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Differential Cav2.1 and Cav2.3 channel inhibition by baclofen and α-conotoxin Vc1.1 via GABAB receptor activation

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Abstract
Neuronal Cav2.1 (P/Q-type), Cav2.2 (N-type), and Cav2.3 (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 (GABAB) receptors (GABABRs) to inhibit Cav2.2 channels. We investigated GABABR-mediated modulation by Vc1.1, a cyclized form of Vc1.1 (c-Vc1.1), and the GABABR agonist baclofen of human Cav2.1 or Cav2.3 channels heterologously expressed in human embryonic kidney cells. 50 µM baclofen inhibited Cav2.1 and Cav2.3 channel Ba2+ currents by ∼40%, whereas c-Vc1.1 did not affect Cav2.1 but potently inhibited Cav2.3, with a half-maximal inhibitory concentration of ∼300 pM. Depolarizing paired pulses revealed that ∼75% of the baclofen inhibition of Cav2.1 was voltage dependent and could be relieved by strong depolarization. In contrast, baclofen or Vc1.1 inhibition of Cav2.3 channels was solely mediated through voltage-independent pathways that could be disrupted by pertussis toxin, guanosine 5′-[β-thio]diphosphate trilithium salt, or the GABABR antagonist CGP55845. Overexpression of the kinase c-Src significantly increased inhibition of Cav2.3 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 Cav2.3 demonstrated that tyrosines 1761 and 1765 within exon 37 are critical for inhibition of Cav2.3 by c-Vc1.1 and are involved in baclofen inhibition of these channels. Remarkably, point mutations introducing specific c-Src phosphorylation sites into human Cav2.1 channels conferred c-Vc1.1 sensitivity. Our findings show that Vc1.1 inhibition of Cav2.3, which defines Cav2.3 channels as potential targets for analgesic α-conotoxins, is caused by specific c-Src phosphorylation sites in the C terminus.

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Differential Ca\textsubscript{V}2.1 and Ca\textsubscript{V}2.3 channel inhibition by baclofen and α-conotoxin Vc1.1 via GABA\textsubscript{B} receptor activation

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Neuronal Ca\textsubscript{V}2.1 (P/Q-type), Ca\textsubscript{V}2.2 (N-type), and Ca\textsubscript{V}2.3 (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\textsubscript{B}) receptors (GABA\textsubscript{B}Rs) to inhibit Ca\textsubscript{V}2.2 channels. We investigated GABA\textsubscript{B}R-mediated modulation by Vc1.1, a cyclized form of Vc1.1 (c-Vc1.1), and the GABA\textsubscript{B}R agonist baclofen of human Ca\textsubscript{V}2.1 or Ca\textsubscript{V}2.3 channels heterologously expressed in human embryonic kidney cells. 50 µM baclofen inhibited Ca\textsubscript{V}2.1 and Ca\textsubscript{V}2.3 channel Ba\textsuperscript{2+} currents by \sim40%, whereas c-Vc1.1 did not affect Ca\textsubscript{V}2.1 but potently inhibited Ca\textsubscript{V}2.3, with a half-maximal inhibitory concentration of \sim300 µM. Depolarizing paired pulses revealed that \sim75% of the baclofen inhibition of Ca\textsubscript{V}2.1 was voltage dependent and could be relieved by strong depolarization. In contrast, baclofen or Vc1.1 inhibition of Ca\textsubscript{V}2.3 channels was solely mediated through voltage-independent pathways that could be disrupted by pertussis toxin, guanosine 5‘-[β-thio]triphosphate trilithium salt, or the GABA\textsubscript{B}R antagonist CGP55845. Overexpression of the kinase c-Src significantly increased inhibition of Ca\textsubscript{V}2.3 by c-Vc1.1. Conversely, coexpression of a catalytically inactive double mutant form of c-Src or pretreatment with a phosphorylation inhibitor pp60c-Src peptide abolished the effect of c-Vc1.1. Site-directed mutational analyses of Ca\textsubscript{V}2.3 demonstrated that tyrosines 1761 and 1765 within exon 37 are critical for inhibition of Ca\textsubscript{V}2.3 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\textsubscript{V}2.1 channels conferred c-Vc1.1 sensitivity. Our findings show that Vc1.1 inhibition of Ca\textsubscript{V}2.3, which defines Ca\textsubscript{V}2.3 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\textsubscript{V}2.1 (P/Q-type), Ca\textsubscript{V}2.2 (N-type), and Ca\textsubscript{V}2.3 (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\textsubscript{V}2.3 channels are less susceptible to direct G protein βγ dimer modulation than Ca\textsubscript{V}2.1 or Ca\textsubscript{V}2.2 (Sheitker et al., 1997), a finding attributed to differences between the N terminus, domain I, and the I–II intracellular linker of Ca\textsubscript{V}2.3 and Ca\textsubscript{V}2.2 channels (Stephens et al., 1998; Simen and Miller, 2000). Nevertheless, carbachol, somatostatin, ATP, and adenosine inhibit exogenous Ca\textsubscript{V}2.3 channels via endogenous receptors in human embryonic kidney (HEK) cells (Mehrke et al., 1997). Interestingly, carbachol, a muscarinic receptor agonist, stimulates or inhibits Ca\textsubscript{V}2.3 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\textsubscript{V}2.3 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 (Cheng and Bekkers, 1999).

