Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Novel Mechanism of Voltage-Gated N-type ($Ca_v2.2$) Calcium Channel Inhibition Revealed Through α-Conotoxin Vc1.1 Activation of the $GABA_B$ Receptor

Thuan G. Huynh, Hartmut Cuny, Paul A. Slesinger and David J. Adams

Health Innovations Research Institute, RMIT University, Melbourne, Victoria 3083 Australia (T.G.H., H.C., D.J.A.)

Peptide Biology Laboratories, The Salk Institute for Biological Studies, La Jolla, CA 92037 USA (P.A.S.)

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Mechanism of $Ca_v2.2$ inhibition revealed by α-conotoxin Vc1.1

Corresponding author:

David J. Adams, Health Innovations Research Institute, RMIT University, PO Box 71,

Bundoora, Victoria 3083, Australia

Email: david.adams@rmit.edu.au

Number of text pages: 37

Number of Tables: 2

Number of figures: 9

Number of References: 60

Number of words in the Abstract: 190

Number of words in the Introduction: 475

Number of words in the Discussion: 1,111

List of non-standard abbreviations:
DRG, dorsal root ganglion
$GABA_BR$, γ-aminobutyric acid type B receptor
GIRK channel, G protein-gated inwardly rectifying potassium channel
GPCR, G protein-coupled receptor
HVA, high voltage-activated
IC_{50}, half-maximal inhibitory concentration
PCT, proximal carboxyl terminus
PTX, pertussis toxin
SSI, steady-state inactivation
$V_{0.5,\ act}$, half-maximal activation voltage
$V_{0.5,\ inact}$, half-maximal inactivation voltage

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Neuronal voltage-gated N-type ($Ca_v2.2$) calcium channels are expressed throughout the nervous system and regulate neurotransmitter release and hence synaptic transmission. They are predominantly modulated via G protein-coupled receptor activated pathways, and the well-characterized Gβγ subunits inhibit $Ca_v2.2$ currents. Analgesic α-conotoxin Vc1.1, a peptide from predatory marine cone snail venom, inhibits $Ca_v2.2$ channels by activating pertussis toxin (PTX)-sensitive $G_{i/o}$ proteins via the $GABA_B$ receptor ($GABA_BR$) and potently suppresses pain in rat models. Using a heterologous $GABA_BR$ expression system, electrophysiology and mutagenesis, we showed α-conotoxin Vc1.1 modulates $Ca_v2.2$ via a different pathway from that of the $GABA_BR$ agonists GABA and baclofen. In contrast to GABA and baclofen, Vc1.1 changes $Ca_v2.2$ channel kinetics by increasing the rate of activation and shifting its half-maximum inactivation to a more hyperpolarized potential. We then systematically truncated the $GABA_{B1a}$ C-terminus and discovered that by removing the proximal carboxyl terminus (PCT) of the $GABA_{B1a}$ subunit, Vc1.1 inhibition of $Ca_v2.2$ currents was significantly reduced. We propose a novel mechanism by which Vc1.1 activates $GABA_BR$ and requires the $GABA_{B1a}$ PCT domain to inhibit $Ca_v2.2$ channels. These findings provide important insights into how $GABA_BRs$ influence $Ca_v2.2$ channel inhibition and nociceptive transmission.

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

The G protein-coupled $GABA_B$ receptor ($GABA_BR$) is widely expressed in the brain and plays an important role in regulating neurotransmission. The neurotransmitter γ-aminobutyric acid (GABA) binds to $GABA_BRs$ leading to activation of $G_{i/o}$ G proteins, which dissociate into activated $G\alpha_{i/o}$-GTP and the Gβγ dimer. $G\alpha_{i/o}$ inhibits adenylyl cyclase while the Gβγ dimer inhibits high voltage-activated (HVA) calcium channels (Menon-Johansson et al., 1993; Ikeda, 1996; Sun and Chiu, 1999) and activates G protein-gated inwardly rectifying potassium (GIRK) channels (Luscher and Slesinger, 2010). The $GABA_BR$ is a potential target for drugs that treat neurological illnesses such as pain, epilepsy, spasticity and psychiatric disorders (Marshall et al., 1999; Bowery et al., 2002). For example, inhibition of presynaptic HVA calcium channels such as $Ca_v2.2$ has been linked to suppression of neurotransmitter release and block of nociception transmission (Winquist et al., 2005; Gribkoff, 2006).

A 16 amino acid peptide derived from venom of the marine cone snail *Conus victoriae,* α-conotoxin Vc1.1 (Clark et al., 2006), potently suppresses neuropathic pain when injected intramuscularly near the site of injury in rat models (Sandall et al., 2003; Satkunanathan et al., 2005; Klimis et al., 2011). Initially, the analgesic effect was attributed to inhibition of neuronal nicotinic acetylcholine receptors (Satkunanathan et al., 2005; Livett et al., 2006; Vincler et al., 2006). However, subsequent studies using rodent dorsal root ganglion (DRG) neurons demonstrated that the analgesic effect of Vc1.1 is more likely due to inhibition of $Ca_v2.2$ channels via $GABA_BR$ activation (Callaghan et al., 2008; Callaghan and Adams, 2010). In support of this, siRNA knockdown of $GABA_BR$ expression in DRG neurons significantly reduced Vc1.1-dependent inhibition of $Ca_v2.2$ (Cuny et al., 2012). In addition, Vc1.1 inhibition of $Ca_v2.2$ can be reconstituted in HEK293 cells co-expressing only $GABA_BRs$ and $Ca_v2.2$ channels (Cuny et al., 2012). Thus, Vc1.1 inhibition minimally requires both $GABA_BR$ subunits, G proteins and $Ca_v2.2$ channels. We have recently shown

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

that Vc1.1 also inhibits $Ca_v2.3$ but not $Ca_v2.1$ channels via $GABA_BR$ activation in HEK293 cells (Berecki et al., 2014). However, little is known about how Vc1.1 activates $GABA_BRs$.

Here, we examined the mechanism of Vc1.1 inhibition in a heterologous expression system that reconstitutes the human $Ca_v2.2/GABA_BR$ signaling pathway (Cuny et al., 2012). We provide further details of the mechanism by which Vc1.1 inhibits $Ca_v2.2$ channels via activation of $GABA_BR$. We discovered that Vc1.1 activation of $GABA_BRs$ occurs via a signaling pathway distinct from that of classical agonists, such as GABA and baclofen. Our findings support an emerging model of signaling bias, which results from allosteric ligand binding to G protein-coupled receptors (GPCRs) at sites different to endogenous ligand binding (Wootten et al., 2013). Numerous studies have shown that agonists do not uniformly activate cellular signaling pathways (biased signaling) and GPCRs produce multiple effector pathways due to their association with secondary messengers, such as G proteins and β-arrestin (Bohn and McDonald, 2010; Rajagopal et al., 2011).

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Clones, cell culture and transfections

Human $GABA_{B1}$ and $GABA_{B2}$ subunit cDNA clones were obtained from OriGene Technologies, Inc., Rockville, MD. Rat $Ca_v2.2$ channel, α_{1B} splice variant e37a and auxiliary subunit β_3 were provided by Dr Diane Lipscombe (Brown University), and the auxiliary subunit $\alpha_2\delta_1$ by Dr Gerald W. Zamponi (University of Calgary). Rat wild-type $GABA_BR$ and the mutant $GABA_{B1a}$-ΔPCT, $GABA_{B1a}$-Δ887, $GABA_{B1a}$-Δ863 (Laviv et al., 2011) and $GABA_{B2}$-R576D (Binet et al., 2007; Boyer et al., 2009) constructs have been described previously. Site-directed mutagenesis on $GABA_{B1a}$ was carried out using the GENEART® Site-Directed Mutagenesis System Kit (Invitrogen Life Technologies) with the following primers: 5'-TCTCATATGGCTCCAGTGCACCAGCCTTGTCAAACCG-3' and 5'-CGGTTTGACAAGGCTGGTGCACTGGAGCCATATGAGA-3' for rat $GABA_{B1a}$-S270A; 5'-CCTTATGCCTGGCTGCGCCTCTGTCTCCACGCTGG-3' and 5'-CCAGCGTGGAGACAGAGGCGCAGCCAGGCATAAGG-3' for human $GABA_{B1a}$-S246A. Correct mutation of the cDNAs was verified by automated DNA sequencing (Australian Genome Research Facility, Melbourne, Australia).

Human Embryonic Kidney 293 cells expressing the SV40 large T-antigen (HEK293T) were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen Australia Pty Ltd., Mt. Waverley, Victoria, Australia) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Australia), 50 IU/ml penicillin and 50 µg/ml streptomycin (Invitrogen, Australia) at 37°C in 5% CO_2. HEK293 cells stably expressing human $Ca_v2.2$ channels (α_{1B}, $\alpha_2\delta1$ and β_3 subunits) were obtained from Merck and cultured according to procedures described previously (Dai et al., 2008).

HEK293T cells were transiently co-transfected by calcium phosphate transfection, as described previously (Cuny et al., 2012), with plasmid cDNAs (3 µg) encoding the rat $Ca_v2.2$

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

channel α_{1B}, $\alpha_2\delta1$ and β_{1b} auxiliary subunits, 5 µg rat wild-type or mutant $GABA_{B1a}R$, $GABA_{B2}R$ and 0.1 µg eGFP. Stable $Ca_v2.2$ expressing HEK293 cells were seeded into 12-well plates and transiently co-transfected with human $GABA_{B1}$ and $GABA_{B2}$ subunits (2 µg each) and 0.2 µg eGFP using Lipofectamine (Invitrogen) according to the manufacturer's protocol.

Two to three days after transfection cells were seeded onto glass coverslips and incubated at 37°C in 5% CO_2 for at least 6 hours before recording. Effects of Vc1.1 or baclofen on the percentage of peak current inhibition were independent of either transfection method or cell line (transiently or stably expressed $Ca_v2.2$) used.

Electrophysiological patch clamp recording

Whole-cell patch clamp recording from transfected HEK293 cells has been described previously (Cuny et al., 2012). Briefly, HEK293 cells were superfused with a solution containing (in mM): NaCl 90, $BaCl_2$ 10, CsCl 5, TEA-Cl 30, $MgCl_2$ 1, D-glucose 10 and HEPES 10, pH 7.4 with TEA-OH. Fire-polished borosilicate patch pipettes (2–3 MΩ tip resistance) were filled with a solution containing (in mM): K-gluconate 120, NaCl 5, $MgCl_2$ 2, EGTA 5, MgATP 2, Na_2GTP 0.6 and HEPES 10, pH 7.2 with CsOH. Typically, cells were voltage-clamped at –90 mV (adjusted for liquid junction potential) and membrane currents elicited by 200 ms step depolarizations to either +5 mV or +10 mV at 0.1 or 0.05 Hz. Whole-cell patch clamp recordings were made using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), controlled by a Clampex 10/ DigiData1332 acquisition system at room temperature (22–23°C). Membrane currents were filtered at 1 or 2 kHz and sampled at 5 or 8 kHz. Leak and capacitive currents were subtracted using a −P/4 pulse protocol. Data were stored digitally on a computer for further analysis. Peak current amplitude in response to the depolarizing pulse was measured once a steady state was achieved (3–5 min).

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Data analysis and statistics

Data were analyzed using Clampfit 10 software (Molecular Devices), 2007 Excel (Microsoft) and Prism 6 (GraphPad Software Inc., La Jolla, CA). Peak current amplitude in response to a depolarization step was calculated and plotted against time. A steady-state peak current was recorded for at least 2–3 min before the drug was applied.

Activation curves were plotted from the I–V relationship by calculating conductance using the equation:

$G = I/(V_t - E_{rev})$

where G is the conductance, V_t the test potential, and E_{rev} the extrapolated reversal potential. The voltage dependence of the peak $Ca_v2.2$ current density was fitted with the Boltzmann function:

$I = G_{max}(V_t - E_{rev})/(1+\exp[(V_{0.5}-V_t)/k])$

where G_{max} is the maximum conductance, $V_{0.5}$ the midpoint or half-maximal activation, and *k* the slope factor.

For steady-state inactivation, data was fitted with a single Boltzmann function:

$I = 1/[1+ \exp((V_{0.5,\ inact} - V_t)/k)]$

where $V_{0.5,\ inact}$ is the midpoint or half-maximal inactivation, and *k* the slope factor.

Using Clampfit 10 (Molecular Devices), activation kinetics were determined from a single exponential fit of the current activating phase. Concentration–response relationships were obtained by plotting normalized current amplitude as a function of drug concentration, and fitted using a logistic equation. All voltage potentials were adjusted for liquid junction potential. Unless stated otherwise, all data are represented as mean ± SEM. The statistical significance between two groups was evaluated by student's *t*-test. One-way analysis of

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

variance (ANOVA) followed by Bonferroni's post hoc test was used to compare three or more groups. Differences were considered statistically significant if $P < 0.05$.

Chemicals and drugs

α-Conotoxin Vc1.1 was synthesized as described previously (Schnolzer et al., 1992; Luo et al., 1998; Clark et al., 2006; Clark et al., 2008). It was provided as a stock concentration of ~ 1 mM in H_2O by Dr Ray Norton (Monash University, Melbourne). Baclofen, GABA and pertussis toxin (PTX) were purchased from Sigma-Aldrich and CGP54626 [*S*-(*R**,*R**)][-3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl]([3,4]-cyclohexylmethyl) phosphinic acid hydrochloride was purchased from Tocris Bioscience (Bristol, UK). All drugs were diluted to the appropriate final concentration and applied via perfusion in the bath solution. Vc1.1 was applied at a concentration of 200 nM for maximum response. As a positive control for functional expression of $GABA_BR$ with $Ca_v2.2$ channels, baclofen was applied after Vc1.1 application. Only cells responding to baclofen with at least 50% peak current inhibition were included in our analysis.

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Comparison of Vc1.1-dependent inhibition with canonical GABA-dependent inhibition of $Ca_v2.2$ channels

Confirming previous reports (Cuny et al., 2012), bath application of Vc1.1 (200 nM) reduced peak Ba^{2+} current (I_{Ba}) amplitude by 45 ± 7% (n = 9) in HEK293 cells stably expressing human $Ca_v2.2$ (α_{1B}, β_{1b} and $\alpha_2\delta$) and co-expressing human $GABA_{B1a/B2}$ receptors (Fig. 1A,B). The concentration-response relationship for Vc1.1 inhibition of $Ca_v2.2$ has an IC_{50} of 19.1 nM, which is approximately ten-fold higher than that reported for rat DRG neurons (Callaghan et al., 2008) (Fig. 1D). This shift most likely reflects differences between human and rat $GABA_BR$. However, typically 200 nM Vc1.1 was used as it gave maximal response with either rat or human $GABA_BR$. Inhibition of I_{Ba} by baclofen was maximal in < 1 min and rapidly reversed upon washout. By contrast Vc1.1 inhibition was slower, reaching steady-state after ~15 min. Furthermore, Vc1.1 inhibition of I_{Ba} was not reversed following washout of Vc1.1 peptide but the amplitude was subsequently further reduced by application of baclofen (Fig. 1B,C). Taken together, these results suggest Vc1.1 inhibition of $Ca_v2.2$ channels occurs via a signaling pathway different from GABA/baclofen-mediated inhibition.

Baclofen and GABA both bind to the extracellular Venus Fly Trap of the $GABA_{B1}$ subunit (Galvez et al., 1999; Galvez et al., 2000). Therefore we introduced an alanine substitution at serine 270 of the $GABA_{B1a}$ subunit to test whether Vc1.1 interacts with this binding site. S270A was shown previously to substantially reduce agonist and antagonist binding affinities to the rat $GABA_BR$ (Galvez et al., 1999). As expected, S270A significantly reduced baclofen inhibition of $Ca_v2.2$-mediated currents (14.7 ± 3.0%, n = 8) compared with wild-type $GABA_BR$ (43.3 ± 8.6%, n = 6; P < 0.05). However, the S270A mutation in $GABA_{B1a}$ did not significantly alter Vc1.1-dependent inhibition of $Ca_v2.2$ currents (47.1 ± 14.4%, n = 7), and was similar to wild-type $GABA_BR$ (52.3 ± 8.6%, n = 6; P = 0.75) (Fig. 2).

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

We therefore tested another mutation, S246A, in the Venus Flytrap domain of $GABA_{B1a}$, which causes complete insensitivity to baclofen and GABA (Galvez et al., 2000). Expression of $GABA_{B1a}$-S246A with $GABA_{B2}$ abolished baclofen-dependent inhibition of $Ca_v2.2$ currents (2.4 ± 4.8%, *n* = 6), as compared with wild type $GABA_BR$ (76.9 ± 12.5%, *n* = 5). In contrast, Vc1.1 continued to inhibit $Ca_v2.2$ currents in cells co-expressing $GABA_{B1a}$-S246A/$GABA_{B2}$ (26.6 ± 7.9%, *n* = 6). Inhibition was to a similar extent as that observed at wild type $GABA_BR$ (30.4 ± 1.8%, *n* = 4), supporting our findings obtained with the S270A mutant. Vc1.1 inhibition of $Ca_v2.2$, however, was strictly dependent on co-expression with the $GABA_BR$ subunits (Fig. 1C) and consistent with previous results (Cuny et al., 2012). These findings raise the possibility that Vc1.1 interacts with a different region of the $GABA_BR$. In rat DRG neurons, the selective $GABA_BR$ antagonists CGP54626 and CGP55845 blocked Vc1.1 inhibition of $Ca_v2.2$ currents (Callaghan et al., 2008), suggesting that these receptor antagonists interfere with Vc1.1 binding. In HEK293 cells expressing $GABA_{B1}$-S270A, CGP54626 was inactive and unable to antagonize Vc1.1 inhibition of $Ca_v2.2$ (*n* = 3; data not shown). This is consistent with previous studies showing the S270A mutation prevents binding of CGP54626 to $GABA_BR$ (Galvez et al., 1999; Geng et al., 2013) and suggests that CGP54626 and CGP55845 occlude Vc1.1 binding to a site physically distinct from S270 and S246 of $GABA_{B1}$.

$GABA_BR$-dependent inhibition of voltage-gated calcium channels has been examined extensively (Tedford and Zamponi, 2006). This form of inhibition typically involves Gβγ subunits binding to Ca_v channels, shifting the voltage-dependence of activation and slowing the activation kinetics. In addition, prepulse potentiation can antagonize Gβγ-dependent inhibition of Ca_v channels (Ikeda, 1996; Weiss et al., 2007). We next examined if these features were also observed with Vc1.1-dependent inhibition. The voltage-dependence and kinetics of activation/inactivation of $Ca_v2.2$ channels were analyzed in the presence of Vc1.1

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

or baclofen. Similar to previous studies (Jones et al., 1997; Colecraft et al., 2000), baclofen shifted the half-maximum activation ($V_{0.5, act}$) of the conductance-voltage relationship by +10.0 mV (+3.7 ± 1.5 mV, *n* = 7) as compared to that of control (–6.3 ± 0.7 mV, *n* = 8) (Fig 3A and Table 1). By contrast, Vc1.1 did not alter the I–V relationship or voltage-dependence of activation with $V_{0.5, act}$ of –5.1 ± 0.9 mV (*n* = 6) (Fig. 3A and Table 1). We also analyzed the activation curves generated from $Ca_v2.2$ tail currents in the presence of baclofen or Vc1.1 (Fig. 3B). The normalized peak tail current amplitudes were fitted with the sum of two Boltzmann functions (Lin et al., 2004; Castiglioni et al., 2006). In the presence of baclofen, $Ca_v2.2$ channels were activated at significantly more depolarized voltages than in control conditions. Baclofen, but not Vc1.1, caused a shift in both the $V_{0.5}$ and *k* of each component of the Boltzmann fit (Table 1 and Fig. 3B).

We next examined whether Vc1.1 inhibition altered the voltage-dependence of steady-state inactivation (SSI) of $Ca_v2.2$. We used a 10 s prepulse at varying potentials to evaluate SSI. This prepulse duration was sufficient to allow complete recovery from inactivation. The inactivation curve obtained in the presence of Vc1.1 gave a half-maximal inactivation ($V_{0.5, inact}$) of –78.8 ± 0.5 mV (*n* = 10), ~13 mV more hyperpolarized than that of control (–65.5 ± 0.6 mV, *n* = 8; $P < 0.05$) (Table 1 and Fig. 3C). Because of this shift in voltage, we examined the extent of Vc1.1-dependent inhibition produced by the shift in SSI. Therefore, we investigated SSI in individual HEK293 cells with pre-pulses of –110, –80 and –50 mV in the absence and presence of Vc1.1. Vc1.1 significantly reduced P2/P1 at both –50 mV and –110 mV ($P < 0.01$, paired *t*-test) (Fig. 3D). The shift in voltage-dependence of inactivation over a wide range of pre-pulse potentials may account, in part, for Vc1.1 inhibition of $Ca_v2.2$ channels. Thus, Vc1.1 significantly affected SSI by shifting the inactivation curve to more hyperpolarized potentials.

