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TITLE PAGE

ARE α9α10 NICOTINIC ACETYLCHOLINE RECEPTORS A PAIN TARGET FOR α-CONOTOXINS?

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RUNNING TITLE PAGE

Running Title: α-Conotoxin Vc1.1 and analogues inhibit α9α10 nAChRs

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Abbreviations: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine binding protein; ACV1, α-conotoxin Vc1.1; HEPES, 4-2-(hydroxyethyl)piperazine-1-ethanesulfonic acid; IC_{50}, half-maximal inhibitory concentration; ES-MS, electrospray-mass spectrometry; RP-HPLC, reverse phase-high performance liquid chromatography; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; HBTU: O-benzotriazole-N,N,N',N'-tetramethyl-uronium; HF, hydrogen fluoride; MBHA, 4-methylbenzhydryl amine; TFA, trifluroacetic acid; DSS, 2,2-dimethyl-2-silapentane- 5-sulfonate sodium salt; DQF-COSY, double quantum filtered-correlation spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy

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ABSTRACT

The synthetic α-conotoxin Vc1.1 (ACV1) is a small disulfide bonded peptide currently in development as a treatment for neuropathic pain. Unlike Vc1.1, the native post-translationally-modified peptide vc1a does not act as an analgesic *in vivo* in rat models of neuropathic pain. Recently, it has been proposed that the primary target of Vc1.1 is the α9α10 nicotinic acetylcholine receptor (nAChR). We show that Vc1.1 and its post-translationally modified analogues vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 are equally potent at inhibiting ACh-evoked currents mediated by α9α10 nAChRs. This suggests that α9α10 nAChRs are unlikely to be the molecular mechanism or therapeutic target of Vc1.1 for the treatment of neuropathic pain.

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The α-conotoxins are a subfamily of peptide toxins isolated from the venom of snails from the *Conus* genus that typically range in size from 12 to 16 amino acids, contain two disulfide bonds in a I-III, II-IV connectivity and have an amidated C-terminus (McIntosh et al., 1999). The α-conotoxins interact with both muscle and neuronal nicotinic acetylcholine receptors (nAChRs), which are now implicated in many neurological disorders, and play a role in analgesia and addiction (Dutton & Craik, 2001; Livett et al., 2004; McIntosh et al., 1999). A PCR screen of cDNAs from the venom ducts of *Conus victoriae* was used to reveal α-conotoxin Vc1.1 (Sandall et al., 2003). The native peptide, designated vc1a, was subsequently identified in the venom of *C. victoriae* using MS analysis and has the two residues, Pro6 and Glu14, post-translationally modified to hydroxyproline and γ-carboxyglutamate, respectively (Jakubowski et al., 2004). Synthetic Vc1.1 is a competitive antagonist of neuronal nAChRs in bovine adrenal chromaffin cells (Clark et al., 2006) and is most potent at recombinant α9α10 nAChRs expressed in *Xenopus* oocytes (Vincler et al., 2006). Vc1.1 also antagonizes the nicotine-induced increase in axonal excitability of unmyelinated C-fiber axons in isolated segments of peripheral human nerves (Lang et al., 2005). Inhibition of nAChRs on unmyelinated peripheral sympathetic and/or sensory axons may alleviate pain associated with small-fiber neuropathies. Surprisingly, synthetic vc1a was not able to antagonize neuronal nAChRs in chromaffin cells and was also found to be inactive in two rat neuropathic pain assays (Livett et al., 2002). In contrast, it has been reported that Vc1.1 alleviates neuropathic pain in three rat models of human neuropathic pain and accelerates the functional recovery of injured neurons (Satkunanathan et al., 2005). Vc1.1 is currently under development as neuropathic pain treatment without a specific receptor target being unequivocally identified (Livett et al., 2004, 2006).

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MATERIALS AND METHODS

Peptide synthesis and oxidative folding. Vc1.1, vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 were synthesized by manual solid-phase peptide synthesis on MBHA amide resin using the in situ neutralisation/HBTU protocol for Boc chemistry (Schnolzer et al., 1992). Cleavage of the peptides from the resin was achieved using HF with p-cresol and p-thiocresol as scavengers (9:0.5:0.5 (v/v) HF:p-cresol:p-thiocresol). The reaction was allowed to proceed at –5 to 0°C for 1.5 h. The HF was then removed under vacuum and the peptides precipitated with ether, filtered, dissolved in 50% acetonitrile containing 0.05% TFA and lyophilised. The crude peptides were purified by RP-HPLC on a Phenomenex C_{18} column using a gradient of 0-80% B (Buffer A – H_2O/0.05% TFA, Buffer B – 90% CH_3CN/10% H_20/0.045% TFA) in 80 minutes and the eluent was monitored at 230 nm. These conditions were used in subsequent purification steps. Analytical RP-HPLC and ES-MS confirmed the purity and molecular mass of the synthesized peptides. The linear peptides were oxidised by dissolving in 0.1M NH_4HCO_3 (pH 8.2) at a concentration of 0.3 mg/ml and stirring overnight at room temperature. The oxidised peptides were then purified by RP-HPLC and the molecular mass confirmed by ES-MS. All four peptides, Vc1.1, vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1, formed almost exclusively a single isomer with a monoisotopic molecular mass of 1809.7, 1866.6, 1821.6 and 1850.9 Da, respectively, determined by ES-MS (see Clark et al., 2006).

