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Author/s:

Berecki, G;McArthur, JR;Cuny, H;Clark, RJ;Adams, DJ

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Differential $Ca_v2.1$ and $Ca_v2.3$ channel inhibition by baclofen and α -conotoxin Vc1.1 via $GABA_B$ receptor activation

Géza Berecki,¹ Jeffrey R. McArthur,¹ Hartmut Cuny,¹ Richard J. Clark,² and David J. Adams¹

¹Health Innovations Research Institute, RMIT University, Melbourne, Victoria 3083, Australia

²School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

Neuronal $Ca_v2.1$ (P/Q-type), $Ca_v2.2$ (N-type), and $Ca_v2.3$ (R-type) calcium channels contribute to synaptic transmission and are modulated through G protein-coupled receptor pathways. The analgesic α -conotoxin Vc1.1 acts through γ -aminobutyric acid type B ($GABA_B$) receptors ($GABA_B$ Rs) to inhibit $Ca_v2.2$ channels. We investigated $GABA_B$ R-mediated modulation by Vc1.1, a cyclized form of Vc1.1 (c-Vc1.1), and the $GABA_B$ R agonist baclofen of human $Ca_v2.1$ or $Ca_v2.3$ channels heterologously expressed in human embryonic kidney cells. 50 μ M baclofen inhibited $Ca_v2.1$ and $Ca_v2.3$ channel Ba^{2+} currents by $\sim 40\%$, whereas c-Vc1.1 did not affect $Ca_v2.1$ but potently inhibited $Ca_v2.3$, with a half-maximal inhibitory concentration of ~ 300 pM. Depolarizing paired pulses revealed that $\sim 75\%$ of the baclofen inhibition of $Ca_v2.1$ was voltage dependent and could be relieved by strong depolarization. In contrast, baclofen or Vc1.1 inhibition of $Ca_v2.3$ channels was solely mediated through voltage-independent pathways that could be disrupted by pertussis toxin, guanosine 5'-[β -thio]diphosphate trilithium salt, or the $GABA_B$ R antagonist CGP55845. Overexpression of the kinase c-Src significantly increased inhibition of $Ca_v2.3$ by c-Vc1.1. Conversely, coexpression of a catalytically inactive double mutant form of c-Src or pretreatment with a phosphorylated pp60c-Src peptide abolished the effect of c-Vc1.1. Site-directed mutational analyses of $Ca_v2.3$ demonstrated that tyrosines 1761 and 1765 within exon 37 are critical for inhibition of $Ca_v2.3$ by c-Vc1.1 and are involved in baclofen inhibition of these channels. Remarkably, point mutations introducing specific c-Src phosphorylation sites into human $Ca_v2.1$ channels conferred c-Vc1.1 sensitivity. Our findings show that Vc1.1 inhibition of $Ca_v2.3$, which defines $Ca_v2.3$ channels as potential targets for analgesic α -conotoxins, is caused by specific c-Src phosphorylation sites in the C terminus.

INTRODUCTION

Presynaptic voltage-gated $Ca_v2.1$ (P/Q-type), $Ca_v2.2$ (N-type), and $Ca_v2.3$ (R-type) voltage-gated calcium channels (VGCCs) mediate nerve-evoked transmitter release. Their modulation by G protein-coupled receptors (GPCRs) is a key factor in controlling neuronal excitability at central and peripheral synapses (Luebke et al., 1993; Takahashi and Momiyama, 1993; Wu et al., 1998; Gasparini et al., 2001). Multiple GPCR-mediated pathways converge on VGCCs, but $Ca_v2.3$ channels are less susceptible to direct G protein $\beta\gamma$ dimer modulation than $Ca_v2.1$ or $Ca_v2.2$ (Shekter et al., 1997), a finding attributed to differences between the N terminus, domain I, and the I–II intracellular linker of $Ca_v2.3$ and $Ca_v2.2$ channels (Stephens et al., 1998; Simen and Miller, 2000). Nevertheless, carbachol, somatostatin, ATP, and adenosine inhibit exogenous $Ca_v2.3$ channels via endogenous receptors in human embryonic kidney (HEK) cells

(Mehrkke et al., 1997). Interestingly, carbachol, a muscarinic receptor agonist, stimulates or inhibits $Ca_v2.3$ currents by distinct signaling pathways in HEK cells (Bannister et al., 2004), whereas the D2 dopamine receptor agonist quinpirole (Page et al., 1998) and μ opioid receptor agonist DAMGO (Ottolia et al., 1998) inhibit $Ca_v2.3$ currents in the *Xenopus laevis* oocyte system. Electrophysiological data suggest that baclofen, a derivative of γ -aminobutyric acid (GABA), inhibits R-type currents in the rat medial nucleus (Wu et al., 1998) and locus coeruleus neurons (Chieng and Bekkers, 1999).

VGCCs are associated with a wide range of pathologies, including pain, and the value of selectively targeting Ca_v2 channels for neuropathic pain treatment is recognized (Altier et al., 2007; Pexton et al., 2011). We have shown that α -conotoxin Vc1.1, a small venom peptide from *Conus victoriae*, inhibits $Ca_v2.2$ channels via $GABA$ type B ($GABA_B$) receptors ($GABA_B$ Rs) in rodent dorsal root ganglion (DRG) neurons (Callaghan et al., 2008; Callaghan and Adams, 2010) and the HEK expression system (Cuny et al., 2012). We also demonstrated

Correspondence to Géza Berecki: geza.berecki@rmit.edu.au

J.R. McArthur's present address is Dept. of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138.

Abbreviations used in this paper: DRG, dorsal root ganglion; GABA, γ -aminobutyric acid; $GABA_B$, GABA type B; $GABA_B$ R, $GABA_B$ receptor; GDP- β -S, guanosine 5'-[β -thio]diphosphate trilithium salt; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; HP, holding potential; I_{Ba} , Ba^{2+} current; PTX, pertussis toxin; VD, voltage dependent; VGCC, voltage-gated calcium channel; VI, voltage independent.

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that Vc1.1 can be used as an analgesic in rat models of neuropathic pain (Klimis et al., 2011). Ca_v2.3 channels are also present in various nociceptors (Fang et al., 2007, 2010) and contribute to pain behavior control by spinal and supraspinal mechanisms (Saegusa et al., 2000; Terashima et al., 2013). However, Ca_v2.3 modulation via GABA_BRs is incompletely characterized and has not been reconstituted in any heterologous expression system. Moreover, few drugs or toxins have specific Ca_v2.3 inhibitory effects (Schneider et al., 2013).

In this study, we hypothesized that α -conotoxin Vc1.1 can modulate Ca_v2.1 and Ca_v2.3 channels via GABA_BR activation. We designed experiments to examine the mechanisms of VGCC Ba²⁺ current (I_{Ba}) inhibition by baclofen and Vc1.1, with emphasis on voltage-dependent (VD) and voltage-independent (VI) pathways, which may be present in these cells. Our data show that Vc1.1 only inhibits Ca_v2.3 channels, despite baclofen efficiently inhibiting both Ca_v2.1 and Ca_v2.3 channels. Using site-directed mutagenesis in combination with functional expression in HEK cells, we demonstrate that c-Src phosphorylation of specific tyrosine residues in the α_1 subunit C terminus is sufficient to mediate Vc1.1 inhibition of Ca_v2.3 channels. A preliminary report of these results, in part, has been presented in abstract form (Berecki, G., J.R. McArthur, and D.J. Adams. 2013. Australian Neuroscience Society Inc. 33rd Annual Meeting. Abstr. ORAL-03-03).

MATERIALS AND METHODS

Cell culture, clones, and transfections

HEK cells containing the SV40 large T antigen (HEK-293T) were cultured at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen), 50 IU/ml penicillin, and 50 μ g/ml streptomycin (Invitrogen). HEK-293 cells, stably expressing human Ca_v2.1 (P/Q-type) channel α_{1A2} splice variant (GenBank accession no. AF004883) or human Ca_v2.3c (R-type) channel α_{1E3} splice variant (also called α_{1E-C} ; GenBank accession no. L29385), were obtained from Merck and cultured according to procedures described previously (Dai et al., 2008). Both cell lines express human $\alpha_{2B}\delta$ -1 (GenBank accession no. M76559) and human β_{3a} (RefSeq accession no. NM_000725) auxiliary subunits, and the human KCNJ4 (Kir2.3; GenBank accession no. U07364) channel (Dai et al., 2008).

The human Ca_v2.1 (P/Q-type) channel, α_{1A} , transcript variant 5 (RefSeq accession no. NM_001174080), cloned into pCMV plasmid, was provided by J. Striessnig (University of Innsbruck, Innsbruck, Austria). The human Ca_v2.3d (R-type) channel (fetal brain α_{1E-d} , splice variant L27745) was provided by T. Schneider (University of Cologne, Cologne, Germany). The wild-type human Ca_v2.3c (α_{1E-C}) channel (GenBank accession no. L29385) and mutant Ca_v2.3c channels, α_{1E-C} (Y1761F) and α_{1E-C} (Y1765F), all cloned into pCDNA3.1 vectors, were purchased from GenScript USA Inc. The α_{1E-d} splice variant is identical in amino acid sequence to α_{1E-C} , except for a 43-amino acid segment (insert III or exon 46) at the C terminus of the α_{1E-d} channel (Pereverzev et al., 2002). Human $\alpha_{2B}\delta$ -1 (RefSeq accession no. NM_000722) and human β_3 channel subunits, transcript variant 1 (RefSeq accession no. NM_000725),

were purchased from OriGene Technologies, Inc. Site-directed mutagenesis of the wild-type human Ca_v2.3d (α_{1E-d}) channel, resulting in α_{1E-d} (Y1761F) or α_{1E-d} (Y1765F), and site-directed mutagenesis of the wild-type human Ca_v2.1 (α_{1A5}) channel, resulting in α_{1A5} (L1852T) or α_{1A5} (Q1852E), was performed with the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies), using the following oligonucleotides: Ca_v2.3(Y1761F)-for, GCATGTG-GCCGCATCCATTTCACTGAGATGTATGAAATG; Ca_v2.3(Y1761F)-rev, CATTTCATACATCTCAGTGAATGGATGCGGCCACATGC; Ca_v2.3(Y1765F)-for, CATTACACTGAGATGTTTGAATGCTGATCTCTC; Ca_v2.3(Y1765F)-rev, GAGATCAGCATTTCAAACATCTCAGTGAATG; Ca_v2.1(L1852T)-for, GCGCGCATGCCTTACACGGACATGTATCAGATG; Ca_v2.1(L1852T)-rev, CATCTGATACATGTCCGTGTAAGGCATGCGGCC; Ca_v2.1(Q1856E)-for, CTTACTGGACATGTATGAGATGCTGAGACACATGTC; Ca_v2.1(Q1856E)-rev, GACATGTGTCTCAGCATCTCATACATGTCCAGGTAAG.