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

We next examined the kinetics of activation after Vc1.1 inhibition of $Ca_v2.2$ channels. We measured the time constant of activation (τ) of $Ca_v2.2$ current in the absence (control) and then presence of baclofen or Vc1.1 (Fig. 4A,B). Baclofen (50 μM) significantly increased τ from 1.63 ± 0.18 ms to 2.13 ± 0.36 ms (n = 7; P = 0.022, paired t-test) (Fig. 4C). These values were comparable to those in previous reports (Bean, 1989; Toselli and Taglietti, 1993). Studies using prepulse facilitation to examine the involvement of $G\beta\gamma$ in Ca_v2 modulation also reported similar changes in kinetics of activation (Doupnik and Pun, 1994; Ikeda, 1996). In contrast, Vc1.1 (100 nM), significantly reduced τ from 1.69 ± 0.25 ms to 1.26 ± 0.23 ms (n = 8; $P < 0.05$). Thus, these results show that Vc1.1 inhibition produces the opposite effect on the kinetics of $Ca_v2.2$ activation; baclofen slows whereas Vc1.1 speeds activation.

Previous studies suggested that binding of $G\beta\gamma$ to the pore-forming $Ca_v\alpha$ subunit and the subsequent slowing of activation are reversed by a strong depolarizing prepulse (Bean, 1989; Ikeda, 1996; Furukawa et al., 1998; Zamponi and Currie, 2012). We therefore compared the effect of prepulse facilitation on GABA- and Vc1.1-dependent inhibition of $Ca_v2.2$ channels. In the absence of agonist, a prepulse (*pp*) to +120 mV led to an increase in the current amplitude evoked by the test pulse (20.9 ± 1.9%, n = 6), compared with the current amplitude recorded in the absence of a prepulse. This observation suggests some basal inhibition of $Ca_v2.2$ occurs in HEK293 cells similar to previous reports of $G\beta\gamma$ modulation of $Ca_v2.2$ in the absence of GPCR activation (Meza and Adams, 1998; McDavid and Currie, 2006). As expected, the prepulse to +120 mV significantly relieved GABA-dependent inhibition of peak current from 64 ± 4% [–*pp*] to 4 ± 5% [+*pp*] (n = 8; $P < 0.05$) (Fig. 5B). Remarkably, a prepulse to +120 mV did not significantly relieve Vc1.1 inhibition, which was 50 ± 11% [–*pp*] compared with 36 ± 6% [+*pp*] (n = 5; P = 0.283) (Fig. 5D). Taken together, these experiments demonstrate Vc1.1 inhibition differs from that of

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

GABA/baclofen modulation of $Ca_v2.2$ channels in voltage-dependence of activation and inactivation, time course of activation, and sensitivity to recovery from prepulse potentiation. We therefore suggest that Vc1.1 inhibits $Ca_v2.2$ channels via a novel mechanism that does not involve Gβγ G proteins.

Unique role for $G_{i/o}$ G proteins in Vc1.1 inhibition of $Ca_v2.2$ channels

To elucidate the signaling pathway(s) underlying Vc1.1-dependent inhibition of $Ca_v2.2$ channels, we first explored the role of PTX-sensitive $G_{i/o}$ G proteins. $G_{i/o}$ G proteins are essential components of GABA inhibition of $Ca_v2.2$ channels via $GABA_B$Rs (Tedford and Zamponi, 2006; Katada, 2012). PTX ADP-ribosylates the $G\alpha_{i/o}$ subunit, uncoupling it from the receptor (Katada, 2012). To examine the potential role of $G_{i/o}$ G proteins in Vc1.1-dependent inhibition of $Ca_v2.2$, we treated HEK293 cells co-expressing human $GABA_B$Rs and $Ca_v2.2$ channels with PTX (3 μg/ml) for 16 hours. As expected, PTX almost completely prevented baclofen-dependent inhibition of $Ca_v2.2$ currents (5.0 ± 2.3%, $n = 4$), as compared to untreated controls (70.7 ± 2.3%, $n = 11$; $P < 0.05$, unpaired *t*-test). Similarly, PTX attenuated Vc1.1 (200 nM) inhibition of peak $Ca_v2.2$ currents (0.9 ± 3.5%, $n = 6$) compared with control (42.0 ± 5.7%, $n = 7$; $P < 0.05$, unpaired *t*-test) (Fig. 6). Therefore, it appears Vc1.1 inhibition of $Ca_v2.2$ channels requires $G_{i/o}$ G protein coupling with $GABA_B$Rs (see also Callaghan et al., 2008).

We next examined whether activating $G\alpha_{i/o}$ through the $GABA_B$R is also needed for Vc1.1-dependent inhibition of $Ca_v2.2$ channels. Previously, Binet et al. (2007) demonstrated that an arginine to aspartate mutation in the third transmembrane segment (TM3) of $GABA_{B2}$ dramatically reduces G protein activation. Consistent with such an effect, baclofen inhibition of peak current amplitude in HEK293 cells co-expressing $GABA_{B2}$-R576D/$GABA_{B1a}$ and $Ca_v2.2$ channels was decreased to 11.2 ± 4.3% ($n = 7$), compared to control peak current

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

amplitude of 64.7 ± 4.0% (n = 6; P < 0.05). Surprisingly, Vc1.1-dependent inhibition persisted in the presence of $GABA_{B2}$-R576D. Here, Vc1.1 decreased peak current amplitude by 35.6 ± 8.8% (n = 8) and this did not differ significantly from that of wild-type $GABA_BR$ (52.9 ± 8.9%, n = 6; P = 0.204) (Fig. 7). Thus, Vc1.1 inhibition of $Ca_v2.2$ channel current involves activation of $GABA_BRs$ through a novel mechanism that requires $G\alpha_{i/o}$ G proteins (i.e., PTX-sensitive) but not the canonical pathway for stimulating $G\alpha_{i/o}$.

Important role for the C-terminal domain of the $GABA_{B1a}$ subunit in Vc1.1 inhibition of $Ca_v2.2$ channels

The observation that Vc1.1 inhibition occurred even with the R576D mutation in $GABA_{B2,}$ shifted our focus to other regions of the $GABA_BR$. The C-terminal domain of the $GABA_{B1a}$ subunit contains an endoplasmic reticulum (ER) retention signal, a coiled-coil domain for binding with $GABA_{B2}$ and shielding the ER retention signal, and a putative domain in the proximal C-terminus (PCT) that interacts with Ca_v channels (Laviv et al., 2011). To examine the role of the coiled-coil domain in Vc1.1 inhibition, we truncated the C-terminus of the $GABA_{B1a}$ receptor subunit from S887 ($GABA_{B1a}$-Δ887). Compared to wild-type $GABA_BR$, co-expression of $GABA_{B1a}$-Δ887 with $GABA_{B2}$ and $Ca_v2.2$ channels did not significantly alter Vc1.1-dependent inhibition of peak current (37.9 ± 5.6%, n = 11 compared to 24.9 ± 6.9%, n = 7; P = 0.171, unpaired t-test). Similarly, baclofen inhibition of peak current was not affected by the $GABA_{B1a}$ Δ887 deletion (62.0 ± 7.3%, n = 7 for Δ877 compared with control 57.5 ± 5.2%, n = 11; P = 0.613) (Fig. 8B,E). Truncating the $GABA_{B1a}$ C-terminal domain at G863 (Δ863), which removes the PCT as well as the coiled-coil domain, significantly reduced Vc1.1 inhibition of peak current (9.5 ± 4.0%, n = 12 for Δ863 compared with control 37.9 ± 5.6%, n = 11; P < 0.05) (Fig. 8C,E). However, baclofen-dependent inhibition was unaffected by the truncation at G863 (Fig. 8C,E).

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

To further explore the role of the PCT, we examined the effect of specifically deleting 21 amino acids within this domain (R857 – S877; ΔPCT) on Vc1.1 inhibition. In HEK293 cells co-expressing $Ca_v2.2$ and $GABA_{B1a}$-ΔPCT/$GABA_{B2}$-wt, Vc1.1 inhibition of peak current was significantly less (5.3 ± 3.9%, $n = 5$) than that of cells co-expressing wild-type $GABA_BR$ (37.9 ± 5.7 %; $n = 11$; $P < 0.05$). In contrast, baclofen inhibition of peak current in cells expressing the $GABA_{B1a}$-ΔPCT mutant was unchanged (60 ± 2.7%, $n = 5$ for ΔPCT compared with 57.5 ± 5.2% for control, $n = 11$; $P > 0.05$) (Fig. 8D,E). These results suggest that the proximal C-terminal domain of $GABA_{B1a}$ is involved in Vc1.1-dependent inhibition of $Ca_v2.2$ channels.

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

α-Conotoxin Vc1.1 and GABA/baclofen differentially inhibit $Ca_v2.2$

The $GABA_BR$ agonists, GABA and baclofen, are known to bind to the $GABA_{B1}$ Venus Fly Trap, activating the heterodimer of $GABA_{B1a/B2}$ and inhibiting $Ca_v2.2$ channels via a pathway involving Gβγ subunits (Galvez et al., 1999). Binding of Gβγ to the $Ca_v\alpha_{1B}$ pore shifts the channels from 'willing' to 'reluctant' gating states, thus changing the channel's inactivation to a hyperpolarized potential and slowing the rate of activation to reduce peak current (Ikeda, 1996). Prepulse potentiation can recover the inhibition produced by Gβγ subunits (Zamponi and Currie, 2013). Using an established HEK293 system that co-expresses $GABA_BR$ and $Ca_v2.2$ channels (Cuny et al., 2012), we discovered the mode of Vc1.1 inhibition is distinctly different from that of GABA/baclofen inhibition of $Ca_v2.2$ channels.

First, GABA and baclofen inhibition of $Ca_v2.2$ via $GABA_BR$ activation is predominantly voltage-dependent (Ikeda, 1996); that is, activation is slowed and shifted to more depolarized potentials. In comparison, Vc1.1 inhibition of $Ca_v2.2$ channels does not shift the voltage-dependence of activation, regardless of whether or not Gβγ is activated, and the kinetics of activation are accelerated following Vc1.1 inhibition. Second, Vc1.1 inhibition of $Ca_v2.2$ via $GABA_BR$ activation cannot be reversed by peptide washout or strong depolarization. These findings indicate that Vc1.1 inhibition of $Ca_v2.2$ channels does not occur via the traditional pathway of activated Gβγ voltage-dependent inhibition.

The slow onset and recovery of Vc1.1 inhibition of $Ca_v2.2$-mediated currents are suggestive of a phosphorylation-dependent mechanism, similar to that of tyrosine kinase-dependent Gβγ-independent inhibition of $Ca_v2.2$-e37a (Gray et al., 2007; Raingo et al., 2007). The $Ca_v2.2$-e37a splice variant is predominantly expressed in nociceptive DRG neurons where it is implicated in nociception transmission. The more prevalent $Ca_v2.2$-e37b variant is widely distributed in the central nervous system (Altier et al., 2007). Interestingly, the one

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

known underlying cause of decreased morphine efficacy is thought to be due to altered $Ca_v2.2$-e37a to $Ca_v2.2$-e37b ratios in DRG neurons (Andrade et al., 2010). However, Vc1.1 affects both $Ca_v2.2$ splice variants as inhibition was observed in both the stable $Ca_v2.2$ cell line (expressing e37b) and in cells transiently co-transfected with $Ca_v2.2$-e37a. This rules out a role for tyrosine kinase phosphorylation at exon 37a in Vc1.1 inhibition. Another potential mechanism of action is $GABA_BR$-activated internalisation of the $GABA_BR$–$Ca_v2.2$ complex. However, it was recently shown that β-arrestin, which is important for receptor internalisation, is not bound to $GABA_BR$ (Sudo et al., 2012). Furthermore, surface biotinylation showed that $GABA_BR$ cell surface expression was not affected by exposure to Vc1.1 (Cuny et al., 2012).

However, one common feature of GABA/baclofen- and Vc1.1-dependent inhibition is the requirement for $G_{i/o}$ G proteins. Pre-treatment with PTX abolished Vc1.1 inhibition of $Ca_v2.2$ channels, indicating that $G_{i/o}$ proteins may be involved. Moreover, we showed previously that GDP-β-S also prevented Vc1.1 inhibition of HVA calcium channel currents (Callaghan et al., 2008; Berecki et al., 2014). However, Vc1.1 inhibited $Ca_v2.2$ channels in cells co-expressing $Ca_v2.2$ and $GABA_BR$ constructs with known GABA/baclofen binding sites removed ($GABA_{B1}$-S270A, $GABA_{B1}$-S246A) or that reduce activation of G proteins ($GABA_{B2}$-R576D). Thus, we conclude that PTX-sensitive G proteins are required for Vc1.1 inhibition but that Vc1.1 appears to act as an allosteric modulator, similar to the biased signaling mechanisms described for other receptors (Wootten et al., 2013; Wisler et al., 2014).

Vc1.1 inhibition of $Ca_v2.2$ via $GABA_BR$ activation is dependent on the $GABA_{B1a}$ subunit PCT domain

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Our findings raise the question of how Vc1.1 activates $GABA_BR$ to inhibit $Ca_v2.2$ channels without activating G proteins. One explanation may be direct contact between the $GABA_BR$ and $Ca_v2.2$ and such interaction could involve G proteins. $GABA_BR$ physically interacts with the GIRK channel to modulate its function via G proteins (Fowler et al., 2007; Ciruela et al., 2010). However, Vc1.1 does not appear to activate GIRK channels (T. Huynh and P. Slesinger, unpublished observation; see also McIntosh et al., 2009).

Laviv et al. (2011) showed functional FRET coupling of $GABA_BR$ with $Ca_v2.2$ via G proteins. This was dependent on the PCT domain of the $GABA_{B1a}$ subunit (Laviv et al., 2011). Also, heterodimeric $GABA_B$ receptors can form higher-ordered oligomers that may regulate $GABA_BR$ function (Comps-Agrar et al., 2011). We hypothesize that $GABA_BR$ and $Ca_v2.2$ form a complex with G proteins and this interaction is dependent on the $GABA_{B1a}$ PCT domain (Fig. 9). Interestingly, deleting the $GABA_{B1a}$ PCT domain reduced Vc1.1, but not GABA/baclofen, inhibition of $Ca_v2.2$ channels. This suggests the PCT domain may couple $GABA_BR$ and $Ca_v2.2$ during Vc1.1 activation of $GABA_BRs$. The PCT domain may act as the anchoring site for the channel and if so, its deletion would prevent the formation of a $GABA_BR$ – $Ca_v2.2$ channel complex. In hippocampal neurons, the PCT domain of the $GABA_B$ receptor was essential for formation of a $Ca_v2.2$ and G protein complex (Laviv et al., 2011). We speculate the PCT domain serves the same role for enabling Vc1.1 inhibition. It will be interesting to investigate whether Vc1.1 produces a decrease in FRET between $GABA_BR$ PCT and $Ca_v2.2$, indicating coupling and physical contact between GPCR and channel.

Physiological significance of bias to $GABA_BR$ signaling

Our results show the PCT domain of the $GABA_{B1a}$ subunit is integral to α-conotoxin Vc1.1 activation of $GABA_BR$ and inhibition of $Ca_v2.2$ channels. In contrast, the PCT is not required

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

for GABA/baclofen Gβγ-facilitated channel inhibition. $GABA_BR$-biased signaling upon Vc1.1 activation is a newly identified mechanism for presynaptic inhibition of neurotransmitter release at central synapses. A comparison of baclofen and Vc1.1-mediated inhibition of $Ca_v2.2$ via $GABA_BR$ activation is summarized in Table 2. It is also distinct from that recently reported for $Ca_v2.3$ inhibition by α-conotoxin Vc1.1. $Ca_v2.3$ inhibition is voltage-independent and mediated by specific c-Src kinase-dependent phosphorylation sites in the C-terminus of the channel (Berecki et al., 2014).

$GABA_BRs$ are widely distributed in the central and peripheral nervous systems. Baclofen, a selective $GABA_BR$ agonist, has been used to treat various neurological disorders, including spasticity, chronic pain and alcoholism (Hudgson and Weightman, 1971; Hering-Hanit, 1999; Balerio and Rubio, 2002; Garbutt and Flannery, 2007). However, it causes numerous side effects due to its indiscriminate activation of multiple $GABA_BR$ signaling pathways (Bowery, 2007). In contrast, α-conotoxin Vc1.1 activates a distinct voltage-independent $GABA_BR$-$Ca_v2.2$ signaling pathway that requires the $GABA_{B1a}$ PCT domain to couple $GABA_BR$ to $Ca_v2.2$ ultimately inhibiting $Ca_v2.2$ channels. This $GABA_BR$-biased signaling mechanism likely contributes to the superior selectivity and analgesic properties of Vc1.1 (Klimis et al., 2011).

Our enhanced understanding of the molecular mechanism of Vc1.1 inhibition of $Ca_v2.2$ channels and its analgesic properties, along with the fact that it did not cause observable side effects in animal models of chronic pain, provides a platform from which analgesic conotoxins could be developed into safe and effective drugs (Satkunanathan et al., 2005; Vincler et al., 2006; Klimis et al., 2011).

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions

Participated in research design: Adams, Slesinger, Huynh, Cuny

Conducted experiments: Huynh, Cuny

Contributed new reagents or analytical tools: Adams, Slesinger

Performed data analysis: Huynh, Cuny, Adams

Wrote or contributed to the writing of the manuscript: Huynh, Cuny, Slesinger, Adams

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

References

Altier C, Dale CS, Kisilevsky AE, Chapman K, Castiglioni AJ, Matthews EA, Evans RM, Dickenson AH, Lipscombe D, Vergnolle N, and Zamponi GW (2007) Differential role of N-type calcium channel splice isoforms in pain. *J Neurosci* **27**: 6363-6373.

Andrade A, Denome S, Jiang YQ, Marangoudakis S, and Lipscombe D (2010) Opioid inhibition of N-type Ca^{2+} channels and spinal analgesia couple to alternative splicing. *Nat Neurosci* **13**: 1249-1256.

Balerio GN and Rubio MC (2002) Baclofen analgesia: involvement of the GABAergic system. *Pharmacol Res* **46**: 281-286.

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* **340**: 153-156.

Berecki G, McArthur JR, Cuny H, Clark RJ, and Adams DJ (2014) Differential $Ca_v2.1$ and $Ca_v2.3$ channel inhibition by baclofen and α-conotoxin Vc1.1 via $GABA_B$ receptor activation. *J Gen Physiol* **143**: 465-479.

Binet V, Duthey B, Lecaillon J, Vol C, Quoyer J, Labesse G, Pin JP, and Prezeau L (2007) Common structural requirements for heptahelical domain function in class A and class C G protein-coupled receptors. *J Biol Chem* **282**: 12154-12163.

Bohn LM and McDonald PH (2010) Seeking ligand bias: Assessing GPCR coupling to β-Arrestins for drug discovery. *Drug Discov Today Technol* **7**: e37-e42.

Bowery N (2007) $GABA_B$ receptor as a potential therapeutic target, in *The GABA Receptors* (Enna SJ and Möhler H eds) pp 289-312, Humana Press.

Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, and Enna SJ (2002) International Union of Pharmacology. XXXIII. Mammalian γ-aminobutyric acid(B) receptors: structure and function. *Pharmacol Rev* **54**: 247-264.

Boyer SB, Clancy SM, Terunuma M, Revilla-Sanchez R, Thomas SM, Moss SJ, and Slesinger PA (2009) Direct interaction of $GABA_B$ receptors with M2 muscarinic receptors enhances muscarinic signaling. *J Neurosci* **29**:15796-15809.

Callaghan B and Adams DJ (2010) Analgesic α-conotoxins Vc1.1 and RgIA inhibit N-type calcium channels in sensory neurons of α9 nicotinic receptor knockout mice. *Channels* **4**: 51-54.

Callaghan B, Haythornthwaite A, Berecki G, Clark RJ, Craik DJ, and Adams DJ (2008) Analgesic α-conotoxins Vc1.1 and Rg1A inhibit N-type calcium channels in rat sensory neurons via $GABA_B$ receptor activation. *J Neurosci* **28**: 10943-10951.

Castiglioni AJ, Raingo J, and Lipscombe D (2006) Alternative splicing in the C-terminus of $Ca_V2.2$ controls expression and gating of N-type calcium channels. *J Physiol* **576**: 119-134.

Ciruela F, Fernandez-Duenas V, Sahlholm K, Fernandez-Alacid L, Nicolau JC, Watanabe M and Lujan R (2010) Evidence for oligomerization between $GABA_B$ receptors and GIRK channels containing the GIRK1 and GIRK3 subunits. *Eur J Neurosci* **32**: 1265-1277.

Clark RJ, Daly NL, Halai R, Nevin ST, Adams DJ, and Craik DJ (2008) The three-dimensional structure of the analgesic α-conotoxin, RgIA. *FEBS Lett* **582**: 597-602.

Clark RJ, Fischer H, Nevin ST, Adams DJ, and Craik DJ (2006) The synthesis, structural characterization, and receptor specificity of the α-conotoxin Vc1.1. *J Biol Chem* **281**: 23254-23263.

Colecraft HM, Patil PG and Yue DT (2000) Differential occurrence of reluctant openings in G-protein–inhibited N- and P/Q-Type calcium channels. *J Gen Physiol* **115**: 175-192.

Comps-Agrar L, Kniazeff J, Norskov-Lauritsen L, Maurel D, Gassmann M, Gregor N, Prezeau L, Bettler B, Durroux T, Trinquet E, and Pin JP (2011) The oligomeric state sets $GABA_B$ receptor signalling efficacy. *EMBO J* **30**: 2336-2349.

Cuny H, de Faoite A, Huynh TG, Yasuda T, Berecki G, and Adams DJ (2012) γ-Aminobutyric acid type B ($GABA_B$) receptor expression is needed for inhibition of N-type ($Ca_v2.2$) calcium channels by analgesic α-conotoxins. *J Biol Chem* **287**: 23948-23957.

