of PeIA (red) and Vc1.1 (blue). Despite the majority of secondary shifts being negative, only residues 6-10 are formally recognised as an α-helix. Similarly, in the structures calculated previously for Vc1.1 only residues 6-12 are recognised as an α-helix despite the negative secondary shifts at the N and C-termini (30). It appears the turn regions at the N- and C-termini of PeIA and Vc1.1 result in the negative secondary shifts present in these regions. (C) Surface representations of PeIA (left) and Vc1.1 (right). Hydrophobic residues are shown in green, cysteines in yellow, polar in cyan, positively charged in blue and negatively charged residues in red.

Figure 3: Effect of α-conotoxin PeIA on nAChRs subtypes expressed in Xenopus oocytes and GABAB-mediated inhibition of N-type Ca\textsuperscript{2+} channels in rat DRG neurons. (A) Representative superimposed currents evoked by ACh in α9α10, α3β2, and α7 nAChRs were activated by 30 µM and 100 µM ACh, respectively, whereas human α7 nAChRs were activated by 200 µM ACh. (B) Concentration-response relationships obtained for the inhibition of ACh-evoked current amplitudes following 5 min incubation of α9α10 (●) and α3β2 (○) nAChRs with PeIA giving IC\textsubscript{50}'s of 54.9 nM and 97.5 nM, respectively. No significant inhibition was observed with concentrations up to 1 µM PeIA at α4β2 (▼), human α7 (▲) and muscle αβγδ (■) nAChRs. Concentration-response data (mean ± SEM, n = 4-7 for each data point) were fitted using the logistic equation (see Materials and Methods). (C) Superimposed depolarization-activated whole-cell Ba\textsuperscript{2+} currents elicited by a voltage steps from a holding potential of −80 mV to −10 mV in the absence (control) and presence of 10 nM PeIA. (D) Concentration-response relationship obtained for inhibition of HVA Ca\textsuperscript{2+} channel currents in DRG neurons by PeIA (n = 4-13 cells per data point) giving an IC\textsubscript{50} of 1.1 nM. Data points represent mean ± S.E.M of normalized peak current amplitude.

Figure 4: Effect of α-conotoxin PeIA on GABAB\textsubscript{m}-mediated inhibition of N-type Ca\textsuperscript{2+} channels in rat DRG neurons. (A) Superimposed depolarization-activated whole-cell Ba\textsuperscript{2+} currents elicited by voltage steps from a holding potential of −80 mV to −10 mV in the absence (control) and presence of 10 nM PeIA. (B) Concentration-response relationship obtained for inhibition of HVA Ca\textsuperscript{2+} channel currents in DRG neurons by PeIA (n = 4-13 cells per data point) giving an IC\textsubscript{50} of 1.1 nM. The effect of PeIA is antagonized in the presence of 100 nM PeIA. (D) Bar graph of relative inhibition of HVA Ca\textsuperscript{2+} channel currents by 100 nM PeIA alone, in the presence of 1 µM CGP55845 alone and after application of 100 nM PeIA in the presence of CGP 55845. Numbers in parentheses reflect the number of cells. PeIA significantly reduced the
HVA Ca$^{2+}$ channel currents compared to CGP55845 and CGP55845 + PeIA in an unpaired t-test, $P < 0.0001$. Cells treated with CGP55845 alone and PeIA + CGP55845 were not significantly different in a paired t-test, $P = 0.312$. 
Figure 1

PeIA  GCCSHPACSVNHPELC*
Vc1.1 GCCSDPRCNYDHPEIC*
RglA  GCCSDPRCRYR----CR

loop 1  loop 2
Figure 2

A

Secondary shift (ppm)

B

N

C

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Figure 4
Structure and activity of α-conotoxin PeIA at nicotinic acetylcholine receptor subtypes and GABAB receptor-coupled N-type calcium channels
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