NMR Spectroscopy. NMR data for Vc1.1, vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 were recorded on samples dissolved in 90% H_2O/10% D_2O at pH 3.5. Bruker ARX 500 and 600 MHz spectrometers were used in the acquisition of data. 2D NMR experiments included DQF-COSY, TOCSY and NOESY, with all spectra recorded at 280 K. All spectra were analysed on Silicon Graphics Indigo (SGI) workstations using XWINNMR 1.3 (Bruker) and Sparky software. Chemical shifts were referenced to DSS at 0 ppm.

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Electrophysiological recordings from nAChRs exogenously expressed in *Xenopus* oocytes. RNA preparation, oocyte preparation and expression of nAChR subunits in *Xenopus* oocytes were performed as described previously (Clark et al., 2006). Plasmids with cDNA encoding the rat α9 and α10 nAChR subunits were kindly provided by Dr. A.B. Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). All oocytes were injected with 1-5 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 and 5 mM pyruvic acid 2-5 days before recording.

Membrane currents were recorded from *Xenopus* oocytes using an automated workstation with eight channels in parallel, including drug delivery and on-line analysis (OpusXpress™ 6000A workstation, Molecular Devices, Union City, CA). Both the voltage recording and current injecting electrodes were pulled from borosilicate glass (GC150T-15, Harvard Apparatus Ltd., Edenbridge, UK) and had resistances of 0.3-1.5 MΩ when filled with 3M KCl. All recordings were conducted at room temperature (20-23°C) using a bath solution of ND96 as described above. During recordings, the oocytes were perfused continuously at a rate of 1.5 ml/min, with 300 s incubation times for the conotoxin. Acetylcholine (ACh; 30 μM) was applied for 2 s at 5 ml/min, with 600 s washout periods between applications. Conopeptides were bath applied and co-applied with the agonist. Cells were voltage clamped at a holding potential of –80 mV. Data were sampled at 500 Hz and filtered at 200 Hz. Peak current amplitude was measured before and following incubation of the peptide.

Neuropathic pain was assessed using partial ligation of the left sciatic nerve (PNL) (Seltzer et al., 1990). Briefly, the left sciatic nerve in the mid thigh region of male Sprague-Dawley rats (250-300 g) was exposed by blunt dissection through the biceps femoris. A 4-0 silk suture was inserted into the left sciatic neve trunk approximately 3 mm proximal to the

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trifurcation of the nerve at the popliteal fossa and was tightly ligated so that the dorsal 30-50% of the nerve thickness was trapped in the ligature.

The effects of the conotoxins on withdrawal thresholds and motor function were assessed between 12-16 days post-PNL in all animals. On the day of drug testing, animals were tested twice pre-injection (30 minutes prior to and just before injection) in order to stabilise pre-injection responses, then at 1, 2 and 4 hours post-injection. Conotoxins were injected in a normal saline vehicle (0.2 ml per animal) intramuscularly in the vicinity of the nerve injury. Mechanical paw withdrawal thresholds (PWT) were measured using a series of von Frey filaments with bending pressures ranging from 0.41-15.1 g. Rats were placed in elevated plastic cages with wire mesh bases. All rats were given 30 min to acclimate to the testing environment. Beginning with the 2 g filament, von Frey hairs were pressed perpendicularly against the plantar surface of the left hind paw and held for 2 seconds. Each von Frey filament was applied 7 times at random locations. A positive response was regarded as the sharp withdrawal of the paw, paw licking, or flinching upon removal of the von Frey filament.

The mechanical PWT was calculated using the up-down paradigm (Chaplan et al., 1994). If the animals responded to all or did not respond to any hairs then the mechanical PWT was assigned as 0.2 or 15 g, respectively. The tester was blind to all treatments.

Statistics. Concentration-response curves for antagonists were fitted by unweighted non-linear regression to the logistic equation:

$$E_x = E_{max}X^n/(X^n + IC_{50}{}^n) \qquad [1]$$

where E_x is the response; X the antagonist concentration; E_{max} the maximal response; n, is the slope factor; and IC_{50}, the concentration of antagonist that gives 50% inhibition of the agonist response. All electrophysiological data were pooled (n = 3-7 for each data point) and represent arithmetic means ± S.E. of the fit. Computation was done using SigmaPlot 9.0

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(Jandel Corporation, San Rafael, CA). Statistical comparisons of mechanical PWT over time were made using a two-way analysis of variance (ANOVA, using time and drug treatment as a within- and between-subjects factors), with post-hoc comparisons over time for individual drug treatment groups using Sidak's adjustment for multiple comparisons (SPSS).