VGCCs are associated with a wide range of pathologies, including pain, and the value of selectively targeting Ca\textsubscript{V}2 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, inhibites Ca\textsubscript{V}2.2 channels via GABA\textsubscript{B}R type B (GABA\textsubscript{B}R) receptors (GABA\textsubscript{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
that Vc1.1 can be used as an analgesic in rat models of neuropathic pain (Klinis et al., 2011). Cav2.3 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, Cav2.3 modulation via GABA_B receptors is incompletely characterized and has not been reconstituted in any heterologous expression system. Moreover, few drugs or toxins have specific Cav2.3 inhibitory effects (Schneider et al., 2013).

In this study, we hypothesized that α-conotoxin Vc1.1 can modulate Ca_{2.1} and Ca_{2.3} channels via GABA_B receptors. We designed experiments to examine the mechanisms of VGCC Ba^{2+} 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_{2.3} channels, despite baclofen efficiently inhibiting both Ca_{2.1} and Ca_{2.3} 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 subunit C terminus is sufficient to mediate Vc1.1 inhibition of Ca_{2.3} 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-05-03).

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% CO_{2} in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen), 50 IU/ml penicillin, and 50 µg/ml streptomycin (Invitrogen). HEK-293 cells, stably expressing human Ca_{2.1} (P/Q-type) channel α_{1A,2} splice variant (GenBank accession no. AF004883) or human Ca_{2.3,2c} (R-type) channel α_{1E,2} splice variant (also called α_{1E,1}; GenBank accession no. L29385), were obtained from Merck and cultured according to procedures described previously (Dai et al., 2008). Both cell lines express human α_{2δ,1} (GenBank accession no. M76559) and human β_{3a} (ReSeq accession no. NM_000725) auxiliary subunits, and the human KCNJ4 (Kir2.3; GenBank accession no. U07364) channel (Dai et al., 2008).

The human Ca_{2.1} (P/Q-type) channel, α_{1A,2} transcript variant 5 (ReSeq accession no. NM_001174080), cloned into pCMV plasmid, was provided by J. Striessnig (University of Innsbruck, Innsbruck, Austria). The human Ca_{2.3,2d} (R-type) channel (fetal brain α_{1E,2} splice variant L27745) was provided by T. Schneider (University of Cologne, Cologne, Germany). The wild-type human Ca_{2.3,2c} (α_{1E,1}) channel (GenBank accession no. L29385) and mutant Ca_{2.3,2c} channels, α_{1E,1} (Y1761F) and α_{1E,1} (Y1765F), all cloned into pCDNA3.1 vectors, were purchased from GenScript USA Inc. The α_{1E,2} splice variant is identical in amino acid sequence to α_{1E,1}, except for a 43-amino acid segment (insert III or exon 46) at the C terminus of the α_{1E,1} channel (Pereruev et al., 2002). Human α_{2δ,1} and human β_{3} channels, splice variant 1 (ReSeq accession no. NM_000725), were purchased from OriGene Technologies, Inc. Site-directed mutagenesis of the wild-type human Ca_{2.1} (α_{1A,2}) channel, resulting in α_{1E,1} (Y1761F) or α_{1E,1} (Y1765F), and site-directed mutagenesis of the wild-type human Ca_{2.1} (α_{1A,2}) channel, resulting in α_{1E,1} (L1852T) or α_{1E,1} (Q1852E), was performed with the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies), using the following oligonucleotides: Ca_{2.1}(Y1761F)-for, GCATGTGCCCAGTTTTTCTCCGATGATGAAATG; Ca_{2.1}(Y1765F)-rev, GACTTCTACATCTCAGTAAATGTAGATGGCAGATCCATGC; Ca_{2.3}(Y1765F)-for, CATTACACTACATCCTGAAATGATGCGGCC; Cav2.3(Y1765F)-rev, GACAGACTGAGATTTGAAATGTCGATCATTCTC; Ca_{2.3}(Y1765F)-rev, CATCAGTCATTGAAGTGGATGCGGCC; Cav2.3(Y1856E)-for, CTTACGCTGACATGATGCGATCTGAGACATCAG; Ca_{2.1}(Q1856E)-rev, GCAGACTGTCATTGAAGTGGATGCGGCC; Cav2.3(Y1856E)-rev, GACAGACTGTCATTGAAGTGGATGCGGCC.

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 in a phenylalanine (Ca_{2.1,2}, leucine to threonine (Ca_{2.1,2}), or glutamine to glutamic acid (Ca_{2.1,2}). All mutations were verified by automated DNA sequencing (Australian Genome Research Facility).

HEK cells stably expressing Ca_{2.1} or Ca_{2.3}c channels were transiently cotransfected with plasmid cDNAs encoding human GABA_{B}R1 (ReSeq accession no. NM_001470; 3 µg; OriGene Technologies, Inc.), human GABA_{B}R2 (ReSeq accession no. NM_005458; 3 µg; OriGene Technologies, Inc.), and enhanced green fluorescent protein (eGFP) reporter gene construct (1 µg; Counting Cells, San Diego, CA) with the kinase, whereas the Y527F mutation abolishes intramolecular interactions between the C-terminal tail and the SH2 domain (Gao et al., 2008).

