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Abstract

α -Conotoxins Vc1.1 and Rg1A are peptides from the venom of marine *Conus* snails that are currently in development as a treatment for neuropathic pain. Here we report that the $\alpha 9\alpha 10$ nicotinic acetylcholine receptor-selective conotoxins Vc1.1 and Rg1A potently and selectively inhibit high-voltage-activated (HVA) calcium channel currents in dissociated DRG neurons in a concentration-dependent manner. The post-translationally modified peptides vc1a and [P6O]Vc1.1 were inactive, as were all other α -conotoxins tested. Vc1.1 inhibited the ω -conotoxin-sensitive HVA currents in DRG neurons but not those recorded from *Xenopus* oocytes expressing CaV2.2, CaV2.1, CaV2.3, or CaV1.2 channels. Inhibition of HVA currents by Vc1.1 was not reversed by depolarizing prepulses but was abolished by pertussis toxin (PTX), intracellular GDP β S, or a selective inhibitor of pp60c-src tyrosine kinase. These data indicate that Vc1.1 does not interact with N-type calcium channels directly but inhibits them via a voltage-independent mechanism involving a PTX-sensitive, G-protein-coupled receptor. Preincubation with a variety of selective receptor antagonists demonstrated that only the GABAB receptor antagonists, [S-(R*,R*)][3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl]([3,4]-cyclohexylmethyl) phosphinic acid hydrochloride (2S)-3[[1-(3,4-dichlorophenyl)-ethyl]amino-2-hydroxypropyl](phenylmethyl) phosphinic acid and phaclofen, blocked the effect of Vc1.1 and Rg1A on Ca²⁺ channel currents. Together, the results identify CaV2.2 as a target of Vc1.1 and Rg1A, potentially mediating their analgesic actions. We propose a novel mechanism by which α -conotoxins Vc1.1 and Rg1A modulate native N-type (CaV2.2) Ca²⁺ channel currents, namely acting as agonists via G-protein-coupled GABAB receptors.

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Analgesic α -Conotoxins Vc1.1 and Rg1A Inhibit N-Type Calcium Channels in Rat Sensory Neurons via GABA_B Receptor Activation

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α -Conotoxins Vc1.1 and Rg1A are peptides from the venom of marine *Conus* snails that are currently in development as a treatment for neuropathic pain. Here we report that the α 9 α 10 nicotinic acetylcholine receptor-selective conotoxins Vc1.1 and Rg1A potently and selectively inhibit high-voltage-activated (HVA) calcium channel currents in dissociated DRG neurons in a concentration-dependent manner. The post-translationally modified peptides vc1a and [P60]Vc1.1 were inactive, as were all other α -conotoxins tested. Vc1.1 inhibited the ω -conotoxin-sensitive HVA currents in DRG neurons but not those recorded from *Xenopus* oocytes expressing Ca_v2.2, Ca_v2.1, Ca_v2.3, or Ca_v1.2 channels. Inhibition of HVA currents by Vc1.1 was not reversed by depolarizing prepulses but was abolished by pertussis toxin (PTX), intracellular GDP β S, or a selective inhibitor of pp60c-src tyrosine kinase. These data indicate that Vc1.1 does not interact with N-type calcium channels directly but inhibits them via a voltage-independent mechanism involving a PTX-sensitive, G-protein-coupled receptor. Preincubation with a variety of selective receptor antagonists demonstrated that only the GABA_B receptor antagonists, [S-(R*,R*)][1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl]([3,4]-cyclohexylmethyl) phosphinic acid hydrochloride (2S)-3[[1-(3,4-dichlorophenyl)-ethyl]amino-2-hydroxypropyl](phenylmethyl) phosphinic acid and phaclofen, blocked the effect of Vc1.1 and Rg1A on Ca²⁺ channel currents. Together, the results identify Ca_v2.2 as a target of Vc1.1 and Rg1A, potentially mediating their analgesic actions. We propose a novel mechanism by which α -conotoxins Vc1.1 and Rg1A modulate native N-type (Ca_v2.2) Ca²⁺ channel currents, namely acting as agonists via G-protein-coupled GABA_B receptors.

Key words: conotoxins; N-type calcium channel; dorsal root ganglion; GABA_B receptor; G-protein; analgesia

Introduction

Conotoxins are small peptides derived from the venom of the *Conus* genus of predatory marine snails (Terlau and Olivera, 2004). Many conotoxins are highly selective antagonists of a diverse range of mammalian ion channels and receptors associated with pain pathways. Thus, conotoxins are attractive as drug leads for treating neuropathic pain. Previous studies have demonstrated the analgesic potential of several different classes of conotoxins (Adams et al., 1999; Livett et al., 2004) and, most recently, antagonists of neuronal nicotinic acetylcholine receptors (nAChRs) have begun to show promise for the treatment of neuropathic pain (Sandall et al., 2003; Lang et al., 2005; Satkunanathan et al., 2005; Vincler et al., 2006). The α -conotoxins competitively inhibit neuronal-type nAChRs (Dutton and Craik,

2001) with varying degrees of selectivity for different nAChR subtypes (McIntosh et al., 1999).

α -Conotoxin Vc1.1, which was first discovered using a PCR screen of cDNAs from the venom ducts of *Conus victoriae* (Sandall et al., 2003), contains two residues, Pro6 and Glu14, that are post-translationally modified to hydroxyproline and γ -carboxyglutamate, respectively, in the native peptide, designated vc1a (Jakubowski et al., 2004). Synthetic Vc1.1 lacking these two modifications is a competitive antagonist of neuronal nAChRs in bovine adrenal chromaffin cells (Clark et al., 2006) and is potent at recombinant α 9 α 10 nAChRs expressed in *Xenopus* oocytes (Vincler et al., 2006; Nevin et al., 2007). Furthermore, Vc1.1 and its post-translationally modified analogs vc1a, [P60]Vc1.1, and [E14 γ]Vc1.1 have been reported to be equally potent at inhibiting ACh-evoked currents mediated by α 9 α 10 nAChRs (Nevin et al., 2007). 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). Blockade of nAChRs on unmyelinated peripheral nerve fibers may have an analgesic effect on unmyelinated sympathetic and/or sensory axons. Interestingly, synthetic vc1a, which incorporates the modified residues, has been reported to inhibit α 9 α 10 (Nevin et al., 2007) but not α 3-containing (Clark et al., 2006) nAChRs expressed in *Xenopus* oocytes and is inactive

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in two rat neuropathic pain assays (Livett et al., 2006). In contrast, 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). α -Conotoxin Rg1A from *Conus regius*, which also selectively inhibits $\alpha 9\alpha 10$ nAChRs (Ellison et al., 2006), has similarly been shown to significantly reduce chronic constriction nerve injury-induced hyperalgesia in rats (Vincler et al., 2006; Vincler and McIntosh, 2007). Thus, although Vc1.1 is currently under development as a treatment for neuropathic pain (Livett et al., 2004, 2006), its specific receptor target has not been unequivocally identified. We show here that Vc1.1 and Rg1A act by an unexpected mechanism not reported previously for any conotoxin.

Materials and Methods

DRG neuron preparation. DRG neurons were enzymatically dissociated from ganglia of 2- to 30-d-old Wistar rats according to standard protocols. Rats were killed by cervical dislocation as approved by the University of Queensland Animal Ethics Committee, the spinal column was hemi-segmented, and the spinal cord was removed. Ganglia were removed and rinsed in cold HBSS (MultiCell). They were minced and incubated in 1 mg/ml collagenase (type 2; 405 U/mg) (Worthington Biochemical) in HBSS at 37°C for ~30 min. After incubation, ganglia were rinsed three times with warm (37°C) DMEM (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, and gently triturated with a fire-polished Pasteur pipette. Cells were plated on glass coverslips, incubated at 37°C in 95% O_2 /5% CO_2 , and used within 4–48 h.