RESULTS

Sequences and structural comparison of α-conotoxin Vc1.1 and its post-translationally modified analogues. In this study we synthesised α-conotoxin Vc1.1, the post-translationally modified native peptide vc1a, and the intermediate analogues [P6O]Vc1.1 and [E14γ]Vc1.1 and compared their structures and selectivity for nAChR subtypes. The sequences and disulfide connectivity are shown in Table 1. [P6O]Vc1.1 and [E14γ]Vc1.1, which contain either a hydroxyproline in position 6 or a γ-carboxyglutamate at position 14, respectively, together with the unmodified peptide Vc1.1 and the fully modified vc1a, provide the full complement of analogues to determine the role of the post-translationally modified residues on activity.

Vc1.1, vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 were found to fold efficiently into the predominant "globular" isomer, which has a I-III, II-IV disulfide connectivity common to all α-conotoxins (see Fig. 1A). Fig. 1B shows a comparison of the NMR αH secondary shifts for the four peptides. This analysis reveals that they are structurally almost identical, with the only chemical shift changes being where there is a change in residue type at positions 6 and/or 14. This similarity in structure is reinforced by the fact the αH secondary shift for [P6O]Vc1.1 matches vc1a at position 6 (as both share a hydroxyproline at this position) and matches Vc1.1 at position 14 (both Glu) and the reverse is the case for [E14γ]Vc1.1. In addition, the chemical shift differences between the Hβ protons of the side chains of comparable residues in each molecule are also similar, suggesting that the side chain

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orientations in all four peptides are also very similar (see Fig 1C). Therefore any differences in the biological targets of these peptides are not due to major changes in the three-dimensional shape of the molecules.

Inhibition of rat α9α10 nAChRs expressed in *Xenopus* oocytes by Vc1.1 and its post-translationally modified analogues. Vc1.1 has been shown previously to inhibit α3-containing nAChRs but only at micromolar concentrations and was inactive at concentrations up to 10 μM at α7, α4-containing and muscle (α1β1γδ) nAChRs expressed in oocytes (Clark et al., 2006). The potency and selectivity of Vc1.1, vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 were investigated for their effects on ACh-evoked membrane currents in *Xenopus* oocytes expressing various nAChRs subunit combinations. Vc1.1 has been shown to act as a competitive antagonist at nAChRs in bovine chromaffin cells (Clark et al., 2006) and in oocytes raising the ACh concentration to 100 μM shifted the Vc1.1 concentration-response curve to the right compared to 30 μM ACh (data not shown). We examined the effects of Vc1.1, vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 on α9α10 nAChRs expressed in *Xenopus* oocytes. Globular Vc1.1 inhibited reversibly α9α10 nAChR-mediated currents in a concentration-dependent manner with an IC_{50} of 64.2 ± 15.0 nM (n = 12) (Fig. 2A) whereas the ribbon isomer (*i.e.* I-IV, II-III disulfide connectivity) was inactive at concentrations up to 10 μM (n = 4). Application of vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 also inhibited reversibly α9α10 nAChRs in a concentration-dependent manner, giving IC_{50}'s of 62.9 ± 5.2 nM, 99.1 ± 29.7 nM, 65.3 ± 14.9 nM (n = 10-12), respectively (Fig. 2B). The inhibition of ACh-evoked currents mediated by α9α10 nAChRs was complete at peptide concentrations ≥ 1 μM, in contrast to that observed for other nAChR subunit combinations (Clark et al., 2006), and the Hill coefficients indicate a unitary toxin:receptor stoichiometry (see Table 2).

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Intramuscular injection of α-conotoxin Vc1.1, but not vc1a or [P6O]Vc1.1, relieves mechanical allodynia in a nerve injury model of neuropathic pain. PNL produced a profound reduction in paw withdrawal threshold from a pre-surgery baseline of 12.9 ± 0.7 g to 0.7 ± 0.1 g (n = 33) 12-16 days after surgery (see Figure 3). As reported previously (Satkunanathan et al., 2005), intramuscular injection of 60 μg Vc1.1 produced significant partial reversal of allodynia associated with nerve injury (Figure 3). By contrast, injections of high doses of vc1a or [P6O]Vc1.1 (60 μg/rat each) had no effect on mechanical allodynia. The small apparent (non-significant) increase in withdrawal threshold observed 4 hours after administration of [P6O]Vc1.1 suggests that this analogue may produce a delayed anti-allodynic effect compared with Vc1.1. At present, we cannot exclude the possibility that a delayed effect of this analogue of Vc1.1 is due to reduced bioavailability.