HEK293T cells were transiently cotransfected with plasmid cDNAs encoding human Ca_{2.1} channel transcript variant 5 (5 µg) or wild-type or mutant human Ca_{2.3}d channels (5 µg), human α_{5δ,1} (1 µg) and human β_{3} (5 µg) auxiliary subunits, human GABA_{B}R1 (3 µg), human GABA_{B}R2 (3 µg), and eGFP (1 µg). In a separate series of experiments, HEK293T cells were transiently coexpressed with plasmid cDNAs encoding rabbit Ca_{2.1} channel (ReSeq accession no. NM_001101693; 5 µg; provided by F. Meunier, 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).

Electrophysiology

Experiments were performed 3–5 d after transfection, using the whole-cell patch-clamp technique. Currents through calcium channels were recorded using barium (Ba^{2+}) as the charge carrier. Cells expressing the proteins of interest were superfused with a solution containing (mM): 110 NaCl, 10 BaCl_{2}, 1 MgCl_{2}, 5 CaCl_{2}, 30 TEA-Cl, 10 p-glucose, and 10 HEPES, pH 7.4 with TEA-OH, at ~600 µl/min. Fire-polished borosilicate patch pipettes with tip resistance values of 2–3 MΩ were filled with an intracellular solution containing (mM): 125 Kglucurate, 2 MgCl_{2}, 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

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concentration of 0.5 or 10 mM. GTP was not used in the intracellular solution to prevent I\(_{\text{Ba}}\) 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 (25–25°C) using Multiclamp 700B amplifiers (Molecular Devices) controlled by Clampfit 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\(_{\text{Ba}}\) was measured for each step and normalized to the cell’s maximal current. Normalized currents were averaged across cells and plotted (mean ± SEM) as a function of voltage. Test depolarizations to 10 mV (in cells coexpressing Ca\(_{\text{V}}\)\(_{1.2}\) channels and GABA\(_{\text{AR}}\)s) or 15 mV (in cells coexpressing Ca\(_{\text{V}}\)\(_{1.2}\) and GABA\(_{\text{BR}}\)s) of 150-ms duration were applied at a frequency of 0.1 Hz from an HP of −80 mV, where I\(_{\text{Ba}}\) was evaluated in the absence and presence of various compounds. I–V 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\(_{\text{Ba}}\) inhibited in the absence of a prepulse (−PP I\(_{\text{Ba}}\)) or presence of a +80-mV prepulse (+PP I\(_{\text{Ba}}\)) was determined according to \([I_{\text{0,-PP}} - I_{\text{0,+PP}}]/I_{\text{0,+PP}}\) × 100, or \([I_{\text{0,-PP}} - I_{\text{0,+PP}}]/I_{\text{0,-PP}}\) × 100, respectively, where I\(_{\text{0,-PP}}\) and I\(_{\text{0,+PP}}\) represent current amplitudes (controls) obtained with or without a prepulse in the absence of a compound, respectively. I\(_{\text{0,-PP}}\) was normalized to I\(_{\text{0,+PP}}\) (lIPP and l\(\text{PP}\)) represent current amplitudes obtained with or without a prepulse in the presence of a compound, respectively. The VI fraction was defined as \((-\text{PP} I_{\text{Ba}})/I_{\text{Ba}}\) whereas the VI fraction was calculated as \((-\text{PP} I_{\text{Ba}}) - VI\).

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\(_{\text{Ba}}\) was routinely tested in HEK cells stably expressing Ca\(_{\text{V}}\)\(_{1.2}\) or Ca\(_{\text{V}}\)\(_{1.3}\) channels and coexpressing GABA\(_{\text{AR}}\)s. In ~5% of all cells tested, I\(_{\text{Ba}}\) inhibition by baclofen was ≤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\(_{\text{Ba}}\) 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-Conotoxin 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 1-III, II-IV arrangement (Clark et al., 2006, 2010; Daly et al., 2011). c-Vc1.1 exhibits better properties than the linear bond in a I–III, II–IV arrangement (Clark et al., 2006, 2010; Daly et al., 2011). Synthetic Vc1.1 and PeIA are 16–amino acid residue bonds in a I–III, II–IV arrangement (Clark et al., 2006, 2010; Daly et al., 2011). Synthetic Vc1.1, PeIA, and the C-terminal regions corresponding to exon 37 (e37) of Ca\(_{\text{V}}\)\(_{2.2}\) 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\(_{\text{Ba}}\) activation was determined from I–V curves fitted to the following transform of a Boltzmann function: I\(_{\text{Ba}}\) = G\(_{\text{max}}\)(V − V\(_{\text{0.5,act}}\))/\{1 + exp[(V − V\(_{\text{0.5,act}}\))/k]\}, where V\(_{\text{0.5,act}}\) is the extrapolated reversal potential, V is the membrane potential, I\(_{\text{Ba}}\) is the peak current elicited by the voltage pulse, G\(_{\text{max}}\) is the maximum conductance, V\(_{\text{0.5,act}}\) 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\(_{\text{+PP}}\)) were normalized to current amplitudes obtained under control conditions (I\(_{\text{0}}\)). Concentration–response curves were obtained by plotting averaged relative peak current amplitude (I/\(I_{\text{0}}\)) against compound concentration and fitting the Hill equation I = I\(_{\text{0}}\){[D]\(h\)/\{[D]\(h\) + [D]\(h\)}] to resulting data, where I\(_{\text{0}}\) 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\(_{\text{V}}\)\(_{2.3}\) channels is irreversible, these experiments do not represent equilibrium steady-state measurements (Christopoulos and Kenakin, 2002).