For all primers, sense and antisense orientations are denoted as "for" and "rev," respectively. The names reflect the position of the tyrosine to be mutated to a phenylalanine (Ca_v2.3), leucine to threonine (Ca_v2.1), or glutamine to glutamic acid (Ca_v2.1). All mutations were verified by automated DNA sequencing (Australian Genome Research Facility).

HEK cells stably expressing Ca_v2.1 or Ca_v2.3c channels were transiently cotransfected with plasmid cDNAs encoding human GABA_BR1 (RefSeq accession no. NM_001470; 3 μ g; OriGene Technologies, Inc.), human GABA_BR2 (RefSeq accession no. NM_005458; 3 μ g; OriGene Technologies, Inc.), and enhanced green fluorescent protein (eGFP) reporter gene construct (1 μ g; provided by J.W. Lynch, The University of Queensland, Brisbane, Australia), using the calcium phosphate precipitation method (Jordan et al., 1996). In separate experiments, pRC-CMV/Src encoding wild-type mouse c-Src or pRC-CMV/Src(K295R/Y527F) double mutant mouse c-Src cDNA (provided by J. Ulrich, University of Iowa, Iowa City, IA) was also included in the above transfection mixture. The K295R mutation in the ATP-binding site inactivates the kinase, whereas the Y527F mutation abolishes intramolecular interactions between the C-terminal tail and the SH2 domain (Gao et al., 1997).

HEK-293T cells were transiently cotransfected with plasmid cDNAs encoding human Ca_v2.1 channel transcript variant 5 (5 μ g) or wild-type or mutant human Ca_v2.3d channels (5 μ g), human $\alpha_{2B}\delta$ -1 (5 μ g) and human β_3 (5 μ g) auxiliary subunits, human GABA_BR1 (3 μ g), human GABA_BR2 (3 μ g), and eGFP (1 μ g). In a separate series of experiments, HEK-293T cells were transiently cotransfected with plasmid cDNAs encoding rabbit Ca_v2.1 channel (RefSeq accession no. NM_001101693; 5 μ g; provided by F. Meunier, The University of Queensland, St. Lucia, Australia), rat $\alpha_2\delta$ -1 (5 μ g; provided by G.W. Zamponi, University of Calgary, Calgary, Canada) and rat β_3 (5 μ g; provided by D. Lipscombe, Brown University, Providence, RI) auxiliary subunits; human GABA_BR1 (3 μ g) and human GABA_BR2 (3 μ g); and eGFP (1 μ g). After transfection, cells were plated on glass coverslips and incubated at 37°C in 5% CO₂ for 6 h. Transfection medium was then replaced with culture medium, and cells were incubated at 30°C in 5% CO₂.

Electrophysiology

Experiments were performed 3–5 d after transfection, using the whole-cell patch-clamp technique. Currents through calcium channels were recorded using barium (Ba²⁺) as the charge carrier. Cells expressing the proteins of interest were superfused with a solution containing (mM): 110 NaCl, 10 BaCl₂, 1 MgCl₂, 5 CsCl, 30 TEA-Cl, 10 D-glucose, and 10 HEPES, pH 7.4 with TEA-OH, at ~600 μ l/min. Fire-polished borosilicate patch pipettes with tip resistance values of 2–3 M Ω were filled with an intracellular solution containing (mM): 125 K-gluconate, 2 MgCl₂, 5 EGTA, 5 NaCl, 4 MgATP, and 10 HEPES, pH 7.25 with CsOH. In a series of experiments, EGTA was included in the intracellular solution at a

concentration of 0.5 or 10 mM. GTP was not used in the intracellular solution to prevent I_{Ba} rundown caused by activation of signaling pathways when the whole-cell recording configuration was established (Raingo et al., 2007). To minimize endogenous currents, the osmolarity of solutions was adjusted with sucrose (310-mOsm extracellular, slightly hypertonic with respect to the 295-mOsm intracellular solution).

Electrophysiological recordings were performed at room temperature (23–25°C) using Multiclamp 700B amplifiers (Molecular Devices) controlled by Clampex 9.2/DigiData 1332 acquisition systems. I-V relationships were recorded from a holding potential (HP) of -80 mV using 100-ms depolarizations from -45 to $+50$ mV, in 5-mV increments. Peak I_{Ba} was measured for each step and normalized to the cell's maximal current. Normalized currents were averaged across cells and plotted (mean \pm SEM) as a function of voltage. Test depolarizations to 10 mV (in cells coexpressing $Ca_v2.3$ channels and $GABA_B$ Rs) or 15 mV (in cells coexpressing $Ca_v2.1$ channels and $GABA_B$ Rs) of 150-ms duration were applied at a frequency of 0.1 Hz from an HP of -80 mV, where I_{Ba} was evaluated in the absence and presence of various compounds.

VD relief of the inhibition was assessed from an HP of -80 mV, using a protocol with a 20-ms prepulse to $+80$ mV, a 5-ms interpulse to -80 mV, and a 40-ms test pulse to $+10$ mV. The percentage of I_{Ba} inhibited in the absence of a prepulse ($-PP I_{Ba}$) or presence of a $+80$ -mV prepulse ($+PP I_{Ba}$) was determined according to $[(I_{0-PP} - I_{PP})/I_{0-PP}] \times 100$, or $[(I_{0+PP} - I_{PP})/I_{0+PP}] \times 100$, respectively, where I_{0+PP} and I_{0-PP} represent current amplitudes (controls) obtained with or without a prepulse in the absence of a compound, respectively. I_{0-PP} was normalized to I_{0+PP} . I_{PP} and I_{-PP} represent current amplitudes obtained with or without a prepulse in the presence of a compound, respectively. The VI fraction was defined as $(+PP I_{Ba}) \times 100/(-PP I_{Ba})$, whereas the VD fraction was calculated as $(-PP I_{Ba}) - VI$.

Membrane currents were filtered at 3 kHz and sampled at 10 kHz. Leak and capacitive currents were subtracted using a $-P/4$ pulse protocol. Peptides and various drugs were prepared from stock solutions, diluted to appropriate final concentration, and applied via perfusion in the bath solution. Data were stored digitally on a computer for further analysis. Current densities were calculated by dividing the normalized current amplitude by the cell capacitance measured at the start of each experiment.

In successive transfections, the magnitude of baclofen inhibition of I_{Ba} was routinely tested in HEK cells stably expressing $Ca_v2.1$ or $Ca_v2.3$ channels and coexpressing $GABA_B$ Rs. In $\sim 5\%$ of all cells tested, I_{Ba} inhibition by baclofen was $\leq 25\%$. In such cases, the results were not included in the analysis or the experiment was discontinued. When evaluating the Vc1.1 concentration dependence of I_{Ba} inhibition, only a maximum of three different Vc1.1 concentrations per cell were tested because of the relatively long time needed to reach maximum inhibition with each Vc1.1 concentration.

Peptides, chemicals, and drugs

α -Conotoxins Vc1.1, cyclized-Vc1.1 (c-Vc1.1), and PeIA were synthesized as described previously (Clark et al., 2006, 2010; Daly et al., 2011). Synthetic Vc1.1 and PeIA are 16-amino acid residue peptides with a characteristic helical region and two disulfide bonds in a I–III, II–IV arrangement (Clark et al., 2006, 2010; Daly et al., 2011). c-Vc1.1 exhibits better properties than the linear Vc1.1 (also known as ACV1), including high chemical stability, resistance to cleavage by proteases, and improved potency to inhibit N-type VGCCs (Clark et al., 2010). Most data on α -conotoxin effects on various Ca_v2 channels were obtained using c-Vc1.1, unless otherwise noted. GABA, baclofen, guanosine 5'-[β -thio]diphosphate trilithium salt (GDP- β -S), and pertussis toxin (PTX) were purchased from Sigma-Aldrich. (2S)-3-[[1S]-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl) phosphinic

acid hydrochloride (CGP55845) and pp60c-Src peptide (521–533) were purchased from Tocris Bioscience.

c-Src phosphorylation site prediction

A publicly available catalog of phosphorylation motifs (http://www.hprd.org/PhosphoMotif_finder; Amanchy et al., 2007) was used to identify Src kinase substrate motifs within $Ca_v2.1$ and $Ca_v2.3$ C-terminal regions corresponding to exon 37 (e37) of $Ca_v2.2$ channel. This catalog does not use algorithms or computational strategies to predict phosphorylation but reports the presence of any literature-derived motifs.

Curve fitting and statistical analysis

Data analysis was performed in Clampfit 9.2 (Molecular Devices) and Origin 9.0 (Microcal Software Inc.). The voltage dependence of I_{Ba} activation was determined from I-V curves fitted to the following transform of a Boltzmann function: $I_{Ba} = G_{max}(V - V_{rev})/[1 + \exp[(V - V_{0.5,act})/k]]$, where V_{rev} is the extrapolated reversal potential, V is the membrane potential, I_{Ba} is the peak current elicited by the voltage pulse, G_{max} is the maximum conductance, $V_{0.5,act}$ is the voltage for half-maximal current activation, and k is the slope factor (Favre et al., 1995). Current amplitudes obtained in the presence of a compound (I) were normalized to current amplitudes obtained under control conditions (I_0). Concentration–response curves were obtained by plotting averaged relative peak current amplitude (I/I_0) against compound concentration and fitting the Hill equation $I = I_0\{[D]^h/(IC_{50}^h + [D]^h)\}$ to resulting data, where I_0 is the maximum peak current amplitude, $[D]$ is the concentration of the compound (drug), IC_{50} is the half-maximal inhibitory concentration, and h is the Hill coefficient (slope). Concentration–response curves are interpreted as functional responses by a ligand (baclofen or c-Vc1.1) against a change in ligand concentration. Results shown in Fig. 2 (B and C) and Table 2 were obtained by applying increasing concentrations of baclofen to the extracellular solution. Because baclofen inhibition of $Ca_v2.3$ channels is irreversible, these experiments do not represent equilibrium steady-state measurements (Christopoulos and Kenakin, 2002).