Electrophysiological methods. The external recording solution for rat DRG neurons contained the following (in mM): 150 tetraethylammonium (TEA)-Cl, 2 BaCl_2 , 10 D-glucose, and 10 HEPES, pH 7.3–7.4. Recording electrodes were filled with an internal solution containing the following (in mM): 140 CsCl, 1 MgCl_2 , 5 MgATP, 0.1 Na-GTP, 5 bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA)- Cs_4 , and 10 HEPES, pH 7.3–7.4, with CsOH, and had resistances of 1–2.5 M Ω . Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique with an Axopatch 200B amplifier (Molecular Devices). Neurons were voltage clamped at a holding potential of -90 mV and Ba^{2+} currents were elicited by step depolarizations, first to -40 mV for 200 ms [for low-voltage-activated (LVA) currents] and then to -10 mV, from a holding potential of -50 mV, for 200 ms [for high-voltage-activated (HVA) currents] at 10 s intervals. In a series of experiments, BaCl_2 was replaced by CaCl_2 . A voltage protocol using step depolarizations from -70 to 0 mV was used when examining HVA currents alone. Test potentials 150 ms in duration were applied every 20 s. To construct current–voltage (*I*–*V*) relationships, neurons were voltage clamped at -100 mV and voltage stepped from -80 mV to $+50$ mV in 10 mV increments. Voltage-dependent inhibition was assessed by applying a prepulse to $+80$ mV for 20 ms in duration before the test pulse. Leak and capacitive currents were subtracted using a $-P/4$ pulse protocol. Currents were generated by a computer using pClamp 9.2 software (Molecular Devices), filtered at 2 kHz, and sampled at 8 kHz by the Digidata 1322A (Molecular Devices). Sampled data were stored digitally on a computer for further analysis.

Oocyte preparation and recordings. Stage V–VI oocytes from *Xenopus laevis* frogs were injected with either cRNA or cDNA encoding N- or P/Q-type voltage-gated Ca^{2+} channels (VGCCs), respectively. N-type channels were examined in oocytes injected with 50 nl of solution containing a mixture of cRNAs encoding rat $\text{Ca}_v2.2$ α_{1B-b} subunit (2.5–5 ng/cell) and rat β_3 subunit (2.5–5 ng/cell) (cDNAs provided by Dr. D. Lipscombe, Brown University, Providence, RI), with or without rabbit $\alpha_2\delta_1$ subunit (2.5–5 ng/cell) (cDNA provided by Dr. F. Hofmann and Dr. N. Klugbauer, Technische Universität München, Munich, Germany). L-type channels were examined in oocytes coinjected with rabbit $\text{Ca}_v1.2$ α_{1C} subunit (5 ng/cell) (cDNA provided by Dr. G. Zamponi, University of Calgary, Calgary, Alberta, Canada) and auxiliary subunits β_3 and $\alpha_2\delta_1$ (see above). For experiments examining P/Q-type and R-type VGCCs,

the oocyte nucleus was injected first with cDNA encoding the rabbit $\text{Ca}_v2.1$ α_{1A} (5 ng/cell) (cDNA provided by Dr. G. Zamponi) or rat $\text{Ca}_v2.3$ α_{1E} (4.5 ng/cell) (cDNA provided by Dr. G. Zamponi) subunit, respectively, followed by cytoplasmic injection of auxiliary subunits β_3 and $\alpha_2\delta_1$ (see above). After injection, oocytes were kept at 18°C for 3–6 d before use.

Before recording, oocytes were injected with 25 nl of 50 mM BAPTA to eliminate the endogenous Ca^{2+} -activated chloride conductance. Depolarization-activated Ca^{2+} channel currents were recorded using Ba^{2+} as a charge carrier and a two-electrode virtual ground voltage-clamp circuit with a GeneClamp 500B amplifier (Molecular Devices). The external bath solution contained the following (in mM): 85 TEA-OH, 5 BaCl_2 , 5 KCl, and 10 HEPES, pH 7.4, with methanesulfonic acid. During recording, oocytes were constantly perfused with bath solution using a gravity-fed perfusion system at a flow rate of ~1.5 ml/min. Electrodes were filled with a solution containing 3 M KCl (0.5–1.0 M Ω). Oocytes were voltage clamped at a holding potential of -80 mV and membrane currents were elicited by 200 ms step depolarization to 0 mV (for $\text{Ca}_v2.2$) or $+20$ mV (for $\text{Ca}_v2.1$) applied every 10 s. To construct *I*–*V* relationships, cells were voltage-clamped at -80 mV and then stepped from -80 mV to $+50$ mV in 10 mV increments. Data were low-pass filtered at 2 kHz, digitized at 10 kHz, leak subtracted online using a $-P/4$ protocol, and analyzed off-line.

Data analysis. Data were analyzed off-line using pClamp 9.2 software (Molecular Devices). Peak Ba^{2+} current amplitude in response to a depolarizing pulse was calculated and plotted against time to monitor stability of the recording. Generally, Ba^{2+} current reached steady state within 2–5 min of initial recording, and any recordings that did not reach steady state were discarded. Drugs were applied via perfusion in the bath solution. Current amplitudes obtained in the presence of the drug were normalized by dividing by the current amplitude obtained under control conditions. Concentration–response relationships were obtained using GraphPad Prism software by plotting normalized current amplitude as a function of drug concentration and were fitted using a logistic equation. Numerical data are presented as mean \pm SEM.

Drugs and chemicals. CGP55845 (2*S*)-3[[1*S*]-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl(phenylmethyl) phosphinic acid, CGP54626 [*S*-(*R**,*R**)]-3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl[[3,4]-cyclohexylmethyl] phosphinic acid hydrochloride], and pp60c-src peptide (521–533) corresponding to its C-terminal regulatory domain were purchased from Tocris Bioscience. The peptide binds pp60c-src at the Src homology 2 domain suppressing its tyrosine kinase activity (Roussel et al., 1991). Phaclofen (3-amino-2-(4-chlorophenyl)propylphosphonic acid) was from BIOMOL. All other drugs were obtained from Sigma-Aldrich and were prepared as concentrated stock solutions which were then diluted in bath solution and applied via perfusion. The ω -conotoxins CVID and MVIIA were prepared as described previously (Lewis et al., 2000). α -Conotoxins Vc1.1, [P6O]Vc1.1, [E14 γ]Vc1.1, vc1a, Rg1A, ImI, MII, BuIA and [A10L]PnIA, were synthesized as reported previously (Gehrmann et al., 1999; Hogg et al., 1999; Blanchfield et al., 2003; Clark et al., 2006, 2008; Jin et al., 2007) and provided as a stock concentration in H_2O of ~1 mM and used at a final concentration of 0.1 nM to 1 μM . Reduced Vc1.1 was prepared by incubation with dithiothreitol (2 mM; 1 h; room temperature) or alkylated with iodoacetamide in 0.1 M ammonium acetate buffer.