Data are mean ± 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\(_{\text{V}}\)\(_{2.1}\)/GABA\(_{\text{AR}}\) 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\(_{\text{V}}\)\(_{2.3}\)d channels in the presence of 0.5 or 10 mM EGTA in the intracellular recording solution. Whole-cell I\(_{\text{Ba}}\) was recorded from HEK cells transiently coexpressing wild-type Ca\(_{\text{V}}\)\(_{2.3}\)d or mutant Ca\(_{\text{V}}\)\(_{2.3}\)d (Y1765F) channels and GABA\(_{\text{AR}}\)s. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201311104/DC1.

### RESULTS

#### Differential inhibition of Ca\(_{\text{V}}\)\(_{2.3}\) and Ca\(_{\text{V}}\)\(_{2.1}\) channels by α-conotoxin Vc1.1 via G protein–coupled GABA\(_{\text{AR}}\)s

We investigated VGCC modulation by baclofen and α-conotoxin Vc1.1 in HEK cells stably expressing Ca\(_{\text{V}}\)\(_{2.1}\) (α\(_{1A,2}\)) or Ca\(_{\text{V}}\)\(_{2.3}\) (α\(_{1E,4}\)) channels and transiently coexpressing GABA\(_{\text{AR}}\)s (Ca\(_{\text{V}}\)\(_{2.1}\)/GABA\(_{\text{AR}}\) cells or Ca\(_{\text{V}}\)\(_{2.3}\)/GABA\(_{\text{AR}}\) cells, respectively). Fig. 1 (A–C) shows typical 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\(_{\text{V}}\)\(_{2.1}\) and Ca\(_{\text{V}}\)\(_{2.3}\) C-terminal regions corresponding to exon 37 (e37) of Ca\(_{\text{V}}\)\(_{2.2}\) channel. This catalog does not use algorithms or computational strategies to predict phosphorylation but reports the presence of any literature-derived motifs.
examples of depolarization-activated whole-cell $I_{Ba}$ in the absence or presence of 200 nM c-Vc1.1 or 50 µM baclofen. In Cav2.1/GABArR cells, c-Vc1.1 did not modulate $I_{Ba}$ but inhibited $I_{Ba}$ in Ca.2.3/GABArR cells. The effect of c-Vc1.1 developed relatively slowly, reached maximum inhibition 3–7 min after the response started, and was irreversible (Fig. 1B). The “linear” α-conotoxin Vc1.1 and α-conotoxin PeIA also inhibited depolarization-activated $I_{Ba}$ in Ca.2.3/GABArR cells (Table 1).

These peptides have been shown to selectively inhibit high voltage-activated N-type calcium channels by acting as G protein-coupled GABArR agonists in rat DRG neurons (Callaghan et al., 2008; Daly et al., 2011). Ca.2.1/GABArR or Ca.2.3/GABArR cells typically responded to baclofen, with relatively fast $I_{Ba}$ inhibition that was completely reversible or weakly reversible/irreversible, respectively (Fig. 1A 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.2.3/GABArR cells. We determined the baclofen concentration dependence of $I_{Ba}$ inhibition for Ca.2.1 and Ca.2.3 channels (Fig. 2, B and D), resulting in relationships described by the Hill equation (Table 2). 50 µM GABA also inhibited ~40% of $I_{Ba}$ in Ca.2.1/GABArR and Ca.2.3/GABArR cells and exhibited IC₅₀ values similar to those obtained with baclofen (Table 2). The c-Vc1.1 concentration dependence of $I_{Ba}$ inhibition in Ca.2.3/GABArR cells (Fig. 2F) resulted in IC₅₀ and Hill coefficient values of 290 ± 0.8 pM and 0.61 ± 0.1, respectively, and defined c-Vc1.1 as a potent Ca.2.3 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 GABArR. These results demonstrate that GABArR expression is needed for baclofen to inhibit Ca.2.1 and Ca.2.3 channels, and for c-Vc1.1 to inhibit Ca.2.3 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 GABArR-mediated inhibition of Ca.2.1 and Ca.2.3 channels

Direct VGCC inhibition by G protein–dependent inhibitory pathways involves VD Gβγ binding to the pore-forming subunit (Bean, 1989; Kasai and Aosaki, 1989; Lipscombe et al., 1989). I-V relationships were recorded in Cav2.1/GABArR or Ca.2.3/GABArR cells typically responded to baclofen, with relatively fast $I_{Ba}$ inhibition that was completely reversible or weakly reversible/irreversible, respectively (Fig. 1A 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.2.3/GABArR cells. We determined the baclofen concentration dependence of $I_{Ba}$ inhibition for Ca.2.1 and Ca.2.3 channels (Fig. 2, B and D), resulting in relationships described by the Hill equation (Table 2). 50 µM GABA also inhibited ~40% of $I_{Ba}$ in Ca.2.1/

