Voltage-Gated R-Type Calcium Channel Inhibition via Human $\mu$-, $\delta$-, and $\kappa$-opioid Receptors Is Voltage-Independently Mediated by G$\beta$$\gamma$ Protein Subunits

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ABSTRACT

Elucidating the mechanisms that modulate calcium channels via opioid receptor activation is fundamental to our understanding of both pain perception and how opioids modulate pain. Neuronal voltage-gated N-type calcium channels (Ca$_{V2.2}$) are inhibited by activation of G protein–coupled opioid receptors (ORs). However, inhibition of R-type (Ca$_{V2.3}$) channels by $\mu$- or $\kappa$-ORs is poorly defined and has not been reported for $\delta$-ORs. To investigate such interactions, we coexpressed human $\mu$-, $\delta$-, or $\kappa$-ORs with human Ca$_{V2.3}$ or Ca$_{V2.2}$ in human embryonic kidney 293 cells and measured depolarization-activated Ba$^{2+}$ currents ($I_{Ba}$). Selective agonists of $\mu$-, $\delta$-, and $\kappa$-ORs inhibited $I_{Ba}$ through Ca$_{V2.3}$ channels by 35%. Ca$_{V2.2}$ channels were inhibited to a similar extent by $\kappa$-ORs, but more potently (60%) via $\mu$- and $\delta$-ORs. Antagonists of $\delta$- and $\kappa$-ORs potentiated $I_{Ba}$ amplitude mediated by Ca$_{V2.3}$ and Ca$_{V2.2}$ channels. Consistent with G protein $\beta_{Y}$ (G$\beta$$\gamma$) interaction, modulation of Ca$_{V2.2}$ was primarily voltage-dependent and transiently relieved by depolarizing pre pulses. In contrast, Ca$_{V2.3}$ modulation was voltage-independent and unaffected by depolarizing pre pulses. However, Ca$_{V2.3}$ inhibition was sensitive to pertussis toxin and to intracellular application of guanosine 5’-[$\beta$-thio]diphosphate trilithium salt and guanosine 5’-[$\gamma$-thio]triphosphate tetralithium salt. Coexpression of G$\beta$$\gamma$–specific scavengers—namely, the carboxyl terminus of the G protein–coupled receptor kinase 2 or membrane-targeted myristoylated-phosducin—attenuated or abolished Ca$_{V2.3}$ modulation. Our study reveals the diversity of OR–mediated signaling at Ca$_{V2.2}$ channels and identifies neuronal Ca$_{V2.3}$ channels as potential targets for opioid analgesics. Their novel modulation is dependent on pre-existing OR activity and mediated by membrane-delimited G$\beta$$\gamma$ subunits in a voltage-independent manner.

Introduction

In presynaptic nerve terminals, Ca$_{V2.2}$ influx through voltage-gated P/Q-type (Ca$_{V2.1}$), N-type (Ca$_{V2.2}$), and R-type (Ca$_{V2.3}$) calcium channels controls neurotransmitter release (Eggermann et al., 2012). Both endogenous opioids and opioid drugs efficiently inhibit Ca$_{V2.1}$ and Ca$_{V2.2}$ channels via G protein–coupled $\mu$-, $\delta$-, and $\kappa$-opioid receptor (OR) activation (Al-Hasani and Bruchas, 2011). This inhibition reduces neuronal excitability and alters nociceptive signaling in various animal pain models. Structurally, inhibition of Ca$_{V2.1}$ and Ca$_{V2.2}$ channels via G protein–coupled receptors (GPCRs) is largely determined by direct interaction of G$\beta$$\gamma$ with the channel pore formed by the $\alpha_{1A}$ or $\alpha_{1B}$ subunit, respectively (Zamponi and Currie, 2013). Previous studies suggest there are considerable differences in the ability of G$\beta$$\gamma$ to regulate Ca$_{V2.2}$ and Ca$_{V2.3}$ channels directly (Toth et al., 1996). However, given the challenge of reliably distinguishing the currents through various Ca$_{V2.2}$ channels in neurons, Ca$_{V2.3}$ channel modulation via various GPCRs is incompletely characterized.