Results

$\alpha 9\alpha 10$ nAChR-selective conotoxins, Vc1.1 and Rg1A, inhibit voltage-gated Ca^{2+} channel currents in rat DRG neurons

In an attempt to account for α -conotoxin Vc1.1-induced analgesia and to elucidate the receptor target and mechanism of action, the effects of the conotoxins, Vc1.1 and Rg1A, were investigated on voltage-gated Ca^{2+} channel currents in rat DRG neurons. Whole-cell Ca^{2+} channel currents of rat DRG neurons are mediated by both LVA and HVA calcium channels (Scroggs and Fox, 1992). The effect of Vc1.1 on Ca^{2+} channel currents recorded from neonatal (2- to 10-d-old) rat DRG neurons was initially assessed using a two-step protocol (see Materials and

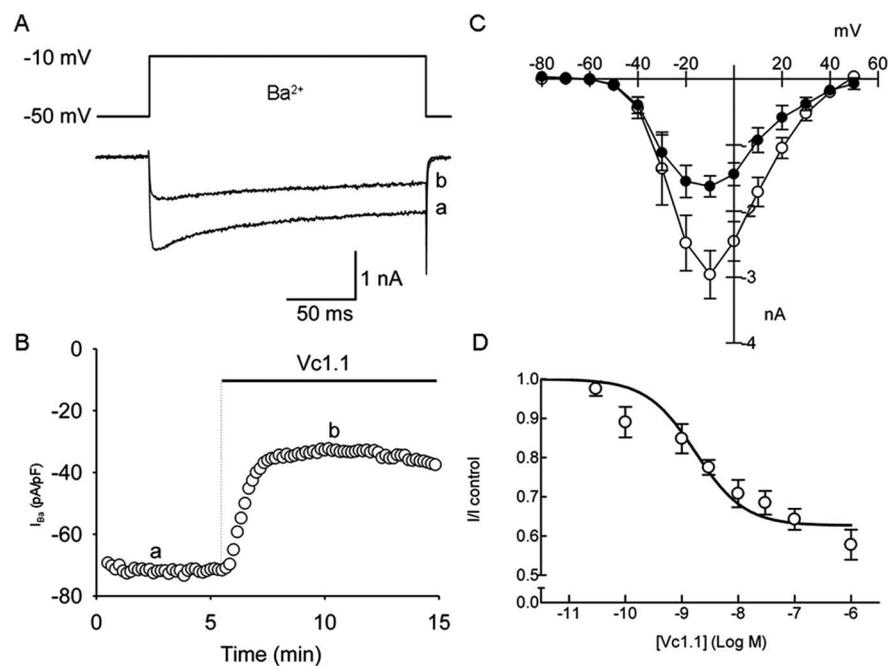


Figure 1. α -Conotoxin Vc1.1 inhibits HVA calcium channels in rat DRG neurons. **A**, Superimposed traces of depolarization-activated whole-cell calcium channel currents recorded using 2 mM Ba^{2+} as the charge carrier, elicited by a voltage step from a holding potential of -50 to 0 mV in the absence (a) and presence (b) of 100 nM Vc1.1. **B**, Corresponding time course of inhibition of peak Ba^{2+} current amplitude by 100 nM Vc1.1. Application of Vc1.1 is indicated by the bar. **C**, Plot of the I - V relationship for peak Ba^{2+} currents in the presence (●) and absence (○) of Vc1.1 (≤ 100 nM) ($n = 16$). **D**, Concentration-response curve for inhibition of calcium channel currents in DRG neurons by Vc1.1. Data points represent mean \pm SEM of normalized peak current amplitude ($n = 8$ –25 cells per data point). Maximum inhibition was $42 \pm 3\%$ with an IC_{50} of 1.7 nM.

Methods). Vc1.1 (1 μM) had no effect on LVA Ca^{2+} channel current amplitude elicited by voltage step from -90 to -40 mV ($97 \pm 12\%$ of control, $n = 10$). In the same cells, Vc1.1 inhibited HVA Ca^{2+} channel currents elicited by a 150 ms voltage step to -10 mV to $56.5 \pm 4.4\%$ of control ($n = 10$) (Fig. 1A,B). Vc1.1 only inhibited VGCC currents evoked by step depolarization positive of -40 mV, demonstrating the lack of effect of Vc1.1 on LVA Ca^{2+} channel currents (Fig. 1C). The effect of Vc1.1 on HVA Ca^{2+} channel currents was therefore assessed using a 150 ms step depolarization from a holding potential of either -70 or -90 mV to the test potential of either -10 or 0 mV at a frequency of 0.05 Hz, with 2 mM Ba^{2+} as the charge carrier. Vc1.1 inhibition of HVA Ca^{2+} channel currents developed with a delay of ~ 2 min and maximum inhibition occurred ~ 7 min after the beginning of the response, independent of the concentration of Vc1.1 ($n = 74$). Inhibition of HVA Ca^{2+} channel currents by Vc1.1 was concentration dependent and the mean concentration-response data gave an IC_{50} of 1.7 nM, with maximum inhibition to $57.8 \pm 3.8\%$ of control occurring at 1 μM ($n = 25$) (Fig. 1D).

Vc1.1 (100 nM) inhibited VGCC currents in $>75\%$ of DRG neurons (120 of 159) tested, including small (<20 μm), medium (20–40 μm), and large (>40 μm) neurons. Only a subset of primary sensory DRG neurons are capsaicin sensitive (Szallasi and Blumberg, 1999). There was no correlation between cell size or capsaicin sensitivity and responsiveness to Vc1.1: 88% of small cells ($n = 17$), 71% of medium cells ($n = 51$), and 76% of large cells ($n = 91$) responded to ≥ 10 nM Vc1.1; 5 of 8 capsaicin-sensitive cells responded to Vc1.1 and 6 of 9 capsaicin-insensitive cells responded to Vc1.1. Vc1.1 (100 nM) also inhibited VGCC currents in 14 of 25 juvenile (11- to 20-d-old) and 4 of 6 adult (>28 -d-old) rats ($67.5 \pm 3.3\%$ and $69.4 \pm 3.2\%$ of control, respectively).

Vc1.1 inhibition of VGCC currents was not reversed during washout. However, reapplication of Vc1.1 produced an equivalent inhibition of HVA Ca^{2+} channel currents in other DRG neurons on the same coverslip ($n = 5$ sets), suggesting that depolarization-induced Ca^{2+} channel activation is required for inhibition by Vc1.1.

To further assess the dependence of Vc1.1 inhibition on activation of HVA Ca^{2+} channel currents, we varied the frequency and duration of the depolarizing pulse in the presence of Vc1.1 (Fig. 2). When the depolarization pulse was shortened to 15 ms with the frequency maintained at 0.05 Hz, 100 nM Vc1.1 inhibited the current to only $88.4 \pm 3.3\%$ of control in all cells tested ($n = 24$). However, when the frequency of the 15 ms pulse was increased to 0.1 Hz, the current was reduced to $51.1 \pm 5.6\%$ of control in all cells tested ($n = 7$) (Fig. 2A,B). Figure 2B also shows the relative inhibition of Ba^{2+} currents by Vc1.1, using the standard depolarizing pulse duration (150 ms) at both 0.05 Hz and 0.1 Hz. The action of Vc1.1 does not depend on a rise in intracellular divalent cation, because the inhibition was also observed with 2 mM Ca^{2+} as the charge carrier. In 9 of 12 cells, Vc1.1 (100 nM) inhibited the HVA Ca^{2+} current amplitude to $61.4 \pm 5.2\%$ of control and was not reversible on washout (>30 min) (Fig. 2C). Furthermore, in fura-2-loaded DRG neurons, bath application of 1 μM Vc1.1 alone failed to elicit any increase in intracellular Ca^{2+} concentration ($n = 14$, data not shown).

Application of α -conotoxin Rg1A (100 nM) also significantly inhibited HVA Ca^{2+} channel currents in rat DRG neurons (11 of 14 responding cells) to $60.6 \pm 4.5\%$ of control (Fig. 3A).

Post-translationally modified analogs of Vc1.1 and other α -conotoxins do not inhibit HVA Ca^{2+} channel currents in DRG neurons

The effects of post-translationally modified analogs of Vc1.1 were examined on HVA Ca^{2+} channel currents from rat DRG neurons under the same conditions as Vc1.1. The native peptide, vc1a, did not inhibit HVA Ca^{2+} channel currents ($100.0 \pm 4.8\%$ of control, $n = 9$); however, the same cells responded to Vc1.1 after washout of vc1a (Fig. 3B,C). [P6O]Vc1.1 had no effect on HVA Ca^{2+} channel currents ($103.7 \pm 7.2\%$ of control, $n = 9$) (Fig. 3C), whereas four of five cells from this test group subsequently responded to Vc1.1 after washout of [P6O]Vc1.1. [E14 γ]Vc1.1 was also without effect ($110.1 \pm 9.2\%$ of control, $n = 7$) (Fig. 3C), but after its removal, all cells of this group tested with Vc1.1 showed a response ($n = 6$). Both reduced Vc1.1 and alkylated Vc1.1 (1 μM) were without effect on HVA Ca^{2+} channel currents, whereby the peak current amplitude was $98.8 \pm 7.1\%$ of control ($n = 10$) and $104.9 \pm 11.4\%$ of control ($n = 3$), respectively (Fig. 3C).