Dai G, Haedo RJ, Warren VA, Ratliff KS, Bugianesi RM, Rush A, Williams ME, Herrington J, Smith MM, McManus OB, and Swensen AM (2008) A high-throughput assay for evaluating state dependence and subtype selectivity of Ca_v2 calcium channel inhibitors. *Assay Drug Dev Technol* **6**: 195-212.

Doupnik CA and Pun RY (1994) G-protein activation mediates prepulse facilitation of Ca^{2+} channel currents in bovine chromaffin cells. *J Membr Biol* **140**: 47-56.

Fowler CE, Aryal P, Suen KF, and Slesinger PA (2007) Evidence for association of $GABA_B$ receptors with Kir_3 channels and regulators of G protein signalling (RGS_4) proteins. *J Physiol* **580**: 51-65.

Furukawa T, Nukada T, Mori Y, Wakamori M, Fujita Y, Ishida H, Fukuda K, Kato S, and Yoshii M (1998) Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal Ca^{2+} channels with G-protein α and βγ subunits. I. Molecular determination. *J Biol Chem* **273**: 17585-17594.

Galvez T, Parmentier ML, Joly C, Malitschek B, Kaupmann K, Kuhn R, Bittiger H, Froestl W, Bettler B, and Pin JP (1999) Mutagenesis and modeling of the $GABA_B$ receptor extracellular domain support a venus flytrap mechanism for ligand binding. *J Biol Chem* **274**: 13362-13369.

Galvez T, Urwyler S, Prezeau L, Mosbacher J, Joly C, Malitschek B, Heid J, Brabet I, Froestl W, Bettler B, Kaupmann K, and Pin JP (2000) Ca^{2+} requirement for high-affinity γ-aminobutyric acid (GABA) binding at $GABA_B$ receptors: involvement of serine 269 of the $GABA_BR1$ subunit. *Mol Pharmacol* **57**: 419-426.

Garbutt JC and Flannery B (2007) Baclofen for alcoholism. *Lancet* **370**: 1884-1885.

Geng Y, Bush M, Mosyak L, Wang F, and Fan QR (2013) Structural mechanism of ligand activation in human $GABA_B$ receptor. *Nature* **504**: 254-259.

Gray AC, Raingo J, and Lipscombe D (2007) Neuronal calcium channels: splicing for optimal performance. *Cell Calcium* **42**: 409-417.

Gribkoff VK (2006) The role of voltage-gated calcium channels in pain and nociception. *Semin Cell Dev Biol* **17**: 555-564.

Hering-Hanit R (1999) Baclofen for prevention of migraine. *Cephalalgia* **19**: 589-591.

Hudgson P and Weightman D (1971) Baclofen in the treatment of spasticity. *Br Med J* **4**: 15-17.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein βγ subunits. *Nature* **380**: 255-258.

Jones LP, Patil PG, Snutch TP, and Yue DT (1997) G-protein modulation of N-type calcium channel gating current in human embryonic kidney cells (HEK 293). *J Physiol* **498**: 601-610.

Katada T (2012) The inhibitory G protein G_i identified as pertussis toxin-catalyzed ADP-ribosylation. *Biol Pharm Bull* **35**: 2103-2111.

Klimis H, Adams DJ, Callaghan B, Nevin S, Alewood PF, Vaughan CW, Mozar CA, and Christie MJ (2011) A novel mechanism of inhibition of high-voltage activated calcium channels by α-conotoxins contributes to relief of nerve injury-induced neuropathic pain. *Pain* **152**: 259-266.

Laviv T, Vertkin I, Berdichevsky Y, Fogel H, Riven I, Bettler B, Slesinger PA, and Slutsky I (2011) Compartmentalization of the $GABA_B$ receptor signaling complex is required for presynaptic inhibition at hippocampal synapses. *J Neurosci* **31**: 12523-12532.

Lin Y, McDonough SI, and Lipscombe D (2004) Alternative splicing in the voltage-sensing region of N-Type $Ca_V2.2$ channels modulates channel kinetics. *J Neurophysiol* **92**: 2820-2830.

Livett BG, Sandall DW, Keays D, Down J, Gayler KR, Satkunanathan N, and Khalil Z (2006) Therapeutic applications of conotoxins that target the neuronal nicotinic acetylcholine receptor. *Toxicon* **48**: 810-829.

Luo S, Kulak JM, Cartier GE, Jacobsen RB, Yoshikami D, Olivera BM, and McIntosh JM (1998) α-conotoxin AuIB selectively blocks α3β4 nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release. *J Neurosci* **18**: 8571-8579.

Luscher C and Slesinger PA (2010) Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. *Nat Rev Neurosci* **11**: 301-315.

Marshall FH, Jones KA, Kaupmann K, and Bettler B (1999) $GABA_B$ receptors - the first 7TM heterodimers. *Trends Pharmacol Sci* **20**: 396-399.

McDavid S and Currie KP (2006) G-proteins modulate cumulative inactivation of N-type ($Ca_V2.2$) calcium channels. *J Neurosci* **26**: 13373-13383.

McIntosh JM, Absalom N, Chebib M, Elgoyhen AB, and Vincler M (2009) α9 nicotinic acetylcholine receptors and the treatment of pain. *Biochem Pharmacol* **78**: 693-702.

Menon-Johansson AS, Berrow N, and Dolphin AC (1993) G_o transduces $GABA_B$-receptor modulation of N-type calcium channels in cultured dorsal root ganglion neurons. *Pflügers Arch* **425**: 335-343.

Meza U and Adams B (1998) G-protein-dependent facilitation of neuronal α_{1A}, α_{1B}, and α_{1E} Ca channels. *J Neurosci* **18**: 5240-5252.

Raingo J, Castiglioni AJ, and Lipscombe D (2007) Alternative splicing controls G protein-dependent inhibition of N-type calcium channels in nociceptors. *Nat Neurosci* **10**: 285-292.

Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, Violin JD, and Lefkowitz RJ (2011) Quantifying ligand bias at seven-transmembrane receptors. *Mol Pharmacol* **80**: 367-377.

Sandall DW, Satkunanathan N, Keays DA, Polidano MA, Liping X, Pham V, Down JG, Khalil Z, Livett BG, and Gayler KR (2003) A novel α-conotoxin identified by gene sequencing is active in suppressing the vascular response to selective stimulation of sensory nerves in vivo. *Biochemistry* **42**: 6904-6911.

Satkunanathan N, Livett B, Gayler K, Sandall D, Down J, and Khalil Z (2005) α-conotoxin Vc1.1 alleviates neuropathic pain and accelerates functional recovery of injured neurones. *Brain Res* **1059**: 149-158.

Schnolzer M, Alewood P, Jones A, Alewood D, and Kent SB (1992) In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int J Pept Protein Res* **40**: 180-193.

Sudo Y, Hojo M, Ando Y, Takada M, Murata H, Kurata S, Nishida N, and Uezono Y (2012) $GABA_B$ receptors do not internalize after baclofen treatment, possibly due to a lack of β-arrestin association: study with a real-time visualizing assay. *Synapse* **66**: 759-769.

Sun BB and Chiu SY (1999) N-type calcium channels and their regulation by $GABA_B$ receptors in axons of neonatal rat optic nerve. *J Neurosci* **19**: 5185-5194.

Tedford HW and Zamponi GW (2006) Direct G protein modulation of Ca_v2 calcium channels. *Pharmacol Rev* **58**: 837-862.

Toselli M and Taglietti V (1993) Baclofen inhibits high-threshold calcium currents with two distinct modes in rat hippocampal neurons. *Neurosci Lett* **164**: 134-136.

Vincler M, Wittenauer S, Parker R, Ellison M, Olivera BM, and McIntosh JM (2006) Molecular mechanism for analgesia involving specific antagonism of α9α10 nicotinic acetylcholine receptors. *Proc Natl Acad Sci U S A* **103**: 17880-17884.

Weiss N, Tadmouri A, Mikati M, Ronjat M, and De Waard M (2007) Importance of voltage-dependent inactivation in N-type calcium channel regulation by G-proteins. *Pflugers Arch* **454**: 115-129.

Winquist RJ, Pan JQ, and Gribkoff VK (2005) Use-dependent blockade of $Ca_v2.2$ voltage-gated calcium channels for neuropathic pain. *Biochem Pharmacol* **70**: 489-499.

Wisler JW, Xiao K, Thomsen AR, and Lefkowitz RJ. (2014) Recent developments in biased agonism. *Curr Opin Cell Biol* **27**: 18-24.

Wootten D, Christopoulos A, and Sexton PM (2013) Emerging paradigms in GPCR allostery: implications for drug discovery. *Nat Rev Drug Discov* **12**: 630-644.

Zamponi GW and Currie KP (2012) Regulation of Ca_V2 calcium channels by G protein-coupled receptors. *Biochim Biophys Acta*. **1828**: 1629-1643.

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

a) This work was supported by the National Health and Medical Research Council (NHMRC) [Program Grant 569927] (D.J.A.) and the US-Israel Binational Science Foundation (P.A.S.). T.G.H. was supported by a University of Queensland Postgraduate Scholarship. D.J.A. is an Australian Research Council (ARC) Australian Professorial Fellow.

b) Huynh, T.G. (2013) *Mechanism of α-conotoxin Vc1.1 inhibition of Cav2.2 channel*. PhD Thesis, Queensland Brain Institute, The University of Queensland.

c) Reprint requests: David J. Adams, Health Innovations Research Institute, RMIT University, PO Box 71, Bundoora, Victoria 3083, Australia. Email: david.adams@rmit.edu.au

d) Present address of P.A.S: Department of Neuroscience, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029 USA.

e) Conflict of Interest: The authors have no conflicting financial interests.

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. α-Conotoxin Vc1.1 inhibits $Ca_v2.2$ channels by activating $GABA_B$Rs in HEK293 cells. (**A**) Representative superimposed current traces from HEK293 cells co-expressing human $GABA_B$R and $Ca_v2.2$ channels in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50 μM) (c). (**B**) Peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent superimposed current traces shown in **(A).** (**C**) Bar graph of inhibition of peak current amplitude by 200 nM Vc1.1 (41.0 ± 5.3%), 50 μM baclofen (61.5 ± 5.7%), 10 μM GABA (69.6 ± 2.3%), and 200 nM Vc1.1 + 50 μM baclofen (78.1 ± 3.6%). Number of experiments is indicated in parenthesis. Data represent mean ± SEM (**$P < 0.01$, ***$P < 0.001$ versus Vc1.1, one-way ANOVA). (**D**) Concentration–response relationship for Vc1.1 inhibition of peak current in HEK293 cells co-expressing $GABA_B$R and $Ca_v2.2$ channels with an IC_{50} of 19.1 nM and Hill slope of –1 (n = 6–7 cells per data point).

Figure 2. Mutation of the GABA/Baclofen binding site does not affect Vc1.1 inhibition of $Ca_v2.2$. (A) Schematic diagram of wild-type and mutated $GABA_{B1a}$ subunits. (B) Representative superimposed current traces obtained from HEK293T cells co-expressing human $GABA_B$R and $Ca_v2.2$ channels in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50 μM) (c). (**C**) Peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent time at which the superimposed current traces were obtained. (**D**) Bar graph comparing Vc1.1 and baclofen inhibition of peak current amplitude. Vc1.1 (200 nM) inhibition of peak current was not affected by the mutation (52.3 ± 8.6% compared with control 47.1 ± 14.4%, $P = 0.75$). However, baclofen (50 μM) inhibition was significantly reduced by the mutation (14.7 ± 3.0

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

% compared with control 43.3 ± 8.6 %). Number of experiments is indicated in parenthesis. Data represent mean ± SEM (**P < 0.01).

Figure 3. Differential effects of Vc1.1 and baclofen on the voltage-dependence of $Ca_v2.2$ channel activation and inactivation in HEK293 cells co-expressing human $Ca_v2.2$ and $GABA_BR$. (**A**) Upper panel: Current-voltage relationships obtained in the absence (n = 7) and presence of baclofen (n = 7) and Vc1.1 (n = 6). Lower panel: Voltage-dependence of $Ca_V2.2$ activation in the absence (control) and presence of Vc1.1 (200 nM) and baclofen (50 µM). $Ca_V2.2$-mediated currents were evoked by step depolarization from a holding potential of –80 mV to +50 mV in 5 mV increments at 0.1 Hz. Baclofen shifted the $V_{0.5}$ activation to more depolarized potentials (+3.7 ± 1.5 mV, n = 7), whereas Vc1.1 did not change $V_{0.5}$ activation (–5.1 ± 0.9 mV, n = 8) compared to control (–6.3 ± 0.7 mV, n = 8). Data represent mean ± SEM. (**B**) Analysis of $Ca_V2.2$ activation using a tail current protocol. Upper panel: representative superimposed whole-cell currents obtained in the absence and presence of baclofen from a cell expressing $Ca_v2.2$ and $GABA_BR$. Tail currents were measured upon repolarization to –60 mV, immediately following depolarizing test pulses from –40 mV to +55 mV in 5 mV increments. Lower panel: $Ca_v2.2$ activation curves obtained from tail currents were fitted with the sum of two Boltzmann functions. Baclofen shifted both $V_{0.5}$ and k, whereas Vc1.1 did not. The parameters of the major Boltzmann function obtained from the best fit to the data were $V_{0.5,\,act}$ = +8.9 ± 2.6 mV and k = 5.8 ± 1.2 mV (n = 8) under control conditions, $V_{0.5,\,act}$ = +7.8 ± 2.1 mV and k = 4.5 ± 2.4 mV (n = 6) in the presence of Vc1.1 and $V_{0.5,\,act}$ = +17.4 ± 3.8 mV and k = 8.4 ± 1.5 mV (n = 4) in the presence of baclofen. (**C**) $Ca_v2.2$ steady-state inactivation (SSI) curves obtained in the absence and presence of Vc1.1 (200 nM) and baclofen (50 µM). Peak current amplitudes following a 10 s prepulse at varying potentials were normalized to the maximum peak current amplitude recorded at +5 mV with a

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

prepulse of –120 mV. $V_{0.5,\ inact}$ was unchanged in the presence of baclofen (–64.3 ± 1.8 mV, *n* = 7) compared to control (–65.5 ± 0.6 mV, *n* = 8), whereas Vc1.1 shifted $V_{0.5,\ inact}$ to –78.8 ± 0.5 mV (*n* = 10) which was significantly different to control ($P < 0.05$, one-way ANOVA). Data represent mean ± SEM. (**D**) SSI of $Ca_v2.2$ channels was further investigated in individual cells in the absence (control) and presence of Vc1.1 (200 nM). Upper left panel: voltage protocol to evaluate SSI of $Ca_v2.2$. Upper right panel: ratio of peak current amplitudes elicited by depolarizing test pulses to +10 mV separated by a 10 s prepulse at –110, –80 and –50 mV (P1/P2) in the absence (control) and presence of Vc1.1. Holding potential –80 mV. In the presence of Vc1.1 and at a prepulse potential of –110 mV, P2/P1 was reduced from 1.33 ± 0.07 to 1.01 ± 0.01 (*n* = 11; $P < 0.01$, paired *t*-test) and at –50 mV from 0.55 ± 0.03 to 0.43 ± 0.03 (*n* = 11; $P < 0.01$). Data represent mean ± SEM. Lower panel: representative superimposed current traces evoked by step depolarization to +10 mV (P2) following 10 s pre-pulses to –110, –80 and –50 mV in the absence (○) and presence of Vc1.1 (■).

Figure 4. Baclofen and Vc1.1 effects on the $Ca_v2.2$ channel activation rate.

Representative superimposed whole-cell Ba^{2+} currents from HEK293 cells expressing $Ca_v2.2$ channels and $GABA_BRs$ obtained in the absence and presence of 50 µM baclofen (**A**) and 200 nM Vc1.1 (**B**). The superimposed red trace represents a single exponential function fitted to the time course of current activation. The superimposed grey trace represents the current recorded in the presence of baclofen and Vc1.1 normalized to control. (**C**) Data sets for activation time constants of $Ca_v2.2$ obtained in the absence ($\tau_{control}$: 1.63 ± 0.18 ms) and presence of baclofen ($\tau_{baclofen}$: 2.13 ± 0.36 ms) ($P < 0.05$, paired *t*-test), and absence ($\tau_{control}$: 1.69 ± 0.25 ms) and presence of Vc1.1 ($\tau_{Vc1.1}$: 1.26 ± 0.23 ms) ($P < 0.05$, paired *t*-test). The

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

time constants of activation of I_{Ba} obtained in the presence of baclofen and Vc1.1 were significantly different ($P < 0.05$, unpaired *t*-test). Data represent mean ± SEM.

Figure 5. Pre-pulse relief of GABA but not Vc1.1 inhibition of $Ca_v2.2$. (**A**) Prepulse protocol and representative superimposed traces of $Ca_v2.2$ currents in HEK293 cells obtained in the absence (control) and presence of GABA (**B**) and Vc1.1 (**C**). The depolarizing prepulse to +120 mV relieved GABA inhibition of peak current amplitude, but did not significantly affect inhibition by Vc1.1. The outward current in response to the 50 ms prepulse to +120 mV has been blanked to better display the inward current. (**D**) Bar graph of GABA's inhibition of average peak current amplitude in the absence (64.0 ± 4.0% [*–pp*]) and presence (4.0 ± 5.0% [*+pp*]) of prepulse. The prepulse significantly relieved peak current amplitude (****P* < 0.001, unpaired *t*-test) in the presence of GABA, but did not change Vc1.1 inhibition of peak current amplitude (50.0 ± 11.0 [*–pp*] to 36.0 ± 6.0% [*+pp*]). Number of experiments is indicated in parenthesis. Data represent mean ± SEM. (**E**) Peak inward current amplitude in response to a depolarizing test pulse to +5 mV plotted as a function of time in the absence and presence of Vc1.1 and GABA as indicated. Closed symbols (●,–pp) represent peak current amplitude without a preceding prepulse whereas open symbols (○, +pp) represent peak current amplitude following prepulse.

Figure 6. PTX prevents α-conotoxin Vc1.1 inhibition of $Ca_v2.2$ channels. (**A**) Representative superimposed current traces obtained in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50 μM) (c) in HEK293 cells pre-incubated in PTX (3 μg/ml) for at least 16 hr. (**B**) Peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent time at which superimposed current traces were obtained. (**C**) Bar graph of Vc1.1 and baclofen inhibition of peak current amplitude with

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

or without PTX pre-treatment. Vc1.1 inhibition was significantly less in cells pre-treated with PTX (0.9 ± 3.5%) than in untreated cells (42.0 ± 5.7%). Similarly, baclofen inhibition was significantly less in cells pre-treated with PTX (5.0 ± 2.3%) than in untreated cells (70.7 ± 2.3%). Number of experiments is indicated in parenthesis. Data represent mean ± SEM (***P < 0.001, unpaired t-test).

Figure 7. Point mutation of arginine 576 to aspartic acid on the $GABA_{B2}$ subunit does not affect Vc1.1 inhibition of $Ca_v2.2$ currents. Currents were recorded in HEK293T cells co-expressing $Ca_v2.2$ with either wild-type $GABA_BR$ or $GABA_{B1a}$-WT/$GABA_{B2}$-R576D and in the presence of Vc1.1 or baclofen. (**A**) Schematic diagram of wild type and mutated $GABA_{B2}$ subunits. (**B**) Representative superimposed current traces obtained in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50 μM) (c). (**C**) Peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent times at which the superimposed current traces were obtained. (**D**) Bar graph comparing Vc1.1 and baclofen inhibition of peak current amplitude with co-expressed wild-type $GABA_BR$ and mutated $GABA_BR$ subunits. Vc1.1 inhibition of peak current was not affected by the mutation ($GABA_{B2}$-R576D, 52.9 ± 8.9%; control 41.1 ± 6.1%) but baclofen inhibition of peak current was significantly reduced ($GABA_{B2}$-R576D, 11.2 ± 4.3%; control 64.8 ± 4.0%). Number of experiments is indicated in parenthesis. Data represent mean ± SEM (***P < 0.001, unpaired t-test).

Figure 8. Vc1.1 inhibition of $Ca_v2.2$ currents involves the PCT domain of the $GABA_{B1a}$ subunit. (**A**) Schematic diagram of wild-type (WT) and mutant $GABA_{B1a}$ subunits. Inward Ba^{2+} currents were recorded from HEK293T cells co-expressing $Ca_v2.2$ channels with $GABA_{B1a}$-Δ887/$GABA_{B2}$-WT (**B**), $GABA_{B1a}$-Δ863/$GABA_{B2}$-WT (**C**) or $GABA_{B1a}$-

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

ΔPCT/$GABA_{B2}$-WT (**D**). Left panel: representative superimposed current traces obtained in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50 μM) (c). Right panel: peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent times at which the superimposed current traces were obtained. (**E**) Vc1.1 inhibition of peak current in HEK293T cells expressing $GABA_{B1a}$-Δ887/$GABA_{B2}$-WT (24.9 ± 6.9%, *n* = 7) was not significantly different from that in wild-type $GABA_B$R expressing cells (37.9 ± 5.6%, *n* = 11). Vc1.1 inhibition of peak current in HEK293T cells expressing $GABA_{B1a}$-Δ863/$GABA_{B2}$-WT (9.5 ± 4.0%, *n* = 12) or $GABA_{B1a}$-ΔPCT/$GABA_{B2}$ (5.3 ± 3.9%, *n* = 5) was significantly different from that in wild-type $GABA_B$R expressing cells (37.9 ± 5.6%, *n* = 11, $P < 0.05$). Baclofen inhibition of peak currents was the same in $GABA_{B1a}$-Δ887/$GABA_{B2}$-WT (62.0 ± 7.3%, *n* = 7), $GABA_{B1a}$-Δ863/$GABA_{B2}$-WT (56.6 ± 3.5%, *n* =10) and $GABA_{B1a}$-ΔPCT/$GABA_{B2}$-WT expressing cells (60.7 ± 2.7%, *n* = 5). Data represent mean ± SEM (**$P < 0.01$, one-way ANOVA).

