Differential Ca₉.2.1 and Ca₉.2.3 channel inhibition by baclofen and α-conotoxin Vc1.1 via GABAᵦ receptor activation

Géza Berecki, Jeffrey R. McArthur, Hartmut Cuny, Richard J. Clark, and David J. Adams

Neuronal Ca₂⁺.1 (P/Q-type), Ca₂⁺.2 (N-type), and Ca₂⁺.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 (GABAᵦ) receptors (GABAᵦRs) to inhibit Ca₂⁺.2 channels. We investigated GABAᵦR-mediated modulation by Vc1.1, a cyclicized form of Vc1.1 (c-Vc1.1), and the GABAᵦR agonist baclofen of human Ca₂⁺.1 or Ca₂⁺.3 channels heterologously expressed in human embryonic kidney cells. 50 μM baclofen inhibited Ca₂⁺.1 and Ca₂⁺.3 channel Ba²⁺ currents by ~40%, whereas c-Vc1.1 did not affect Ca₂⁺.1 but potently inhibited Ca₂⁺.3, with a half-maximal inhibitory concentration of ~300 μM. Depolarizing paired pulses revealed that ~75% of the baclofen inhibition of Ca₂⁺.1 was voltage dependent and could be relieved by strong depolarization. In contrast, baclofen or Vc1.1 inhibition of Ca₂⁺.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 GABAᵦR antagonist CGP55845. Overexpression of the kinase c-Src significantly increased inhibition of Ca₂⁺.3 by c-Vc1.1. Conversely, coexpression of a catalytically inactive double mutant form of c-Src or pretreatment with a phosphorylation. pp60c-Src peptide abolished the effect of c-Vc1.1. Site-directed mutational analyses of Ca₂⁺.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 Ca₂⁺.1 channels conferred c-Vc1.1 sensitivity. Our findings show that Vc1.1 inhibition of Ca₂⁺.3, which defines Ca₂⁺.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 Ca₂⁺.1 (P/Q-type), Ca₂⁺.2 (N-type), and Ca₂⁺.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 Ca₂⁺.3 channels are less susceptible to direct G protein βγ dimer modulation than Ca₂⁺.1 or Ca₂⁺.2 (Shekter et al., 1997), a finding attributed to differences between the N terminus, domain I, and the I–II intracellular linker of Ca₂⁺.3 and Ca₂⁺.2 channels (Stephens et al., 1998; Simen and Miller, 2000). Nevertheless, carbachol, somatostatin, ATP, and adenosine inhibit exogenous Ca₂⁺.3 channels via endogenous receptors in human embryonic kidney (HEK) cells (Mehrke et al., 1997). Interestingly, carbachol, a muscarinic receptor agonist, stimulates or inhibits Ca₂⁺.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 Ca₂⁺.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 Ca₂⁺.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, inhibits Ca₂⁺.2 channels via GABAᵦ type B (GABAᵦ) receptors (GABAᵦRs) 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 (Klimis et al., 2011). Ca,2.3 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,2.3 modulation via GABArR is incompletely characterized and has not been reconstituted in any heterologous expression system. Moreover, few drugs or toxins have specific Ca,2.3 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 GABArR 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,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 α1 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% CO2 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-293T cells were transiently cotransfected with plasmid cDNAs encoding human Ca,2.1 (RefSeq accession no. NM_001470; 3 µg; OriGene Technologies, Inc.), human Ca,2.3d (RefSeq accession no. NM_005458; 3 µg; OriGene Technologies, Inc.), and enhanced green fluorescent protein (eGFP) reporter gene construct (1 µg; 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,2.1 channel transcript variant 5 (5 µg) or wild-type or mutant human Ca,2.3d channels (5 µg), human α,β1 (5 µg) and human β3 (5 µg) auxiliary subunits, human GABAaR1 (3 µg), human GABAaR2 (3 µg), and eGFP (1 µg). In a separate series of experiments, HEK-293T cells were transiently cotransfected with plasmid cDNAs encoding rabbit Ca,2.1 channel (RefSeq accession no. NM_001010693; 3 µg; provided by F. Meunier, The University of Queensland, St. Lucia, Australia), rat α,β1–5 (5 µg; provided by G.W. Zamponi, University of Calgary, Calgary, Canada) and rat β3 (5 µg; provided by D. Lipscombe, Brown University, Providence, RI) auxiliary subunits; human GABAaR1 (3 µg) and human GABAaR2 (3 µg) and eGFP (1 µg). 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 CaCl2, 30 TEA-Cl, 10 d-glucose, and 10 HEPES, pH 7.4 with TEA-OH, at 30°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.
concentration of 0.5 or 10 mM. GTP was not used in the intracellular solution to prevent Ib 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 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 Ib 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 Cav2.3 channels and GABAARs) or 15 mV (in cells coexpressing Cav2.1 channels and GABAARs) of 150-ms duration were applied at a frequency of 0.1 Hz from an HP of −80 mV, where Ib 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 Ib, inhibited in the absence of a prepulse (−PP Ib), or presence of a +80-mV prepulse (+PP Ib), was determined according to [(I-I0)/I0] × 100, or [(I−PP − I0)/I0] × 100, respectively, where I0 represents current amplitudes (controls) obtained with or without a prepulse in the absence of a compound, respectively. Ib−PP was normalized to Ib0+PP and I0+PP represent current amplitudes obtained with or without a prepulse in the presence of a compound, respectively. The VI fraction was defined as +PP Ib0/−PP Ib0, whereas the VD fraction was calculated as (−PP Ib0) − 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 Ib was routinely tested in HEK cells stably expressing Cav2.1 or Cav2.3 channels and coexpressing GABAARs. In ~5% of all cells tested, Ib 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 Ib 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 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 Cav2 channels were obtained using c-Vc1.1, unless otherwise noted. GABA, baclofen, guanosine 5′-[β-thio]diphosphate trilithium salt (GBP-βS), and pertussis toxin (PTX) were purchased from Sigma-Aldrich. (2S)-3-[(1S)-1-(3,4-dichlorophenyl)ethylamino-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 Ca2.1 and Ca2.3 C-terminal regions corresponding to exon 37 (c37) of Ca2.2 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 E0 activation was determined from I-V curves fitted to the following transform of a Boltzmann function: E0 = Gmax(V − Vrev)/(1 + exp[(V − V0.5)/k]), where Vrev is the extrapolated reversal potential, V is the membrane potential, I0 is the peak current elicited by the voltage pulse, Gmax is the maximum conductance, V0.5 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 (I0). Concentration–response curves were obtained by plotting averaged relative peak current amplitude (I/I0) against compound concentration and fitting the Hill equation I = I0[D]h/(IC50 + [D]h) to resulting data, where I0 is the maximum peak current amplitude, [D] is the concentration of the compound (drug), IC50 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 Ca2.3 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 Ca2.1/GABAAR 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 Ca2.3d channels in the presence of 0.5 or 10 mM EGTA in the intracellular recording solution. Whole-cell Ib was recorded from HEK cells transiently coexpressing wild-type Ca2.3d or mutant Ca2.3d (Y1765F) channels and GABAARs. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201311104/DC1.