The effects of other 4/7 α -conotoxins (MII and [A10L]PnIa) as well as the 4/3 α -conotoxin ImI, and the 4/4 α -conotoxin BuIa were examined to ascertain whether inhibition of HVA Ca^{2+} channel currents was a common characteristic of α -conotoxins.

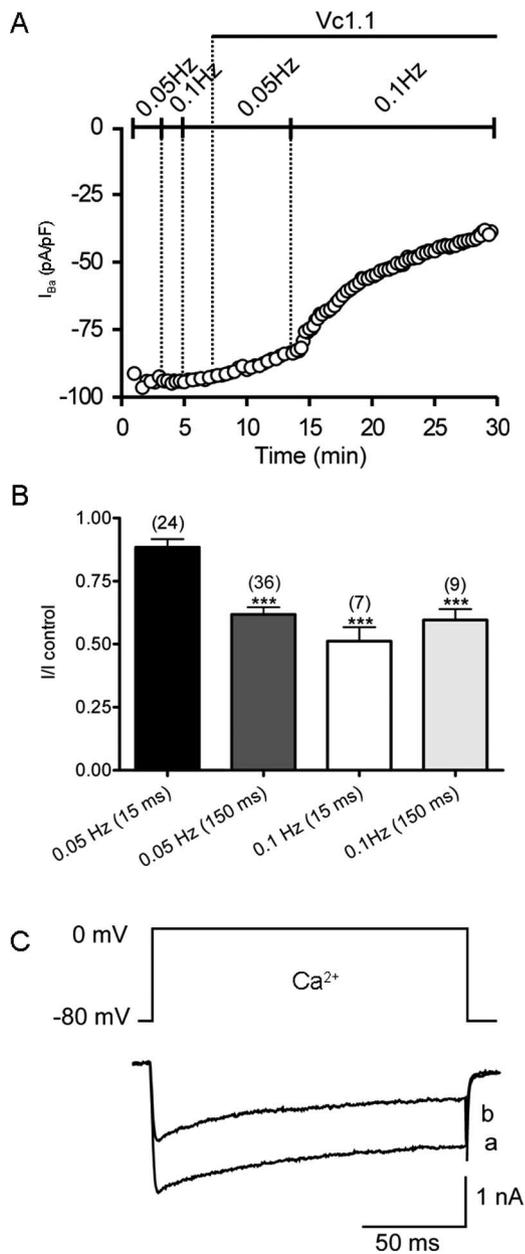


Figure 2. Vc1.1 inhibition of HVA calcium channel currents is use dependent. **A**, Representative time course of peak Ba^{2+} current amplitude recorded using a 15 ms step depolarization from a holding potential of -80 mV to a test potential of -10 mV at a frequency of either 0.05 or 0.1 Hz, as indicated, in the absence and presence of 100 nM Vc1.1. **B**, Bar graph of the relative inhibition of HVA calcium channel currents by 100 nM Vc1.1 during a 15 ms depolarization at a frequency of either 0.05 Hz ($n = 24$) or 0.1 Hz ($n = 7$ responsive cells of 7 tested), and a pulse duration of 150 ms and frequency of either 0.05 Hz ($n = 36$ responsive cells of 50 tested) or 0.1 Hz ($n = 9$ responsive cells of 12 tested). $***p < 0.001$ unpaired t test. **C**, Superimposed traces of depolarization-activated whole-cell Ca^{2+} channel currents recorded using 2 mM Ca^{2+} as the charge carrier, elicited by a voltage step from a holding potential of -80 to 0 mV in the absence (a) and presence (b) of 100 nM Vc1.1.

α -Conotoxins ImI, MII, BuIA, and [A10L]PnIA applied at a concentration of either 100 nM or 1 μM did not inhibit HVA Ca^{2+} channel currents in DRG neurons (ImI, $95.5 \pm 5.1\%$ of control, $n = 7$; MII, $93.2 \pm 1.6\%$ of control, $n = 3$; BuIA, $97.5 \pm 7.9\%$ of control, $n = 8$; [A10L]PnIA, $91.0 \pm 4.1\%$ of control, $n = 10$) (Fig. 3C). Another conopeptide structurally related to the 4/7 α -conotoxins, ρ -TIA, an allosteric α_1 -adrenoreceptor antagonist (Sharpe et al., 2003), also failed to inhibit HVA Ca^{2+} channel

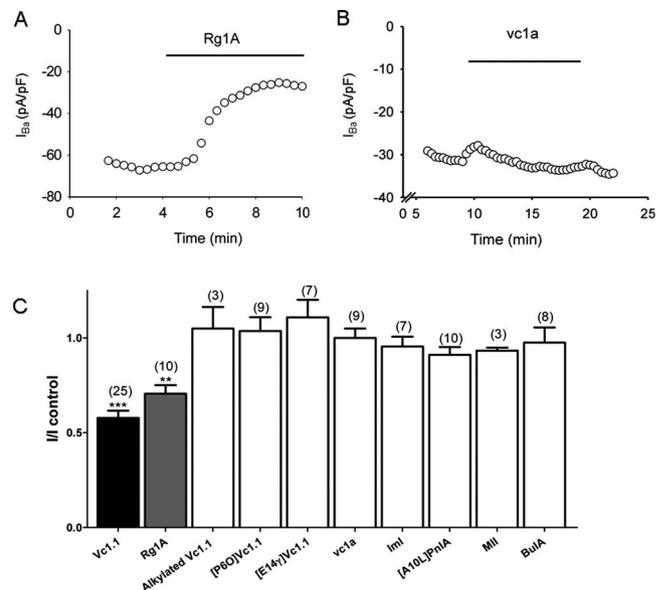


Figure 3. Vc1.1 post-translationally modified analogs and other α -conotoxins are without effect on HVA calcium channel currents. **A**, Representative time course of peak Ba^{2+} current amplitude in the presence of 100 nM Rg1A. The holding potential was -80 mV and the test potential -10 mV. **B**, Representative time course of peak Ba^{2+} current amplitude in the presence of 1 μM vc1a. **C**, Bar graph of relative inhibition of HVA calcium channel currents by α -conotoxins Rg1A, ImI, [A10L]PnIA, MII, BuIA, alkylated Vc1.1, Vc1.1, and analogs. Vc1.1 (1 μM) inhibited peak Ba^{2+} current amplitude in 25 of 30 cells tested. $***p = 0.0001$ compared with control cells. Rg1A (100 nM) inhibited peak Ba^{2+} current amplitude in 11 of 14 cells tested. $**p = 0.0016$ compared with control cells. Numbers in parentheses reflect the number of cells.

currents in DRG neurons when bath applied at 100 nM ($n = 6$, data not shown).

Vc1.1 inhibits the N-type component of HVA Ca^{2+} channel currents in DRG neurons

The selective N-type Ca^{2+} channel blockers, ω -conotoxins CVID and MVIIA, were applied to determine which component of the whole-cell HVA Ca^{2+} channel currents Vc1.1 inhibited in DRG neurons. Bath application of 200 nM CVID reduced peak Ca^{2+} channel current amplitude to $54.4 \pm 4.8\%$ of control ($n = 6$). Application of 1 μM Vc1.1 in the presence of CVID (200 nM) produced no further reduction of the HVA Ca^{2+} channel current amplitude ($51.7 \pm 5.1\%$ of control, $n = 6$) (Fig. 4A–C). Figure 4C also shows the result of the converse experiment, that is, the average response of cells to CVID (200 nM) after Vc1.1 (≥ 100 nM) application. Similar results were obtained with bath application of 100 nM ω -conotoxin MVIIA, with $51.6 \pm 5.6\%$ of the current remaining after application of MVIIA and 50.4 \pm 0.1% remaining in the presence of MVIIA and 100 nM Vc1.1 ($n = 5$) (data not shown). These data suggest that Vc1.1 inhibits the N-type Ca^{2+} channel in DRG neurons.