Data are mean \pm SEM (n , number of experiments). Statistical analyses were performed in Sigma Plot 11.0 (Systat Software, Inc.) using Student's t test for two groups or one-way ANOVA with Bonferroni post-hoc testing for multiple comparisons. When one-way ANOVA failed, Kruskal–Wallis one-way ANOVA on ranks with Tukey test for multiple comparisons was used. Differences were considered statistically significant at $P < 0.05$.

Online supplemental material

Table S1 shows the parameters of the Boltzmann fits to I-V and G-V curves in $Ca_v2.1$ / $GABA_B$ R cells in the presence of 0.5 or 10 mM EGTA in the intracellular recording solution. Fig. S1 shows the voltage dependence of baclofen inhibition of $Ca_v2.3d$ channels in the presence of 0.5 or 10 mM EGTA in the intracellular recording solution. Whole-cell I_{Ba} was recorded from HEK cells transiently coexpressing wild-type $Ca_v2.3d$ or mutant $Ca_v2.3d$ (Y1765F) channels and $GABA_B$ Rs. The online supplemental material is available at <http://www.jgp.org/cgi/content/full/jgp.201311104/DC1>.

RESULTS

Differential inhibition of $Ca_v2.3$ and $Ca_v2.1$ channels by α -conotoxin Vc1.1 via G protein–coupled $GABA_B$ Rs

We investigated VGCC modulation by baclofen and α -conotoxin Vc1.1 in HEK cells stably expressing $Ca_v2.1$ (α_{1A2}) or $Ca_v2.3c$ (α_{1E-c}) channels and transiently expressing $GABA_B$ Rs ($Ca_v2.1$ / $GABA_B$ R cells or $Ca_v2.3$ / $GABA_B$ R cells, respectively). Fig. 1 (A–C) shows typical

examples of depolarization-activated whole-cell I_{Ba} in the absence or presence of 200 nM c-Vc1.1 or 50 μ M baclofen. In $Ca_v2.1/GABA_B R$ cells, c-Vc1.1 did not modulate I_{Ba} but inhibited I_{Ba} in $Ca_v2.3/GABA_B R$ cells. The effect of c-Vc1.1 developed relatively slowly, reached maximum inhibition 3–7 min after the response started, and was irreversible (Fig. 1 B). The “linear” α -conotoxin Vc1.1 and α -conotoxin PeIA also inhibited depolarization-activated I_{Ba} in $Ca_v2.3/GABA_B R$ cells (Table 1).

These peptides have been shown to selectively inhibit high voltage-activated N-type calcium channels by acting as G protein-coupled $GABA_B R$ agonists in rat DRG neurons (Callaghan et al., 2008; Daly et al., 2011). $Ca_v2.1/GABA_B R$ or $Ca_v2.3/GABA_B R$ cells typically responded to baclofen, with relatively fast I_{Ba} inhibition that was completely reversible or weakly reversible/irreversible, respectively (Fig. 1, A and C, and Table 1). In most experiments, applying baclofen after c-Vc1.1 exposure further suppressed a small fraction (<10%) of I_{Ba} in $Ca_v2.3/GABA_B R$ cells. We determined the baclofen concentration dependence of I_{Ba} inhibition for $Ca_v2.1$ and $Ca_v2.3$ channels (Fig. 2, B and D), resulting in relationships described by the Hill equation (Table 2). 50 μ M GABA also inhibited \sim 40% of I_{Ba} in $Ca_v2.1/$

$GABA_B R$ and $Ca_v2.3/GABA_B R$ cells and exhibited IC_{50} values similar to those obtained with baclofen (Table 2). The c-Vc1.1 concentration dependence of I_{Ba} inhibition in $Ca_v2.3/GABA_B R$ cells (Fig. 2 F) resulted in IC_{50} and Hill coefficient values of 290 ± 0.8 pM and 0.61 ± 0.1 , respectively, and defined c-Vc1.1 as a potent $Ca_v2.3$ channel inhibitor (Table 2). Fig. 2 (A and C) and Table 1 summarize the average I_{Ba} inhibition by baclofen, GABA, Vc1.1, c-Vc1.1, and PeIA in the absence and presence of $GABA_B R$ s. These results demonstrate that $GABA_B R$ expression is needed for baclofen to inhibit $Ca_v2.1$ and $Ca_v2.3$ channels, and for c-Vc1.1 to inhibit $Ca_v2.3$ channels. Moreover, the decreased response to baclofen after c-Vc1.1's effect is consistent with an overlap between the intracellular signaling mechanisms induced by these two compounds (Figs. 1 B and 2 E).

Voltage dependence of $GABA_B R$ -mediated inhibition of $Ca_v2.1$ and $Ca_v2.3$ channels

Direct VGCC inhibition by G protein-dependent inhibitory pathways involves VD $G\beta\gamma$ binding to the pore-forming subunit (Bean, 1989; Kasai and Aosaki, 1989; Lipscombe et al., 1989). I-V relationships were recorded

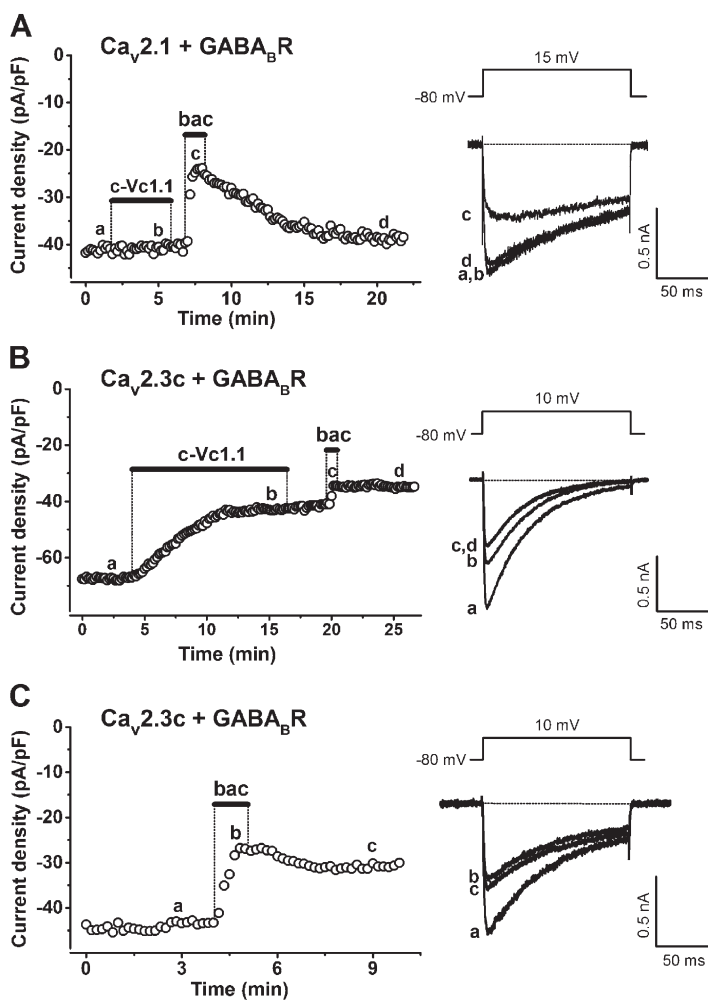


Figure 1. Effects of α -conotoxin c-Vc1.1 and baclofen (bac) on stably expressed human $Ca_v2.1$ (α_{1A-2}) or human $Ca_v2.3c$ (α_{1E-c}) channels in the presence of transiently expressed human $GABA_B R$ subunits R1 and R2 ($GABA_B R$). (A and B) 50 μ M baclofen inhibits $Ca_v2.1$ or $Ca_v2.3c$ channel currents, whereas 200 nM c-Vc1.1 only inhibits $Ca_v2.3c$ currents. Bars indicate c-Vc1.1 or baclofen application. I_{Ba} was evoked by 150-ms depolarizations to 10 mV ($Ca_v2.3c$) or 15 mV ($Ca_v2.1$), applied every 10 s from an HP of -80 mV (voltage inset). Peak current amplitudes were plotted as a function of time. Representative I_{Ba} traces (right) are shown at the times indicated by lowercase letters. Horizontal dotted line represents zero-current level. Note that $Ca_v2.1$ current inhibition by baclofen is reversible on wash-out (A), whereas baclofen or c-Vc1.1 irreversibly inhibits $Ca_v2.3c$ currents (B). (C) 50 μ M baclofen inhibits I_{Ba} in $Ca_v2.3/GABA_B R$ cells. Experimental procedures are similar to those in A or B.

TABLE 1

Summary of I_{Ba} inhibition by baclofen, GABA, or α -conotoxins in the presence or absence of GABA_BRs in HEK cells stably expressing human Ca_v2.1 or Ca_v2.3c channels

Agonist	I_{Ba} inhibition (%)			
	Ca _v 2.1 and GABA _B R	Ca _v 2.1 alone	Ca _v 2.3c and GABA _B R	Ca _v 2.3c alone
Baclofen (50 μ M)	39.9 \pm 2.6 (17)	0 (5)	39.5 \pm 4.3 (11)	0 (6)
GABA (50 μ M)	38.4 \pm 2.7 (7)	ND	39.3 \pm 4.1 (9)	ND
Vc1.1 (200 nM)	0 (9)	ND	25.5 \pm 4.2 (9)	ND
c-Vc1.1 (200 nM)	0 (10)	0 (6)	34.8 \pm 2.9 (16)	0 (5)
PeIA (200 nM)	0 (3)	ND	27.0 \pm 3.0 (3)	ND

Values represent mean \pm SEM; *n*, number of experiments in parentheses; ND, not determined.

in the absence and presence of baclofen in Ca_v2.1/GABA_BR cells and Ca_v2.3/GABA_BR cells, or c-Vc1.1 in Ca_v2.3/GABA_BR cells. The biophysical properties of ion permeation through Ca_v2.1 and Ca_v2.3 channels stably expressed in HEK cells have been characterized previously (Dai et al., 2008). We evaluated any depolarizing shift in the midpoint of activation ($V_{0.5,act}$) of these channels, which may indicate the presence of direct G $\beta\gamma$ modulation, by fitting I-V relationships to a modified Boltzmann function (see Materials and methods; Fig. 3 A). Fits of the normalized I-Vs revealed that $V_{0.5,act}$ shifted slightly

from 6.18 \pm 0.3 mV (*n* = 8; control) to 7.40 \pm 0.58 mV (*n* = 8; baclofen), but the difference was not statistically significant (*P* = 0.083) in Ca_v2.1/GABA_BR cells. However, it should be noted that the inhibition of Ca_v2.1/GABA_BR cells by baclofen (31.1 \pm 2.6%) is slightly less than the inhibition shown in Fig. 2 A and Table 1. This is probably because of spontaneous I_{Ba} recovery from inhibition that occurred even in the continuous presence of baclofen and could cause the underestimation of the $V_{0.5,act}$ positive shift in these experiments. To reduce the contribution of recovery in this process, we