RESULTS
Differential inhibition of Ca2.3 and Ca2.1 channels by α-conotoxin Vc1.1 via G protein–coupled GABAARs
We investigated VGCC modulation by baclofen and α-conotoxin Vc1.1 in HEK cells stably expressing Ca2.1 (α1Aα2) or Ca2.3c (α1Eα2) channels and transiently coexpressing GABAARs (Ca2.1/GABAAR cells or Ca2.3/GABAAR cells, respectively). Fig. 1 (A–C) shows typical
examples of depolarization-activated whole-cell \( I_{Na} \) in the absence or presence of 200 nM c-Vc1.1 or 50 µM baclofen. In Ca\(_{2.1}\)/GABA\(_R\) cells, c-Vc1.1 did not modulate \( I_{Na} \) but inhibited \( I_{Na} \) in Ca\(_{2.3}\)/GABA\(_R\) 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” \( \alpha \)-conotoxin Vc1.1 and \( \alpha \)-conotoxin PeIA also inhibited depolarization-activated \( I_{Na} \) in Ca\(_{2.3}\)/GABA\(_R\) 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\(_R\) agonists in rat DRG neurons (Callaghan et al., 2008; Daly et al., 2011). Ca\(_{2.1}\)/GABA\(_R\) or Ca\(_{2.3}\)/GABA\(_R\) cells typically responded to baclofen, with relatively fast \( I_{Na} \) 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 \( I_{Na} \) in Ca\(_{2.3}\)/GABA\(_R\) cells. We determined the baclofen concentration dependence of \( I_{Na} \) 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 \( I_{Na} \) in Ca\(_{2.1}\)/GABA\(_R\) and Ca\(_{2.5}\)/GABA\(_R\) cells and exhibited IC\(_{50}\) values similar to those obtained with baclofen (Table 2). The c-Vc1.1 concentration dependence of \( I_{Na} \) inhibition in Ca\(_{2.3}\)/GABA\(_R\) 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\(_{2.3}\) channel inhibitor (Table 2). Fig. 2 (A and C) and Table 1 summarize the average \( I_{Na} \) inhibition by baclofen, GABA, Vc1.1, c-Vc1.1, and PeIA in the absence and presence of GABA\(_R\)s. These results demonstrate that GABA\(_R\) 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 GABA\(_R\)-mediated inhibition of Ca\(_{2.1}\) and Ca\(_{2.3}\) channels