In humans, Ca$_{V2.2}$ channels represent a validated therapeutic target for management of chronic pain. Comparatively, Ca$_{V2.1}$ channels play limited roles in afferent pain pathways (Bourinet et al., 2014). Ca$_{V2.3}$ channels are expressed in many regions of the central nervous system, where they contribute

ABBREVIATIONS: BNTX, 7-benzylideneametralenoxale; Ca$_{2.2}$x, neuronal voltage-gated calcium channel; CTAP, D-Phe-cyc[Cys-Tyr-D-Trp-Arg-Thr-Phe-Pro-NH$_2$]; DAMGO, [D-Ala$_2$]-N-Me-Pen$_3$-enkephalin; DRG, dorsal root ganglion; GDP-\(\beta\)-S, guanosine 5’-[\(\beta\)-thio]diphosphate trilithium salt; GNTI, 5’-[(S)-guanidinyl-1-7(S)-thio]triphosphate tetralithium salt. Coexpression of G$\beta$$\gamma$–specific scavengers—namely, the carboxyl terminus of the G protein–coupled receptor kinase 2 or membrane-targeted myristoylated-phosducin—attenuated or abolished Ca$_{V2.3}$ modulation. Our study reveals the diversity of OR–mediated signaling at Ca$_{V2.2}$ channels and identifies neuronal Ca$_{V2.3}$ channels as potential targets for opioid analgesics. Their novel modulation is dependent on pre-existing OR activity and mediated by membrane-delimited G$\beta$$\gamma$ subunits in a voltage-independent manner.
to neuronal excitability, memory, and learning (Burestedt et al., 2003). They are also present in somatosensory neurons of the peripheral ganglia (Fang et al., 2007), implicating them as components of pain pathways. Genetic research approaches confirmed this, with Ca,2.3 knockout mice exhibiting reduced pain perception (Saegusa et al., 2000; Yang and Stephens, 2009). In pharmacological studies, intrathecal delivery of the Ca,2.3 channel blocker SNX-482, a toxin from the tarantula Hysterocrates gigas, produces analgesia in animal models of neuropathic pain (Matthews et al., 2007; Terashima et al., 2013). Despite the clear functional importance of Ca,2.3 channels in pain pathways, the mechanisms by which they are modulated by GPCRs have not yet been adequately examined (Rittenhouse, 2014). It is known that the Ca,2.3 channel is regulated by muscarinic (Bannister et al., 2004), dopamine (Page et al., 1998), and GABAB (Berecki et al., 2014) receptors. From reconstitution in heterologous expression systems, it is also known that Ca,2.3 activity is weakly modulated via μ- and κ-ORs (Ottolia et al., 1998; Simen and Miller, 1998) but not δ-ORs; lack of Ca,2.3 modulation via μ-OR has also been reported (Bourinet et al., 1996). The current consensus is that, compared with Ca,2.2, Ca,2.3 channels are only weakly modulated via GPCRs. This differential regulation is attributed to variations in the N-terminal, domain I, and intracellular linker I-II regions of the αβ1B compared with the αβ1E subunit (Simen and Miller, 1998; Stephens et al., 1998).

The goal of the present study is to comprehensively and comparatively assess the modulation of human Ca,2.3 and Ca,2.2 calcium channels via human μ-, δ-, or κ-OR activation. Our data suggest membrane-delimited Gγ subunits mediate signal transduction not only between ORs and Ca,2.2 channels, but also between ORs and Ca,2.3. However, in contrast with the primarily voltage-dependent modulation of Ca,2.2 channels, Ca,2.3 modulation is voltage-independent. We also reveal that δ- or κ-OR, but not μ-OR, activation directly potentiates depolarization-activated Ba⁺⁺ currents (I Ba) through Ca,2.2 and Ca,2.3 channels in the absence of δ- or κ-OR agonists. Using Gγ protein–specific scavengers, we demonstrate that membrane-delimited Gβγ subunits mediate signal transduction not only between ORs and Ca,2.2 channels, but also between ORs and Ca,2.3 in a voltage-independent manner. Delineating the diversity of OR-mediated signaling at Ca,2.2 channels will enhance our understanding of pain perception and of how opioids modulate pain.