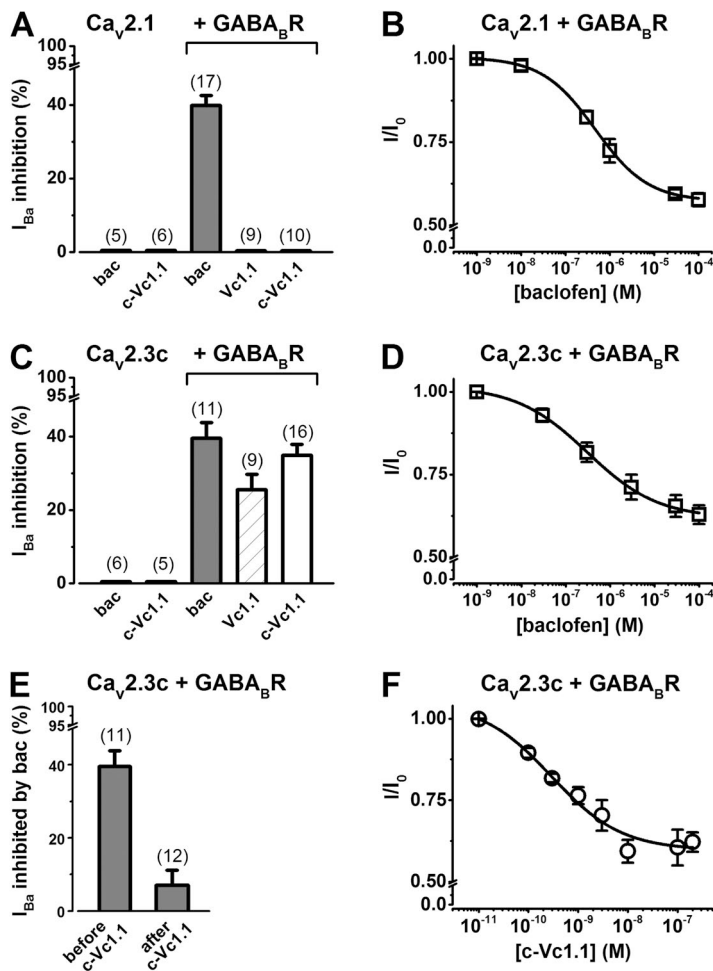


Figure 2. Stably expressed human Ca_v2.1 ($\alpha_{1A,2}$) or human Ca_v2.3c ($\alpha_{1E,c}$) channel inhibition by baclofen (bac) and α -conotoxin Vc1.1 in the absence and presence of transiently expressed human GABA_BR. (A and C) Bar graphs showing average I_{Ba} inhibition through Ca_v2.1 (A) or Ca_v2.3c (C) channels by 50 μ M baclofen, 200 nM Vc1.1, or 200 nM c-Vc1.1. Numbers in parentheses indicate the number of experiments. (E) Average I_{Ba} inhibited by 50 μ M baclofen applied before or after 200 nM c-Vc1.1; “before c-Vc1.1” data are replotted from C. (B, D, and F) Concentration-dependent inhibition of I_{Ba} through Ca_v2.1 and Ca_v2.3c channels by baclofen (B and D) and Ca_v2.3c channels by c-Vc1.1 (F). See IC_{50} values in Table 2.

TABLE 2

Summary of half-maximal inhibitory concentration (IC_{50}) and Hill slope values in HEK cells stably expressing human $Ca_v2.1$ or $Ca_v2.3c$ channels and transiently coexpressing $GABA_B$ Rs

Agonist	$Ca_v2.1$ and $GABA_B$ R		$Ca_v2.3c$ and $GABA_B$ R	
	IC_{50} nM	Hill slope	IC_{50} nM	Hill slope
Baclofen	470 ± 34 (5)	0.73 ± 0.05	330 ± 72 (7)	0.75 ± 0.14
GABA	388 ± 17 (4)	0.76 ± 0.20	328 ± 102 (4)	0.75 ± 0.24
c-Vc1.1	—	—	0.29 ± 0.08 (4-7) ^a	0.61 ± 0.14

Values represent mean \pm SEM; *n*, number of experiments in parentheses; see details of the fitting procedure in Materials and methods.

^aEach data point (Fig. 2 F) was obtained from four to seven individual experiments.

also investigated baclofen inhibition of I_{Ba} in $Ca_v2.1/GABA_B$ cells, evoked by voltage ramps. To assess any effect of divalent cation buffering on I-V relationships, we included 0.5 or 10 mM EGTA in the intracellular solution (Fig. 4 and Table S1). Voltage ramps in the absence and presence of baclofen resulted in $V_{0.5,act}$ values similar to those obtained with voltage steps (Fig. 3 A).

In $Ca_v2.3/GABA_B$ cells, the $V_{0.5,act}$ values were -3.44 ± 0.67 mV ($n = 8$) in the presence of baclofen and -0.44 ± 0.46 mV ($n = 7$) for c-Vc1.1 compared with 0.67 ± 0.24 mV ($n = 15$; control). In these experiments, baclofen caused a significant hyperpolarizing shift of $V_{0.5,act}$ ($P < 0.001$ vs. control; one-way ANOVA). However, $V_{0.5,act}$ was not altered by c-Vc1.1 ($P = 0.223$).

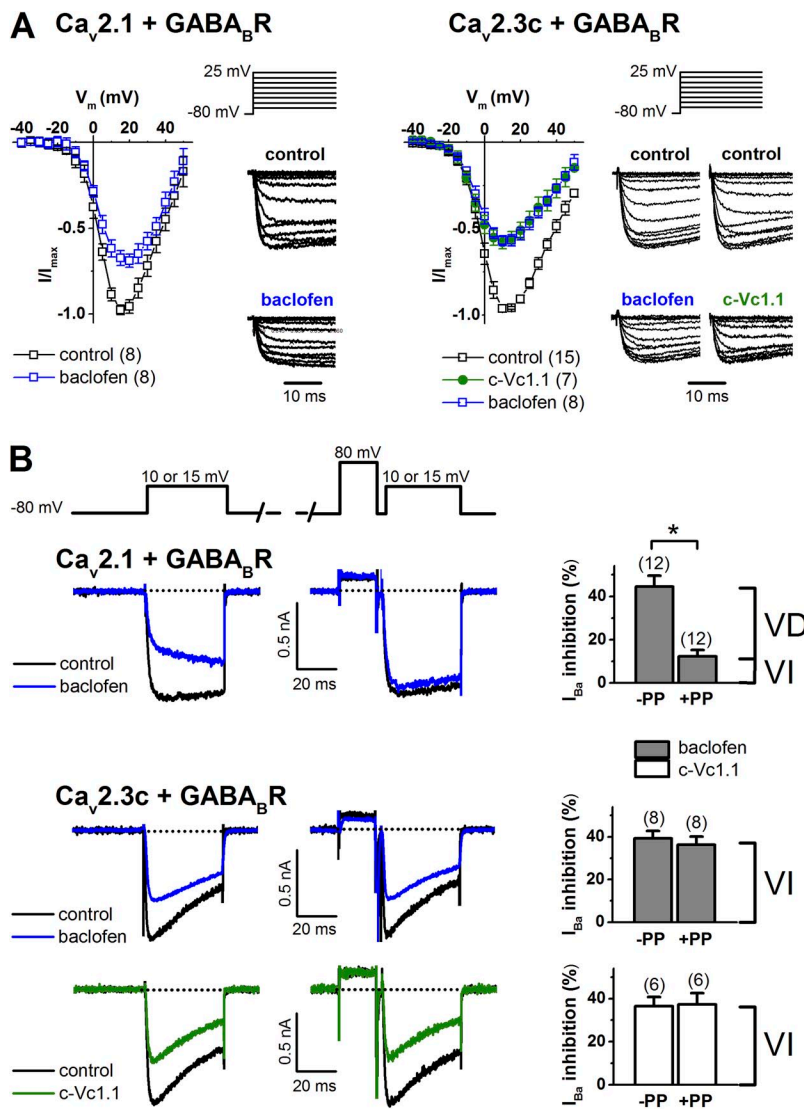


Figure 3. Voltage dependence of $Ca_v2.1$ (α_{1A-2}) and stably expressed $Ca_v2.3c$ channel inhibition via $GABA_B$ activation. (A) I-V relationships in $Ca_v2.1/GABA_B$ cells (left) in the absence (control) and presence of 50 μ M baclofen, and in $Ca_v2.3/GABA_B$ cells (right) in the absence (control) or presence of 50 μ M baclofen or 200 nM c-Vc1.1. (Insets) Voltage protocol and representative normalized current traces (only 23 ms of the 100-ms traces are shown). See Table S1 for $V_{0.5,act}$ values. (B) $Ca_v2.1$ channel inhibition via $GABA_B$ is VD, whereas that of $Ca_v2.3c$ is VI. Representative 15-mV depolarization-activated inward I_{Ba} from $Ca_v2.1/GABA_B$ cells (top) in the absence (control) or presence of 50 μ M baclofen, without (-PP) or after the application of a depolarizing prepulse to +80 mV (+PP). (Bottom) Representative 10-mV depolarization-activated I_{Ba} from $Ca_v2.3/GABA_B$ cells in the absence (control) or presence of 50 μ M baclofen or 200 nM c-Vc1.1. Dotted lines indicate zero-current level. The voltage protocol (top inset) is described in Materials and methods. (Right) Summary of I_{Ba} inhibition in the absence or presence of a prepulse. Data are mean \pm SEM (paired Student's *t* test; *, $P < 0.001$ vs. control [-PP] in $Ca_v2.1/GABA_B$ cells). The number of experiments is in parentheses. VD, voltage dependent; VI, voltage independent.