Figure 9. Schematic diagram of Vc1.1 and baclofen signaling pathways. Baclofen (blue) inhibits $Ca_v2.2$ channels by binding to the Venus Fly Trap on the $GABA_{B1}$ subunit. This activates the G protein complex in a signaling process involving R576 on the $GABA_{B2}$ subunit and results in Gβγ binding to the $Ca_v2.2$ channel. Vc1.1 (red) inhibits $Ca_v2.2$ channels by binding to an as yet unidentified site on the $GABA_B$R triggering inhibition of $Ca_v2.2$ channels by non-activated G proteins. Vc1.1 inhibition of $Ca_v2.2$ involves the PCT domain of the $GABA_{B1a}$ subunit.

Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1. Voltage-dependence of $Ca_v2.2$ channels in HEK293 cells co-expressing $GABA_BRs$ in the absence (control) and presence of Vc1.1 or baclofen.

	Activation G-V		Activation I_{tail}-V		Steady-state Inactivation	
	$V_{0.5,\ act}$	k	$V_{0.5,\ act}$ [a]	k[a]	$V_{0.5,\ inact}$	k
Control	–6.3 ± 0.7 (8)	4.4 ± 0.6	8.9 ± 2.6 (8)	5.8 ± 1.2	–65.5 ± 0.6 (8)	–8.0 ± 0.5
Baclofen	3.7 ± 1.5 (7)[b]	8.6 ± 1.4[b]	17.4 ± 3.8 (4)	8.4 ± 1.5	–64.3 ± 1.8 (7)	–11.0 ± 1.8
Vc1.1	–5.1 ± 0.9 (8)	6.1 ± 0.8	7.8 ± 2.1 (6)	4.5 ± 2.4	–78.8 ± 0.5 (10)[c]	–9.1 ± 0.5

Data represent mean ± SEM. Number of experiments is indicated in parenthesis. $V_{0.5,\ act}$ is half-maximum activation (mV), $V_{0.5,\ inact}$ is half-maximum inactivation (mV); and k is the slope factor.

[a]These parameters were obtained from the major component of the double Boltzmann fit of I_{tail}-V.

[b]$V_{0.5,\ act}$ and k obtained in the presence of baclofen are statistically different from control ($P < 0.05$, one-way ANOVA).

[c]$V_{0.5,\ inact}$ obtained in the presence of Vc1.1 is statistically different from control ($P < 0.05$, one-way ANOVA).

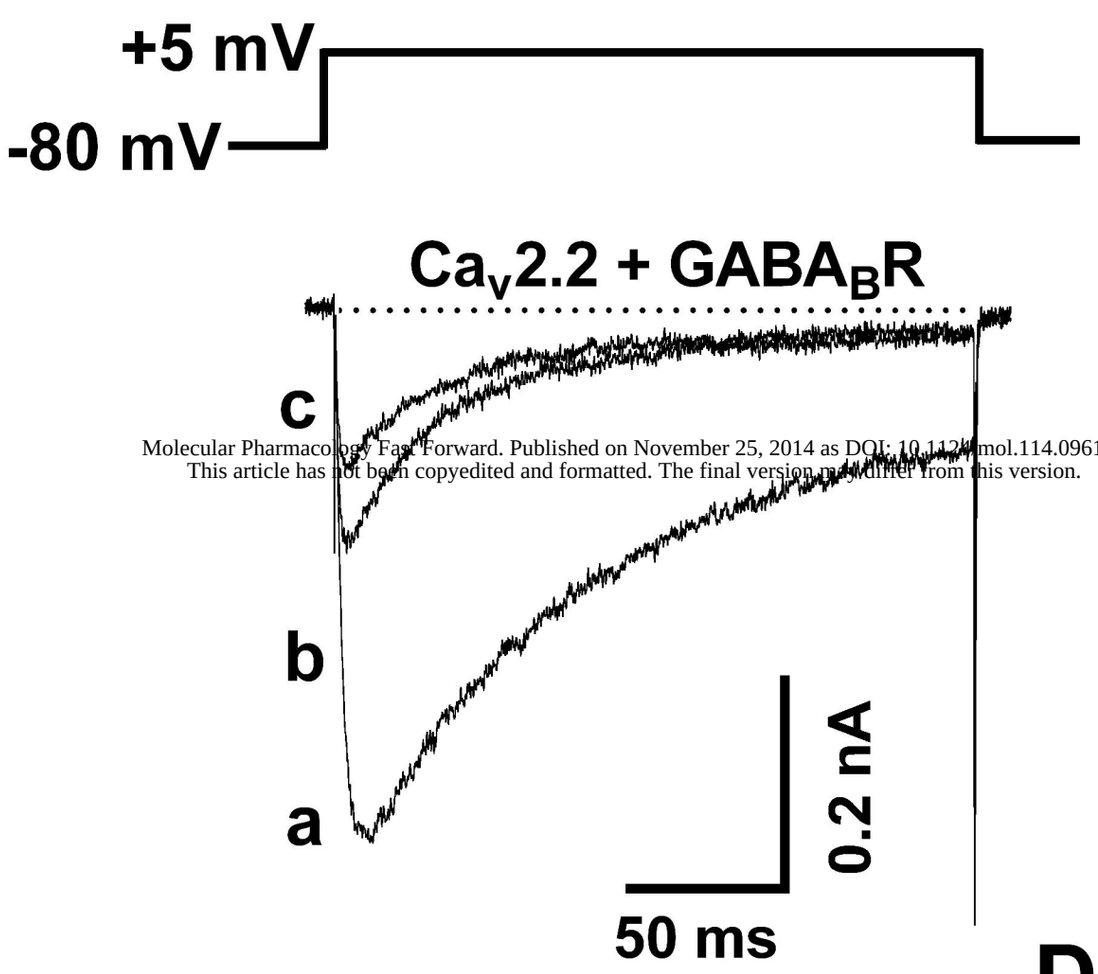
Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

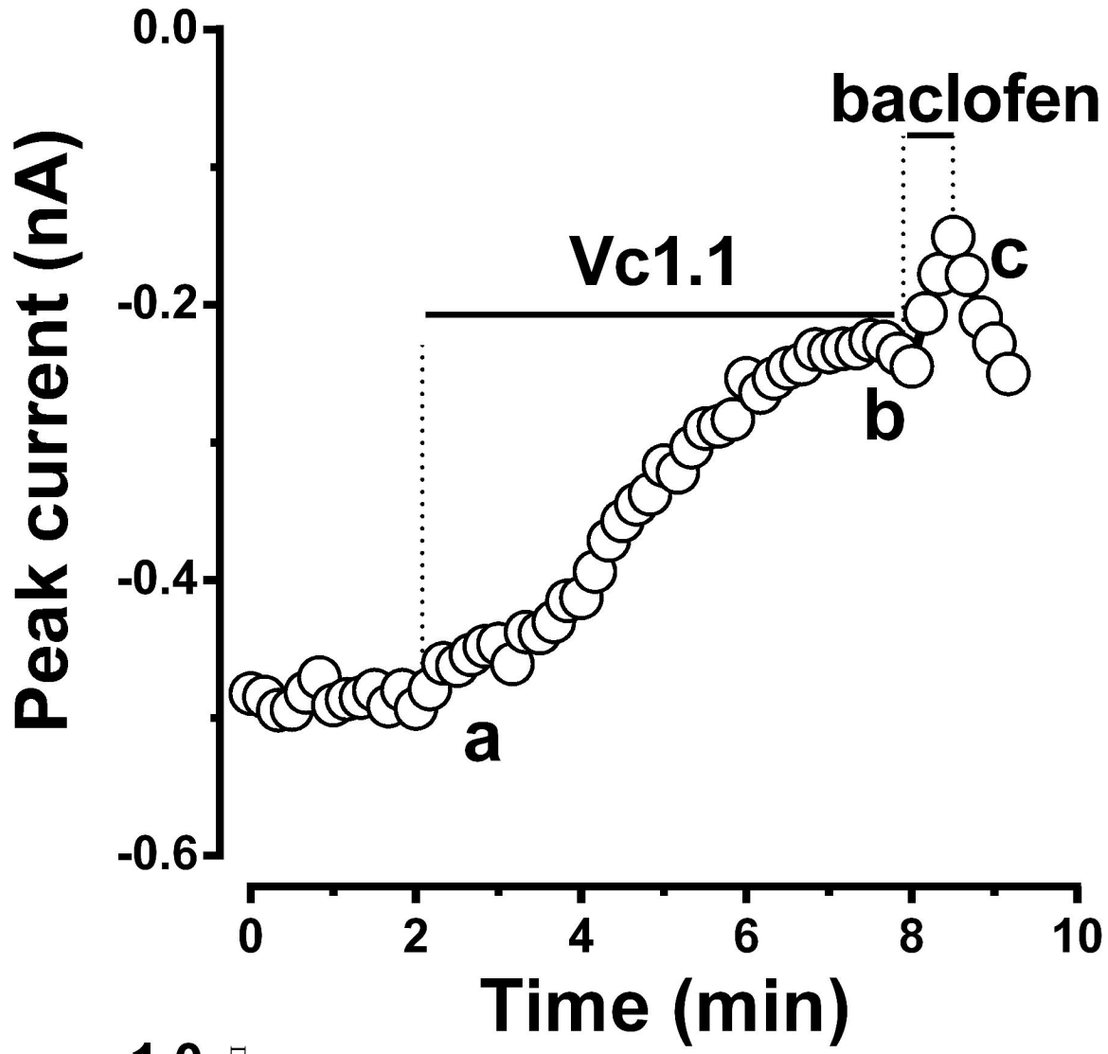
Table 2. Comparison of baclofen- and Vc1.1-mediated inhibition of $Ca_v2.2$ via $GABA_BR$ activation.

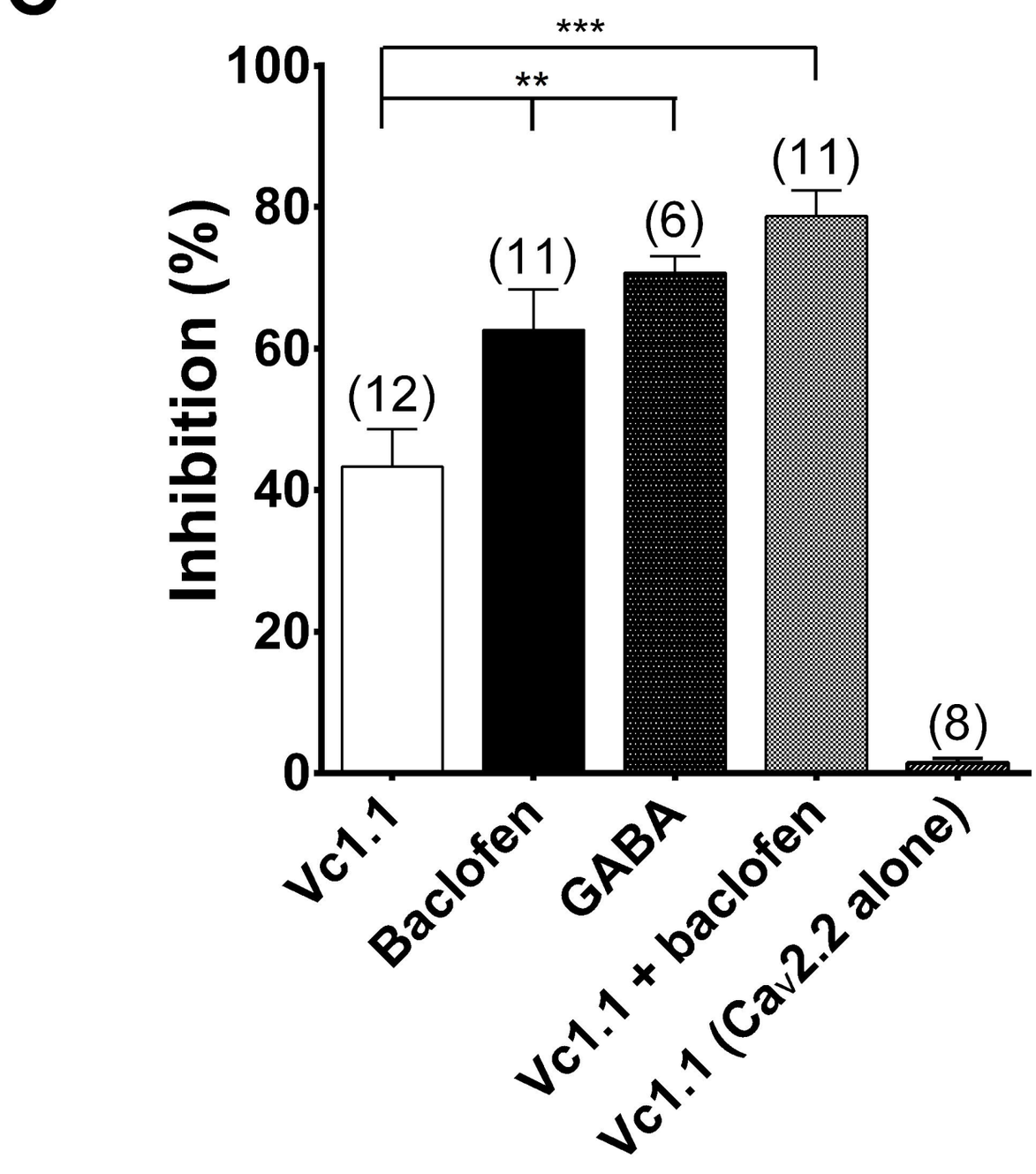
Property	Baclofen	Vc1.1
Onset	Fast (ms)	Slow (s)
$V_{0.5}$ for activation	+10 mV shift	No change
$V_{0.5}$ for inactivation	No change	–13 mV shift
Activation kinetics	Slower	Faster
Prepulse sensitivity	Yes	No
Frequency dependence	Yes	No
Involvement of PCT	No	Yes
Pertussis toxin sensitivity	Yes	Yes
GDP-β-S sensitivity[a]	Yes	Yes

[a]Intracellular GDP-β-S antagonized the inhibitory effect of baclofen and Vc1.1 on HVA calcium channel currents (Callaghan et al., 2008; Berecki et al., 2014).

Figure 1







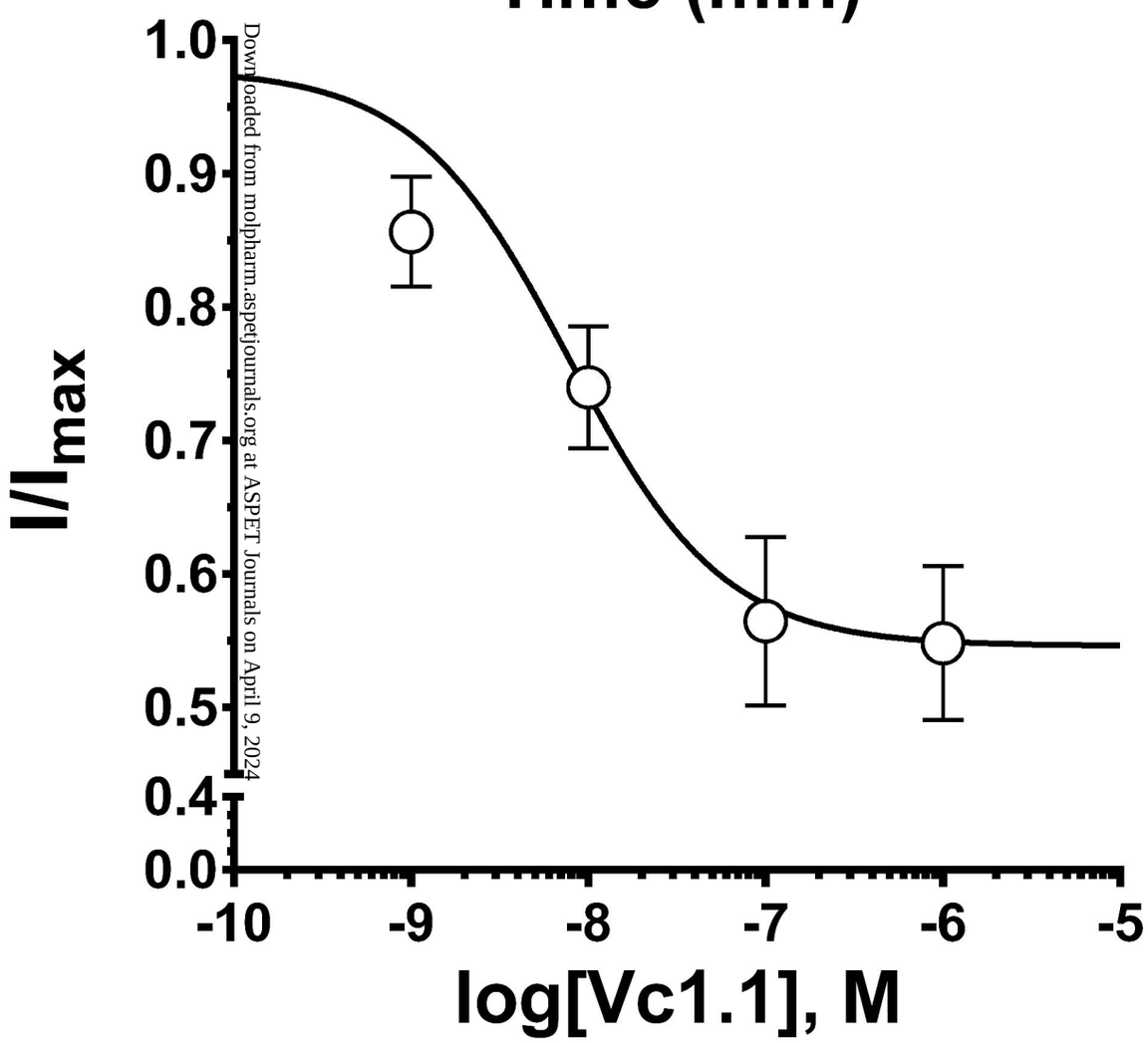
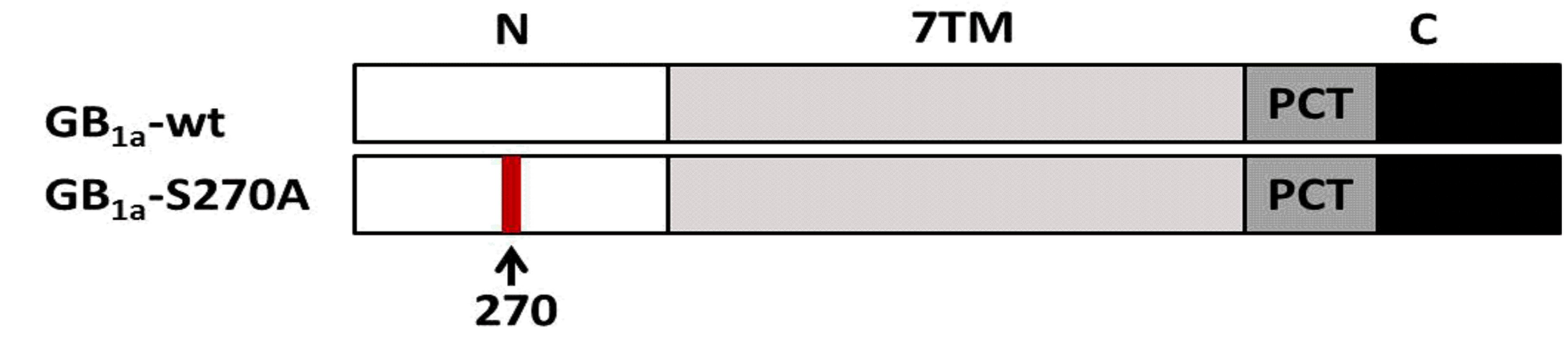
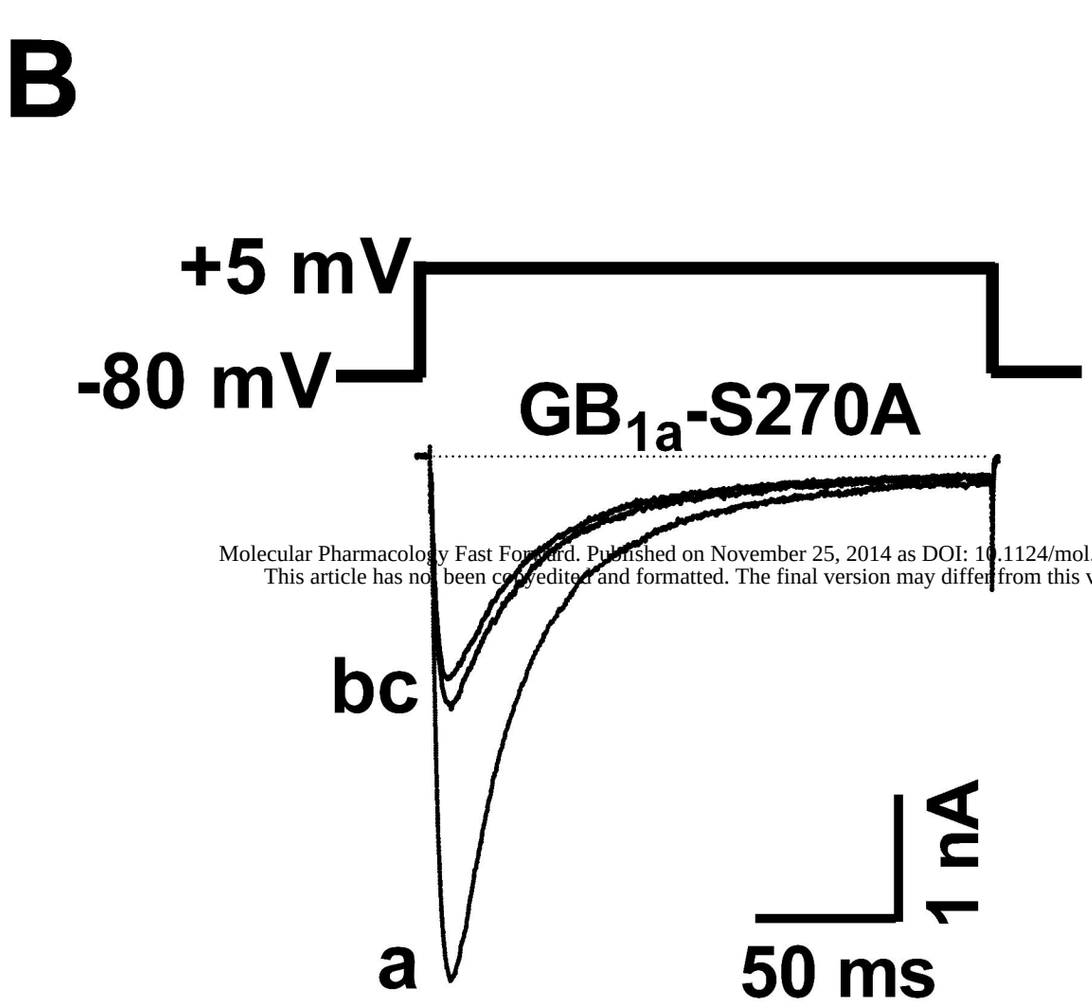
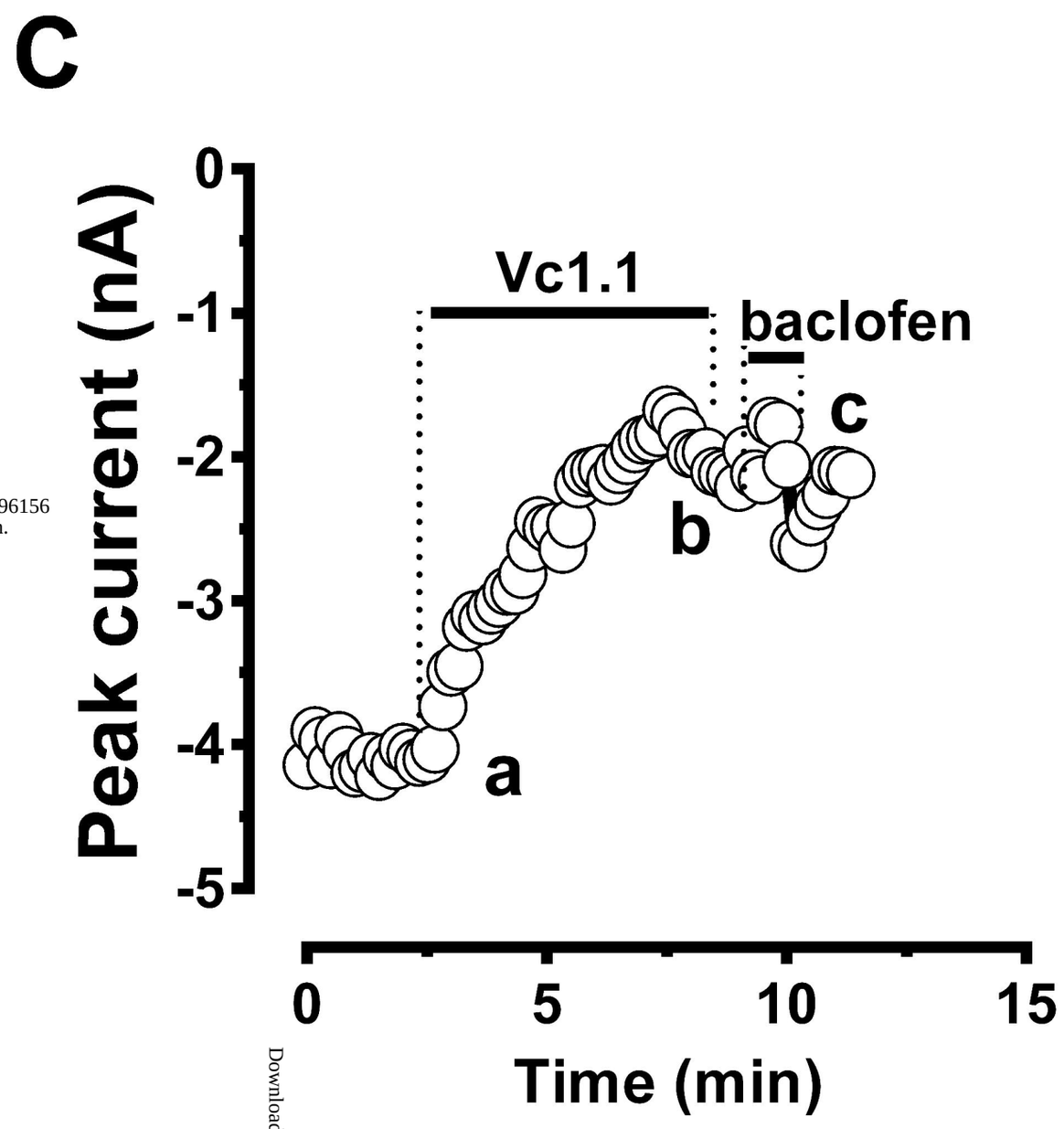