DISCUSSION

Although the analgesic conopeptide Vc1.1 has been reported previously to exhibit selectivity for α3-containing nAChRs (Clark et al., 2006), we demonstrate here that it is approximately 100-fold more potent for α9α10 nAChRs (see also Vincler et al., 2006), and produces a significant partial reversal of allodynia associated with nerve injury. Similarly, the post-translationally modified peptides vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 inhibit α9α10 nAChRs with equivalent potencies to Vc1.1. In contrast, vc1a and [P6O]Vc1.1 at concentrations up to 10 μM failed to inhibit ACh-evoked currents mediated by α1β1γδ, α3β2, α3β4, α4β2, α4β4 and α7 nAChRs subunit combinations expressed in oocytes (Clark et al, 2006), and had no effect on mechanical allodynia. The lack of activity of vc1a on these neuronal nAChR subtypes is consistent with findings reported previously in bovine chromaffin cells and other rat models of neuropathic pain (Livett et al., 2002), however, vc1a is equally potent with Vc1.1 as an antagonist of α9α10 nAChRs.

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A comparison of the NMR chemical shifts for vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1 with those for Vc1.1 showed that the post-translationally modified analogues are structurally analogous to Vc1.1 and therefore variations in biological activity between these peptides is due to the side chain modifications and not a structural perturbation. Substitution of Pro6 in Vc1.1 to hydroxyproline in [P6O]Vc1.1 results in a loss of activity at α3-containing nAChR subtypes (Clark et al., 2006), but the current study shows that activity at the α9α10 nAChR subtype is not negated by this mutation. Recently, the crystal structure of the α-conotoxin ImI, which has an identical loop 1 to Vc1.1, in complex with acetylcholine binding protein (AChBP), a soluble homolog of the ligand binding domain of the nAChR, has been reported (Ulens et al., 2006). This structure revealed that Pro6 in ImI makes van der Waal contacts with Tyr53 and Ile116 of the AChBP. The α3 nAChR subunit also has an Ile at the corresponding position to Ile116 in the AChBP. Therefore, it seems likely that a hydroxyproline in position 6 of the conotoxin could disrupt key hydrophobic interactions with the α3 subunit. In contrast, in both the α9 and α10 nAChR subtypes, the residue corresponding to Ile116 is an aspartic acid. Therefore, mutation of Pro6 in Vc1.1 to hydroxyproline may be counteracted by interactions between the new hydroxyl group and the aspartic acid in the α9 and α10 subunits. This is further supported by work that found that substitution of Pro6 with Hyp in α-conotoxin PnIB caused a dramatic reduction in activity on the α7 nAChR subtype (Quirim et al., 2000). The α7 nAChR subtype also has a hydrophobic residue (leucine) at the position corresponding to Ile116 in the AChBP. The mutation of Glu14 to a γ-carboxyglutamic acid is a more conservative residue change so it is not surprising that it has little effect on activity. In addition, the crystal structures of α-conotoxins with the AChBP shows that this residue is more solvent exposed and is less important for receptor interactions.

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In conclusion, synthetic vc1a and the partially modified homologues [P6O]Vc1.1 and [E14γ]Vc1.1 are all active at α9α10 nAChRs, but not at any of the other nAChR subtypes studied (Clark et al., 2006). Recent studies of α-conotoxins Vc1.1 and RgIA have also attributed the acute analgesia produced by these conotoxins to the antagonism of α9α10 nAChRs (Ellison et al. 2006; Vincler et al. 2006; Vincler & McIntosh, 2007). However, given that Vc1.1, but not vc1a nor its analogue [P6O]Vc1.1, were able to inhibit a vascular response to pain and reduce chronic pain in several animal models of human neuropathy (Lang et al., 2005; Livett et al., 2002, 2006), it is highly unlikely that α9α10 nAChRs are the molecular mechanism or therapeutic target of Vc1.1 for the treatment of neuropathic pain.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Structural comparison of Vc1.1 and its post-translationally modified analogues. ***A***, The three dimensional structure of Vc1.1 showing the α-helical region and "globular" disulfide connectivity: Cys^{I}-Cys^{III} and Cys^{II}-Cys^{IV}. The side chains of Pro6 (green) and Glu14 (red), the two residues subject to modification, are highlighted. ***B***, The αH secondary shifts for Vc1.1 (blue), vc1a (red), [P6O]Vc1.1 (green) and [E14γ]Vc1.1 (purple). The peptides have almost identical secondary shifts, indicating that the structures are very similar. The changes highlighted by the arrows and boxes for Pro/Hyp6 and Glu/Gla14 are local effects confined to the points of substitution. ***C***, βH chemical shift differences for Vc1.1 (blue), vc1a (red), [P6O]Vc1.1 (green) and [E14γ]Vc1.1 (purple). Values for Gly1 and Ile15 are not shown as they do not have two β protons. The similarity in most values for all four molecules suggests that the side chains are in comparable orientations. The boxes highlight the βH chemical shift differences for the modified residues. As for the αH secondary shifts, there is a pairing of the trends based on the residue type at positions 6 or 14.