We evaluated if baclofen inhibition of I_{Ba} in $Ca_v2.1/GABA_B$ R and $Ca_v2.3/GABA_B$ R cells, and c-Vc1.1 inhibition of I_{Ba} in $Ca_v2.3/GABA_B$ R cells, could be reversed by strong depolarization. A +80-mV prepulse of 20-ms duration was applied before the test pulse to relieve any VD component of G protein-mediated I_{Ba} inhibition (Fig. 3 B). In both cells, shortening (10 ms) or prolonging (50 ms) the prepulse or interpulse (10 ms) did not change I_{Ba} facilitation. Applying +120-mV prepulses only added ~5% facilitation in $Ca_v2.1/GABA_B$ R cells, without changing I_{Ba} relief with $Ca_v2.3/GABA_B$ R cells (not depicted). The inhibitory effect of baclofen was associated with a large (73 ± 4%) VD component in $Ca_v2.1/GABA_B$ R cells. In contrast, the effect of baclofen and Vc1.1 was solely mediated by a VI pathway in $Ca_v2.3/GABA_B$ R cells, which clearly indicates that intracellular signaling does not involve the classical G protein $\beta\gamma$ dimer ($G\beta\gamma$) binding to the pore-forming $Ca_v2.3$ channel subunit. Alternatively, $G\beta\gamma$ could bind with high affinity to the $Ca_v2.3$ channel in a VI manner.

c-Vc1.1 inhibition of $Ca_v2.3$ channels involves $G\alpha_{i/o}$ subunits and c-Src kinase

We evaluated the VI pathway leading to $Ca_v2.3$ channel modulation by determining the fraction of I_{Ba} that could be inhibited under various experimental conditions (Fig. 5). In HEK cells stably expressing $Ca_v2.3c$ channels ($Ca_v2.3$ cells) or $Ca_v2.3$ cells coexpressing $GABA_B$ R R2 subunits, neither 200 nM c-Vc1.1 nor 50 μ M baclofen inhibits I_{Ba} , indicating that a fully functional $GABA_B$ R heterodimer is needed for proper signaling. In $Ca_v2.3/GABA_B$ R cells, the selective $GABA_B$ R antagonist CGP55845 (1 μ M) did not change I_{Ba} amplitude or kinetics but strongly antagonized I_{Ba} inhibition by c-Vc1.1 and reduced the effect of baclofen by ~60% compared with control. This confirmed that $GABA_B$ R needed to be activated for c-Vc1.1 and baclofen inhibitory effects to occur. When the hydrolysis-resistant GDP analogue GDP- β -S (500 μ M) was added to the intracellular recording solution, Vc1.1 and baclofen inhibitory effects were almost identically reduced. Overnight treatment

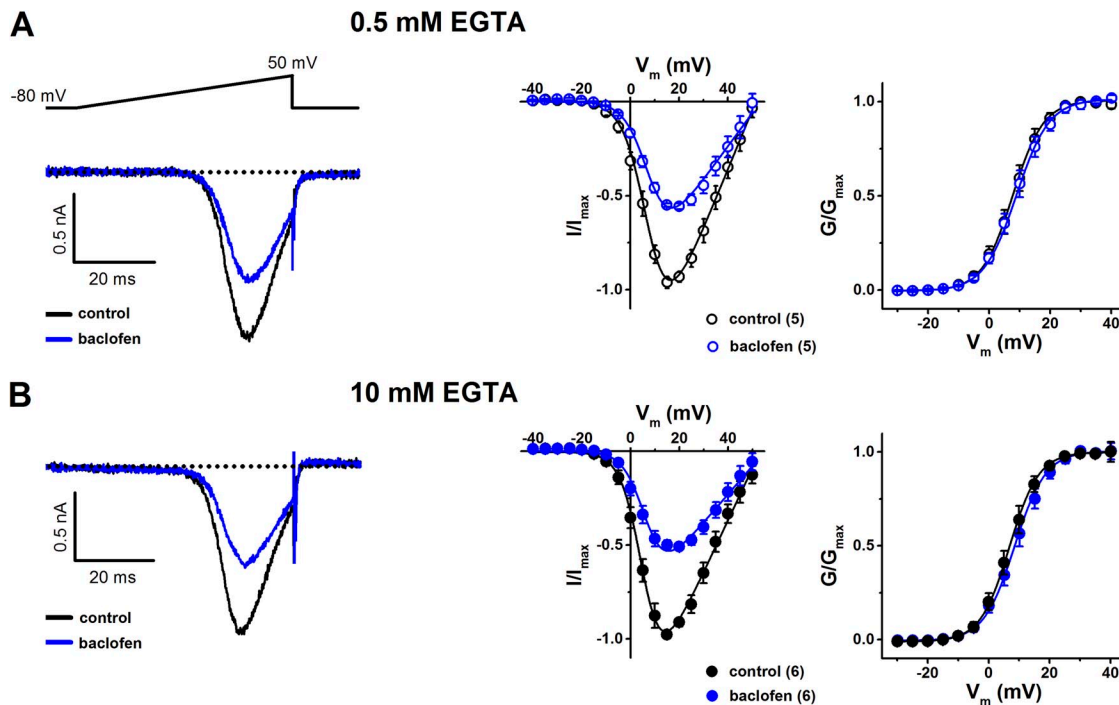


Figure 4. Effects of baclofen on stably expressed human $Ca_v2.1$ ($\alpha_{1A,2}$) channels in the presence of transiently expressed human $GABA_B$ Rs ($Ca_v2.1/GABA_B$ R cells). (A) Baclofen-inhibition of I_{Ba} in the presence of 0.5 mM EGTA in the intracellular recording solution. 50 μ M baclofen reversibly inhibited I_{Ba} by $38.5 \pm 3.9\%$ ($n = 5$). (Left) Representative currents in the absence (control) and presence of baclofen, elicited by voltage ramps to +50 mV from an HP of -80 mV at 0.1 Hz. Dotted line represents zero-current level. (Middle) I-V relationships in the absence and presence of baclofen. Current amplitudes were determined from voltage ramps at selected membrane potentials (V_m). Solid lines are fits of the modified Boltzmann equation to normalized I-V relationships (see Materials and methods). (Right) Voltage dependence of activation determined from G-V relationships. Relative conductance (G/G_{max}) was calculated as $I_{Ba}/(V_m - V_{rev})$, where V_{rev} is the reversal potential of the whole-cell current and plotted as a function of V_m . The normalized G-V relationships were fitted with a Boltzmann function, $G = G_{max}/(1 + \exp((V_{0.5,act} - V_m)/k))$, where $V_{0.5,act}$ is the potential at which the conductance is half-maximally activated, and k is the slope factor. (B) Similar experimental procedures and data representation as shown in A, with 10 mM EGTA in the intracellular recording solution. Baclofen reversibly inhibited I_{Ba} by $41.8 \pm 4.7\%$ ($n = 6$). See Table S1 for $V_{0.5,act}$ (voltage for half-maximal current activation) and k (slope factor) values resulting from experiments shown in A and B.

with 1 $\mu\text{g}/\text{ml}$ PTX abolished c-Vc1.1 and baclofen inhibitory pathway(s) in $\text{Ca}_v2.3/\text{GABA}_B\text{R}$ cells, suggesting that the effects were mediated by G_i and/or G_o proteins.

We previously showed that Vc1.1 inhibition of N-type ($\text{Ca}_v2.2$) calcium channel currents can be blocked by a phosphorylated synthetic pp60c-Src peptide (Callaghan et al., 2008). This is probably a result of pp60c-Src binding to the SH2 domain of native c-Src protein in rat DRG neurons. Therefore, we examined in more detail the role of c-Src in the GABA_BR -mediated $\text{Ca}_v2.1$ or $\text{Ca}_v2.3$ channel inhibition by baclofen and c-Vc1.1 in the HEK expression system (Fig. 6). We changed endogenous HEK cell c-Src protein levels (Luttrell et al., 1999) by including cDNAs of wild-type or mutant c-Src in our expression system. In $\text{Ca}_v2.1/\text{GABA}_B\text{R}$ cells, wild-type c-Src protein overexpression or inclusion of the pp60c-Src peptide (50 μM) in the intracellular solution did not affect baclofen inhibition of I_{Ba} . However, in $\text{Ca}_v2.3/\text{GABA}_B\text{R}$ cells, wild-type c-Src protein overexpression dramatically increased the fraction of I_{Ba} inhibited by c-Vc1.1 compared with control.

To further evaluate the effect of c-Src on $\text{Ca}_v2.3/\text{GABA}_B\text{R}$ cells, we overexpressed the K295R/Y527F c-Src double mutant, which is kinase inactive and functions as a dominant-negative inhibitor of wild-type c-Src (Gao et al., 1997). This construct reduced the effect of baclofen compared with control and abolished c-Vc1.1's inhibitory effect. The effect of K295R/Y527F c-Src was recapitulated with the pp60c-Src peptide, suggesting that c-Src kinase activity is needed for VI inhibition of I_{Ba} by c-Vc1.1 and baclofen (Fig. 6, A and B).

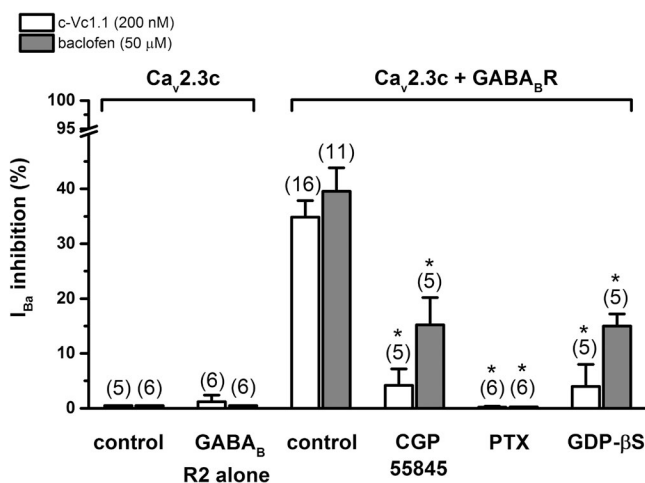


Figure 5. c-Vc1.1 and baclofen inhibit stably expressed $\text{Ca}_v2.3c$ calcium channels via G protein-coupled GABA_BR s. Neither compound inhibits I_{Ba} in the absence of GABA_BR s (see also Fig. 2 C) or the absence of GABA_BR R1 subunits. 1 μM CGP55845 or 500 μM GDP- β -S significantly reduces c-Vc1.1 and baclofen responses, respectively. A 24-h pretreatment with 1 $\mu\text{g}/\text{ml}$ PTX abolishes the effect of c-Vc1.1 or baclofen. Data are mean \pm SEM (one-way ANOVA; *, $P < 0.001$ vs. controls; $\text{Ca}_v2.3/\text{GABA}_B\text{R}$ cells with c-Vc1.1 or baclofen, respectively).