Figure 1. Effects of α-conotoxin c-Vc1.1 and baclofen (bac) on stably expressed human Ca.2.1 (α1A2) or human Ca.2.3c (α1E1) channels in the presence of transiently expressed human GABArR subunits R1 and R2 (GABAβγ). (A and B) 50 µM baclofen inhibits Ca.2.1 or Ca.2.3c channel currents, whereas 200 nM c-Vc1.1 only inhibits Ca.2.3c currents. Bars indicate c-Vc1.1 or baclofen application. $I_{Ba}$ was evoked by 150-ms depolarizations to 10 mV (Ca.2.3c) or 15 mV (Ca.2.1), 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.2.1 current inhibition by baclofen is reversible on washout (A), whereas baclofen or c-Vc1.1 irreversibly inhibits Ca.2.3c currents (B). (C) 50 µM baclofen inhibits $I_{Ba}$ in Ca.2.3/GABArR cells. Experimental procedures are similar to those in A or B.
in the absence and presence of baclofen in Ca_{2.1}/GABAB_{R} cells and Ca_{2.3}/GABAB_{R} cells, or c-Vc1.1 in Ca_{2.3}/GABAB_{R} cells. The biophysical properties of ion permeation through Ca_{2.1} and Ca_{2.3} 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_{βγ} 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 ± 0.3 mV (n = 8; control) to 7.40 ± 0.58 mV (n = 8; baclofen), but the difference was not statistically significant (P = 0.083) in Ca_{2.1}/GABAB_{R} cells. However, it should be noted that the inhibition of Ca_{2.1}/GABAB_{R} cells by baclofen (31.1 ± 2.6%) is slightly less than the inhibition shown in Fig. 2 A and Table 1. This is probably because of spontaneous IBa 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

### Table 1

Summary of IBa inhibition by baclofen, GABA, or α-conotoxins in the presence or absence of GABAB_{Rs} in HEK cells stably expressing human Ca_{2.1} or Ca_{2.3c} channels

| Agonist     | Ca_{2.1} and GABAB_{R} (%) | Ca_{2.1} alone (%) | Ca_{2.3c} and GABAB_{R} (%) | Ca_{2.3c} alone (%) |
|-------------|---------------------------|--------------------|------------------------------|---------------------|
| Baclofen (50 µM) | 39.9 ± 2.6 (17)          | 0 (5)              | 39.5 ± 4.3 (11)              | 0 (6)              |
| GABA (50 µM)    | 38.4 ± 2.7 (7)           | ND                 | 39.3 ± 4.1 (9)               | ND                 |
| Vc1.1 (200 nM)  | 0 (9)                    | ND                 | 25.5 ± 4.2 (9)               | ND                 |
| c-Vc1.1 (200 nM)| 0 (10)                  | 0 (6)              | 34.8 ± 2.9 (16)              | 0 (5)              |
| PeIA (200 nM) | 0 (3)                    | ND                 | 27.0 ± 3.0 (3)               | ND                 |

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

Figure 2. Stably expressed human Ca_{2.1} (α_{1A-2}) or human Ca_{2.3c} (α_{1E-c}) channel inhibition by baclofen (bac) and α-conotoxin Vc1.1 in the absence and presence of transiently expressed human GABAB_{R}. (A and C) Bar graphs showing average IBa inhibition through Ca_{2.1} (A) or Ca_{2.3c} (C) channels by 50 µM baclofen, 200 nM Vc1.1, or 200 nM c-Vc1.1. Numbers in parentheses indicate the number of experiments. (E) Average IBa inhibited by 50 µM 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 IBa through Ca_{2.1} and Ca_{2.3c} channels by baclofen (B and D) and Ca_{2.3c} channels by c-Vc1.1 (F). See IC_{50} values in Table 2.
In Cav2.3/GABABR cells, the $V_{0.5,\text{act}}$ values were $-3.44 \pm 0.67$ mV ($n = 8$) in the presence of baclofen and $-0.44 \pm 0.46$ mV ($n = 7$) for c-Vc1.1 compared with $0.67 \pm 0.24$ mV ($n = 15$; control). In these experiments, baclofen caused a significant hyperpolarizing shift of $V_{0.5,\text{act}}$ ($P < 0.001$ vs. control; one-way ANOVA). However, $V_{0.5,\text{act}}$ was not altered by c-Vc1.1 ($P = 0.223$).

also investigated baclofen inhibition of $I_{Ba}$ in Ca$_{\text{v2.1}}$/GABABR 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 $V_{0.5,\text{act}}$ values similar to those obtained with voltage steps (Fig. 3 A).

In Ca$_{\text{v2.3}}$/GABABR cells, the $V_{0.5,\text{act}}$ values were $-3.44 \pm 0.67$ mV ($n = 8$) in the presence of baclofen and $-0.44 \pm 0.46$ mV ($n = 7$) for c-Vc1.1 compared with $0.67 \pm 0.24$ mV ($n = 15$; control). In these experiments, baclofen caused a significant hyperpolarizing shift of $V_{0.5,\text{act}}$ ($P < 0.001$ vs. control; one-way ANOVA). However, $V_{0.5,\text{act}}$ was not altered by c-Vc1.1 ($P = 0.223$).

| Agonist  | Cav2.1 and GABABR | Cav2.3c and GABABR |
|---------|-------------------|--------------------|
|         | $nM$              | Hill slope         | $nM$ | 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)* | 0.61 ± 0.14 |