Materials and Methods

Cells and Clones. Human embryonic kidney 293 (HEK293) cells stably coexpressing the human Ca,2.3 (R-type) channel (αβ1E; major neuronal splice variant; GenBank accession no. L29385) or human Cav2.2 (N-type) channel (αβ1I, splice variant; M91472.1) and human αβ2δ-1 (M76559), human β3a (NM_0000725) auxiliary subunits, and human KCNJ4 channel (Kir2.3; U07364) were obtained from Merck (Kenilworth, NJ) and cultured according to procedures described previously (Dai et al., 2008). Cells were transiently cotransfected using calcium phosphate precipitation as described previously (Berecki et al., 2014). Transfection with 1.5 μg of plasmids carrying cDNAs of human μ- or δ-, or κ-ORs (NM_001145279.1, NM_000911.3, and NM_000912.2, respectively; OriGene Technologies, Inc., Rockville, MD) and enhanced green fluorescent protein (0.5 μg) resulted in cells expressing Ca,2.3 or Ca,2.2/μ-, δ-, or κ-OR unless indicated otherwise. In another series of experiments, plasmids encoding a 1506-bp C-terminal fragment of the North American opossum G protein kinase 2 (GRK-CT, 3 μg; AF087455; kindly provided by Dr. Paul R. Albert, University of Ottawa, Ottawa, ON, Canada) or a myristoylated bovine phosducin (m-Phos, 5 μg; GB M33529; kindly provided by Dr. Nathan Dascal, Tel Aviv University, Tel Aviv, Israel) were included in transfections. In separate experiments, HEK293 cells stably expressing the Simian virus 40 large T antigen (HEK293ST) were transiently cotransfected with human αβ1E (3 μg), human αβ2δ-1 (M76559; 5 μg), human β3a (NM_000724; 1.5 μg), or human β3a and human μ-, δ-, or κ-OR receptors and enhanced green fluorescent protein cDNAs, as described earlier. After transfections, cells were plated on glass coverslips and cultured as described previously (Berecki et al., 2014).

Electrophysiology. Three to five days after transfection, depolarization-activated I Ba were recorded in the whole-cell patch clamp configuration using superfused (~600 μM/min) extracellular bath solution containing 10 mM BaCl₂, 100 mM NaCl, 1 mM MgCl₂, 5 mM CaCl₂, 30 mM TEA-Cl, 10 mM d-glucose, and 10 mM HEPES adjusted to a pH of 7.4 with TEA-OH, at room temperature (23–25°C). The intracellular pipette solution contained 125 mM K-glucolate, 2 mM MgCl₂ 5 mM EGTA, 5 mM NaCl, 4 mM MgATP, and 10 mM HEPES adjusted to a pH of 7.25 with CsOH. GTP was not included in the pipette solution to prevent I Ba rundown caused by activation of signaling pathways when the whole-cell recording configuration was established (Ruingo et al., 2007). Data were recorded with a Multi-clamp 700B amplifier (Molecular Devices, Sunnyvale, CA) controlled by a Clampex 9.2/DigiData 1322 acquisition system (Molecular Devices). Fire-polished borosilicate patch pipettes typically exhibited resistance values of 1.5–2.5 MΩ, and series resistances were uncompensated >80% in all cases. Membrane currents were filtered at 3 kHz and sampled at 10 kHz. Leak and capacitive currents were subtracted using a P/4 pulse protocol.

