Analgesic α-conotoxins Vc1.1 and RgIA inhibit N-type calcium channels in sensory neurons of α9 nicotinic receptor knockout mice

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Alpha-conotoxins Vc1.1 and RgIA are peptides from the venom of marine Conus snails that are currently in development as a treatment for neuropathic pain. We have reported previously that the α9α10 nicotinic acetylcholine receptor (nAChR) selective-conotoxins Vc1.1 and RgIA potently and selectively inhibit high voltage-activated (HVA) N-type calcium channel currents in dissociated neurons from rat dorsal root ganglia (DRG). Our data indicated that Vc1.1 does not interact directly with N-type Ca2+ channels but inhibits them via GABA_B receptor activation. The present study investigated Vc1.1 and RgIA inhibition of N-type Ca2+ channels currents in DRG neurons of wild-type and α9 knockout (KO) mice to determine if the α9 nAChR was necessary for inhibition of the Ca2+ channel current. Application of Vc1.1 (100 nM) inhibited N-type Ca2+ channel currents to 69.2 ± 3.5% of control in DRG neurons isolated from wild-type mice. In >70% of DRG neurons isolated from the α9 KO mice, both Vc1.1 and RgIA selectively inhibited N-type Ca2+ channel currents with an IC50 of 24.6 nM and 22.4 nM, respectively. The GABAB receptor antagonist CGP55845 (1 μM) antagonized the effect of Vc1.1 and RgIA on the N-type calcium channels in α9 KO mice. RT-PCR and western blot analysis confirmed the absence of the α9 nAChR in mice carrying a null mutation for the nAChR α9 gene. These results demonstrate that the inhibition of N-type Ca2+ channel currents by Vc1.1 and RgIA is not mediated by the expression of α9α10 nAChRs in DRG neurons.

Key words: conotoxin, nicotinic receptors, α9 knockout mice, N-type calcium channel, dorsal root ganglion, GABA_B receptor, analgesia

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Results

To assess if the inhibition of Ca²⁺ channel currents in rat DRG neurons by Vc1.1 and RgIA required the expression of α9 nAChR subunit, we investigated the effect of Vc1.1 and RgIA on Ca²⁺ channel currents recorded from wild-type and α9 KO adult mouse DRG neurons. Vc1.1 (100 nM) inhibited HVA Ca²⁺ channel currents elicited by a voltage step to -10 mV to 69.0 ± 3.6% of control in 55% (11/20) of DRG neurons isolated from wild-type mice. As shown previously in rat DRG neurons, the inhibition of Ca²⁺ channel currents by Vc1.1 was also blocked by selective GABAB receptor antagonists. In wild-type mouse DRG neurons unresponsive to Vc1.1, the GABAB receptor agonist, baclofen (100 μM), also failed to inhibit the Ca²⁺ channel current (109 ± 7.4%, n = 10 of control).

RT-PCR analysis was carried out on mRNA extracted from pooled ganglia of 3 wild-type and 3 α9 KO mice using primers that would produce a 594 base pair product if α9 was expressed. Figure 1A shows that DRG neurons collected from wild-type mice possess a fragment of the predicted size and confirmed the absence of α9 mRNA in KO mice. Neurons collected from rat DRG also expressed α9 nAChR mRNA (data not shown). GAPDH was used as a control to verify the quality of the RNA/cDNA. Since α9 nAChR mRNA was detected in wild-type DRG neurons we examined protein levels by western blotting. No protein was recognized by the α9 nAChR antibody in 50 μg membrane enriched DRG fractions prepared from α9 KO and wild-type mice. The α9 nAChR antibody detected several bands in 15 μg membrane enriched wild-type mouse brain fractions. Control western blots using an actin antibody (lower) on the same quantities of protein as above of wild-type mouse brain and DRG from wild-type and α9 KO mice.