Direct VGCC inhibition by G protein–dependent inhibitory pathways involves VD GB\(_{3,4}\) binding to the pore-forming subunit (Bean, 1989; Kasai and Aosaki, 1989; Lipscombe et al., 1989). I-V relationships were recorded...
in the absence and presence of baclofen in Ca_{2.1}/
GABA_{R} cells and Ca_{2.3}/GABA_{R} cells, or c-Vc1.1 in
Ca_{2.3}/GABA_{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} modula-
tion, 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}/GABABR cells. How-
ever, it should be noted that the inhibition of Ca_{2.1}/
GABA_{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 pres-
ence of baclofen and could cause the underestimation
of the V_{0.5,act} positive shift in these experiments. To re-
duce the contribution of recovery in this process, we

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<th>Ca_{2.3c} and GABA_{R}</th>
<th>Ca_{2.3c} alone</th>
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<td>Baclofen (50 µM)</td>
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<td>c-Vc1.1 (200 nM)</td>
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<td>0 (6)</td>
<td>34.8 ± 2.9 (16)</td>
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<td>PeIA (200 nM)</td>
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<td>27.0 ± 3.0 (3)</td>
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Values represent mean ± SEM; n, number of experiments in parentheses; ND, not determined.

![Figure 2](https://example.com/figure2.png)

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 GABA_{R}. (A and C) Bar graphs showing
average I_{Ba} 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 I_{Ba} 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 I_{Ba} 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.
also investigated baclofen inhibition of $I_{Ba}$ in Ca$_{2.1}$/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 $V_{0.5,act}$ values similar to those obtained with voltage steps (Fig. 3 A). In Ca$_{2.3}$/GABAbR cells, the $V_{0.5,act}$ 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 $V_{0.5,act}$ ($P < 0.001$ vs. control; one-way ANOVA). However, $V_{0.5,act}$ was not altered by c-Vc1.1 ($P = 0.223$).
We evaluated if baclofen inhibition of $I_{Ba}$ in Ca_{2.1}/GAB_{A2}R and Ca_{2.3}/GAB_{A2}R cells, and c-Vc1.1 inhibition of $I_{Ba}$ in Ca_{2.3}/GAB_{A2}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}/GAB_{A2}R cells, without changing $I_{Ba}$ relief with Ca_{2.3}/GAB_{A2}R cells (not depicted). The inhibitory effect of baclofen was associated with a large (73 ± 4%) VD component in Ca_{2.1}/GAB_{A2}R cells. In contrast, the effect of baclofen and Vc1.1 was solely mediated by a VI pathway in Ca_{2.3}/GAB_{A2}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_{2.3} channel subunit. Alternatively, $G_{\beta\gamma}$ could bind with high affinity to the Ca_{2.3} channel in a VI manner.