Figure 2







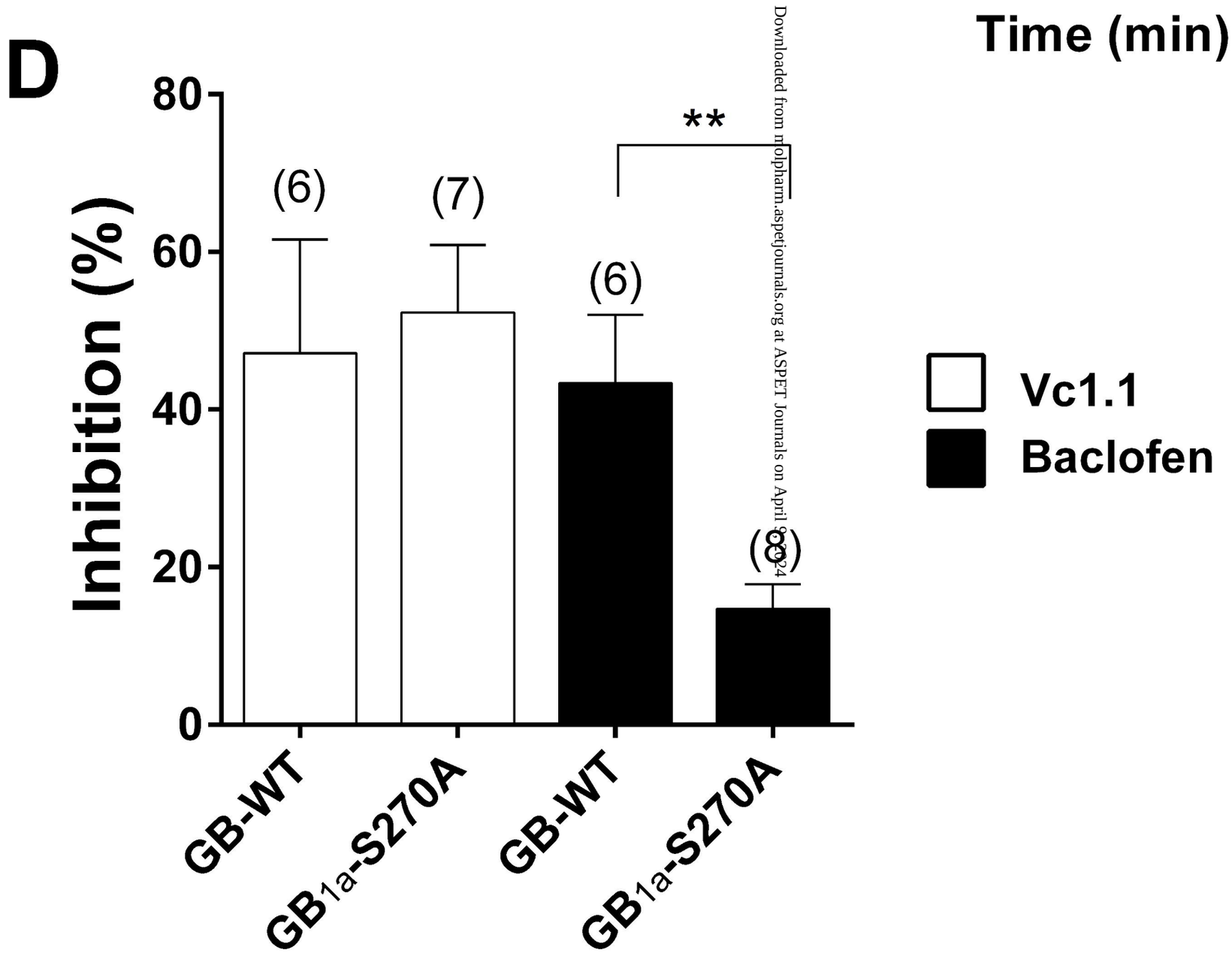
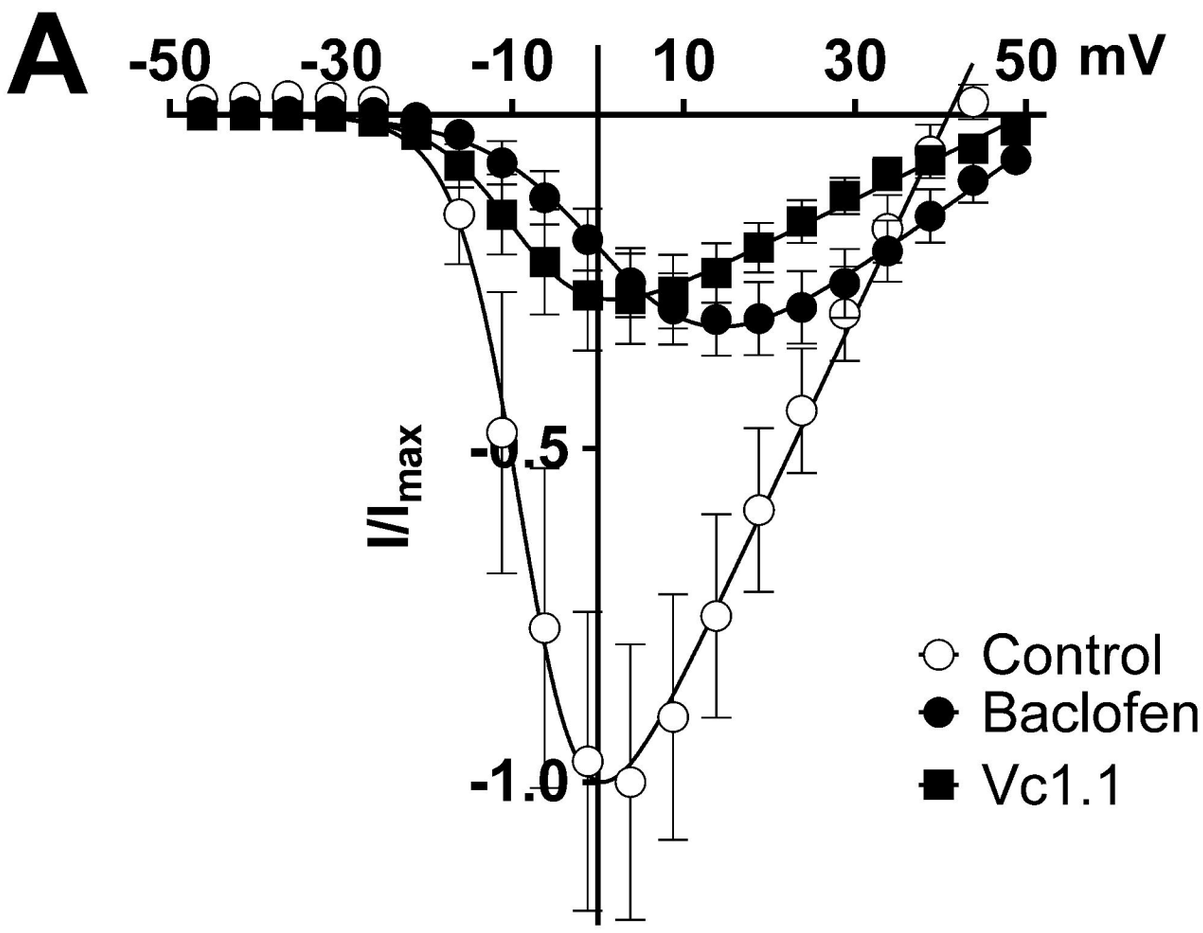
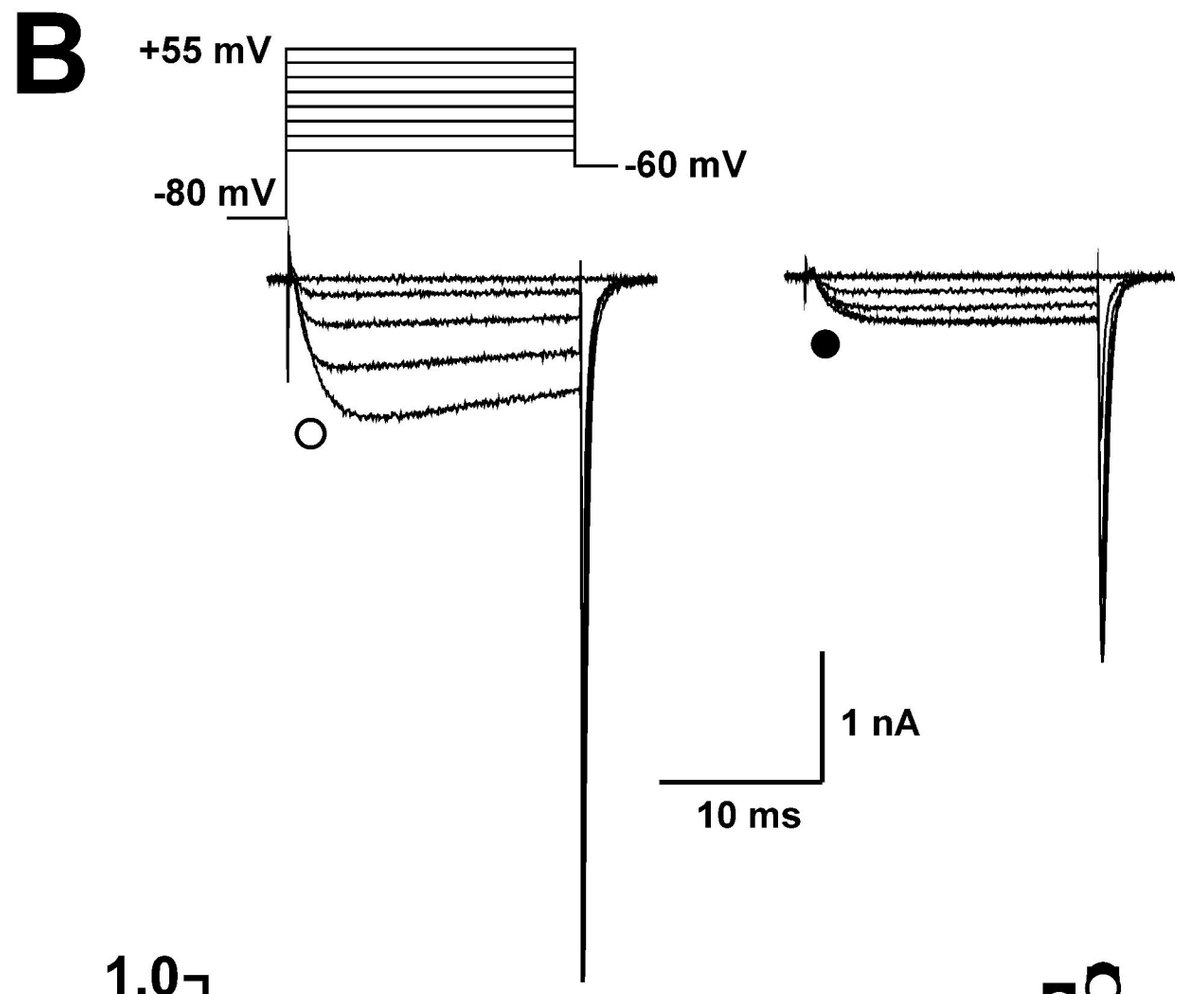
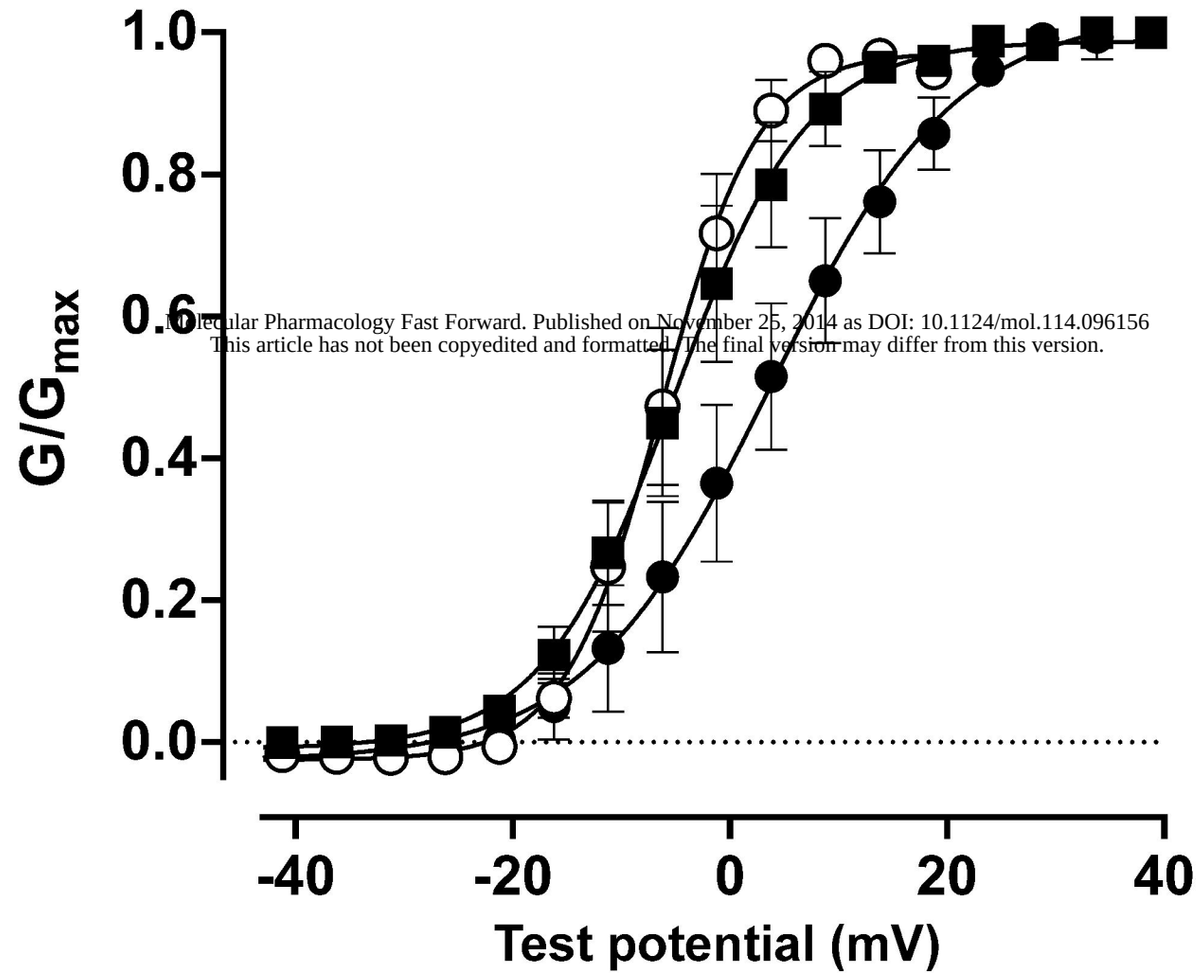
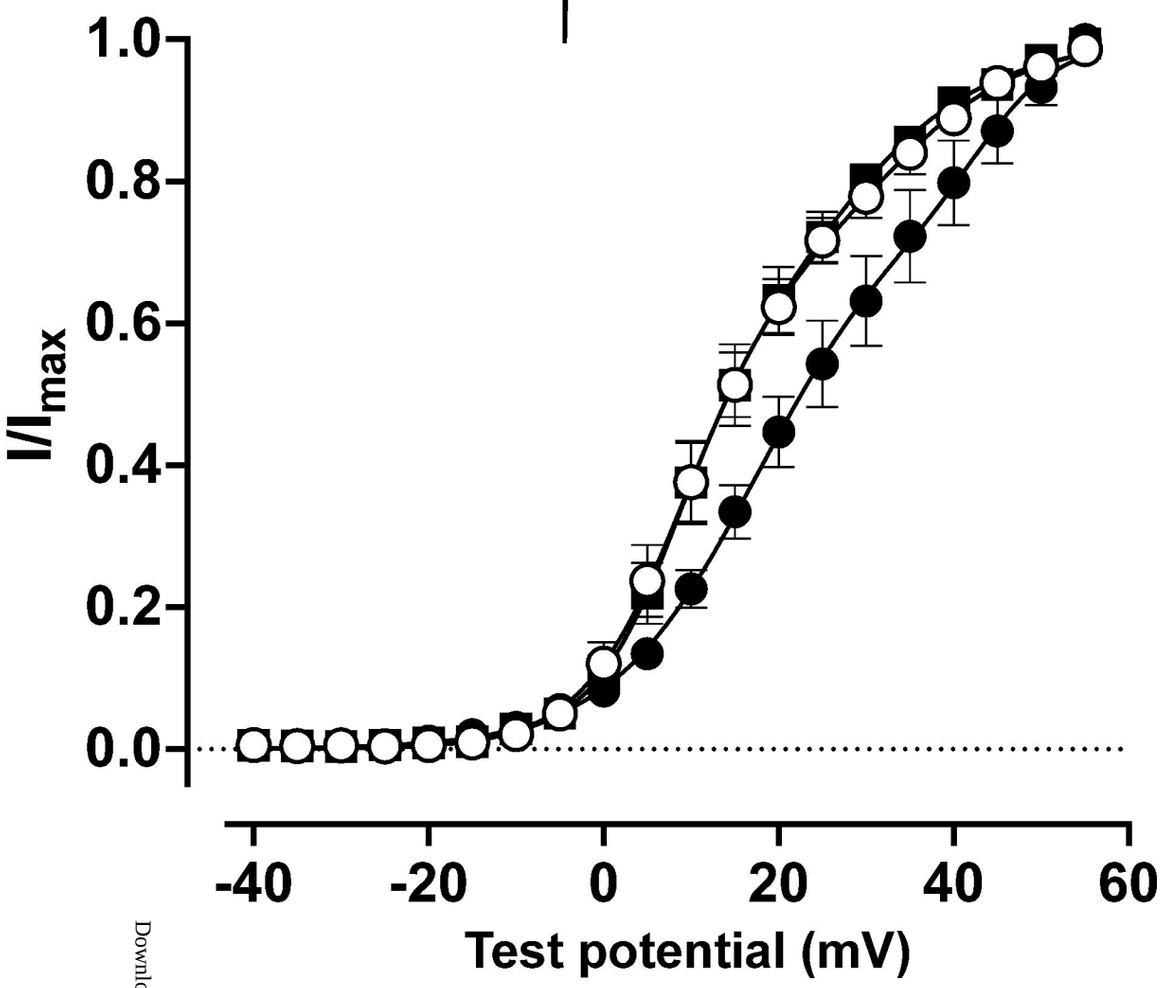