Discussion

Our previous study identified N-type calcium channels of rat DRG neurons as an indirect target of the α-conotoxins Vc1.1 and RgIA. These α-conotoxins did not inhibit the N-type Ca²⁺ channels directly but the effects could be antagonized by inhibiting G protein hydrolysis, c-Src activity and GABA_B receptor activation. N-type Ca²⁺ channels can be modulated indirectly by a variety of G protein-coupled receptors (GPCRs) via second messenger molecules in a voltage-independent manner or modulated directly in a voltage-dependent manner via the G-protein βγ subunit. In some cases, GPCRs activate both voltage-dependent and voltage-independent pathways. Vc1.1 inhibited N-type Ca²⁺ channels in a voltage-independent manner only; despite the fact that activation of the GABA_B receptor by baclofen inhibits N-type Ca²⁺ channels also via the voltage-dependent pathway.10 Given that Vc1.1 and RgIA have been reported to not displace [3H]-CGP54626 binding to HEK293T cells transiently transfected with GABA_B1 and GABA_B2 subunits, the precise binding site and mechanism of action of these α-conotoxins remain to be elucidated. The most striking aspect of these two peptides is that they have been shown to be potent antagonists of α9α10 nAChRs.2,5 Our previous study indicated that activation of acetylcholine receptors was not necessary for α-conotoxin inhibition of N-type Ca²⁺ channels. Antagonists of nicotinic and muscarinic AChR, such as mecamylamine, hexamethonium, atropine and desensitizing concentrations of ACh did not antagonize the effect of Vc1.1. α10 nAChRs cannot form homomeric receptors and only forms heteromeric receptors with the α9 receptor.12 The present study examined whether the

Figure 1. (A) PCR analysis of α9 transcript in DRG neurons of wild-type and KO mice. Total RNA (200 ng) from DRG neurons of wild-type and α9 KO mice was used with oligonucleotides specific to the α9 subunit. RT-PCR analysis using primers for GAPDH was used as a control for the RNA isolation and cDNA synthesis from both the wild-type and KO mice. (B) Western blots using an α9 subunit antibody (upper) to detect the presence of α9 protein in 50 μg membrane enriched DRG fractions prepared from α9 KO and wild-type mice. The α9 nAChR antibody detected several bands in 15 μg membrane enriched wild-type mouse brain fractions. Control western blots using an actin antibody (lower) on the same quantities of protein as above of wild-type mouse brain and DRG from wild-type and α9 KO mice.
presence of α9α10 nAChRs was necessary for N-type Ca2+ channel inhibition by Vc1.1 and RgIA. The binding of these α-conotoxins to α9α10 nAChRs may be a prerequisite to GABA\textsubscript{B} receptor activation and the possible internalization of the receptor/Ca2+ channel complex.\textsuperscript{13} We showed that the inhibition of N-type Ca2+ channels by these α-conotoxins was not species dependent and confirmed that the effect was inhibited by GABA\textsubscript{B} receptor antagonists in mice. Furthermore, we demonstrated through the use of α9 KO mice that expression of α9α10 nAChRs cannot be involved in mediating the inhibition of N-type Ca2+ channels by Vc1.1 and RgIA. These data support our original report\textsuperscript{8} that α-conotoxins Vc1.1 and RgIA inhibit N-type calcium channels in DRG sensory neurons via the GABA\textsubscript{B} receptor signalling pathway.

**Materials and Methods**

**Dorsal root ganglion (DRG) neuron preparation.** Knockout (KO) mice of the background strain 129/SvEv lacking the α9 nAChR subunit were obtained from Dr. Douglas Vetter (Tufts Univ, Boston MA). DRG neurons were enzymatically dissociated from ganglia of adult α9 nAChR knockout mice and wild-type 129/SvEv and CBA mice according to standard protocols. Mice were killed by cervical dislocation as approved by the University of Queensland Animal Ethics Committee. Isolated mouse DRG neurons were prepared following the same protocol as used previously to prepare rat DRG neurons.\textsuperscript{8}