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_{2.3c} channels (Ca_{2.3} cells) or Ca_{2.3} cells coexpressing GAB_{A2}R R2 subunits, neither 200 nM c-Vc1.1 nor 50 µM baclofen inhibits $I_{Ba}$, indicating that a fully functional GAB_{A2}R heterodimer is needed for proper signaling. In Ca_{2.3}/GAB_{A2}R cells, the selective GAB_{A2}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 GAB_{A2}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

Figure 4. Effects of baclofen on stably expressed human Ca_{2.1} (α_{1A-2}) channels in the presence of transiently expressed human GAB_{A2}Rs (Ca_{2.1}/GAB_{A2}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, $G = G_{max}/(1 + \exp((V_m - V_{0.5,act} - V_{rev})/k))$, 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.
with 1 µg/ml PTX abolished c-Vc1.1 and baclofen inhibitory pathway(s) in Ca_{2.3}/GABA_{B}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 (Ca_{2.2}) 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_{B}R-mediated Ca_{2.1} or Ca_{2.3} 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 Ca_{2.1}/GABA_{B}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 Ca_{2.3}/GABA_{B}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 Ca_{2.3}/GABA_{B}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).

Tyrosines 1761 and 1765 are needed in the C terminus for c-Src phosphorylation of Ca_{2.3} channels

Alternative splicing of Ca_{2.1}, Ca_{2.2}, and Ca_{2.3} 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 Ca_{2.2}[e37a] channel occurred via VD and VI pathways. In HEK cells coexpressing GABA_{B}Rs and Ca_{2.2}[e37a] channels, the baclofen-induced VI component required a tyrosine (Y) residue in e37a to be phosphorylated (Raingo et al., 2007).

Alignment of the Ca_{2.2} channel e37a and e37b regions with the corresponding e37 regions in human Ca_{2.3c}, Ca_{2.3d}, and Ca_{2.1} (α_{1A2} or α_{1A3}), and rabbit Ca_{2.1} 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 Ca_{2.3} 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 Ca_{2.3} and rabbit Ca_{2.1}, but not in human Ca_{2.1} channels. In both human Ca_{2.3c} and Ca_{2.3d} 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 Ca_{2.1}, the second Y residue followed by alanine (A) also represents a Src motif described in the literature. In contrast, in the human Ca_{2.1} splice variants α_{1A2} or α_{1A3}, 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_{B}Rs and transiently expressing Ca_{2.3c} or Ca_{2.3d} splice variants, or human or rabbit Ca_{2.1} channels. In patch-clamp experiments, 200 nM c-Vc1.1 inhibited human Ca_{2.3c} and Ca_{2.3d} channels but did not affect human Ca_{2.3c} (α_{1A3}) (Fig. 7 B and Table 3). This confirmed previous results in HEK cells stably expressing Ca_{2.1} (α_{1A2}) or Ca_{2.3c} channels in the presence of GABA_{B}Rs (Figs. 1 and 2). In all experiments, 50 µM baclofen inhibited I_{Ba}. As predicted, c-Vc1.1 also inhibited rabbit Ca_{2.3} 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 Ca_{2.3d} or Ca_{2.3c} demonstrated that the Y1761F mutation completely abolished c-Vc1.1 inhibition of I_{Ba}, and the Y1765F mutation significantly reduced
the c-Vc1.1 inhibition (Fig. 7B 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.2.3d (Y1765F), also reduced baclofen inhibition of I\textsubscript{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\textsubscript{Ba} through Ca.2.3d (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\textsubscript{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 Ca.2.1 (α\textsubscript{1A-5}) (L1852T) and Ca.2.1 (α\textsubscript{1A-5}) (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.2.1 (L1852T) or Ca.2.1 (Q1856E) channel mutants (Fig. 7B 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.2.3 and Ca.2.1 channels. However, it remains possible that other residues are also involved in mediating baclofen’s inhibition of Ca.2.3 channels.

**DISCUSSION**

In this study, we efficiently reconstituted human Ca.2.1 and Ca.2.3 channel modulation via human G protein–coupled GABA\textsubscript{R}s. Baclofen, a GABA\textsubscript{R} agonist, inhibited I\textsubscript{Ba} through both channels; however, α-conotoxin Vc1.1 only inhibited Ca.2.3 channels. The effect of Vc1.1 on Ca.2.3 channels was completely VI and depended on the presence of specific c-Src phosphorylation sites in the C terminus of the human α\textsubscript{1E} (Ca.2.3). These results define Ca.2.3 channels as new targets for analgesic α-conotoxins.