Tyrosines 1761 and 1765 are needed in the C terminus for c-Src phosphorylation of $\text{Ca}_v2.3$ channels

Alternative splicing of $\text{Ca}_v2.1$, $\text{Ca}_v2.2$, and $\text{Ca}_v2.3$ genes creates channels with distinct kinetic, pharmacological, and modulatory properties (Bourinet et al., 1999; Bell et al., 2004; Fang et al., 2007; Gray et al., 2007). It has been reported that GPCR-mediated inhibition of the nociceptor-specific $\text{Ca}_v2.2[\text{e}37\text{a}]$ channel occurred via VD and VI pathways. In HEK cells coexpressing GABA_BR s and $\text{Ca}_v2.2[\text{e}37\text{a}]$ channels, the baclofen-induced VI component required a tyrosine (Y) residue in e37a to be phosphorylated (Raingo et al., 2007).

Alignment of the $\text{Ca}_v2.2$ channel e37a and e37b regions with the corresponding e37 regions in human $\text{Ca}_v2.3c$, $\text{Ca}_v2.3d$, and $\text{Ca}_v2.1$ (α_{1A2} or α_{1A5}), and rabbit $\text{Ca}_v2.1$ channels, indicated a degree of structural conservation and the presence of tyrosine kinase consensus sites (Fig. 7 A). We hypothesized that Y residues within e37 at the proximal C terminus in $\text{Ca}_v2.3$ channels could serve as substrates for phosphorylation by c-Src. Using a publicly available catalog of phosphorylation motifs (Amanchy et al., 2007), we identified putative c-Src kinase phosphorylation sites in human $\text{Ca}_v2.3$ and rabbit $\text{Ca}_v2.1$, but not in human $\text{Ca}_v2.1$ channels. In both human $\text{Ca}_v2.3c$ and $\text{Ca}_v2.3d$ splice variants, the Y1761 and Y1675 (as numbered in GenBank accession no. L29385) are followed by a threonine (T) or glutamic acid (E), respectively, as are potential substrates for Src kinases. Remarkably, in rabbit $\text{Ca}_v2.1$, the second Y residue followed by alanine (A) also represents a Src motif described in the literature. In contrast, in the human $\text{Ca}_v2.1$ splice variants α_{1A2} or α_{1A5} , the consensus Y1851 and Y1855 residues (as numbered in RefSeq accession no. NM_001174080) lack the neighboring amino acids that are needed to generate known Src kinase substrates for phosphorylation (Amanchy et al., 2007).

We conducted a structure–function study in the e37 region to identify the amino acid residues responsible for the different sensitivity to c-Vc1.1. We also tested their contribution to c-Src-mediated inhibition in HEK cells transiently coexpressing GABA_BR s and transiently expressing $\text{Ca}_v2.3c$ or $\text{Ca}_v2.3d$ splice variants, or human or rabbit $\text{Ca}_v2.1$ channels. In patch-clamp experiments, 200 nM c-Vc1.1 inhibited human $\text{Ca}_v2.3c$ and $\text{Ca}_v2.3d$ channels but did not affect human $\text{Ca}_v2.1$ (α_{1A5}) (Fig. 7 B and Table 3). This confirmed previous results in HEK cells stably expressing $\text{Ca}_v2.1$ (α_{1A2}) or $\text{Ca}_v2.3c$ channels in the presence of GABA_BR s (Figs. 1 and 2). In all experiments, 50 μM baclofen inhibited I_{Ba} . As predicted, c-Vc1.1 also inhibited rabbit $\text{Ca}_v2.1$ channels, likely because of the presence of a putative c-Src phosphorylation site in the C terminus (Fig. 7 A).

Mutational analyses of the e37 region in the proximal C termini of $\text{Ca}_v2.3d$ or $\text{Ca}_v2.3c$ demonstrated that the Y1761F mutation completely abolished c-Vc1.1 inhibition of I_{Ba} , and the Y1765F mutation significantly reduced

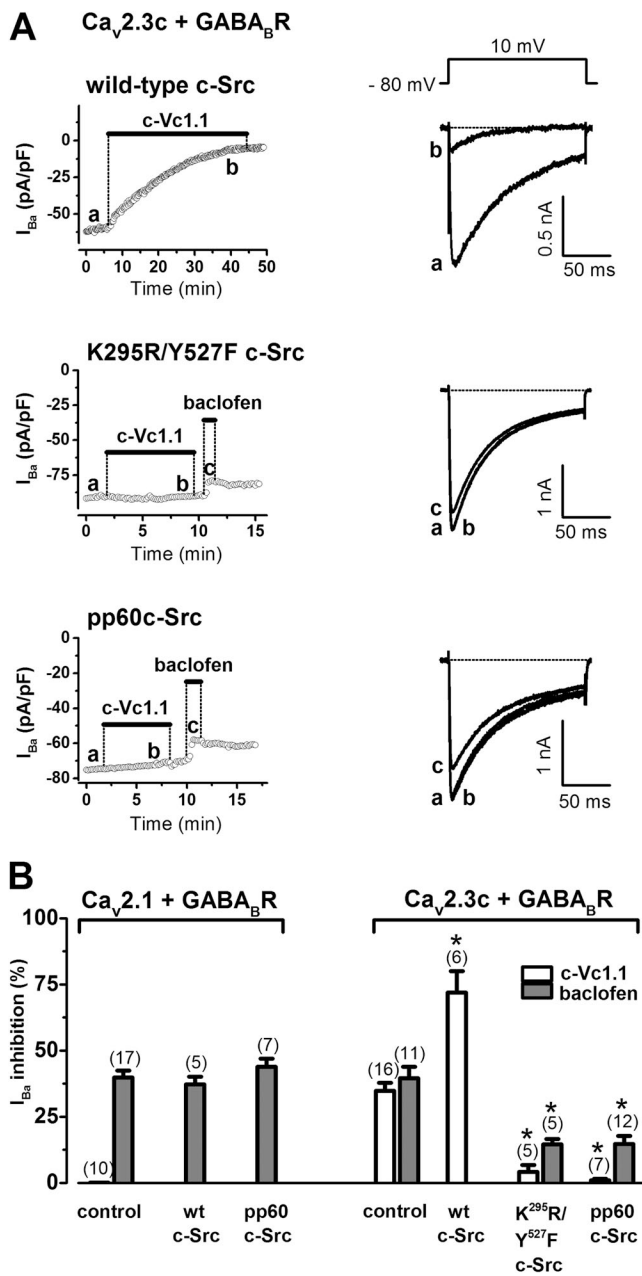


Figure 6. The role of c-Src proteins in GABA_B-mediated inhibition by c-Vc1.1 in HEK cells stably expressing human Ca_v2.3c channels and GABA_BRs. (A) Time course of I_{Ba} through Ca_v2.3c channels in the absence (control) and presence of 200 nM c-Vc1.1 or 50 μM baclofen. Bars indicate c-Vc1.1 or baclofen application. Overexpression of wild-type c-Src protein increases the I_{Ba} fraction inhibited by c-Vc1.1 (top). Overexpression of the double mutant c-Src (K295R/Y527F) (middle) or pretreatment with the phosphorylated pp60c-Src peptide (50 μM) (bottom) abolishes the effect of c-Vc1.1 and reduces baclofen inhibition of I_{Ba}, respectively. Representative current traces (right) are shown at the times indicated by lowercase letters (see Fig. 1 B for control). I_{Ba} was evoked by 150 ms, 0.1-Hz depolarizations to 10 mV, from an HP of -80 mV (voltage inset). Peak current amplitudes were plotted as a function of time. Horizontal dotted lines indicate zero-current levels. (B) Average data (±SEM) of I_{Ba} inhibition by c-Vc1.1 or baclofen after pp60c-Src peptide (50 μM) pretreatment or wild-type c-Src coexpression in Ca_v2.1/GABA_BR or Ca_v2.3/GABA_BR

the c-Vc1.1 inhibition (Fig. 7 B and Table 3). These results suggest that tyrosines 1761 and 1765 are critical for mediating the effects of Vc1.1. Interestingly, these mutants, except the Ca_v2.3d (Y1765F), also reduced baclofen inhibition of I_{Ba}, which indicates that these Y residues are also involved in baclofen signaling. We explored how mutation affects the VI component of inhibition, in the absence and presence of a depolarizing prepulse, via baclofen inhibition of I_{Ba} through Ca_v2.3d (Y1765F) channels. Experiments were performed with either 0.5 or 10 mM EGTA in the intracellular recording solution to (a) identify any effects of intracellular divalent cations on I_{Ba} facilitation (Zühlke et al., 1999), and (b) rule out modulation by phospholipids (Delmas et al., 2005) (Fig. S1). The results showed that the effect of baclofen was solely mediated by a VI pathway, independent of a classical Gβγ binding.

We also generated human Ca_v2.1 (α_{1A5}) (L1852T) and Ca_v2.1 (α_{1A5}) (Q1856E) channel mutants. Remarkably, the introduced putative c-Src phosphorylation sites conveyed sensitivity to c-Vc1.1 in these channels. Baclofen modulation was not affected by the Ca_v2.1 (L1852T) or Ca_v2.1 (Q1856E) channel mutants (Fig. 7 B and Table 3). Collectively, these data suggest that specific c-Src phosphorylation sites in the C terminus are needed for α-conotoxin c-Vc1.1 inhibition of Ca_v2.3 and Ca_v2.1 channels. However, it remains possible that other residues are also involved in mediating baclofen's inhibition of Ca_v2.3 channels.

DISCUSSION

In this study, we efficiently reconstituted human Ca_v2.1 and Ca_v2.3 channel modulation via human G protein-coupled GABA_BRs. Baclofen, a GABA_BR agonist, inhibited I_{Ba} through both channels; however, α-conotoxin Vc1.1 only inhibited Ca_v2.3 channels. The effect of Vc1.1 on Ca_v2.3 channels was completely VI and depended on the presence of specific c-Src phosphorylation sites in the C terminus of the human α_{1E} (Ca_v2.3). These results define Ca_v2.3 channels as new targets for analgesic α-conotoxins.