Action of Vc1.1 on expressed $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ channels in oocytes

To assess whether Vc1.1 directly inhibits HVA Ca^{2+} channels, the α subunits of $\text{Ca}_v2.1$ (P/Q-type) and $\text{Ca}_v2.2$ (N-type) channels were expressed in *Xenopus* oocytes together with the auxiliary subunits $\beta_3/\alpha_2\delta_1$ in the ratio 1:1:1. Bath application of 1 μM Vc1.1 did not significantly inhibit the Ba^{2+} current amplitude for oocytes expressing $\text{Ca}_v2.2$ channels ($91.3 \pm 2.2\%$ of control, $n = 8$) (Fig. 5A, C). Analogous to $\text{Ca}_v2.2$ -expressing oocytes, 1 μM Vc1.1 did not inhibit Ba^{2+} currents mediated by $\text{Ca}_v2.1$ channels

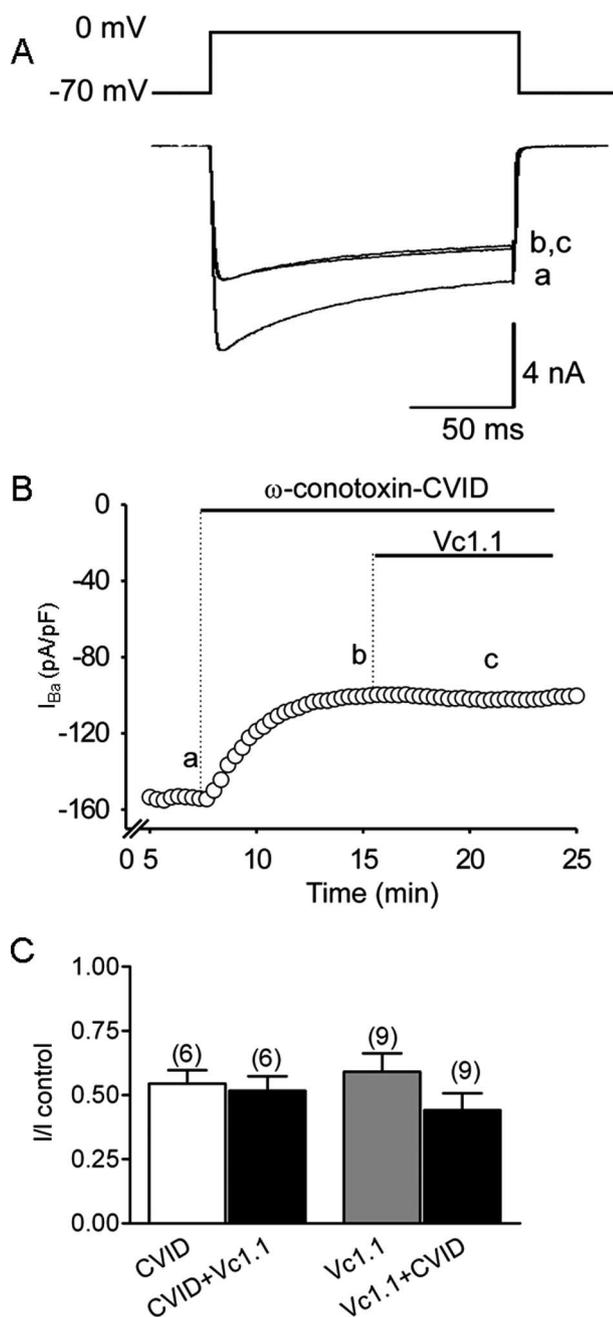


Figure 4. Vc1.1 inhibits N-type calcium channels in rat DRG neurons. **A**, Superimposed traces of depolarization-activated whole-cell Ba^{2+} currents under control conditions (a), in the presence of 200 nM ω -conotoxin CVID (b), and in the presence of CVID and 1 μ M Vc1.1 (c). **B**, Representative time course of peak Ba^{2+} current amplitude in the presence of the selective N-type Ca^{2+} channel inhibitor ω -conotoxin CVID followed by application of Vc1.1. The letters a–c indicate the time points at which the superimposed currents were obtained. **C**, Bar graph of relative inhibition of HVA calcium channel currents by 200 nM CVID alone, after application of 1 μ M Vc1.1 in the presence of CVID, in the presence of Vc1.1 alone, and after application of CVID in the presence of Vc1.1. Numbers in parentheses reflect the number of cells.

(88.1 \pm 8.2% of control, $n = 4$) (Fig. 5B,C). Raising the Vc1.1 concentration to 10 μ M similarly failed to inhibit the Ba^{2+} currents mediated by either $Ca_v2.1$ or $Ca_v2.2$ channels expressed in oocytes. A similar lack of effect of Vc1.1 on $Ca_v2.3$ (R-type) and $Ca_v1.2$ (L-type) channels expressed in *Xenopus* oocytes was also observed (data not shown). To investigate whether the auxiliary subunits influence the sensitivity of recombinant Ca^{2+} channels to Vc1.1 (Yasuda et al., 2004), oocytes were injected with the

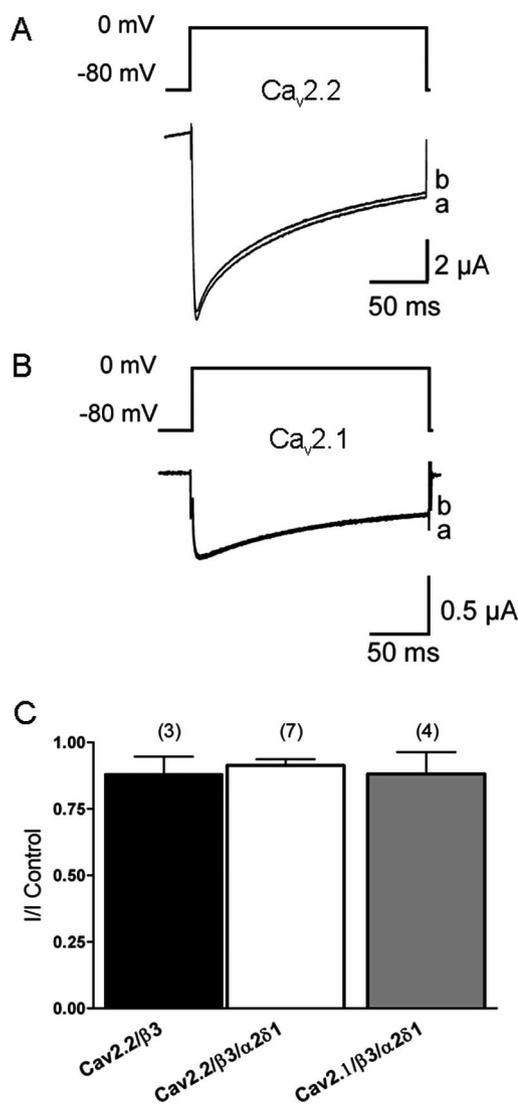


Figure 5. Vc1.1 does not inhibit Ba^{2+} currents through recombinant N- and P/Q-type channels expressed in *Xenopus* oocytes. **A**, Superimposed current traces from an oocyte expressing $Ca_v2.2 \alpha_{1B-d}/\beta_3/\alpha_2\delta_1$ (N-type) calcium channels under control conditions (a) and in the presence of 1 μ M Vc1.1 (b). **B**, Superimposed currents from an oocyte expressing $Ca_v2.1 \alpha_{1E}/\beta_3/\alpha_2\delta_1$ (P/Q-type) calcium channels in the absence (control) (a) and presence (b) of 1 μ M Vc1.1. **C**, Bar graph of relative current amplitude obtained for expressed N- and P/Q-type calcium channels in the presence of 1 μ M Vc1.1. Numbers in parentheses reflect the number of cells.

subunit combination $Ca_v2.2/\beta_3$ in a 1:1 ratio. Although cells expressing $Ca_v2.2/\beta_3$ elicited smaller Ca^{2+} channel currents than those expressed by $Ca_v2.2/\beta_3/\alpha_2\delta_1$ channels, the Ba^{2+} current amplitude was not appreciably changed (88 \pm 6.5% of control, $n = 3$) in the presence of 1 μ M Vc1.1 (Fig. 5C). Together, these data indicate that Vc1.1 does not interact with either $Ca_v2.1$ (P/Q-type) or $Ca_v2.2$ (N-type) channels directly, and that Vc1.1 modulation of HVA Ca^{2+} channel currents occurs via an indirect mechanism.