**Ca.2 channels and chronic pain**

It is well established that Ca.2.2 channel inhibition by antagonists or via GPCRs produces analgesia in animals and humans (Altier and Zamponi, 2004). GABA\textsubscript{R}-mediated inhibition of Ca.2.1 or Ca.2.2 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

Figure 6. The role of c-Src proteins in GABA\textsubscript{R}-mediated inhibition by c-Vc1.1 in HEK cells stably expressing human Ca.2.3c channels and GABA\textsubscript{R}s. (A) Time course of I\textsubscript{Ba} through Ca.2.3c 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\textsubscript{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\textsubscript{Ba}, respectively. Representative current traces (right) are shown at the times indicated by lowercase letters (see Fig. 1B for control). I\textsubscript{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\textsubscript{Ba} inhibition by c-Vc1.1 or baclofen after pp60c-Src peptide (50 μM) pretreatment or wild-type c-Src coexpression in Ca.2.1/GABA\textsubscript{R} or Ca.2.3/GABA\textsubscript{R} cells, respectively, or double mutant c-Src (K295R/Y527F) coexpression in Ca.2.3/GABA\textsubscript{R} 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 Cav2,2,2,2 channels by acting as G protein–coupled GABABR 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 Cav2,3 channels as potential targets for drugs that treat chronic pain (Saegusa et al., 2000; Qian et al., 2013). The anti-nociceptive role of Cav2,3 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). 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 Ca2+ 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 Cav2,1 and Cav2,3 pathways (Dolphin and Scott, 1986; Diversé-Pierluissi et al., 1997). We showed that a subset of α-conotoxins, including Vc1.1, also selectively inhibit Cav2,2,2,2 channels by acting as G protein–coupled GABABR 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).

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GABA<sub>B</sub>R 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<sub>2.1</sub> channel–expressing cells, baclofen inhibition was strong and could be relieved by a 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>2.1</sub> channels (Currie and Fox, 1997). Our results on the voltage dependence of inhibition are consistent with previous studies demonstrating baclofen inhibition of Cav<sub>2.1</sub> 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>2.1</sub>/GABA<sub>B</sub>R or Ca<sub>2.3</sub>/GABA<sub>B</sub>R cells. However, in Ca<sub>2.1</sub>/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>βγ</sub> binding to the α<sub>1A</sub> (Ca<sub>2.1</sub>) subunit (Figs. 3 B). Interestingly, baclofen only caused a small and statistically insignificant shift of the I-V and G-V relationships in Ca<sub>2.1</sub>/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>2.1</sub> channel I-V relationship, suggesting possible differences between Ca<sub>2.1</sub> and Ca<sub>2.2</sub> channel–modulating membrane-delimited pathways. In Ca<sub>2.3</sub>/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>2.3</sub> channels via GABA<sub>B</sub>Rs. However, it has been shown that the rat brain α<sub>1E<sub>long</sub></sub> splice variant could be inhibited in a VD manner via D2 dopamine receptors (Page et al., 1998). Our alignment of the human α<sub>1E<sub>c</sub></sub> or α<sub>1E<sub>d</sub></sub> and rat α<sub>1E<sub>long</sub></sub> splice variants (not depicted) indicate that the N-terminal sequence responsible for VD inhibition of rat α<sub>1E<sub>long</sub></sub> variant is present in human α<sub>1E<sub>c</sub></sub> and α<sub>1E<sub>d</sub></sub>. Therefore, future experiments should determine whether or not human Ca<sub>2.3</sub> 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<sub>2.3</sub> channel inhibition by baclofen or c-Vc1.1 could be disrupted by GDP-β-S, a GDP analogue that keeps Gα permanently associated with G<sub>βγ</sub>. 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 proteins of the G<sub>i/Go</sub> superfamily in cells expressing Ca<sub>2.3</sub> channels (Fig. 5). Analysis of the VI pathway in Ca<sub>2.3</sub>/GABA<sub>B</sub>R cells indicated that signaling mechanisms that contribute to Ca<sub>2.3</sub> 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