Ca_v2 channels and chronic pain

It is well established that Ca_v2.2 channel inhibition by antagonists or via GPCRs produces analgesia in animals and humans (Altier and Zamponi, 2004). GABA_B-mediated inhibition of Ca_v2.1 or Ca_v2.2 channels in various neurons is well documented (Cox and Dunlap, 1992; Mintz and Bean, 1993; Lambert and Wilson, 1996) and has been shown to involve VD and VI second

cells, respectively, or double mutant c-Src (K295R/Y527F) coexpression in Ca_v2.3/GABA_BR cells (*, P < 0.001 vs. controls; one-way ANOVA). The number of experiments is in parentheses.

messenger pathways (Dolphin and Scott, 1986; Diversé-Pierluissi et al., 1997). We showed that a subset of α -conotoxins, including Vc1.1, also selectively inhibit $Ca_v2.2$ channels by acting as G protein-coupled $GABA_B$ R agonists (Callaghan et al., 2008; Callaghan and Adams, 2010; Clark et al., 2010; Daly et al., 2011). This mechanism may help relieve nerve injury-induced neuropathic pain (Klimis et al., 2011).

Studies involving pharmacological and genetic approaches have also established $Ca_v2.3$ channels as potential targets for drugs that treat chronic pain (Saegusa et al., 2000; Qian et al., 2013). The anti-nociceptive role of $Ca_v2.3$ channels was demonstrated in rat dorsal horn

neurons (Matthews et al., 2007), and their inhibition was associated with high efficiency opioid therapy without tolerance (Yokoyama et al., 2004). $Ca_v2.3$ channels are ubiquitously expressed in the central and peripheral nervous systems, but their physiological roles and modulation is not well understood. They typically conduct a small proportion of whole-cell Ca^{2+} current and are difficult to isolate in neurons (Schneider et al., 2013).

Baclofen and α -conotoxin Vc1.1 differentially inhibit $Ca_v2.1$ and $Ca_v2.3$ channels

Our results demonstrate that baclofen or GABA inhibits I_{Ba} to a similar extent in $Ca_v2.1/GABA_B$ R and $Ca_v2.3/$

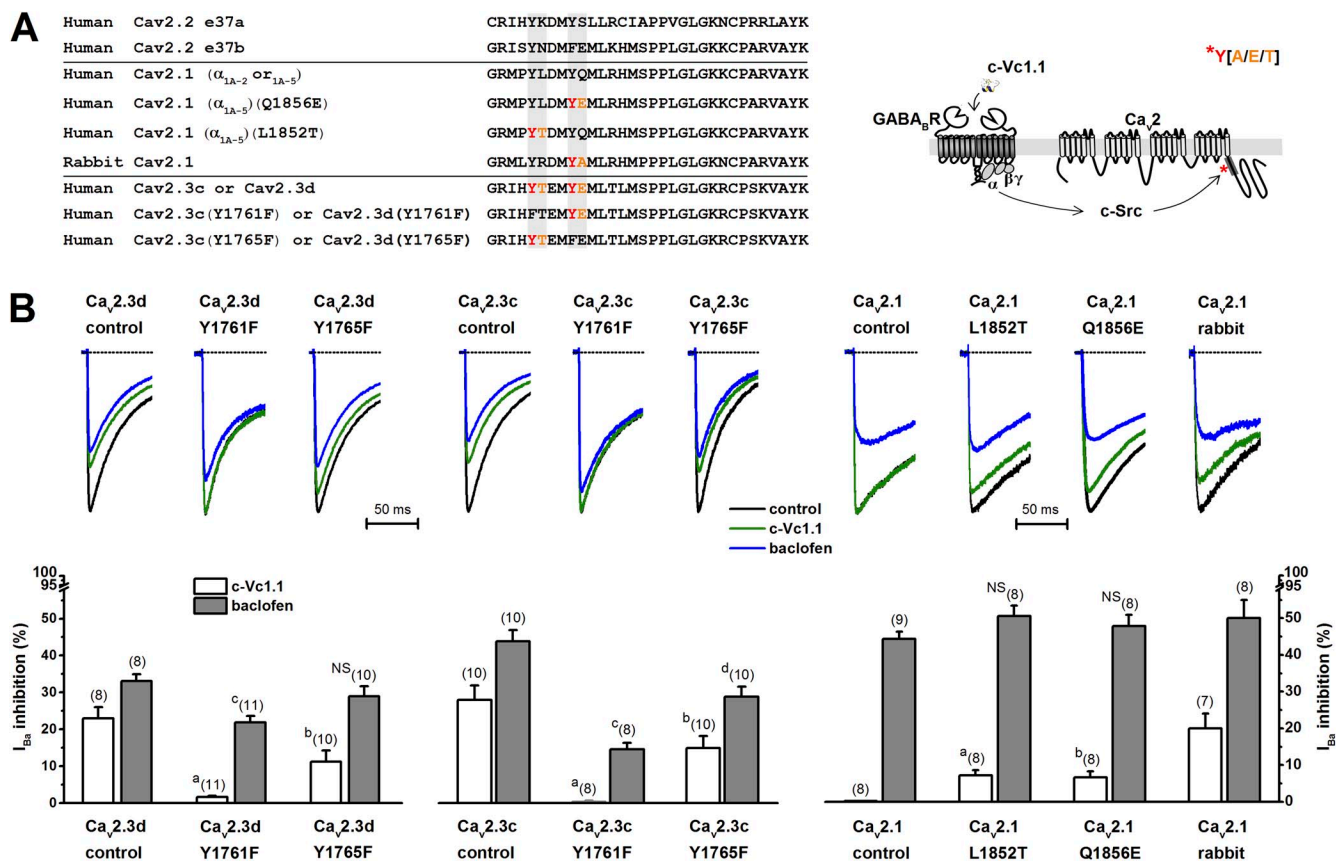


Figure 7. Kinase-dependent phosphorylation of the intracellular proximal C terminus is needed for VI $Ca_v2.3$ channel inhibition by c-Vc1.1. (A) Amino acid sequence alignments of e37 regions in splice variants of $Ca_v2.2$, $Ca_v2.1$, and $Ca_v2.3$ and mutant $Ca_v2.1$ and $Ca_v2.3$ genes. Y (red), followed by A, E, or T (orange), is shown as a possible substrate for c-Src phosphorylation. (Right) Proposed model for c-Vc1.1-induced intracellular signaling leading to Ca_v2 channel inhibition. Asterisk indicates phosphorylation. (B) Average I_{Ba} inhibition in the presence of 200 nM c-Vc1.1 or 50 μ M baclofen in HEK 293T cells transiently cotransfected with wild-type human $Ca_v2.3d$ (α_{1E-d}) and $Ca_v2.3c$ (α_{1E-c}); $Ca_v2.1$ (α_{1A-5}); rabbit $Ca_v2.1$ channel subunits; human e37 mutants $Ca_v2.3d$ (Y1761F), $Ca_v2.3d$ (Y1765F), $Ca_v2.3c$ (Y1761F), $Ca_v2.3c$ (Y1765F), $Ca_v2.1$ (α_{1A-5}) (L1852T), or $Ca_v2.1$ (α_{1A-5}) (Q1852E); auxiliary human Ca_v channel subunits $\alpha_{2\beta}$, δ -1 and β_3 ; and human $GABA_B$ R subunits. Representative normalized I_{Ba} traces (current insets) of wild-type or mutant channels are shown in the absence (control) or presence of 200 nM c-Vc1.1 or 50 μ M baclofen (only 65 ms of the 150-ms current traces are plotted). I_{Ba} was evoked by depolarizations to +10 mV ($Ca_v2.3c$) or 15 mV ($Ca_v2.1$), applied at 0.1 Hz from an HP of -80 mV. Dotted lines indicate zero-current levels. Bar graphs show mean \pm SEM; n, number of experiments in parentheses. Labels a, b, and c denote statistically significant differences between various mutants and control; NS, not significantly different from wild-type modulation. See Table 3 for statistical evaluation of the data.

GABA_BR cells (Figs. 1 and 2). Throughout this study, we used cells with similar electrophysiological characteristics and applied supra-maximal doses of baclofen (50 μM) or c-Vc1.1 (200 nM) to make sure receptors were fully activated and rule out the possibility that differences were caused by cell variability.

In Ca_v2.1 channel-expressing cells, baclofen inhibition was VD and could be relieved by a strong depolarizing prepulse, reflecting transient dissociation of G protein βγ subunits from the channel. The 20-ms prepulses and 5-ms interpulses were considered suitable for VD I_{Ba} relief in Ca_v2.1 channels (Currie and Fox, 1997). Our results on the voltage dependence of inhibition are consistent with previous studies demonstrating baclofen inhibition of Ca_v2.1 channels in adrenal chromaffin cells and cerebellar Purkinje neurons (Mintz and Bean, 1993; Currie and Fox, 1997). We did not analyze the time course of I_{Ba} activation in the presence of baclofen or c-Vc1.1 in Ca_v2.1/GABA_BR or Ca_v2.3/GABA_BR cells. However, in Ca_v2.1/GABA_BR cells, the time course of I_{Ba} activation considerably slowed in the presence of baclofen when compared with control. This is a hallmark of VD Gβγ binding to the α_{1A} (Ca_v2.1) subunit (Fig. 3 B). Interestingly, baclofen only caused a small and statistically insignificant shift of the I-V and G-V relationships in Ca_v2.1/GABA_BR cells (Figs. 3 A and 4), which was independent of the intracellular EGTA concentration. Bourinet et al. (1996) also reported a similar slight positive shift of the μ opioid receptor-activated Ca_v2.1 channel I-V relationship, suggesting possible differences between Ca_v2.1 and Ca_v2.2 channel-modulating membrane-delimited pathways. In Ca_v2.3/GABA_BR cells, the

time course of I_{Ba} activation in the presence of baclofen or c-Vc1.1 seemed unaffected. In these cells, c-Vc1.1 did not affect the I-V relationship, but baclofen caused a hyperpolarizing I-V shift, which suggests that there may be an additional signaling mechanism. Our results show that neither baclofen nor Vc1.1 elicits VD inhibition of Ca_v2.3 channels via GABA_BRs. However, it has been shown that the rat brain α1E_{long} splice variant could be inhibited in a VD manner via D2 dopamine receptors (Page et al., 1998). Our alignment of the human α_{1E-c} or α_{1E-d} and rat α1E_{long} splice variants (not depicted) indicate that the N-terminal sequence responsible for VD inhibition of rat α1E_{long} variant is present in human α_{1E-c} and α_{1E-d}. Therefore, future experiments should determine whether or not human Ca_v2.3 channels can be inhibited via D2 receptors in a VD manner. VD modulation may depend on the type of GPCR and specific signal transduction mechanism elicited by the GPCR-specific ligand.