**Total RNA isolation and RT-PCR.** Total RNA was prepared from dorsal root ganglia of wild-type and α9 KO mice using the NucleoSpin Total RNA isolation kit (Macherey-Nagel Inc.,) as per manufacturer’s instructions. 200 ng of total RNA was used for cDNA synthesis and PCR amplification using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen) as per manufacturer’s instructions. The PCR primers used to amplify α9 transcripts were A903 (GenBank accession number U12336; nt 424–448, 5’TGG AGG CCG GAC ATT GTC CTA TAC-3’), A904 (nt 995-1018, 5’GAT CAA GGC CAT GGT AGC AGC TAT GTA-3’) as previously used by Vetter et al.\textsuperscript{14} Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were purchased from Quantace Ltd., (Alexandria, Australia).

**Western blotting.** Dorsal root ganglia from wild-type and KO mice as well as brain tissue from wild-type mice were homogenized on ice using a Dounce homogenizer followed by several passages through a hypodermic syringe fitted with a 21-gauge needle. The homogenization buffer contained: 1% Nonidet P-40, 1% Triton X-100, 0.1 M Tris-HCl (pH adjusted to 7.4 at 4°C), 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and Complete Mini Protease Inhibitors EDTA-free (Roche Diagnostics GmbH, Germany). The resultant homogenate was centrifuged for 5 min at 1,500 xg (4°C) to precipitate out nuclei and unbroken cells. This centrifugation procedure was repeated and the supernatant was separated from the pellet. The supernatant was next centrifuged for 90 min at 17,000 xg at 4°C. A 40 μl volume of solubilization buffer was added to this pellet, and the resulting suspension was incubated for 30 min at 4°C to extract integral membrane proteins. The solubilization buffer contained: 1% Nonidet P-40, 1% Triton X-100, 0.1 M Tris-HCl (pH adjusted to 7.4 at 4°C), 0.3 M, NaCl, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, Complete Mini Protease Inhibitors EDTA-free, adjusted to pH 7.4 at 4°C. Sample protein concentrations were analyzed using the BCA Protein Assay kit (Pierce). SDS-PAGE separation

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**Figure 2. α-Conotoxins Vc1.1 and RgIA inhibit HVA calcium channels in DRG neurons of α9 KO mice.** (A) Superimposed traces of depolarization-activated whole-cell calcium channel currents in α9 nAChR KO mice recorded using Ba2+ as the charge carrier, elicited by a voltage step from a holding potential of -80 mV to -10 mV in the absence and presence of 100 nM Vc1.1. (B) Superimposed traces of depolarization-activated whole-cell Ca2+ channel currents recorded in α9 KO mice in the absence and presence of 100 nM RgIA. (C) Concentration-response relationships obtained for inhibition of calcium channel currents in isolated DRG neurons of α9 KO mice by Vc1.1 and RgIA (n ≥ 4 cells/data point). Data points represent mean ± S.E.M of normalized current amplitude. Maximum inhibition of whole-cell current amplitude by Vc1.1 and RgIA was 43 ± 0.5% and 45.8 ± 0.9% with IC\textsubscript{50}s of 24.6 and 22.4 nM, respectively. (D) Bar graph of the antagonism by the specific GABA\textsubscript{B} receptor antagonist CGP55845A (1 μM) of the inhibition of whole-cell Ca2+ channel currents recorded in α9 KO mice by Vc1.1 (n = 6) and RgIA (n = 3).
was carried out using NuPAGE Novex Bis-Tris Mini Gels. Protein bands were subsequently transferred to PVDF membrane, blocked for 1 h with 3.5% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 and processed with either anti-\(\alpha\text{-conotoxin} \, \text{Vc1.1} \) or anti-\(\alpha\text{-conotoxin} \, \text{RglA} \) (Sigma) and subsequently with anti-goat horseradish peroxidase-conjugated secondary antibody (donkey; Quantum Scientific, Qld Australia) and anti-rabbit horseradish peroxidase-conjugated secondary antibody (goat; Invitrogen), respectively. PVDF membranes were developed using Super Signal West Pico (Pierce).