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

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

Figure 3. Voltage dependence of Ca$_{\text{v2.1}}$ ($\alpha_{\text{Ca2.1}}$) and stably expressed Ca$_{\text{v2.3c}}$ channel inhibition via GABABR activation. (A) I-V relationships in Ca$_{\text{v2.1}}$/GABABR cells (left) in the absence (control) and presence of 50 µM baclofen, and in Ca$_{\text{v2.3c}}$/GABABR cells (right) in the absence (control) or presence of 50 µM 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 $V_{0.5,\text{act}}$ values. (B) Ca$_{\text{v2.1}}$ channel inhibition via GABABR is VD, whereas that of Ca$_{\text{v2.3c}}$ is VI. Representative 15-mV depolarization-activated inward $I_{Ba}$ from Ca$_{\text{v2.1}}$/GABABR cells (top) in the absence (control) or presence of 50 µM baclofen, without ($-\text{PP}$) or after the application of a depolarizing prepulse to +80 mV (+PP). (Bottom) Representative 10-mV depolarization-activated $I_{Ba}$ from Ca$_{\text{v2.3c}}$/GABABR cells in the absence (control) or presence of 50 µM 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 ± SEM (paired Student’s $t$ test; *, $P < 0.001$ vs. control [$-\text{PP}$] in Ca$_{\text{v2.1}}$/GABABR cells). The number of experiments is in parentheses. VD, voltage dependent; VI, voltage independent.
We evaluated if baclofen inhibition of $I_{Ba}$ in Ca_{2.1}/GABA_{A}R and Ca_{2.3}/GABA_{A}R cells, and c-Vc1.1 inhibition of $I_{Ba}$ in Ca_{2.3}/GABA_{A}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_{2.1}/GABA_{A}R cells, without changing $I_{Ba}$ relief with Ca_{2.3}/GABA_{A}R cells (not depicted). The inhibitory effect of baclofen was associated with a large (73 ± 4%) VD component in Ca_{2.1}/GABA_{A}R cells. In contrast, the effect of baclofen and Vc1.1 was solely mediated by a VI pathway in Ca_{2.3}/GABA_{A}R cells, which clearly indicates that intracellular signaling does not involve the classical G protein βγ dimer (Gβγ) binding to the pore-forming Ca_{2.3} channel subunit. Alternatively, Gβγ could bind with high affinity to the Ca_{2.3} channel in a VI manner.

**Figure 4.** Effects of baclofen on stably expressed human Ca_{2.1} (α_{1A-2}) channels in the presence of transiently expressed human GABA_{A}Rs (Ca_{2.1}/GABA_{A}R cells). (A) Baclofen-inhibition of $I_{Ba}$ in the presence of 0.5 mM EGTA in the intracellular recording solution. 50 µM baclofen reversibly inhibited $I_{Ba}$ by 38.5 ± 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 ± 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.

We evaluated the VI pathway leading to Ca_{2.3} 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_{2.3} channels (Ca_{2.3} cells) or Ca_{2.3} cells coexpressing GABA_{B}R R2 subunits, neither 200 nM c-Vc1.1 nor 50 µM baclofen inhibits $I_{Ba}$, indicating that a fully functional GABA_{B}R heterodimer is needed for proper signaling. In Ca_{2.3}/GABA_{B}R cells, the selective GABA_{B}R antagonist CGP55845 (1 µM) 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 µM) was added to the intracellular recording solution, Vc1.1 and baclofen inhibitory effects were almost identically reduced. Overnight treatment
with 1 µg/ml PTX abolished c-Vc1.1 and baclofen inhibitory pathway(s) in Ca2.3/GABAaR cells, suggesting that the effects were mediated by G4 and/or G6 proteins.

We previously showed that Vc1.1 inhibition of N-type (Ca2.2) 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 GABAaR-mediated Ca2.1 or Ca2.3 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 Ca2.1/GABAaR 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 Iba. However, in Ca2.3/GABAaR cells, wild-type c-Src protein overexpression dramatically increased the fraction of Iba inhibited by c-Vc1.1 compared with control.

To further evaluate the effect of c-Src on Ca2.3/GABAaR 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 on baclofen-induced VI channel inhibition by baclofen and c-Vc1.1 (Fig. 6, A and B).

Tyrosines 1761 and 1765 are needed in the C terminus for c-Src phosphorylation of Ca2.3 channels

Alternative splicing of Ca2.1, Ca2.2, and Ca2.3 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 Ca2.2[e37a] channel occurred via VD and VI pathways. In HEK cells coexpressing GABAaRs and Ca2.2[e37a] channels, the baclofen-induced VI component required a tyrosine (Y) residue in e37a to be phosphorylated (Raingo et al., 2007).

Alignment of the Ca2.2 channel e37a and e37b regions with the corresponding e37 regions in human Ca2.3c, Ca2.3d, and Ca2.1 (α1A2 or α1A3), and rabbit Ca2.1 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 Ca2.3 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 Ca2.3 and rabbit Ca2.1, but not in human Ca2.1 channels. In both human Ca2.3c and Ca2.3d 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 Ca2.1, the second Y residue followed by alanine (A) also represents a Src motif described in the literature. In contrast, in the human Ca2.1 splice variants α1A2 or α1A3, 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 GABAaRs and transiently expressing Ca2.3c or Ca2.3d splice variants, or human or rabbit Ca2.1 channels. In patch-clamp experiments, 200 nM c-Vc1.1 inhibited human Ca2.3c and Ca2.3d channels but did not affect human Ca2.1 (α1A3) (Fig. 7B and Table 3). This confirmed previous results in HEK cells stably expressing Ca2.1 (α1A2) or Ca2.3c channels in the presence of GABAaRs (Figs. 1 and 2). In all experiments, 50 µM baclofen inhibited Iba. As predicted, c-Vc1.1 also inhibited rabbit Ca2.1 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 Ca2.3d or Ca2.3c demonstrated that the Y1761F mutation completely abolished c-Vc1.1 inhibition of Iba, and the Y1765F mutation significantly reduced
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 Ca2.3d (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 Ca2.3d (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 G\(\beta\)\(\gamma\) binding.