Figure 2. Inhibition of rat α9α10 nAChRs expressed in *Xenopus* oocytes by Vc1.1 and its post-translationally modified analogues vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1.
A, Representative superimposed traces of ACh-evoked currents in the absence (solid line) and presence Vc1.1 and vc1a (broken lines). Oocytes were voltage clamped at –80 mV and membrane currents were evoked with 30 μM ACh in the absence and presence of the peptide.
B, Concentration-response curves obtained for the inhibition of ACh-evoked current amplitude following 300 s incubation of Vc1.1 and its analogues, Vc1.1 (●), vc1a (■), [P6O]Vc1.1 (▲) and [E14γ]Vc1.1 (**x**). Concentration-response data for the antagonists were fit using equation [1].

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Figure 3. Intramuscular injection of α-conotoxin Vc1.1 (60 μg) (●) relieves mechanical allodynia in a nerve injury model of neuropathic pain but vc1a (■) and [P6O]Vc.1.1 (▲) are both inactive at high doses (60 μg/ rat). ***A***, time-response relationship of mechanical paw withdrawal response. ** $p < 0.002$ for Vc1.1 versus baseline, n = 6-11 animals per group. ***B***, Area under the curve for the anti-allodynic effect, ** $p < 0.001$ for Vc1.1.

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Table 1. The sequences of Vc1.1, vc1a, [P6O]Vc1.1 and [E14γ]Vc1.1.

Peptide	Sequence																
		I	II					III						IV			
Vc1.1	G	C	C	S	D	P	R	C	N	Y	D	H	P	E	I	C	*
vc1a	G	C	C	S	D	**O**	R	C	N	Y	D	H	P	**γ**	I	C	*
[P6O]Vc1.1	G	C	C	S	D	**O**	R	C	N	Y	D	H	P	E	I	C	*
[E14γ]Vc1.1	G	C	C	S	D	P	R	C	N	Y	D	H	P	**γ**	I	C	*
				Loop 1					Loop 2								

Positions subject to modification are highlighted in light grey, with hydroxyproline and γ-carboxyglutamic acid represented in bold font. The CysI-CysIII and CysII-CysIV disulfide bonds are indicated and the loops between CysII and CysIII and CysIII and CysIV are named Loops 1 and 2 respectively. The asterisk indicates C-terminal amidation.

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Table 2. Inhibition of recombinant α9α10 nAChR subunits expressed in *Xenopus* oocytes by Vc1.1 and post-translationally modified peptides.

	IC_{50} (nM)	**n_H**	**n**
Vc1.1	64.2 ± 15.0	1.1 ± 0.2	12
vc1a	62.9 ± 5.2	1.3 ± 0.1	11
[P6O] Vc1.1	99.1 ± 29.7	0.7 ± 0.1	10
[E14γ]Vc1.1	65.3 ± 14.9	1.2 ± 0.3	10

Membrane currents evoked with 30 μM ACh; Pooled data (n = 3-7 for each data point) for curve fitting; IC_{50}, half-maximal inhibitory concentration; n_H, Hill coefficient; n, number of oocytes; γ, gamma-carboxyglutamic acid.