### Table 3

<table>
<thead>
<tr>
<th>Ca&lt;sub&gt;2&lt;/sub&gt; channel</th>
<th>c-Vc1.1 (200 nM)</th>
<th>Baclofen (50 µM)</th>
<th>I&lt;sub&gt;ba&lt;/sub&gt; inhibition (%)</th>
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<tr>
<td>Human Ca&lt;sub&gt;2.3d&lt;/sub&gt;</td>
<td>23 ± 2.9 (8)</td>
<td>35.1 ± 1.8 (8)</td>
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<td>Human Ca&lt;sub&gt;2.3d&lt;/sub&gt; (Y1761F)</td>
<td>1.7 ± 0.3 (11)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.9 ± 1.8 (11)&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Human Ca&lt;sub&gt;2.3d&lt;/sub&gt; (Y1765F)</td>
<td>11.3 ± 3.0 (10)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NN28.9 ± 2.7 (10)</td>
<td></td>
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<tr>
<td>Human Ca&lt;sub&gt;2.3c&lt;/sub&gt;</td>
<td>28.0 ± 4.0 (10)</td>
<td>44.0 ± 3.0 (10)</td>
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<tr>
<td>Human Ca&lt;sub&gt;2.3c&lt;/sub&gt; (Y1761F)</td>
<td>0.37 ± 0.3 (8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.6 ± 1.8 (8)&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Human Ca&lt;sub&gt;2.3c&lt;/sub&gt; (Y1765F)</td>
<td>15.0 ± 3.2 (10)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.9 ± 2.7 (10)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Human Ca&lt;sub&gt;2.1&lt;/sub&gt;</td>
<td>0 (8)</td>
<td>44.4 ± 1.9 (9)</td>
<td></td>
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<tr>
<td>Human Ca&lt;sub&gt;2.1&lt;/sub&gt; (L1852T)</td>
<td>7.2 ± 1.4 (8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NN50.6 ± 2.8 (8)</td>
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<tr>
<td>Human Ca&lt;sub&gt;2.1&lt;/sub&gt; (Q1856E)</td>
<td>6.6 ± 1.7 (8)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NN47.9 ± 3.0 (8)</td>
<td></td>
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<tr>
<td>Rabbit Ca&lt;sub&gt;2.1&lt;/sub&gt;</td>
<td>*20.0 ± 4.0 (7)</td>
<td>*50.0 ± 5.0 (8)</td>
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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>2.1</sub> (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>2.1</sub> or Ca<sub>2.3c</sub> 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>2.1</sub> or Ca<sub>2.3c</sub> channels in the presence of transiently coexpressed GABA<sub>B</sub>Rs (see Table 1). *P < 0.001 versus Ca<sub>2.3d</sub> with c-Vc1.1; P < 0.001 versus Ca<sub>2.3c</sub> with c-Vc1.1; and P = 0.002 versus human Ca<sub>2.1</sub> with c-Vc1.1.

**P = 0.003 versus Ca<sub>2.3d</sub> with baclofen; P = 0.006 versus Ca<sub>2.3c</sub> with c-Vc1.1; and P = 0.002 versus human Ca<sub>2.1</sub> with c-Vc1.1.

**P = 0.003 versus Ca<sub>2.3d</sub> with baclofen and P < 0.001 versus Ca<sub>2.3c</sub> with baclofen.
peptide abolished c-Vc1.1 inhibition of I\text{Ba} (Fig. 6). This suggested that Ca\text{,}2\text{,}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\text{,}2\text{,}2 channels involves direct Go\text{,}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\text{,}2\text{,}3 channels involves direct G\text{I}/G\text{o} activation of c-Src.

In Ca\text{,}2\text{,}1 and Ca\text{,}2\text{,}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\text{,}2\text{,}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\text{,}2\text{,}3 splice variants (Williams et al., 1994) with similar biophysical properties (Pereverzev et al., 2002). Of these, Ca\text{,}2\text{,}3c represents the major neuronal type variant, which is dominantly expressed in the adult central nervous system (Schneider et al., 2013), whereas Ca\text{,}2\text{,}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\text{,}2\text{,}3e was also identified in nociceptive trigeminal ganglion and DRG neurons together with Ca\text{,}2\text{,}3a (Fang et al., 2007, 2010). Importantly, the e37 region containing the putative c-Src phosphorylation sites can be identified in all Ca\text{,}2\text{,}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\textsuperscript{2\text+} channel \(\alpha\text{,}1\text{c} \text{subunit (Dubuis et al., 2006)}.\) 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\(_{\text{A}}\)-mediated inhibition of Ca\text{,}2\text{,}2 channels by baclofen (Raingo et al., 2007) and Vc1.1 (Callaghan et al., 2008).