We also generated human Ca2.1 (\(\alpha_{1A5}\)) (L1852T) and Ca2.1 (\(\alpha_{1A5}\)) (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 Ca2.1 (L1852T) or Ca2.1 (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 \(\alpha\)-conotoxin c-Vc1.1 inhibition of Cav2.3 and Ca2.1 channels. However, it remains possible that other residues are also involved in mediating baclofen’s inhibition of Ca2.3 channels.

**DISCUSSION**

In this study, we efficiently reconstituted human Ca2.1 and Ca2.3 channel modulation via human G protein-coupled GABA\(\beta\)Rs. Baclofen, a GABA\(\beta\)R agonist, inhibited I_{ba} through both channels; however, \(\alpha\)-conotoxin Vc1.1 only inhibited Ca2.3 channels. The effect of Vc1.1 on Ca2.3 channels was completely VI and depended on the presence of specific c-Src phosphorylation sites in the C terminus of the human \(\alpha_{1E}\) (Ca2.3). These results define Ca2.3 channels as new targets for analgesic \(\alpha\)-conotoxins.

**Ca2 channels and chronic pain**

It is well established that Ca2.2 channel inhibition by antagonists or via GPCRs produces analgesia in animals and humans (Altier and Zamponi, 2004). GABA\(\beta\)R-mediated inhibition of Ca2.1 or Ca2.2 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
neurons (Matthews et al., 2007), and their inhibition was associated with high efficiency opioid therapy without tolerance (Yokoyama et al., 2004). Cav2.3 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 Cav2.1 and Cav2.3 channels

Our results demonstrate that baclofen or GABA inhibits IBa to a similar extent in Ca\(v\)2.1/GABA\(_{B}\)R and Ca\(v\)2.3/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\(v\)2.2 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\(v\)2.3 channels as potential targets for drugs that treat chronic pain (Saegusa et al., 2000; Qian et al., 2013). The anti-nociceptive role of Ca\(v\)2.3 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\(v\)2.3 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 Cav2.1 and Cav2.3 channels

Our results demonstrate that baclofen or GABA inhibits IBa to a similar extent in Ca\(v\)2.1/GABA\(_{B}\)R and Ca\(v\)2.3/
GABA<sub>B</sub>R cells (Figs. 1 and 2). Throughout this study, we used cells with 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<sub>v</sub>2.1 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<sub>Ba</sub> relief in Ca<sub>v</sub>2.1 channels (Currie and Fox, 1997). Our results on the voltage dependence of inhibition are consistent with previous studies demonstrating baclofen inhibition of Ca<sub>v</sub>2.1 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<sub>Ba</sub> activation in the presence of baclofen or c-Vc1.1 in Ca<sub>v</sub>2.1/GABA<sub>B</sub>R or Ca<sub>v</sub>2.3/GABA<sub>B</sub>R cells. However, in Ca<sub>v</sub>2.1/GABA<sub>B</sub>R cells, the time course of I<sub>Ba</sub> activation considerably slowed in the presence of baclofen when compared with control. This is a hallmark of VD G<sub>Bγ</sub>y binding to the α<sub>1A</sub> (Ca<sub>v</sub>2.1) subunit (Fig. 3 B). Interestingly, baclofen only caused a small and statistically insignificant shift of the I-V and G-V relationships in Ca<sub>v</sub>2.1/GABA<sub>B</sub>R 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<sub>v</sub>2.1 channel I-V relationship, suggesting possible differences between Ca<sub>v</sub>2.1 and Ca<sub>v</sub>2.2 channel–modulating membrane-delimited pathways. In Ca<sub>v</sub>2.3/GABA<sub>B</sub>R cells, the time course of I<sub>Ba</sub> 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<sub>v</sub>2.3 channels via GABA<sub>B</sub>Rs. However, it has been shown that the rat brain α<sub>1Elong</sub> splice variant could be inhibited in a VD manner via D<sub>2</sub> dopamine receptors (Page et al., 1998). Our alignment of the human α<sub>1Ec</sub> or α<sub>1Ed</sub> and rat α<sub>1Elong</sub> splice variants (not depicted) indicate that the N-terminal sequence responsible for VD inhibition of rat α<sub>1Elong</sub> variant is present in human α<sub>1Ec</sub> and α<sub>1Ed</sub>. Therefore, future experiments should determine whether or not human Ca<sub>v</sub>2.3 channels can be inhibited via D<sub>2</sub> 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<sub>v</sub>2.3 channel inhibition by baclofen or c-Vc1.1 could be disrupted by GDP-β-S, a GDP analogue that keeps G<sub>α</sub> permanently associated with G<sub>Bγ</sub>y. In all cases, PTX treatment abolished baclofen and c-Vc1.1 inhibition of I<sub>Ba</sub>, indicating that GABA<sub>B</sub>Rs couple with G<sub>α</sub>s of the G<sub>i/Go</sub> superfamily in cells expressing Ca<sub>v</sub>2.3 channels (not depicted). Analysis of the VI pathway in Ca<sub>v</sub>2.3/GABA<sub>B</sub>R cells indicated that signaling mechanisms that contribute to Ca<sub>v</sub>2.3 channel inhibition, downstream of G protein subunits, involve c-Src kinase activation. For example, wild-type c-Src overexpression increased I<sub>Ba</sub> inhibition, whereas the dominant-negative double mutant c-Src or the pp60c-Src