Vc1.1 inhibits HVA Ca^{2+} channel currents via a G-protein-coupled receptor mechanism and src tyrosine kinase

The role of G-proteins in mediating Vc1.1 inhibition of HVA Ca^{2+} channel currents in DRG neurons was examined by replacing GTP with GDPβS, a nonhydrolyzable GDP analog, in the intracellular recording solution. GDPβS antagonized the effect of Vc1.1 on Ca^{2+} channel currents, with 96.8 \pm 4.5% of current

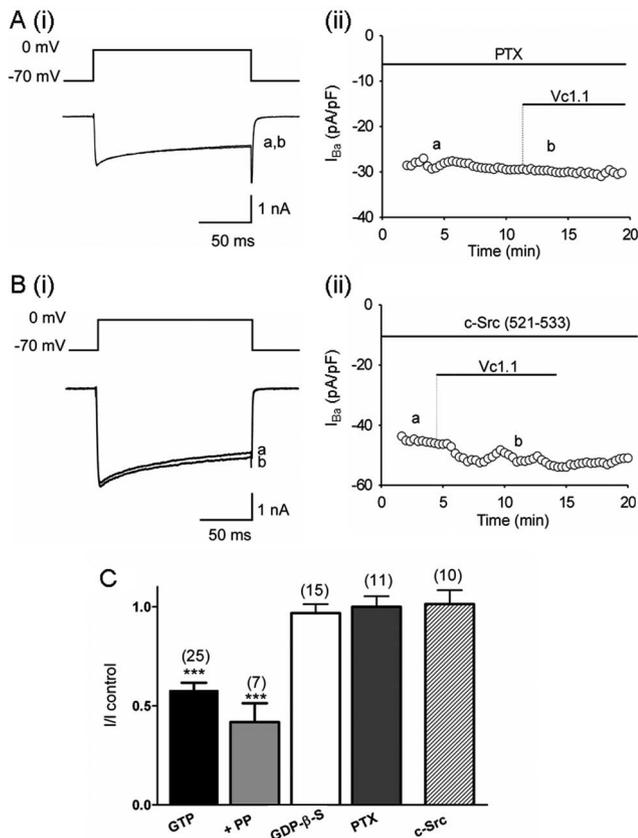


Figure 6. Vc1.1 inhibition of N-type calcium channel currents is mediated by G-protein-coupled receptor and tyrosine kinase activation. **A(i)**, Superimposed traces in the absence (a) and presence (b) of $1 \mu\text{M}$ Vc1.1 in cells incubated overnight in PTX ($3 \mu\text{g/ml}$). Cells were recorded under continual perfusion of PTX (200 ng/ml). **(ii)**, Corresponding time course. **B(i)**, Superimposed traces in the absence (a) and presence (b) of Vc1.1 ($1 \mu\text{M}$) in cells recorded with pp60c-src peptide (521–533) inhibitory peptide ($100 \mu\text{M}$) included in the recording pipette. **(ii)**, Corresponding time course. **C**, Bar graph of relative inhibition of HVA calcium channel currents by Vc1.1 ($1 \mu\text{M}$) in regular recording solution (GTP), when a prepulse to $+80 \text{ mV}$ (+PP) was applied 10 ms before the test pulse, when GTP was replaced with GDP β S, after overnight incubation in PTX, or when pp60c-src inhibitory peptide was included in the pipette recording solution. $***p = 0.0001$ compared with control. Numbers in parentheses reflect the number of cells.

remaining after addition of 100 nM Vc1.1 in the presence of GDP β S ($n = 15$) (Fig. 6C).

These results suggest that Vc1.1 inhibits N-type Ca^{2+} channel currents in DRG neurons via a G-protein-coupled receptor (GPCR) mechanism. Vc1.1 inhibition also persisted after application of depolarizing prepulses to $+80 \text{ mV}$, suggesting a voltage-independent inhibitory pathway ($n = 7$) (Fig. 6C). Incubation of cells in medium containing $3 \mu\text{g/ml}$ pertussis toxin (PTX) for 24 h antagonized the inhibition of VGCC currents by Vc1.1 ($99.9 \pm 4.5\%$ of control, $n = 11$) (Fig. 6A,C). Together, these results suggest that the mechanism by which Vc1.1 inhibits HVA Ca^{2+} channel currents involves a PTX-sensitive GPCR of the $\text{G}\alpha_{i/o}$ subfamily.

Nonreceptor tyrosine kinases have been implicated in voltage-independent inhibition of N-type Ca^{2+} channel currents initiated by activation of GPCR pathways (Diversé-Pierluissi et al., 1997; Strock and Diversé-Pierluissi, 2004; Raingo et al., 2007). We therefore tested the ability of Vc1.1 to inhibit HVA Ca^{2+} channel currents when a selective peptide inhibitor of pp60c-src tyrosine kinase (Roussel et al., 1991) ($100 \mu\text{M}$) was included in the intracellular recording solution. Vc1.1 had no effect on HVA

Table 1. Receptor antagonists tested for reversal of the inhibition of N-type calcium channel currents by α -conotoxin Vc1.1

Antagonist (concentration)	Target receptor	Antagonist of Vc1.1 inhibition	Percentage inhibition of I_{ba} by Vc1.1 (n)
Mecamylamine ($3 \mu\text{M}$)	nAChR	—	29.2 ± 8.6 (3)
ACh ($100 \mu\text{M}$)	nAChR	—	28.4 ± 4.5 (7)
Atropine ($1 \mu\text{M}$)	mAChR	—	27.2 ± 10.4 (4)
Prazosin ($10 \mu\text{M}$)	α_1 -Adrenergic	—	41.3 ± 4.8 (3)
Yohimbine ($10 \mu\text{M}$)	α_2 -Adrenergic	—	35.8 ± 11.5 (3)
Naloxone ($10 \mu\text{M}$)	μ -Opioid	—	50.1 ± 12.4 (3)
Bicuculline ($30 \mu\text{M}$)	GABA _A	—	32.9 ± 7.0 (10)
Phaclofen ($50 \mu\text{M}$)	GABA _B	+	4.6 ± 4.4 (9)
CGP 55845 ($1 \mu\text{M}$)	GABA _B	+	6.8 ± 4.5 (14)
CGP 54626 ($1 \mu\text{M}$)	GABA _B	+	7.4 ± 2.3 (4)

+, Reversal of Vc1.1 inhibition; —, no effect; mAChR, muscarinic acetylcholine receptor.

Ca^{2+} channel currents in the presence of the pp60c-src peptide ($101.39 \pm 6.9\%$ of control, $n = 10$) (Fig. 6B,C).