Chemicals. [D-Ala², NMe-Phe⁴, Gly-ol⁷]-enkephalin (DAMGO), naloxonazine dихiодрилозо (naloхозине), D-Phe-tyr[Cys-Tyr-D-Trp-Arg-Thr-Phe]-NH₂ (CTAP), (+)-4-[(R)-(1S,2S)-(25R,4R)-1S,2S-2,5-dimethyl-1-piperazinyl]-3-methoxybenzylYL-N,V-diethylbenzamide (SNAC90), 7-benzylidenenaltrexone maleate (BNTX), (2S,3R)-trans-4-Allyl-2,5-dihydro-6a,10b-dimethyl-4,10-dioxo-2H-naphtho[2,1-π]pyran-7-carboxylic acid methyl ester (salvinorin-A), and 5‘-guanydino-1-(7-cyclopentylpropyl)aminomethyl-6,7-dehydro-4,6,5-epoxy-3,14-dihydroxy-6,7,2-3-indolomorphinan dihydrochloride (GNTI) were purchased from Tocris Bioscience (Bristol, UK). Various drugs were prepared from stock solutions and added to the bath solution at concentrations specified in the Results section. Pertussis toxin was purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). In some series of experiments, guanosine 5‘-β-thiophosphate trilithium salt (GDP-β-S, 500 nM; Sigma-Aldrich), guanosine 5‘-γ-thiophosphate tetralithium salt (GDP-γ-S, 200 nM; Sigma-Aldrich), and pp60c-Src peptide (50 μM; Tocris) were included in the pipette solution.

Curve Fitting and Statistical Analysis. Data were analyzed offline using Clampfit 9.2 (Molecular Devices) and Origin 9.0 (Microcal Software Inc., Northampton, MA). I Ba amplitude, voltage dependence of I Ba activation, concentration-response curves, and voltage-independent fractions of the inhibited I Ba were determined and interpreted as described previously (Berecki et al., 2014). The percentage of I Ba modulation was defined as I/I 0, where I represents the peak I Ba amplitude obtained in the presence of a agonist, and I 0 is the peak I Ba amplitude under control conditions. Total I Ba modulation was estimated as the sum of the percent of inhibition (agonist response) and percent of potentiation (antagonist response). Steady-state inactivation was determined by analyzing 10-mV step-elicted I Ba amplitude values from a holding potential of −80 mV and a 2-second conditioning step (to potentials between −110 and +40 mV) at 0.066 Hz. Normalized I Ba amplitudes were plotted as a function of the conditioning voltages, and data were fitted using the Boltzmann equation, I/I max = 1/1 + exp((V−V 1/2,inact)/k), where V and V 1/2,inact are the conditioning and the half-maximal inactivation potential, respectively, and k is a slope factor. Voltage-dependent relief of inhibition was assessed using a protocol described previously.
Cav2.3 antagonist GNTI after k-time course of potentiation of current densities in the presence of neither opioid receptor agonists nor antagonists (Sal-A) washed out Ca v2.3/κ-OR and Ca v2.2/κ-OR cells. Supplemental Fig. 2 shows that, in the absence of opioid receptors, OR antagonists and agonists do not modulate Ca v2.3 or Ca v2.2 channels in HEK293 cells. Supplemental Fig. 3 demonstrates the effects of varying the parameters of the voltage protocol to investigate voltage-dependent relief of inhibition in HEK293 cells stably expressing Ca v2.2 or Ca v2.3 channels and transiently coexpressing μ-ORs. Supplemental Table 1 shows average P2/P1 values in HEK293 cells coexpressing Ca v2.2 or Ca v2.3 channels and various ORs.