| Ca<sub>v</sub>2 channel | c-Vc1.1 (200 nM) | Baclofen (50 µM) |
|----------------------|-----------------|-----------------|
| Human Ca<sub>v</sub>2.3d | 23 ± 2.9 (8) | 35.1 ± 1.8 (8) |
| Human Ca<sub>v</sub>2.3d (Y1761F) | 1.7 ± 0.3 (11)* | 21.9 ± 1.8 (11)* |
| Human Ca<sub>v</sub>2.3d (Y1765F) | 11.3 ± 3.0 (10)<sup>b</sup> | 28.9 ± 2.7 (10) |
| Human Ca<sub>v</sub>2.3c | 28.0 ± 4.0 (10) | 44.0 ± 3.0 (10) |
| Human Ca<sub>v</sub>2.3c (Y1761F) | 0.37 ± 0.3 (8)<sup>*</sup> | 14.6 ± 1.8 (8)<sup>†</sup> |
| Human Ca<sub>v</sub>2.3c (Y1765F) | 15.0 ± 3.2 (10)<sup>b</sup> | 28.9 ± 2.7 (10)<sup>©</sup> |
| Human Ca<sub>v</sub>2.1 | 0 (8) | 44.4 ± 1.9 (9) |
| Human Ca<sub>v</sub>2.1 (L1852T) | 7.2 ± 1.4 (8)<sup>†</sup> | 50.6 ± 2.8 (8) |
| Human Ca<sub>v</sub>2.1 (Q1856E) | 6.6 ± 1.7 (8)<sup>μ</sup> | 47.9 ± 3.0 (8) |
| Rabbit Ca<sub>v</sub>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<sub>v</sub>2.1 (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<sub>v</sub>2.1 or Ca<sub>v</sub>2.3c channels and transiently coexpressed GABA<sub>B</sub>Rs (above) is similar (within the statistical margin of error) to that obtained with stably expressed human Ca<sub>v</sub>2.1 or Ca<sub>v</sub>2.3c channels in the presence of transiently coexpressed GABA<sub>B</sub>Rs (see Table 1).

<sup>a</sup> P < 0.001 versus Ca<sub>v</sub>2.3d with c-Vc1.1; P < 0.001 versus Ca<sub>v</sub>2.3c with c-Vc1.1; and P = 0.002 versus human Ca<sub>v</sub>2.1 with c-Vc1.1.
<sup>b</sup> P = 0.003 versus Ca<sub>v</sub>2.3d with baclofen; P = 0.008 versus Ca<sub>v</sub>2.3c with baclofen; and P = 0.002 versus human Ca<sub>v</sub>2.1 with baclofen.
<sup>c</sup> P = 0.003 versus Ca<sub>v</sub>2.3d with baclofen and P < 0.001 versus Ca<sub>v</sub>2.3c with baclofen.
<sup>d</sup> P < 0.001 versus Ca<sub>v</sub>2.3c with baclofen.

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peptide abolished c-Vc1.1 inhibition of I_{Ba} (Fig. 6). This suggested that Ca_{2.3} 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_{2.2} channels involves direct Go_{a} activation of Src kinase (Diversé-Pierluissi et al., 1997). However, further studies are needed to elucidate whether baclofen or Vc1.1 inhibition of Ca_{2.3} channels involves direct G_{i}/G_{o} activation of c-Src.

In Ca_{2.1} and Ca_{2.2} 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_{2.3} 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_{2.3} splice variants (Williams et al., 1994) with similar biochemical properties (Pereverzev et al., 2002). Of these, Ca_{2.3c} represents the major neuronal type variant, which is dominantly expressed in the adult central nervous system (Schneider et al., 2013), whereas Ca_{2.3d}, 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_{2.3e} was also identified in nociceptive trigeminal ganglion and DRG neurons together with Ca_{2.3a} (Fang et al., 2007, 2010). Importantly, the e37 region containing the putative c-Src phosphorylation sites can be identified in all Ca_{2.3} 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 α1C 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_{2.2} channels by baclofen (Raingo et al., 2007) and Vc1.1 (Callaghan et al., 2008).

Therapeutic implications of Ca_{2.3} channel inhibition

Few studies have examined Cav_{2.3} (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 α9α10 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 GABABR, its development is likely to remain. It remains to be established if analgesic α-conotoxins can be used as specific Ca_{2.2}[e37a] and Ca_{2.3} 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_{2.3} channels that involves GABABRs. We systematically examined the intracellular pathways and elucidated the molecular details that determine ε-Src phosphorylation of the Ca_{2.1} and Ca_{2.3} channel C termini. Although the physiological significance of kinase-mediated Ca_{2.3} channel inhibition is unclear, it may have long-term influence over Ca^{2+}-dependent intracellular signaling, exocytosis, and/or gene transcription in neurons.

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