Vc1.1 inhibits HVA Ca^{2+} channel currents in DRG neurons via GABA_B receptor

To investigate further the identity of the primary target for Vc1.1, a number of different receptor antagonists were tested for their ability to antagonize the effect of Vc1.1 (Table 1). Antagonists of neuronal nAChRs, mecamylamine and hexamethonium, and desensitizing concentrations of ACh were bath applied $\sim 5 \text{ min}$ before application of Vc1.1 and in all cases failed to antagonize the inhibition of N-type VGCC currents in DRG neurons by Vc1.1. Similarly, the muscarinic acetylcholine receptor antagonist, atropine, the α_1 - and α_2 -adrenergic receptor antagonists, prazosin and yohimbine, respectively, the μ -opioid receptor antagonist, naloxone, and the GABA_A receptor antagonist, bicuculline, all failed to antagonize the effect of Vc1.1 in the majority of cells tested.

Numerous studies have shown that activation of GABA_B receptors leads to inhibition of N-type Ca^{2+} channel currents in central and peripheral neurons (Menon-Johansson et al., 1993; Guyon and Leresche, 1995; Fujikawa et al., 1997; Harayama et al., 1998; Richman et al., 2005; Castro et al., 2007; Raingo et al., 2007), but conotoxins have not previously been contemplated to act on this pathway. The inhibition of N-type Ca^{2+} channel currents after activation of GABA_B receptors has been shown to occur in both a voltage-dependent and a voltage-independent manner, to be PTX-sensitive, and to involve c-Src (Richman et al., 2005; Raingo et al., 2007). Several GABA_B receptor antagonists were tested for their ability to antagonize Vc1.1 inhibition of HVA Ca^{2+} channel currents in DRG neurons. Application of $1 \mu\text{M}$ Vc1.1 in the presence of the GABA_B receptor antagonists phaclofen ($50 \mu\text{M}$) and CGP55485 ($1 \mu\text{M}$) did not significantly reduce the HVA Ca^{2+} channel current in DRG neurons [$95.5 \pm 4.4\%$ ($n = 9$) and $93.2 \pm 4.5\%$ ($n = 14$) of control, respectively] (Fig. 7A,B). RgIA (100 nM) also failed to inhibit HVA Ca^{2+} channel current in the presence of CGP55485 ($92.5 \pm 3.5\%$ of control, $n = 5$) (data not shown). Together, these data indicate that Vc1.1 and RgIA inhibit HVA Ca^{2+} channel currents via activation of the GABA_B receptor in DRG neurons.

The GABA_B receptor agonist baclofen has previously been shown to inhibit HVA Ca^{2+} channel currents in neonatal rat DRG neurons (Dolphin and Scott, 1987, 1990; Menon-Johansson et al., 1993; Fujikawa et al., 1997). We examined whether the effects of Vc1.1 and baclofen were additive in inhibition of the HVA Ca^{2+} channel current. The inhibition produced after addition of 10 – $30 \mu\text{M}$ baclofen ($63.6 \pm 10.6\%$ of

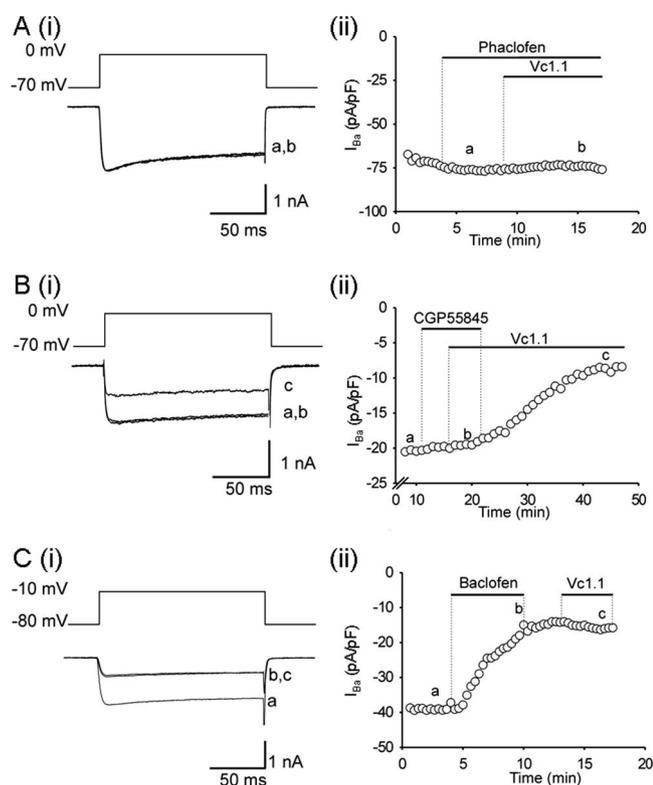


Figure 7. GABA_B receptor activation mediates Vc1.1 inhibition of N-type calcium channel currents in rat DRG neurons. **A(i)**, Superimposed traces of depolarization-activated whole-cell Ba²⁺ currents in the presence of the GABA_B inhibitor phaclofen (50 μM) followed by application of 100 nM Vc1.1. **(ii)**, Corresponding time course. Numbers on the plot indicate which points were used for sample traces in **A(i)**. **B(i)**, Superimposed traces of depolarization-activated whole-cell Ba²⁺ currents in the presence of another GABA_B inhibitor CGP55845 (1 μM) (a), in the presence of CGP55845 and Vc1.1 (100 nM) (b), and in the presence of Vc1.1 alone (100 nM) (c). **(ii)**, Corresponding time course. Numbers on the plot indicate which points were used for sample traces in **B(i)**. **C(i)**, Superimposed traces of depolarization-activated whole-cell Ba²⁺ currents in the absence (a), in the presence of the GABA_B agonist baclofen (30 μM) (b), and in the presence of 100 nM Vc1.1 (c). **(ii)**, Corresponding time course. Numbers on the plot indicate which points were used for sample traces in **C(i)**.

control, $n = 4$) was not significantly greater than that produced by 100 nM Vc1.1 alone ($61.2 \pm 15.9\%$ of control, $n = 4$) in the same cells. Similarly, application of Vc1.1 (100 nM) after addition of 30 μM baclofen did not further inhibit HVA Ba²⁺ current amplitude ($63.6 \pm 7.2\%$ of control, $n = 5$) compared with baclofen alone ($64.6 \pm 6.6\%$ of control, $n = 5$) (Fig. 7C). Under the present recording conditions (150 ms depolarizing pulse to -10 mV applied at a frequency of 0.05 Hz), the time to maximum inhibition (9.3 ± 1.2 min) and reversal ($<5\%$ after 10 min wash-out) by 30 μM baclofen ($n = 11$) was significantly slower than when the depolarizing pulse was shortened to 15 ms or the frequency of depolarization was increased to 0.1 Hz (time to maximum inhibition occurred in 2.8 ± 0.9 min and complete recovery within 5 min after washout of baclofen, $n = 5$).

In subsequent experiments, we observed that in $\sim 70\%$ of oocytes expressing recombinant Ca_v2.2 (N-type) but not Ca_v2.1 (P/Q-type), Ca_v2.3 (R-type), or Ca_v1.2 (L-type) calcium channels, application of baclofen (100 μM) inhibited Ba²⁺ currents (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). We therefore tested the effect of α -conotoxins Vc1.1 and Rg1A in these oocytes. Both Vc1.1 and Rg1A inhibited Ba²⁺ currents through recombinant Ca_v2.2 channels in baclofen-responding oocytes ($n \geq 28$). Preincubation of oocytes

with the selective GABA_B receptor antagonist CGP55845 (1 μM) abolished the inhibition of Ba²⁺ currents observed with 1 μM Vc1.1 ($n = 4$) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Discussion

To date, the α -conotoxins have been classified as competitive nAChR antagonists (Dutton and Craik, 2001; Livett et al., 2006). The present study is the first to identify the N-type calcium channel of mammalian DRG neurons as a target of the α -conotoxins Vc1.1 and Rg1A. To the best of our knowledge, this study is also the first to describe conotoxin modulation of an ion channel via a G-protein receptor-activated pathway.