**Results**

**Inhibition of Ca v2.3 Channels Occurs via Agonist-Dependent and Agonist-Independent OR Activation.** In HEK293 cells stably expressing Ca v2.3 channels and transiently coexpressing μ-, δ-, or κ-ORs (Ca v2.3/μ-, δ-, or κ-OR cells), the selective OR agonists DAMGO and SNC80 reversibly inhibited ∼35% of peak IBa amplitude via μ- and δ-ORs, respectively. In comparison, Sal-A rapidly and irreversibly reduced IBa by ∼30% via κ-OR activation (Fig. 1A). These inhibitions were concentration-dependent and were described by the Hill equation, resulting in half-maximal inhibitory concentration (IC 50) values of 9.3 ± 0.5, 76.1 ± 19, and 4.2 ± 0.6 nM for DAMGO, SNC80, and Sal-A, respectively (Fig. 1A). IBa inhibition produced by selective μ-, δ-, or κ-OR agonists was fully antagonized by the broad-spectrum μ-OR antagonist naloxone (1 μM) or only partially antagonized by the selective δ- and κ-OR antagonists GNTI (100 nM) and GNTI (100 nM), respectively. Remarkably, BNTX and GNTI, but not naloxone, potentiated IBa through Ca v2.3 channels and transiently coexpressing μ-ORs. Supplemental Table 1 shows average P2/P1 values in HEK293 cells coexpressing Ca v2.2 or Ca v2.3 channels and various ORs. Overall, these results suggest BNTX and GNTI act as inverse agonists and relieve IBa inhibition through either Ca v2.3 or Ca v2.2 channels. In these cells, the sum of the inhibited and potentiated IBa fraction represents total modulation (Fig. 2B; Table 1).

**Inhibition of Ca v2.3 Channels via ORs Is Voltage-Independent.** We evaluated the current-voltage relationships (I = V) and steady-state inactivation in Ca v2.3/μ-, δ-, or κ-OR cells. In the absence and presence of agonists or antagonists, the midpoint of activation (V 0.5,act) and the midpoint of inactivation (V 0.5,inact) values, respectively, remained largely unchanged (Table 2). Ca v2.2 channel inhibition via various ORs involves direct and voltage-dependent Gbg binding to the channel pore-forming subunit, and inhibition can be transiently relieved by strong depolarizing pulses (Bourinet et al., 1996; Simen and Miller, 1998). Figure 3 shows Ca v2.2 channel modulation via various ORs was primarily voltage-dependent and could be relieved by depolarizing pulses. In the absence of agonists (control), pulses resulted in P2/P1 ratios >1, suggesting facilitation of IBa may be associated with constitutive or tonic OR activity (Fig. 3, A and B). The P2/P1 value was relatively high in control Ca v2.2/κ-OR cells and remained unaltered in the presence of Sal-A. In comparison, DAMGO or SNC80 significantly increased the P2/P1 ratios in Ca v2.2/δ- or δ-OR cells, respectively (Fig. 3B; Supplemental Table 1). Remarkably, OR-mediated Ca v2.3 channel inhibition was voltage-independent and was not affected by depolarizing pulses. This is indicated by the P2/P1 < 1 ratios that remained unchanged in the absence or presence of OR agonists (Fig. 3, A and B; Supplemental Table 1). Previous studies indicated that inactivation typically obscures facilitation of Ca v2.3 current amplitudes (Toth et al., 1996; Meza and Adams, 1998). Consequently, the smaller IBa amplitudes during P2 compared with those during P1 are attributable to voltage-dependent inactivation that occurs during the prepulse in Ca v2.3-OR cells. To minimize IBa inactivation during a prepulse, and unmask any prepulse-induced facilitation, we transiently coexpressed Ca v2.3 (α1Eδ3), α1Eδ5-1, and Ca v2.2 subunits, and μ-, δ-, or κ-ORs in HEK293T cells (Ca v2.3/α1Eδ5-1 + β2 + OR cells), and studied the voltage dependence of IBa in the absence or presence of OR agonists (Fig. 3, C and D; Supplemental Table 1). The results suggest that depolarizing pulses have a negligible effect on IBa facilitation through the Ca v2.3 channel during inhibition via ORs. Next, in Ca v2.2/μ-OR and Ca v2.3/μ-OR cells, we altered the duration between the prepulse and P2 (Δt1) or the prepulse duration...
To determine $I_{Ba}$ facilitation in the absence and presence of DAMGO while altering these parameters (Supplemental Fig. 3). In Cav2.2/μ-OR cells, increasing $\Delta t1$ progressively reduced the degree of $I_{Ba}$ facilitation, whereas increasing $\Delta t2$ initially resulted in an increase in $I_{Ba}$ facilitation that peaked at $\Delta t2 = 20$ ms and then decreased. In Cav2.3/μ-OR cells, altering $\Delta t1$...
had negligible effects on the P2/P1 ratio, whereas increasing ΔT2 progressively reduced the P2/P1 ratio, confirming that I_{Ba} inactivation during prepulse obscures I_{Ba} facilitation through Ca_{2.3} channels. For both channels, P2/P1 values followed a similar trend when using a +120-mV depolarizing prepulse instead of +80 mV (not shown). Ca_{2.3} channel inhibition via ORs evidently does not follow the conventional G_{βγ} signaling paradigm associated with the change in the voltage dependence that opens Ca_{2.2} channels (Bean, 1989).