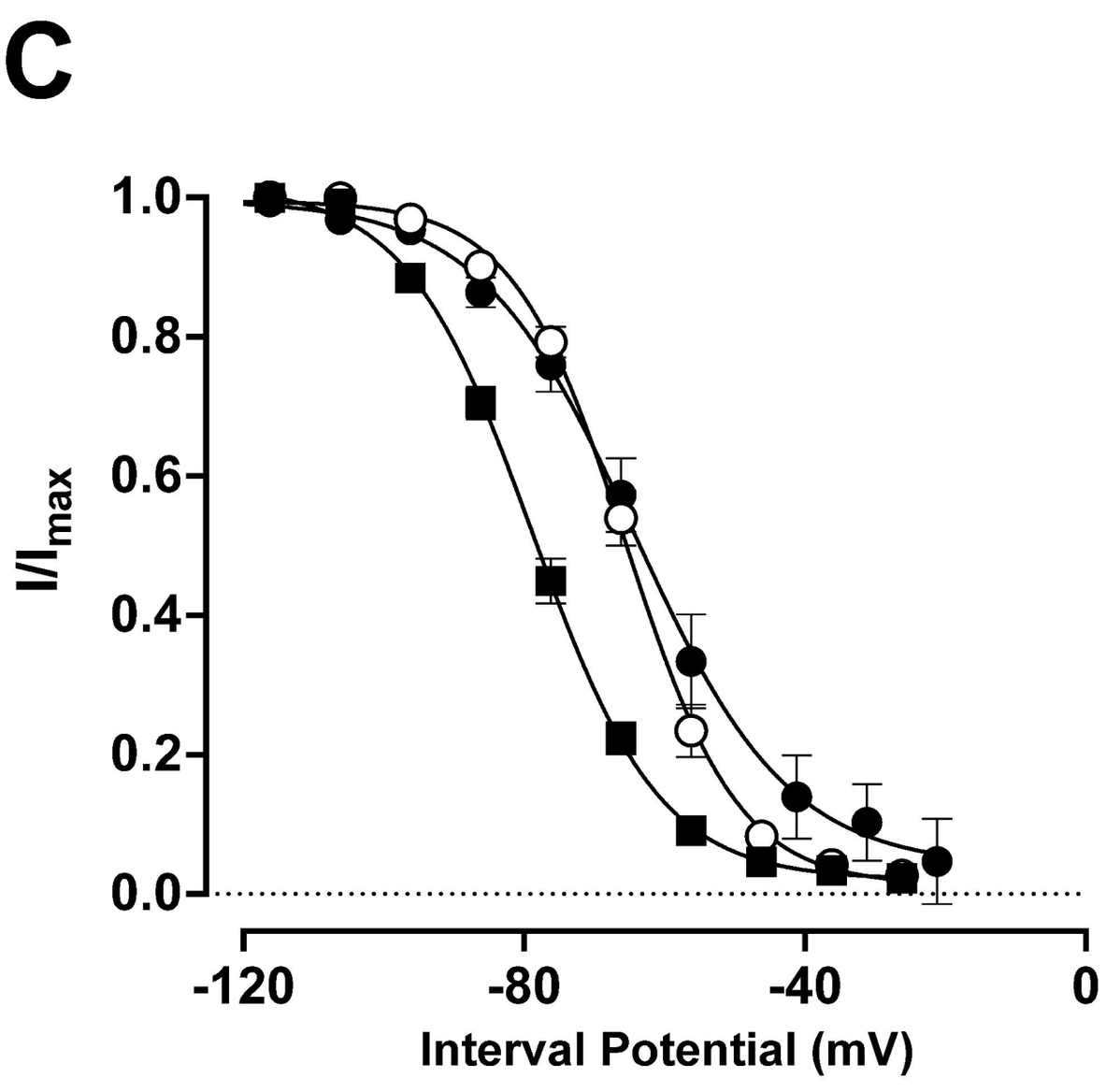
Figure 3











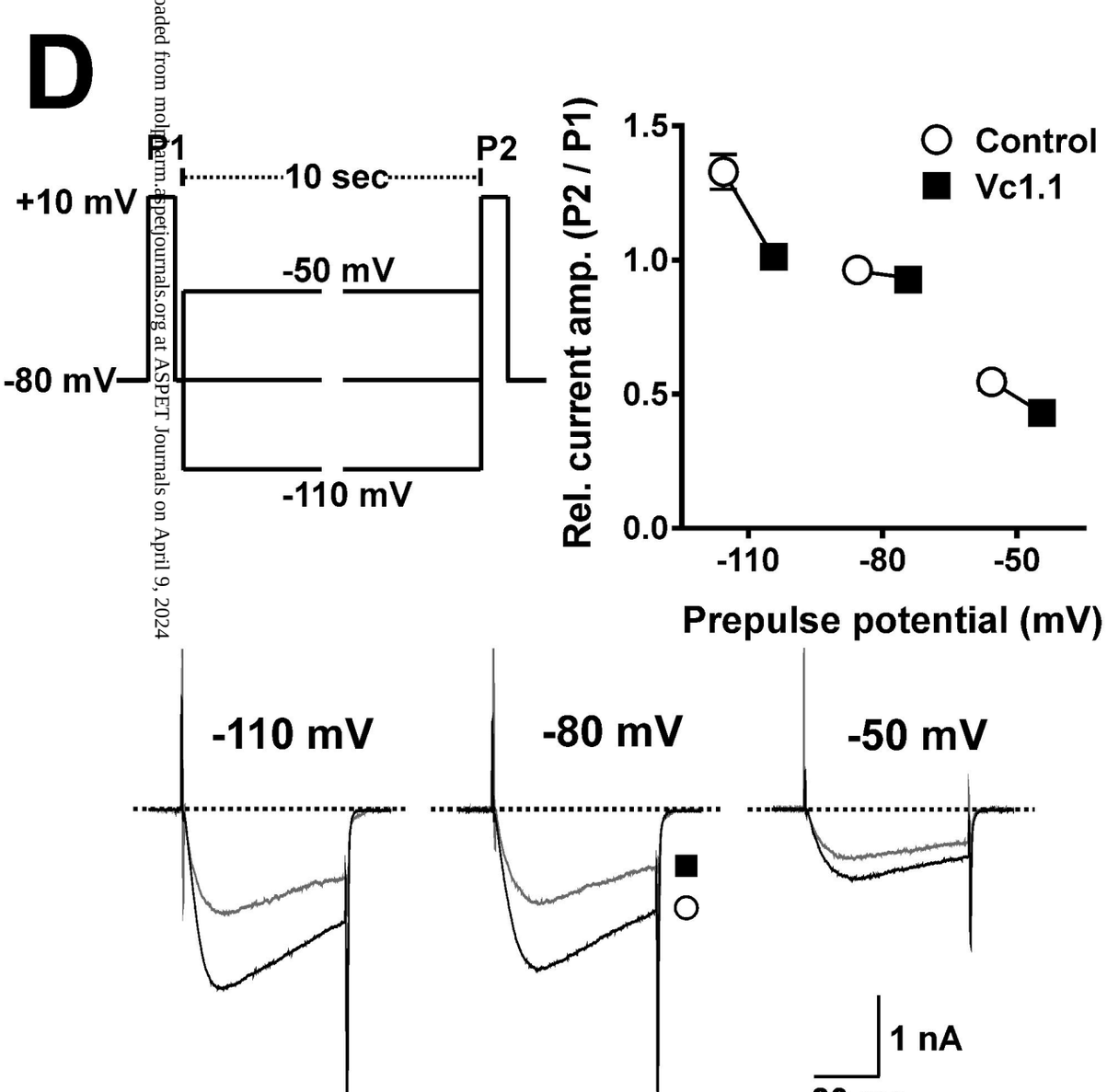
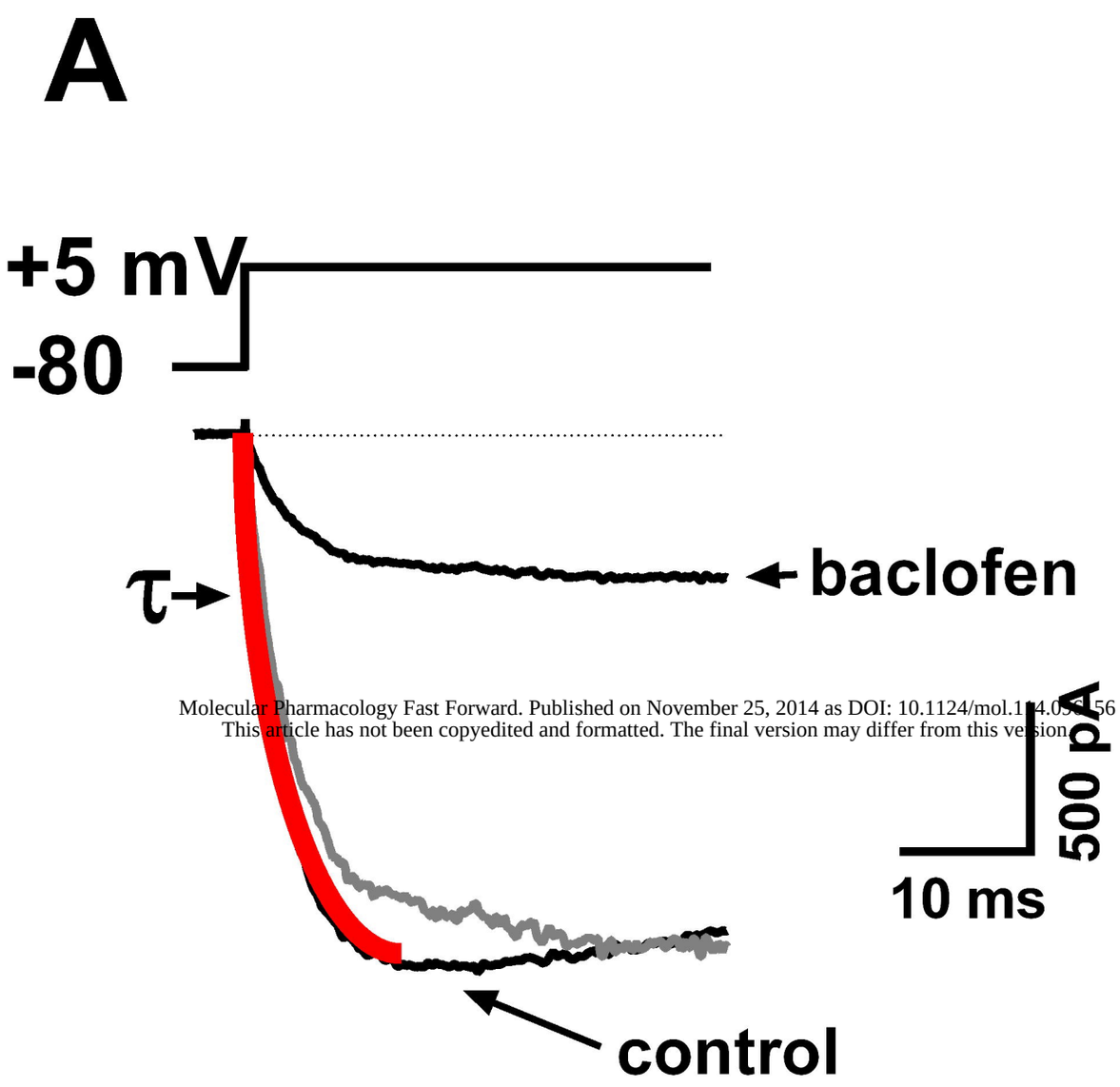
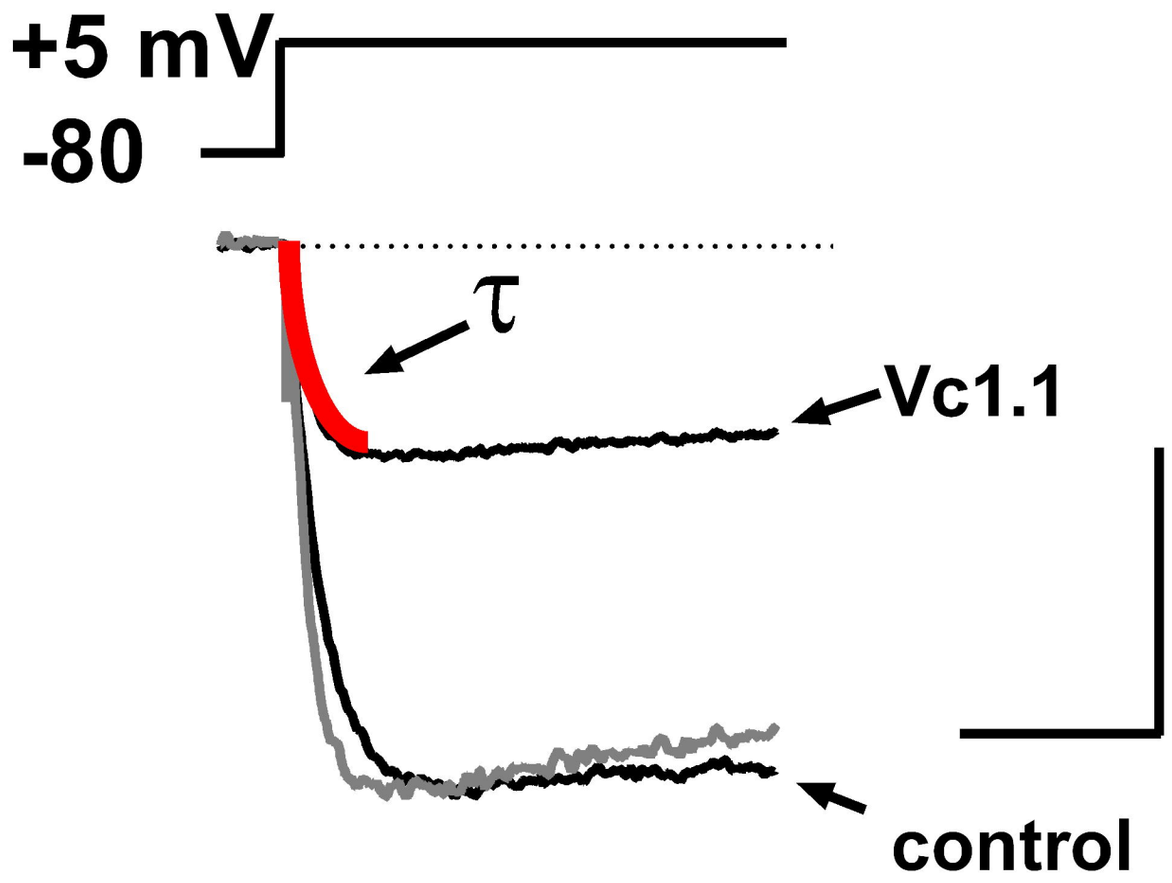
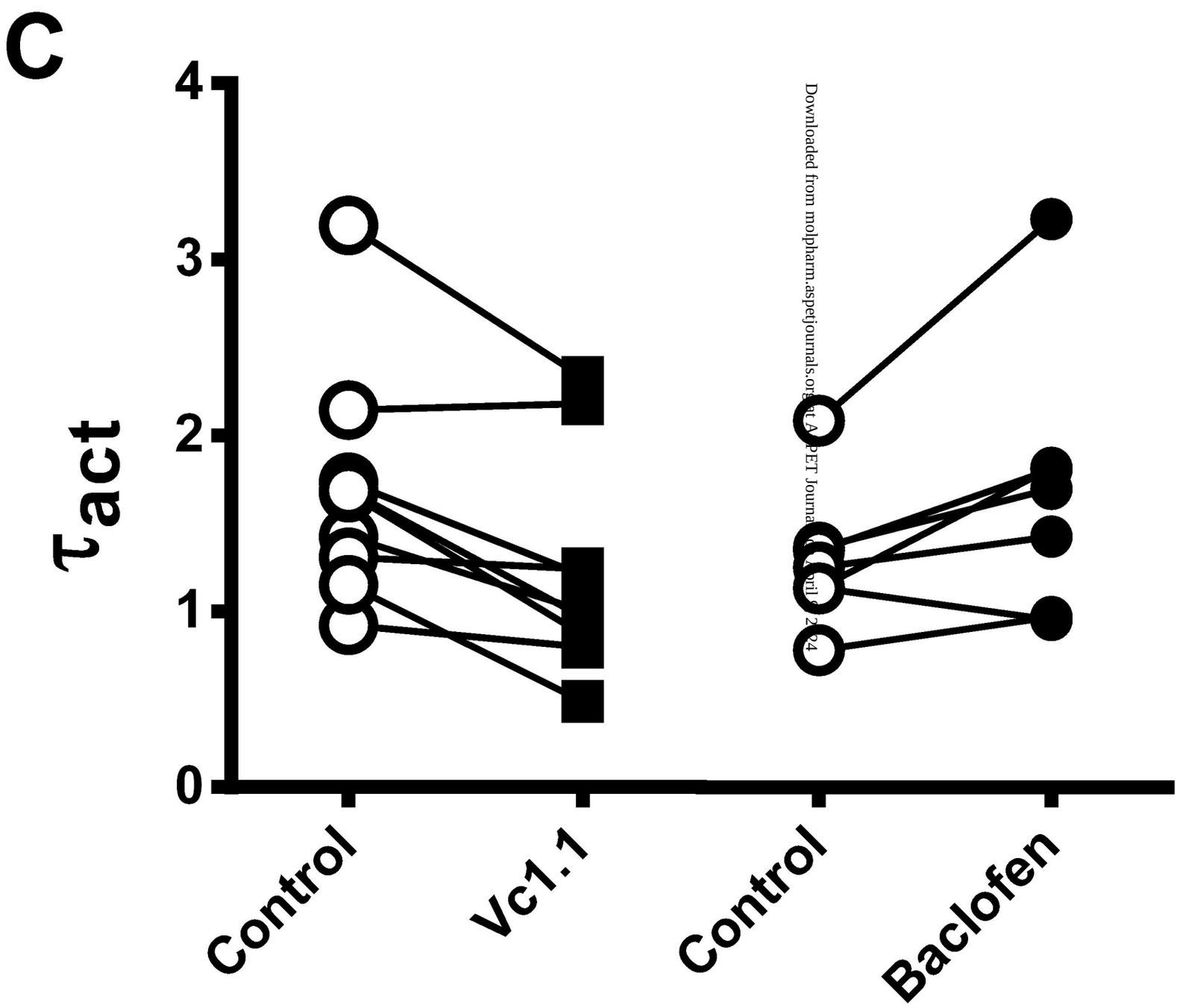