Fig.1

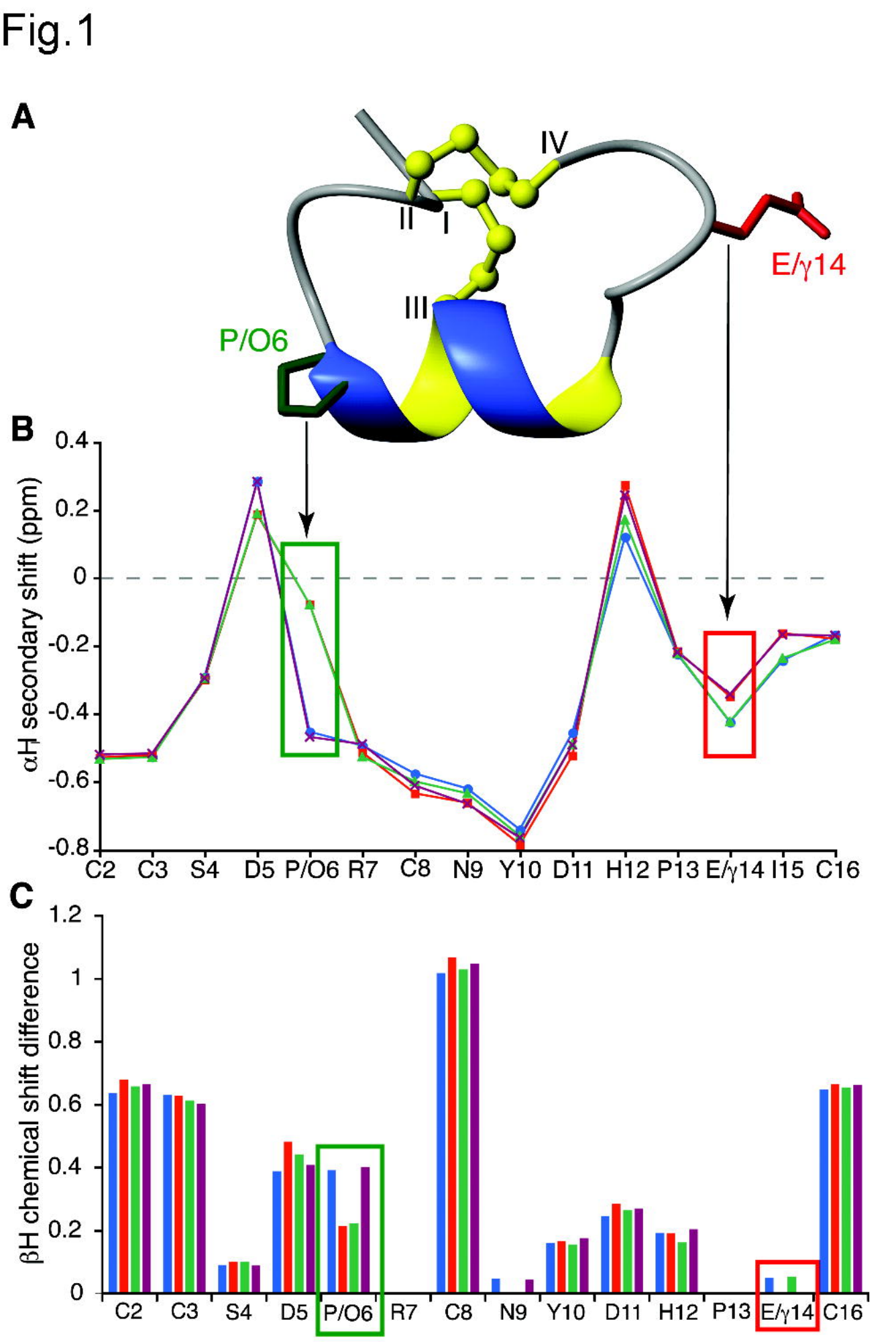
A
IV
II
I
III
E/γ14
P/O6
B
αH secondary shift (ppm)
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
C2 C3 S4 D5 P/O6 R7 C8 N9 Y10 D11 H12 P13 E/γ14 I15 C16
C
βH chemical shift difference
1.2
1
0.8
0.6
0.4
0.2
0
C2 C3 S4 D5 P/O6 R7 C8 N9 Y10 D11 H12 P13 E/γ14 C16

Fig.2

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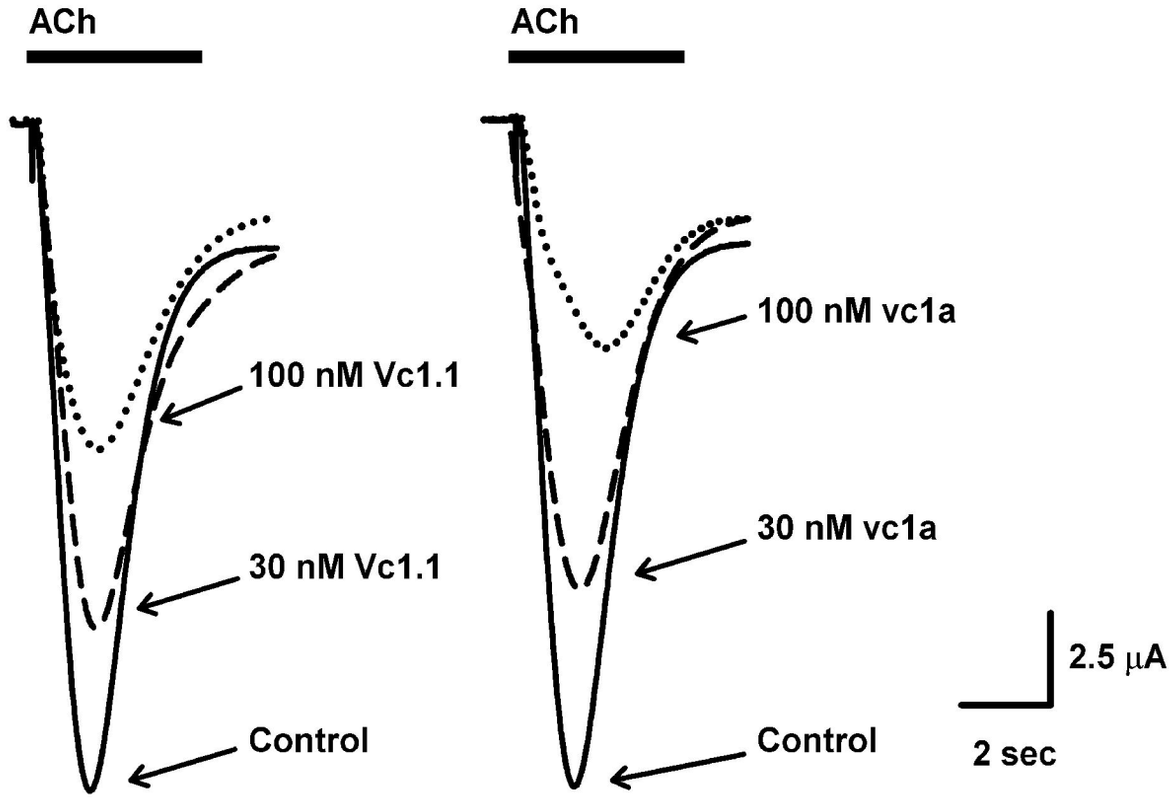