Vc1.1 and Rg1A are of great interest as analgesic drugs because of their effectiveness in peripheral nerve-injured rats (Satkunanathan et al., 2005; Vincler et al., 2006). Although these peptides have been shown to be potent antagonists of $\alpha 9\alpha 10$ nAChRs, post-translationally modified analogs of Vc1.1, [P6O]Vc1.1, and vcl1a are ineffective in assays of neuropathic pain (Livett et al., 2006; Nevin et al., 2007). However, they are equipotent to Vc1.1 as $\alpha 9\alpha 10$ nAChRs antagonists, indicating that this nAChR is not the therapeutic target of Vc1.1 (Nevin et al., 2007).

N-type calcium channels are an appealing target of Vc1.1 because they are well known to play a central role in the detection and transmission of nociceptive stimuli in DRG neurons (Altier and Zamponi, 2004). Several studies have highlighted the importance of N-type calcium channels in neuropathic pain: N-type α_{1B} channel knock-out mice have a decreased response to neuropathic pain (Hatakeyama et al., 2001; Kim et al., 2001; Saegusa et al., 2001), there is an upregulation of N-type α_{1B} as well as $\alpha_2\delta$ subunits in rat nerve injury models (Abe et al., 2002; Cizkova et al., 2002; Yokoyama et al., 2003), and currently used treatments, or treatments being developed, for pain relief include direct (ω -conotoxin MVIIA, also known as Prialt) and indirect (morphine) inhibitors of N-type channels (McGivern, 2006).

HVA Ca²⁺ channel currents of rat DRG neurons include contributions from N-, P/Q-, L-, and R-type calcium channels (Scroggs and Fox, 1992). The present study indicates that Vc1.1 targets solely the N-type component of the whole-cell Ca²⁺ current, because it had no effect in the presence of the N-type Ca²⁺ channel blockers, ω -conotoxins CVID and MVIIA. Vc1.1 inhibition of N-type Ca²⁺ channel current was irreversible after wash-out of up to 30 min, but subsequent cells patched from the same coverslip were as sensitive to Vc1.1 as the initial cell exposed to Vc1.1. This suggests that Vc1.1 inhibition of N-type current is dependent on membrane depolarization. Consistent with this hypothesis, Vc1.1 had no effect on HVA Ca²⁺ currents in DRG neurons when the duration of the depolarization pulse was shortened to one-tenth of its initial duration with the frequency maintained at 0.05 Hz. Vc1.1 inhibition was only observed with this short depolarizing pulse when the frequency was increased to 0.1 Hz.

DRG neurons have been shown to express a splice variant Ca_v2.2 that differs at exon 37 (Bell et al., 2004) from the peripheral splice variant we expressed, but extensive studies reported here indicate that the reason Vc1.1 is ineffective on recombinant N-type Ca²⁺ channels is that it is acting via a G-protein receptor-activated pathway. Our results suggest that the GPCR responsible is the GABA_B receptor: first, Vc1.1 inhibition of N-type Ca²⁺ current was antagonized by selective GABA_B receptor antagonists, and second, similar to previous studies of the GABA_B receptor agonist baclofen in rat or chick DRG neurons (Dolphin and Scott, 1987; Richman and Diversé-Pierluissi, 2004; Raingo et

al., 2007), Vc1.1 inhibition of N-type Ca^{2+} currents in rat DRG neurons was antagonized when GTP was replaced in the patch pipette with GDP β S, when the cells were incubated overnight with PTX, and when c-Src tyrosine kinase activity was inhibited. Activation of the GABA_B receptor has been shown to inhibit N-type Ca^{2+} channel current by voltage-dependent (relieved by a strong depolarizing prepulse) and voltage-independent pathways, and to be readily reversible after washout of baclofen (Richman and Diversé-Pierluissi, 2004; Raingo et al., 2007). No relief of Vc1.1 inhibition was observed after depolarizing prepulses to +80 mV. Although baclofen inhibition during prolonged depolarizations (150 ms, 0.05 Hz) was also irreversible, baclofen inhibition of N-type Ca^{2+} current during short depolarization pulses (15 ms, 0.05 Hz) was rapidly reversible after washout of baclofen (our unpublished observations). These results suggest that Vc1.1 does not inhibit the N-type current via the fast, G $\beta\gamma$ -mediated, membrane-delimited, voltage-dependent pathway associated with GABA_B receptor activation. Rather, it activates a pathway that remains to be fully elucidated but involves pp60c-src tyrosine kinase and leads to a maintained inhibition of the N-type Ca^{2+} channel current, possibly as a result of persistent binding of Vc1.1 to the GABA_B receptor. Alternatively Vc1.1 may interact differently with the GABA_B receptor than baclofen, which binds to the venus fly trap motif of the GABA_{B1} subunit, leading to its closure and activation of the receptor (for review, see Emson, 2007).

Vc1.1 was without effect on recombinant $\text{Ca}_v2.1$ (P/Q-type), $\text{Ca}_v1.2$ (L-type), and $\text{Ca}_v2.3$ (R-type) channels expressed in *Xenopus* oocytes. However, in subsequent experiments we found that baclofen activation of endogenous GABA_B receptors in batches of *Xenopus* oocytes mediates inhibition of Ba^{2+} current through recombinant $\text{Ca}_v2.2$ (N-type) channels. In oocytes expressing endogenous GABA_B receptors, α -conotoxins Vc1.1 and Rg1A also inhibit recombinant $\text{Ca}_v2.2$ channels via agonist activation of a GABA_B receptor-signaling pathway in *Xenopus* oocytes (Yang et al., 2001).

The characteristics of the G-protein-induced inhibition of voltage-dependent N-type calcium channels by Vc1.1 and Rg1A activation of GABA_B receptors is reminiscent of the agonist-induced internalization of N-type calcium channels observed in rat DRG neurons (Altier et al., 2006) and embryonic chick DRG neurons (Puckerin et al., 2006; Tomblor et al., 2006). Prolonged exposure of opioid receptor-like receptor 1 (ORL1) receptors to the agonist nociceptin has been shown to trigger the internalization of the ORL1 receptors and $\text{Ca}_v2.2$ channels coexpressed in human embryonic kidney tsA-201 cells (Altier et al., 2006), which is accompanied by a tonic inhibition of calcium entry. Baclofen, a GABA_B agonist, has also been shown to induce the rapid removal of ω -conotoxin GVIA-sensitive (N-type) calcium channels from the plasma membrane of DRG neurons (Tomblor et al., 2006). A mechanism proposed for the internalization of voltage-dependent calcium channels involves the selective binding of β -arrestin 1 to the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor)-binding region of the calcium channel whereby, on GABA_B receptor activation, receptors are recruited to the arrestin-channel complex and internalized (Puckerin et al., 2006). Furthermore, β -arrestins have been shown to be required to target nonreceptor tyrosine kinase c-Src to constitutively active G-protein-coupled μ -opioid receptors in DRG neurons, resulting in their internalization (Walwyn et al., 2007). It is likely, therefore, that α -conotoxin activation of GABA_B receptors and the inhibition of N-type calcium channels in DRG neurons is mediated by a mechanism similar to that

reported previously for internalization of GPCR-channel complexes.

In conclusion, our results identify $\text{Ca}_v2.2$ as a target of the analgesic α -conotoxins Vc1.1 and Rg1A. We propose a novel mechanism by which these α -conotoxins modulate N-type calcium channel currents in DRG neurons, that is, acting as agonists via G-protein-coupled GABA_B receptors. The activation of GABA_B receptors by agonists such as baclofen is well established to produce antinociceptive and antiallostatic actions in chronic pain models (Schuler et al., 2001; Bettler et al., 2004). Therefore, the activation of GABA_B receptors by Vc1.1 and Rg1A and subsequent inhibition of $\text{Ca}_v2.2$ (N-type) channels is most likely the mechanism mediating their analgesic properties, despite their recently reported modulation, at lower efficacy, of the $\alpha 9\alpha 10$ nAChR (Vincler et al., 2006; Vincler and McIntosh, 2007).

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