The VI pathway leading to Ca_v2.3 channel inhibition by baclofen or c-Vc1.1 could be disrupted by GDP-β-S, a GDP analogue that keeps Gα permanently associated with Gβγ. In all cases, PTX treatment abolished baclofen and c-Vc1.1 inhibition of I_{Ba}, indicating that GABA_BRs couple with G proteins of the G_i/G_o superfamily in cells expressing Ca_v2.3 channels (Fig. 5). Analysis of the VI pathway in Ca_v2.3/GABA_BR cells indicated that signaling mechanisms that contribute to Ca_v2.3 channel inhibition, downstream of G protein subunits, involve c-Src kinase activation. For example, wild-type c-Src overexpression increased I_{Ba} inhibition, whereas the dominant-negative double mutant c-Src or the pp60c-Src

TABLE 3

Summary of I_{Ba} inhibition by baclofen and c-Vc1.1 in HEK 293T cells transiently coexpressing GABA_BRs and Ca_v2.1 or Ca_v2.3 channels

Ca _v 2 channel	I _{Ba} inhibition (%)	
	c-Vc1.1 (200 nM)	Baclofen (50 μM)
Human Ca _v 2.3d	23 ± 2.9 (8)	33.1 ± 1.8 (8)
Human Ca _v 2.3d (Y1761F)	1.7 ± 0.3 (11) ^a	21.9 ± 1.8 (11) ^c
Human Ca _v 2.3d (Y1765F)	11.3 ± 3.0 (10) ^b	^{NS} 28.9 ± 2.7 (10)
Human Ca _v 2.3c	28.0 ± 4.0 (10)	44.0 ± 3.0 (10)
Human Ca _v 2.3c (Y1761F)	0.37 ± 0.3 (8) ^a	14.6 ± 1.8 (8) ^c
Human Ca _v 2.3c (Y1765F)	15.0 ± 3.2 (10) ^b	28.9 ± 2.7 (10) ^d
Human Ca _v 2.1	0 (8)	44.4 ± 1.9 (9)
Human Ca _v 2.1 (L1852T)	7.2 ± 1.4 (8) ^a	^{NS} 50.6 ± 2.8 (8)
Human Ca _v 2.1 (Q1856E)	6.6 ± 1.7 (8) ^b	^{NS} 47.9 ± 3.0 (8)
Rabbit Ca _v 2.1	[#] 20.0 ± 4.0 (7)	[#] 50.0 ± 5.0 (8)

Values represent mean ± SEM; n, number of experiments in parentheses. NS, not significantly different from wild-type modulation. One-way ANOVA with Bonferroni post-hoc testing was used to test for statistically significant differences except when comparing the effect of c-Vc1.1 on wild-type or mutant Ca_v2.1 (one-way ANOVA on ranks with Tukey test). Data marked with a hash symbol were not included in the statistical analysis. Note that the percentage of inhibition with transiently expressed human Ca_v2.1 or Ca_v2.3c channels and transiently coexpressed GABA_BRs (above) is similar (within the statistical margin of error) to that obtained with stably expressed human Ca_v2.1 or Ca_v2.3c channels in the presence of transiently coexpressed GABA_BRs (see Table 1).

^aP < 0.001 versus Ca_v2.3d with c-Vc1.1; P < 0.001 versus Ca_v2.3c with c-Vc1.1; and P = 0.002 versus human Ca_v2.1 with c-Vc1.1.

^bP = 0.003 versus Ca_v2.3d with c-Vc1.1; P = 0.008 versus Ca_v2.3c with c-Vc1.1; and P = 0.002 versus human Ca_v2.1 with c-Vc1.1.

^cP = 0.003 versus Ca_v2.3d with baclofen and P < 0.001 versus Ca_v2.3c with baclofen.

^dP < 0.001 versus Ca_v2.3c with baclofen.

peptide abolished c-Vc1.1 inhibition of I_{Ba} (Fig. 6). This suggested that $Ca_v2.3$ channels are a potential c-Src substrate. It has been demonstrated that certain protein tyrosine kinases can be direct effectors of G proteins (Bence et al., 1997), and GABA inhibition of $Ca_v2.2$ channels involves direct $G\alpha_o$ activation of Src kinase (Diversé-Pierluissi et al., 1997). However, further studies are needed to elucidate whether baclofen or Vc1.1 inhibition of $Ca_v2.3$ channels involves direct G_i/G_o activation of c-Src.

In $Ca_v2.1$ and $Ca_v2.2$ channels, intracellular N and C termini and cytoplasmic loops connecting domains I–IV have been shown to interact with other proteins and are targeted by second messenger pathways, including phosphorylation by kinases (Zamponi and Currie, 2013). Many of these interaction or modulatory sites can also be identified in $Ca_v2.3$ channels (Schneider et al., 2013). Furthermore, alternative splicing, recognized as a mechanism for creating functional diversity in VGCCs (Gray et al., 2007), results in a series of $Ca_v2.3$ splice variants (Williams et al., 1994) with similar biophysical properties (Pereverzev et al., 2002). Of these, $Ca_v2.3c$ represents the major neuronal type variant, which is dominantly expressed in the adult central nervous system (Schneider et al., 2013), whereas $Ca_v2.3d$, the variant cloned from human fetal brain (Schneider et al., 1994), shows minor *in vivo* expression in the adult brain (Pereverzev et al., 2002). Interestingly, the endocrine splice variant $Ca_v2.3e$ was also identified in nociceptive trigeminal ganglion and DRG neurons together with $Ca_v2.3a$ (Fang et al., 2007, 2010). Importantly, the e37 region containing the putative c-Src phosphorylation sites can be identified in all $Ca_v2.3$ splice variants.

There is evidence of multiple Src interaction sites in various VGCCs. For example, Src interacts with both the II–III linker and C-terminal tail regions of the L-type Ca^{2+} channel α_1c subunit (Dubuis et al., 2006). c-Src kinases also appear to be pre-associated with N-type VGCCs, efficiently modulating their function (Schiff et al., 2000). In addition, c-Src kinases have been implicated in the $GABA_B$ R-mediated inhibition of $Ca_v2.2$ channels by baclofen (Raingo et al., 2007) and Vc1.1 (Callaghan et al., 2008).

To date, $GABA_B$ R-mediated modulation of $Ca_v2.3$ channels has not been reconstituted in expression systems, and c-Src phosphorylation of $Ca_v2.3$ channels has not been demonstrated. Conserved Y residues within the e37 region can be identified across all Ca_v2 family members and appear to be key substrates for phosphorylation by various tyrosine kinases. Our analysis of the e37 regions in $Ca_v2.1$ and $Ca_v2.3$ channels predicted Src kinase motifs in human $Ca_v2.3$ channels. These motifs were absent in human $Ca_v2.1$ (Fig. 7). Although phosphorylation site prediction can be error prone, it is a useful tool to determine whether e37 regions contain sequence contexts typical of c-Src motifs described previously in the literature (Amanchy et al., 2007). It is

generally accepted that the amino acid sequence motif around the tyrosine residue and three-dimensional structure of the substrate proteins contribute to phosphorylation site specificity (Pawson et al., 2001). Our structure–function studies confirmed that Y[A/E/T] sequences are the likely c-Src phosphorylation substrates and represent key switches for the molecular mechanisms involved. The lack of any effect of Vc1.1 on human $Ca_v2.1$ channels agrees with the absence of a c-Src motif in the e37 region and marginal VI component in cells expressing these channels. Interestingly, c-Src motif Y[K] can also be identified in the $Ca_v2.2$ [e37a] channel, whereas such a motif is absent in $Ca_v2.2$ [e37b]. Therefore, further studies are required to dissect the effects of Vc1.1 on $Ca_v2.2$ channel splice variants. Our results also suggest that the effects of baclofen on $Ca_v2.3$ channels are, at least partially, controlled by c-Src phosphorylation. Given that PTX completely abolishes the inhibition by baclofen, an additional PTX-sensitive pathway is probably also involved. Future studies should be aimed at directly correlating $Ca_v2.3$ channel phosphorylation and dephosphorylation with inhibition and (the absence of) recovery, respectively. Clearly, further experiments are also needed to confirm that c-Src kinase directly phosphorylates $Ca_v2.3$ channels *in vivo*.

Therapeutic implications of $Ca_v2.3$ channel inhibition

Few studies have examined $Ca_v2.3$ (R-type) channel modulation in neurons, where a combination of specific inhibitors is needed to completely block various VGCCs while preserving the R-type calcium channel. In thalamocortical neurons, R-type current modulation by baclofen has been demonstrated and could be antagonized by CGP55845 (Guyon and Leresche, 1995). In rat DRG neurons, we also observed R-type current inhibition by baclofen in the presence of specific L-, N- and P/Q-type channel blockers (not depicted). However, further studies are needed to demonstrate what contribution the R-type current component makes to the whole-cell calcium conductance inhibited by Vc1.1 in nociceptive neurons.

$GABA_B$ R activation produces anti-nociceptive effects in animal models of acute or chronic pain (Pan et al., 2008; Bowery, 2010). Baclofen is mainly injected into the spine to manage spasticity and neuropathic pain and as an adjuvant analgesic for relieving cancer pain (Zuniga et al., 2000; Yomiya et al., 2009). Its oral dose must be carefully regulated because of possible side effects. Vc1.1 does not compete with baclofen for binding to receptors, but it targets the interface between the $GABA_B$ R ectodomains (see Adams and Berecki, 2013). Vc1.1 was tested in human clinical trials, but its development was discontinued because of its lack of potency at human $\alpha_9\alpha_{10}$ nicotinic acetylcholine receptor, which was proposed to be the molecular target (McIntosh et al., 2009). However, with the emergence

of new α -conotoxin-based pharmacological tools that act on neuronal VGCCs via the GABA_BR, its development is likely to resume. It remains to be established if analgesic α -conotoxins can be used as specific Ca_v2.2[e37a] and Ca_v2.3 channel inhibitors for the treatment of chronic pain.

In conclusion, we identified a previously unrecognized mechanism of α -conotoxin Vc1.1 and baclofen inhibition of Ca_v2.3 channels that involves GABA_BRs. We systematically examined the intracellular pathways and elucidated the molecular details that determine c-Src phosphorylation of the Ca_v2.1 and Ca_v2.3 channel C termini. Although the physiological significance of kinase-mediated Ca_v2.3 channel inhibition is unclear, it may have long-term influence over Ca²⁺-dependent intracellular signaling, exocytosis, and/or gene transcription in neurons.

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