**Electrophysiological methods.** The recording solutions and experimental protocols used were the same as described previously.8

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**References**  
1. Sandall DW, Satkunanathan N, Keays DA, Polidano MA, Liping X, Pham V, et al. A novel \(\alpha\text{-conotoxin} \) identified by gene sequencing is active in suppressing the vascular response to selective stimulation of sensory nerves in vivo. Biochemistry 2003; 42:6904-11.
2. Ellison M, Haberlandt C, Gomez-Casati ME, Watkins M, Elgoyhen AB, McIntosh JM, et al. \(\alpha\text{-RglA} \): a novel conotoxin that specifically and potently blocks the \(\alpha\text{9}\alpha\text{10} \) nAChR. Biochemistry 2006; 45:1511-7.
3. Satkunanathan N, Livett B, Gayler K, Sandall D, Down J, Khalil Z. \(\alpha\text{-conotoxin} \, \text{Vc1.1} \) alleviates neuropathic pain and accelerates functional recovery of injured neurones. Brain Res 2005; 1059:149-58.
4. Vincler M, Wittenauer S, Parker R, Ellison M, Olivera BM, McIntosh JM. Molecular mechanism for analgesia involving specific antagonism of \(\alpha\text{9}\alpha\text{10} \) nicotinic acetylcholine receptors. Proc Natl Acad Sci USA 2006; 103:17880-4.
5. Nevin ST, Clark RJ, Klimis H, Christie MJ, Craik DJ, Adams DJ. Are \(\alpha\text{9}\alpha\text{10} \) nicotinic acetylcholine receptors a pain target for \(\alpha\text{-conotoxins} \)? Mol Pharmacol 2007; 72:1406-10.
6. Elgoyhen AB, Johnson DJ, Boulter J, Vetter DE, Heinemann S. \(\alpha\text{9} \) an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 1994; 79:705-15.
7. Sgard F, Charpentier E, Bertrand S, Walker N, Caput D, Graham D, et al. A novel human nicotinic receptor subunit, \(\alpha\text{10} \), that confers functionality to the \(\alpha\text{9} \) subunit. Mol Pharmacol 2002; 61:150-9.
8. Callaghan B, Haythorowtwaite A, Berecki G, Clark RJ, Craik DJ, Adams DJ. Analgesic alpha-conotoxins Vc1.1 and RglA inhibit N-type calcium channels in rat sensory neurons via \(\text{GABA}_\beta \) receptor activation. J Neurosci 2008; 28:10943-51.
9. Tedford HW, Zamponi GW. Direct \(\text{G protein} \) modulation of \(\text{Cav2 calcium channels} \). Pharmacol Rev 2006; 58:837-62.
10. Raingo J, Castiglioni AJ, Lipscombe D. Alternative splicing controls \(\text{G protein} \)-dependent inhibition of N-type calcium channels in nociceptors. Nat Neurosci 2007; 10:285-92.
11. McIntosh JM, Absalom N, Chebib M, Elgoyhen AB, Vincler M. \(\alpha\text{9} \) nicotinic acetylcholine receptors and the treatment of pain. Biochem Pharmacol 2009; 78:693-702.
12. Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J. \(\alpha\text{10} \): a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. Proc Natl Acad Sci USA 2001; 98:3501-6.
13. Tombler E, Cabanilla NJ, Carman P, Permaul N, Hall JJ, Richman RW, et al. \(\text{G protein} \)-induced trafficking of voltage-dependent calcium channels. J Biol Chem 2006; 281:1827-39.
14. Vetter DE, Libermer MC, Mann J, Barhanin J, Boulter J, Brown MC, et al. Role of \(\alpha\text{9} \) nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. Neuron 1999; 23:93-103.