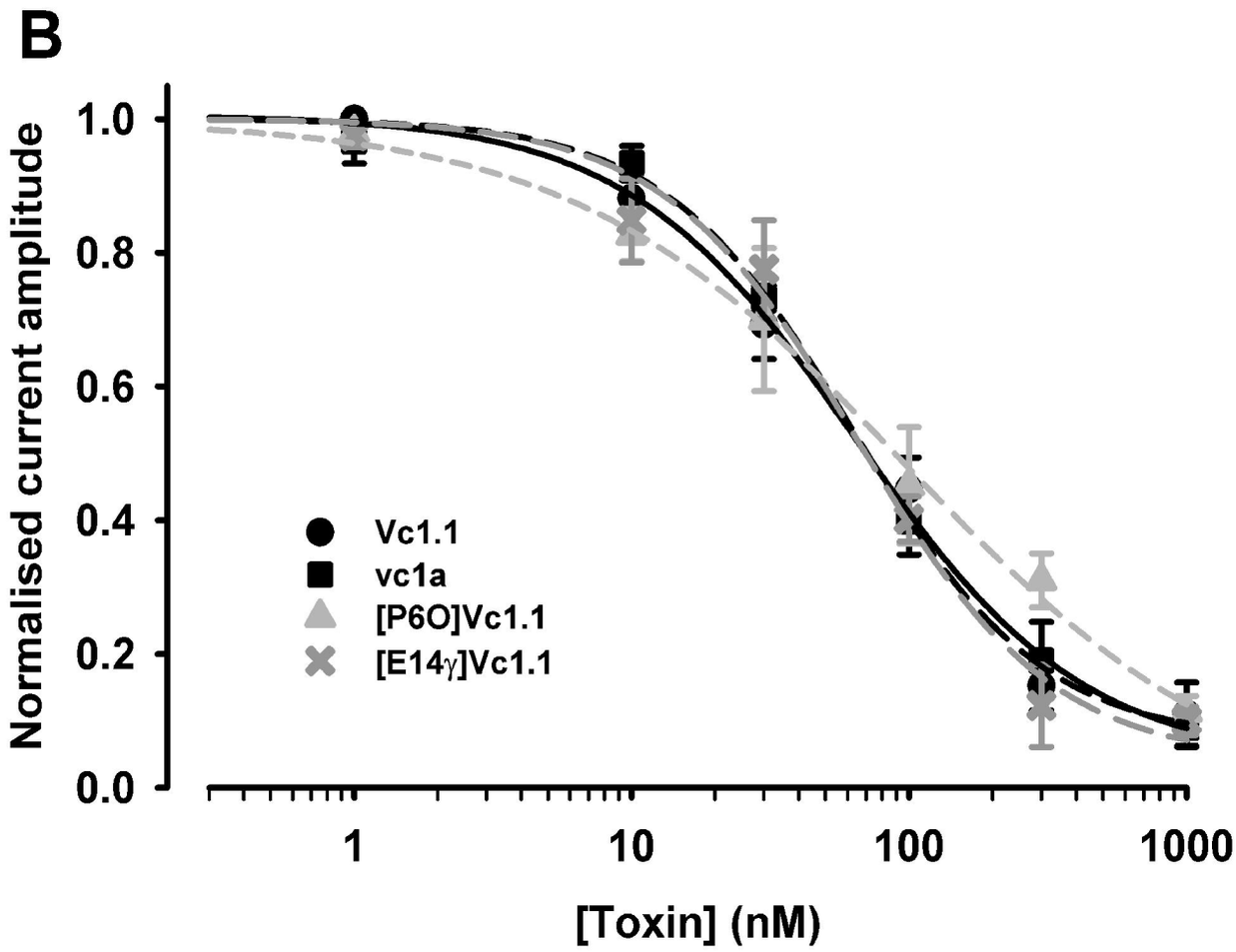
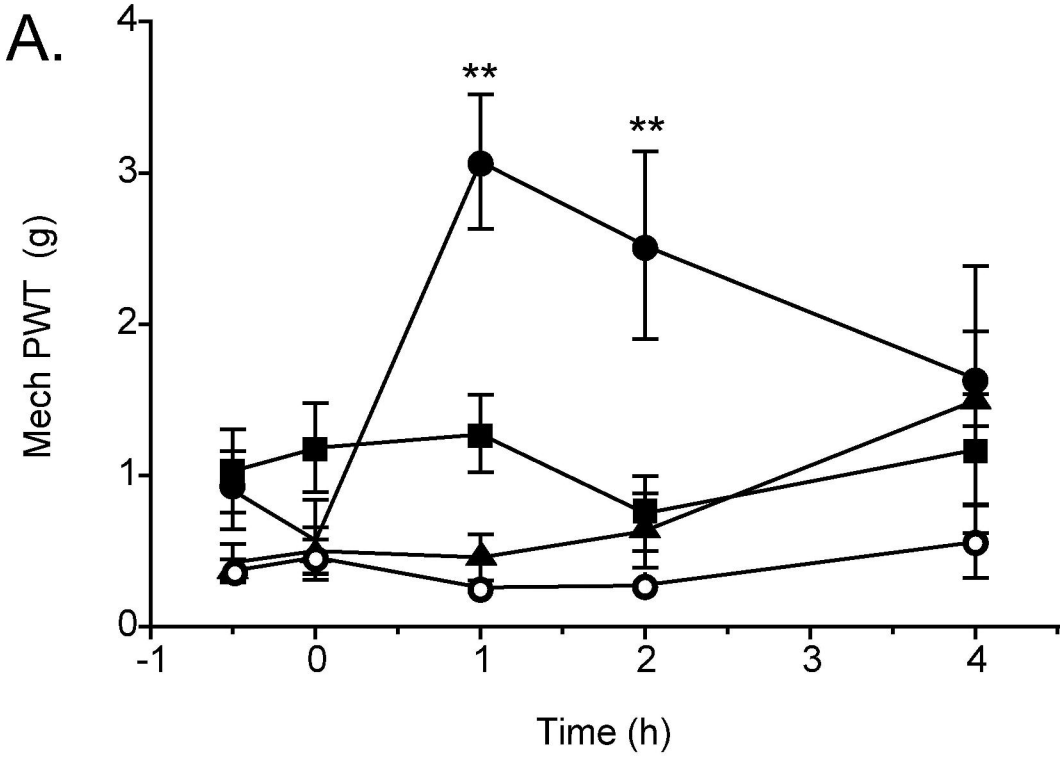
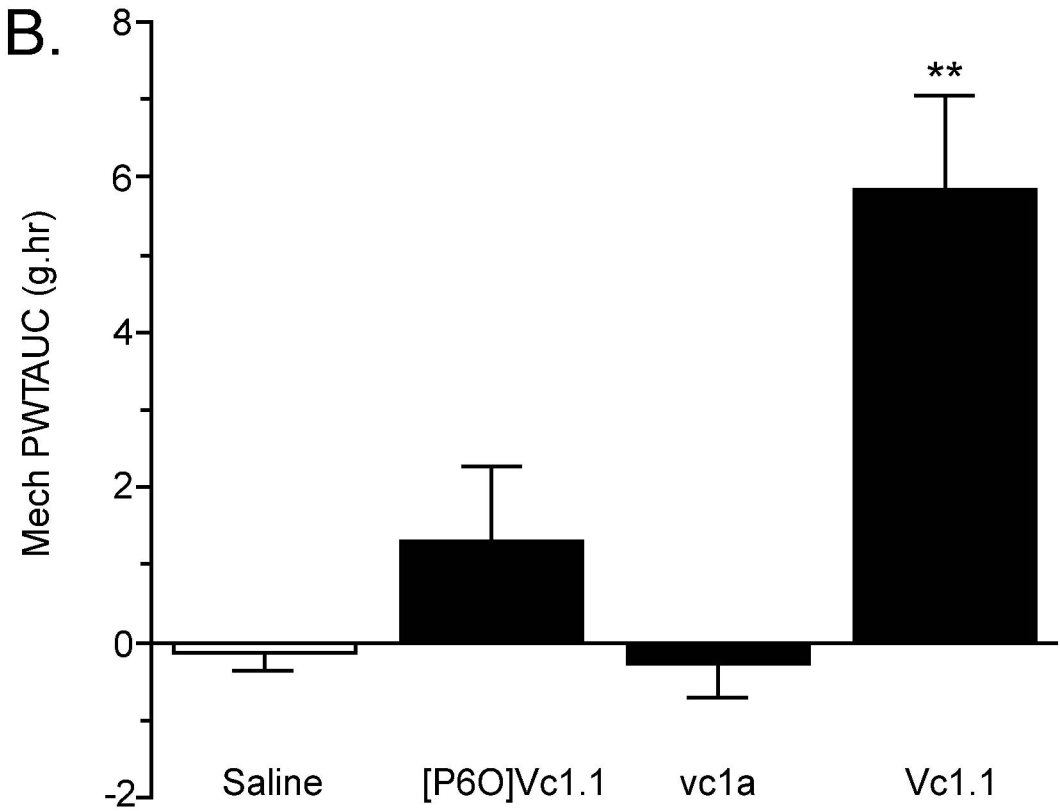


Fig. 3


A.
Mech PWT (g)
Time (h)
**
**
4
3
2
1
0
-1
0
1
2
3
4


B.
Mech PWTAUC (g.hr)
**
8
6
4
2
0
-2
Saline
[P6O]Vc1.1
vc1a
Vc1.1