Figure 4

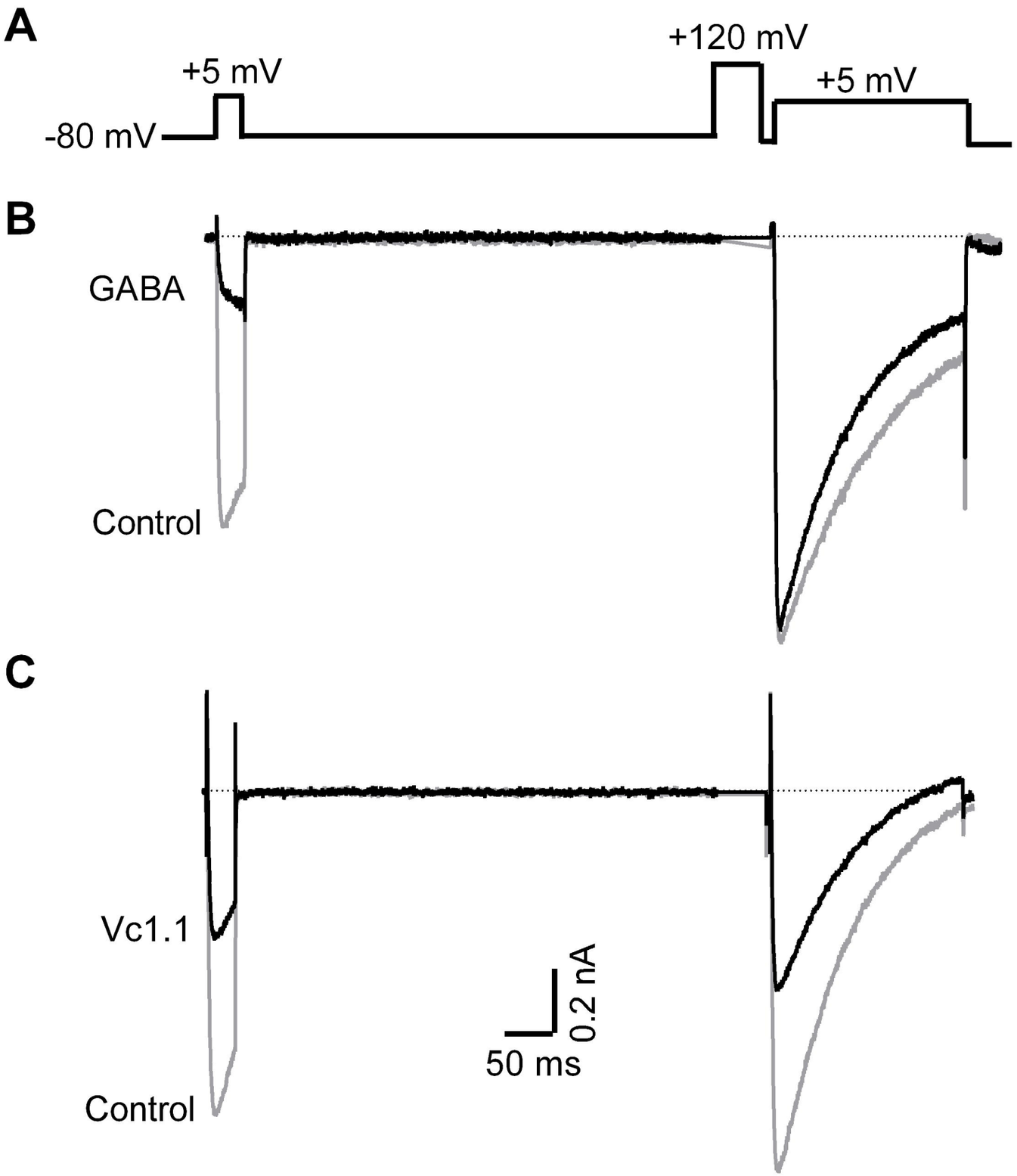


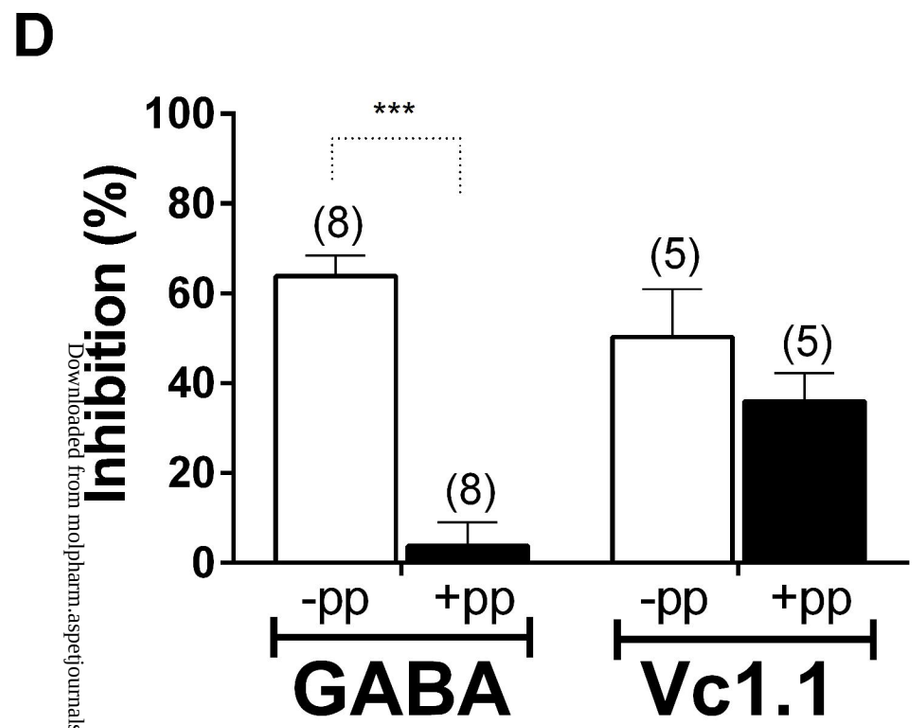




Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.095356
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5





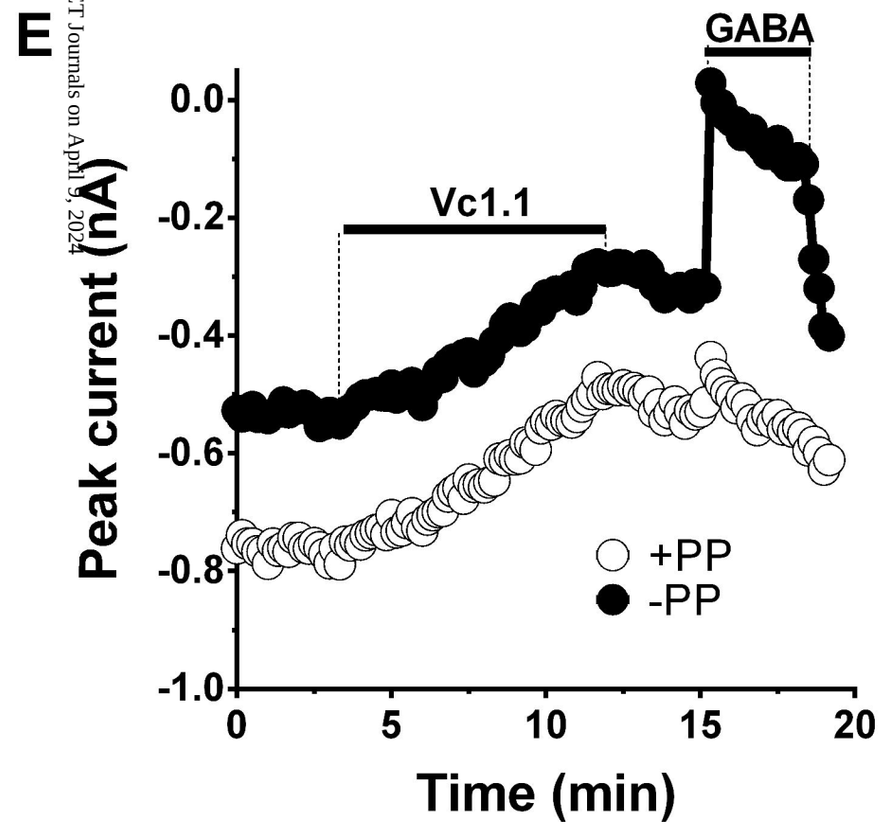
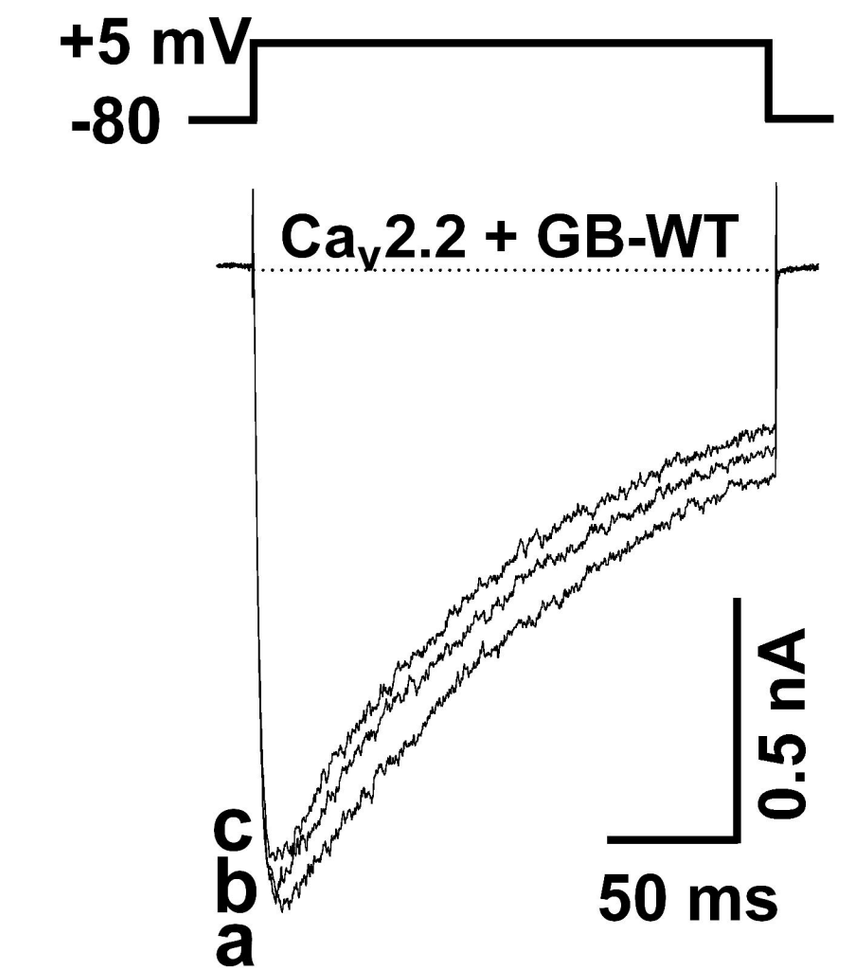
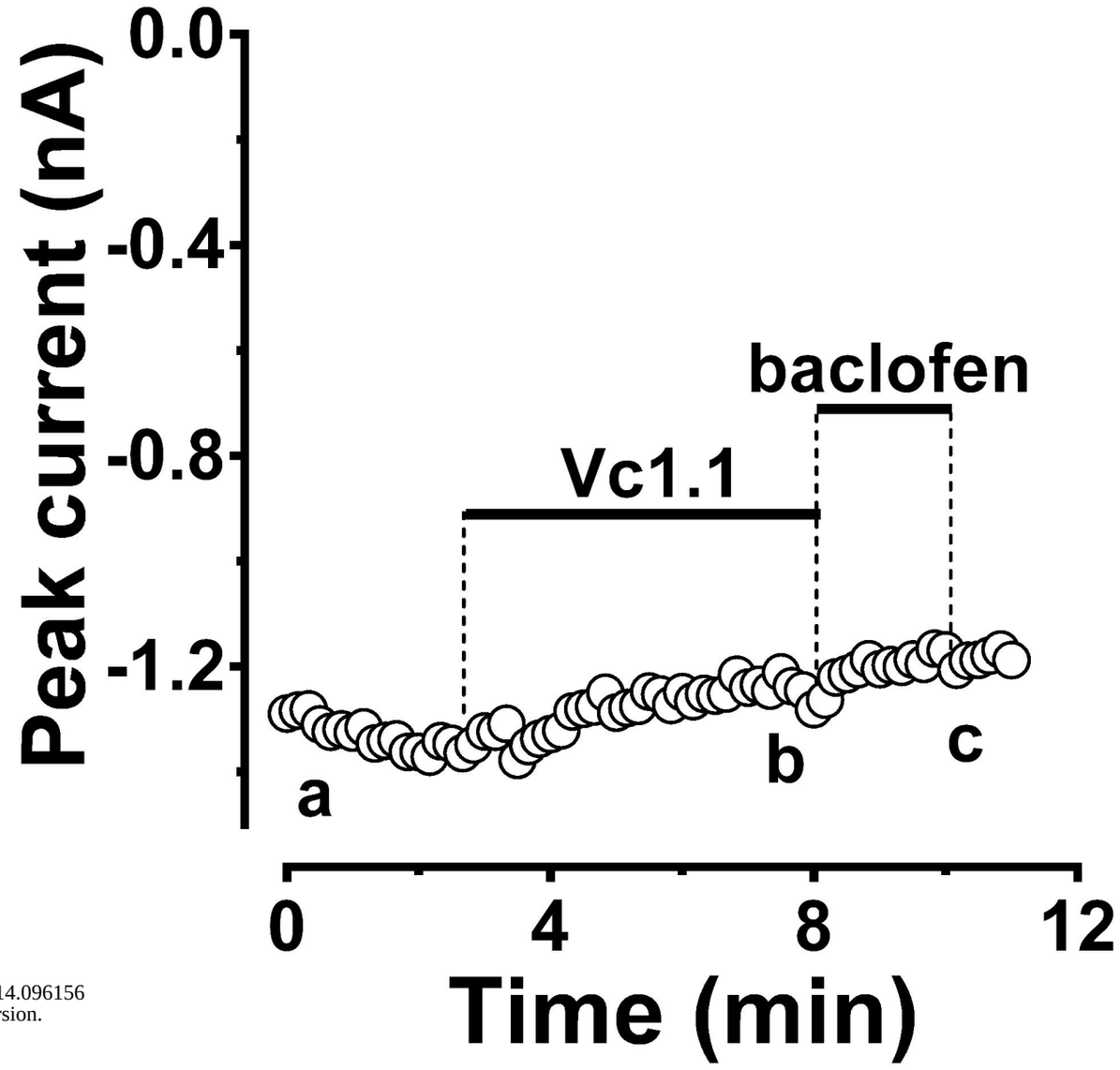


Figure 6





Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.