To date, GABA\(_{\text{A}}\)-mediated modulation of Ca\text{,}2\text{,}3 channels has not been reconstituted in expression systems, and c-Src phosphorylation of Ca\text{,}2\text{,}3 channels has not been demonstrated. Conserved Y residues within the e37 region can be identified across all Ca\text{,}2 family members and appear to be key substrates for phosphorylation by various tyrosine kinases. Our analysis of the e37 regions in Ca\text{,}2\text{,}1 and Ca\text{,}2\text{,}3 channels predicted Src kinase motifs in human Ca\text{,}2\text{,}3 channels. These motifs were absent in human Ca\text{,}2\text{,}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\text{,}2\text{,}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\text{,}2\text{,}3[e37a] channel, whereas such a motif is absent in Cav2.2[e37b]. Therefore, further studies are required to dissect the effects of Vc1.1 on Ca\text{,}2\text{,}2 channel splice variants. Our results also suggest that the effects of baclofen on Ca\text{,}2\text{,}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\text{,}2\text{,}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\text{,}2\text{,}3 channels in vivo.

**Therapeutic implications of Ca\text{,}2\text{,}3 channel inhibition**

Few studies have examined Cav2.3 (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\(_{\text{A}}\) 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\(_{\text{A}}\)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 GABA B receptors. We systematically examined the intracellular pathways and elucidated the molecular details that determine c-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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Figure S1. Voltage dependence of baclofen inhibition of human Cav2.3d (Y1765F) channel via GABAB<sub>R</sub> activation. In HEK cells transiently coexpressing Cav2.3d or Cav2.3d (Y1765F) channels and GABAB<sub>R</sub>s, EGTA does not affect the voltage dependence of inhibition in the absence or presence of 50 µM baclofen. (A and B) Representative I<sub>Ba</sub> traces in the absence (control) and presence of baclofen before (left) and after (middle) a depolarizing prepulse to +80 mV (inset protocols), with intracellular solution containing 0.5 or 10 mM EGTA. Currents were normalized to peak I<sub>Ba</sub> amplitude obtained with a prepulse in the absence of baclofen; dotted lines represent zero-current level. (Right) Summary of I<sub>Ba</sub> inhibition in the absence and presence of a prepulse, −PP and +PP, respectively. Data are mean ± SEM; the number of experiments is in parentheses. NS, +PP inhibition not significantly different from −PP inhibition. VI, voltage-independent fraction.

Table S1
Parameters derived from Boltzmann fits to I-V and G-V curves in Cav2.1/GABAB<sub>R</sub> cells in the presence of 0.5 or 10 mM EGTA in the intracellular recording solution

<table>
<thead>
<tr>
<th>EGTA</th>
<th>Condition</th>
<th>V&lt;sub&gt;0.5, act&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>k&lt;sub&gt;act&lt;/sub&gt;</th>
<th>V&lt;sub&gt;0.5, act&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>k&lt;sub&gt;b&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td></td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
</tr>
<tr>
<td>0.5</td>
<td>Control (5)</td>
<td>6.50 ± 0.9</td>
<td>4.4 ± 0.6</td>
<td>7.68 ± 0.4</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Baclofen (5)</td>
<td>7.97 ± 0.8</td>
<td>4.6 ± 0.3</td>
<td>8.91 ± 0.3</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>Control (6)</td>
<td>6.47 ± 0.6</td>
<td>4.3 ± 0.2</td>
<td>6.79 ± 0.3</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Baclofen (6)</td>
<td>7.67 ± 0.9</td>
<td>4.4 ± 0.4</td>
<td>8.37 ± 0.4</td>
<td>5.1 ± 0.2</td>
</tr>
</tbody>
</table>

The number of experiments is in parentheses. Values represent mean ± SEM.

<sup>a</sup>These parameters were obtained from Boltzmann fits of I-V relations.

<sup>b</sup>These parameters were obtained from Boltzmann fits of G-V relations.