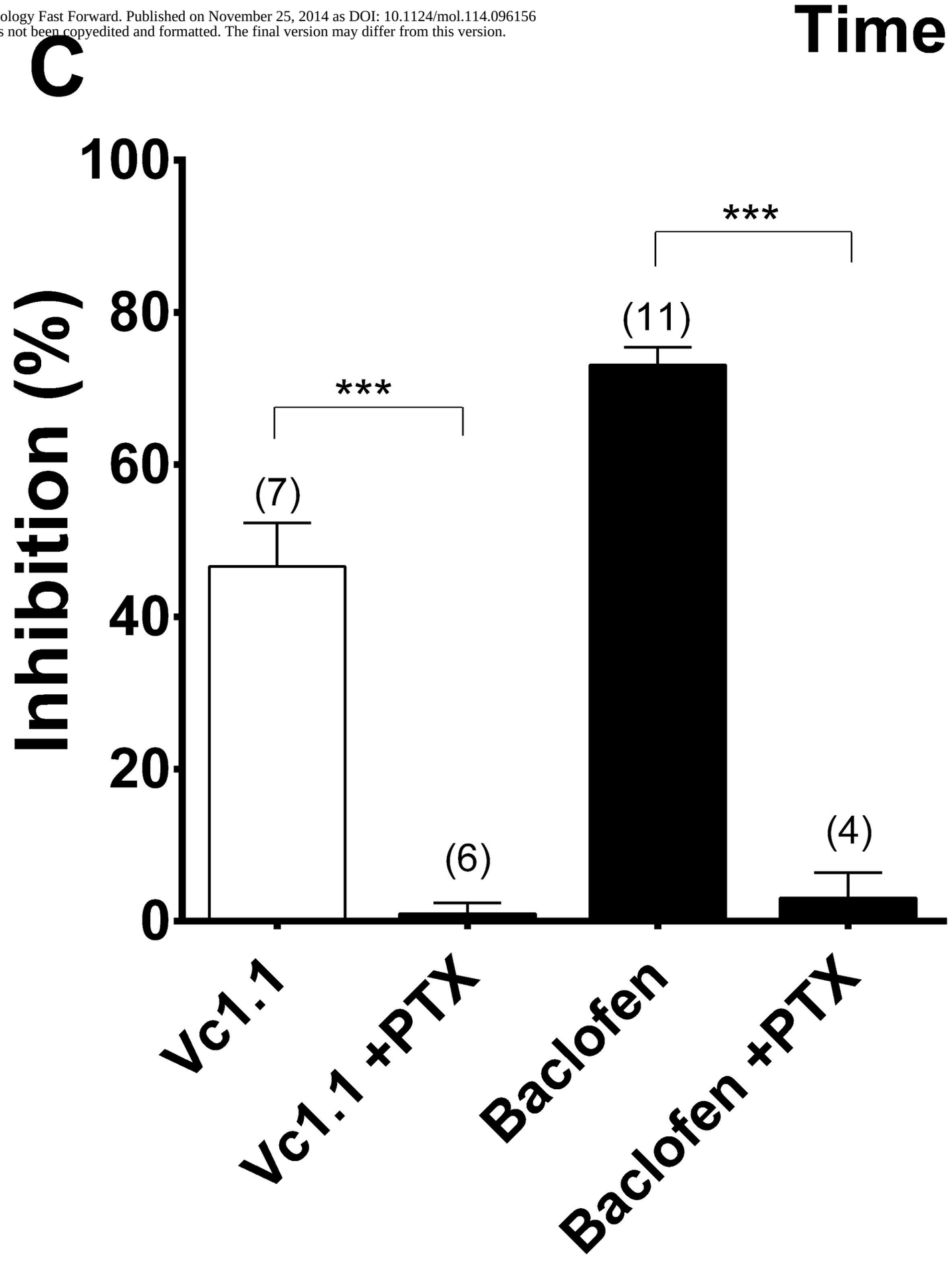


Figure 7

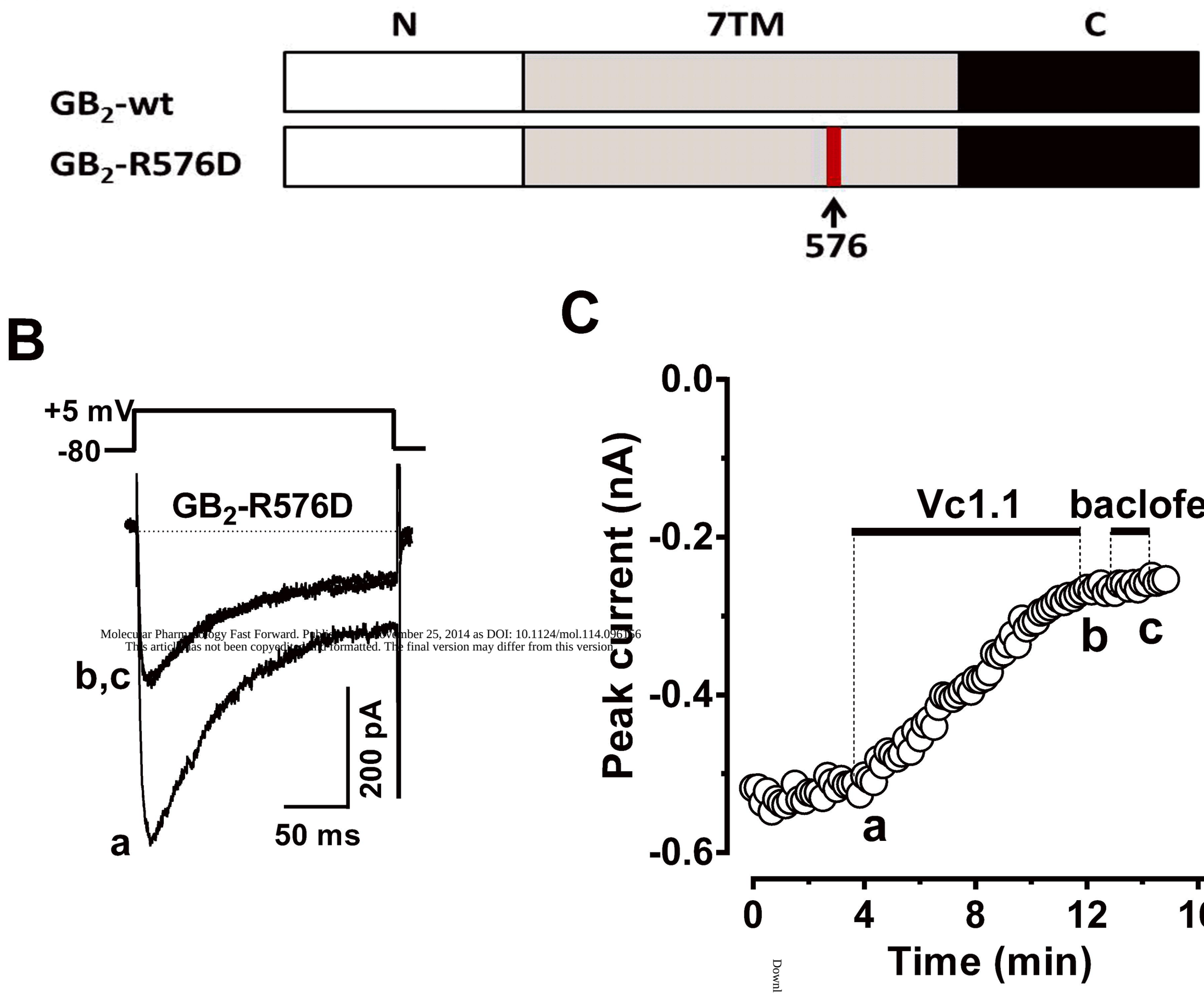
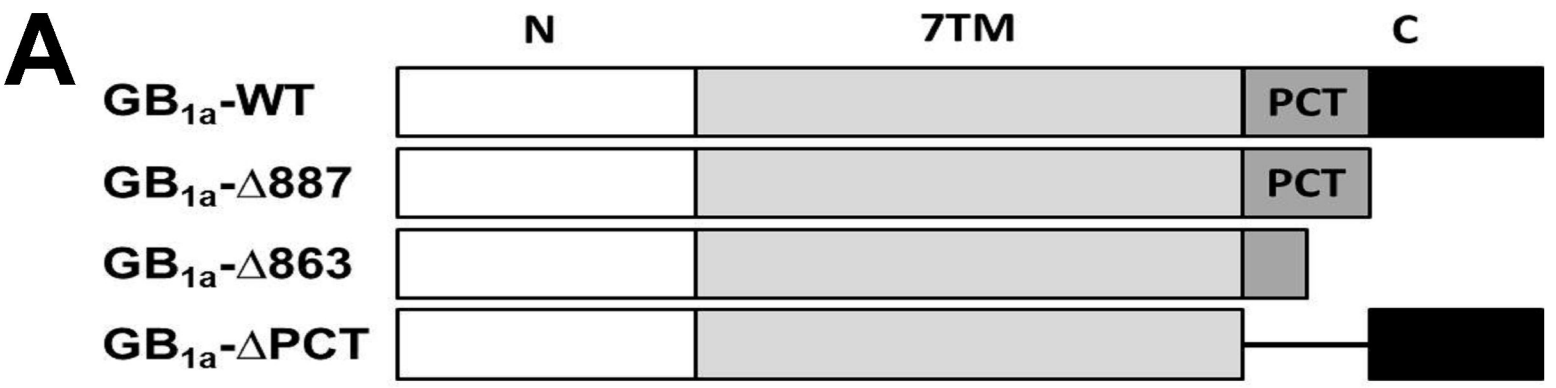
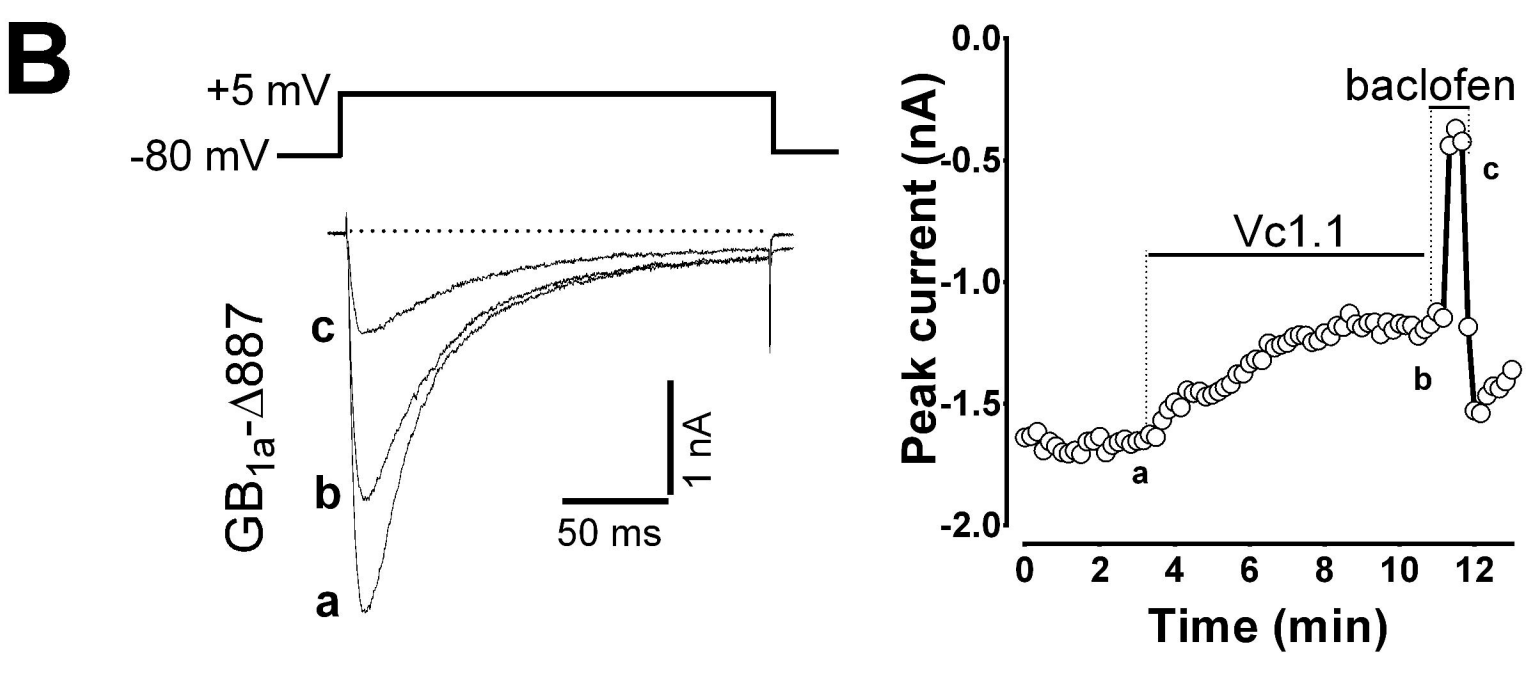
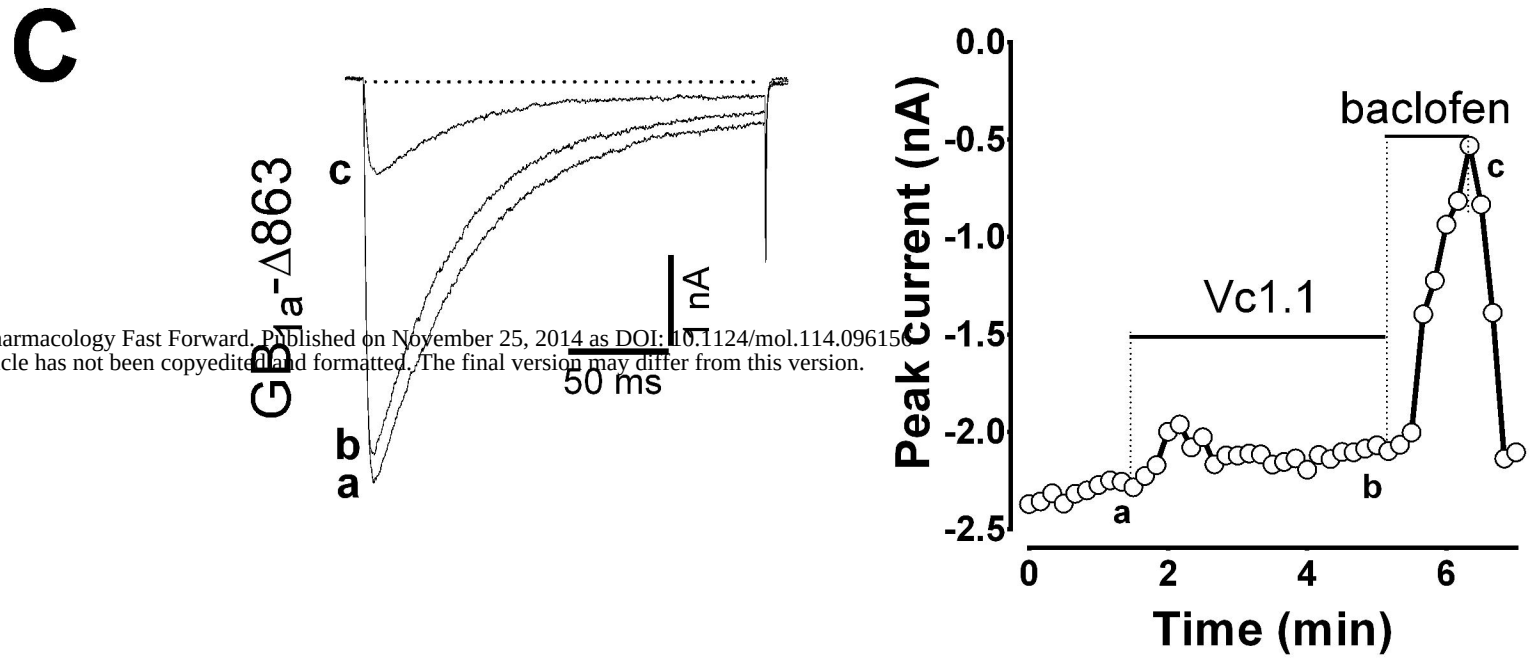


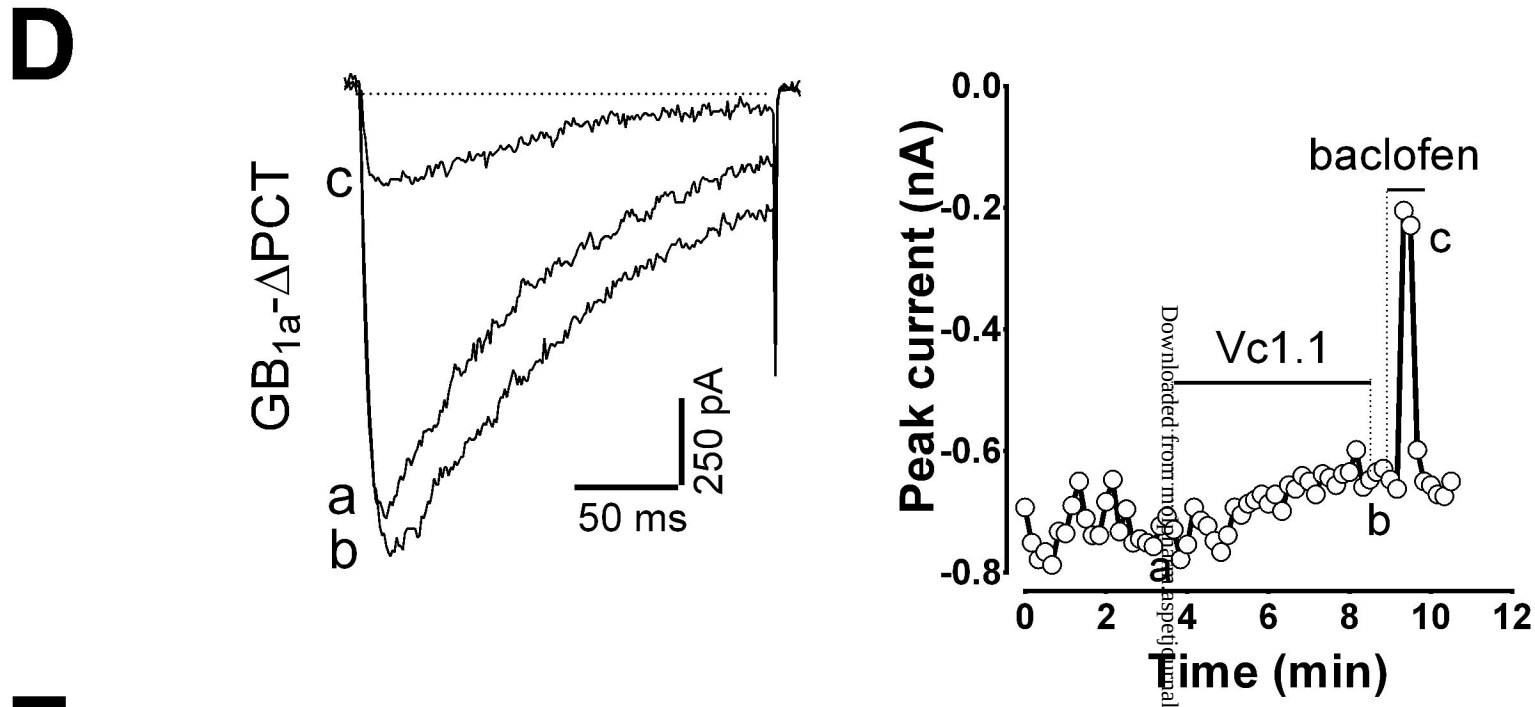
Figure 8







Molecular Pharmacology Fast Forward. Published on November 25, 2014 as DOI: 10.1124/mol.114.096156
This article has not been copyedited and formatted. The final version may differ from this version.



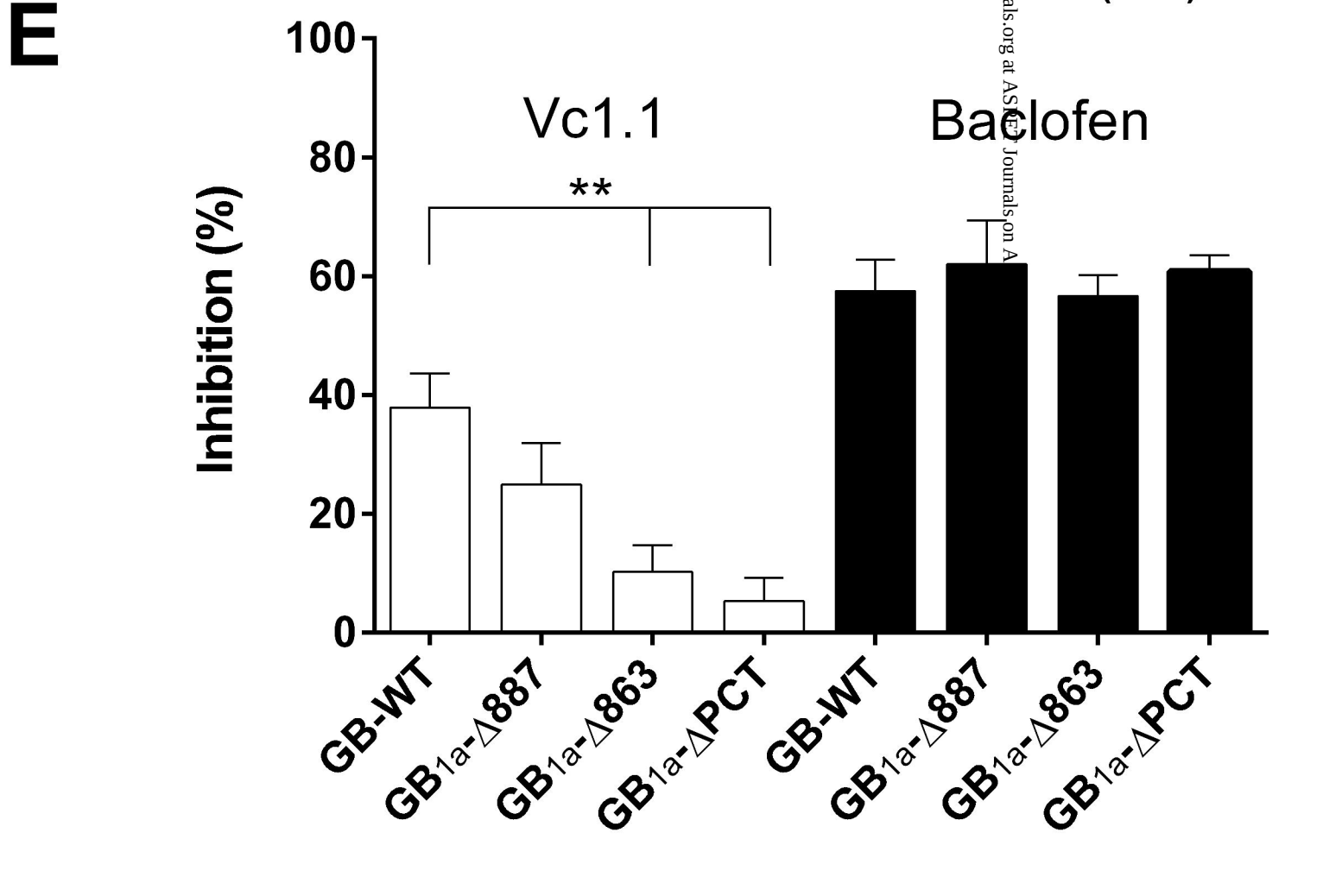
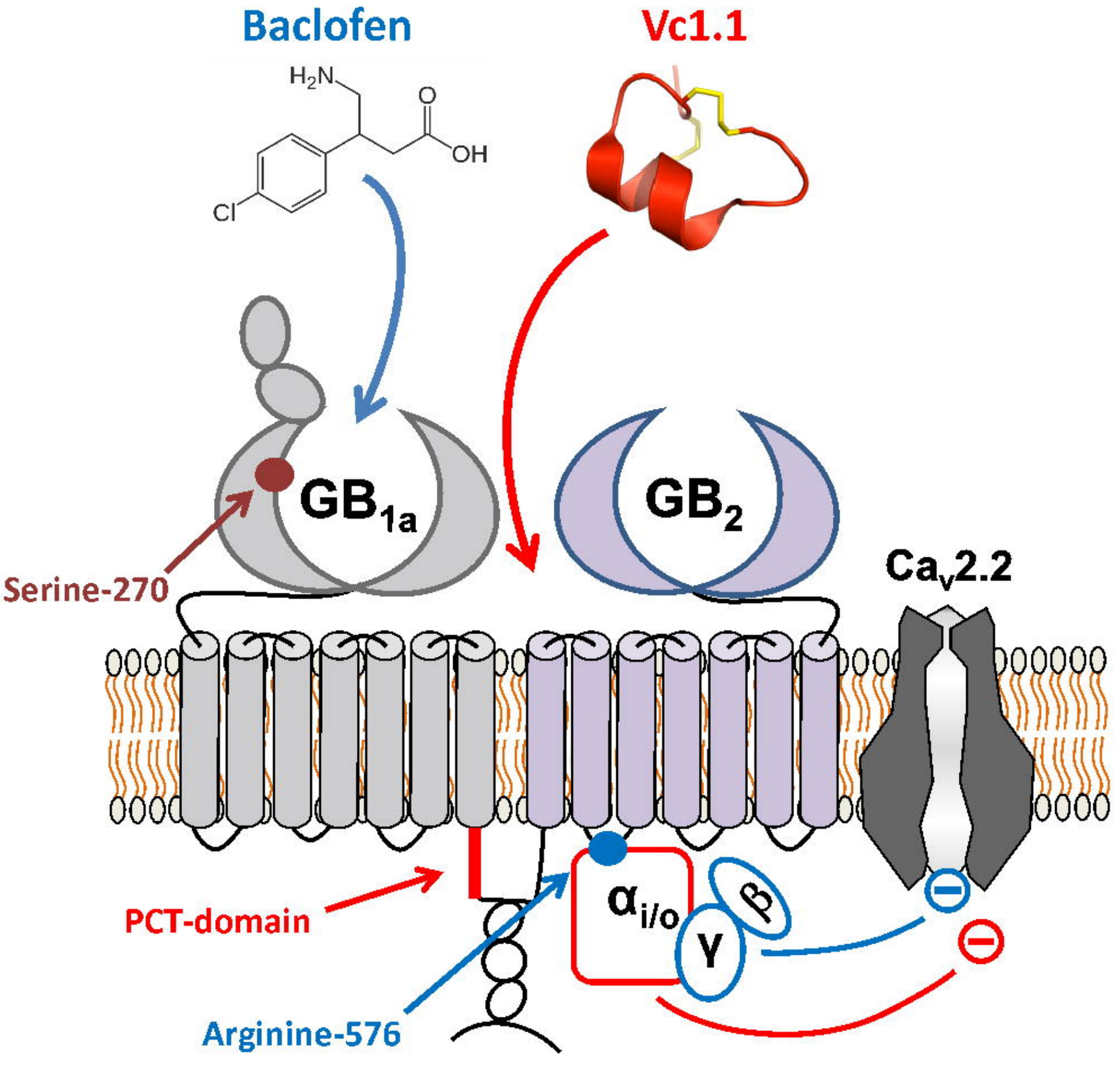


Figure 9


Baclofen
Vc1.1
H_2N
O
OH
Cl
GB_{1a}
GB_2
Serine-270
$Ca_V2.2$
PCT-domain
$\alpha_{i/o}$
β
γ
Arginine-576