G_{βγ} Scavengers Disrupt Signal Transduction between ORs and Ca_{2.3} Channels. Functional interaction between Ca_{β} subunits and G_{βγ} is known to limit GPCR-mediated inhibition of Ca_{2} channels (Dolphin, 2012). We next investigated the effects of Ca_{β} subunits on inhibition of I_{Ba} in HEK293T cells transiently coexpressing μ, δ, or κ-ORs, α_{1E,3}, and α_{2δ}-1, and in the absence or presence of the Ca_{β2} or Ca_{β3} subunits. Specific OR agonists inhibited I_{Ba} to a similar extent as control, indicating Ca_{β} has no role (Fig. 4A). In Ca_{2.3}/μ-, δ-, or κ-OR cells, I_{Ba} inhibition was sensitive to intracellular application of hydrolysis-resistant GDP-β-S and GTP-γ-S, and was abolished by pertussis toxin. These results suggest activation of the Go_{i/o} subunit is also required for I_{Ba} inhibition. Various protein kinase inhibitors, known to modify the activities of c-Src (pp60-c-Src, stauroseporine), protein kinase A, protein kinase C, Ca_{2}^{2+}/calmodulin-dependent protein kinase II (stauroseporine), and phosphatidylinositol

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**TABLE 1**

Percentages of I_{Ba} modulation by opioid receptor agonists and antagonists in Ca_{2.3}/μ-, δ-, or κ-OR cells and Ca_{2.2}/μ-, δ-, or κ-OR cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Agonist</th>
<th>Antagonist</th>
<th>Total Modulation (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca_{2.3}/μ-OR</td>
<td>35.2 ± 1.1 (34)</td>
<td>-7.3 ± 3.0 (8)</td>
<td>35.2^b</td>
</tr>
<tr>
<td>Ca_{2.3}/δ-OR</td>
<td>35.9 ± 1.6 (22)</td>
<td>18.2 ± 4.7 (7)</td>
<td>54.1</td>
</tr>
<tr>
<td>Ca_{2.3}/κ-OR</td>
<td>31.2 ± 2.7 (27)</td>
<td>23.8 ± 5.4 (6)</td>
<td>55</td>
</tr>
<tr>
<td>Ca_{2.2}/μ-OR</td>
<td>52.8 ± 3.8 (11)</td>
<td>-1.3 ± 2.4 (5)</td>
<td>52.8^b</td>
</tr>
<tr>
<td>Ca_{2.2}/δ-OR</td>
<td>60.9 ± 4.9 (9)</td>
<td>77.3 ± 7.4 (6)</td>
<td>138.2</td>
</tr>
<tr>
<td>Ca_{2.2}/κ-OR</td>
<td>22.6 ± 4.9 (12)</td>
<td>72.0 ± 8.9 (5)</td>
<td>94.0</td>
</tr>
</tbody>
</table>

^aNaloxone slightly inhibited I_{Ba} in Ca_{2.3}/μ-OR cells (see also Fig. 2).

^bTotal modulation in Ca_{2.3} or Ca_{2.2}/μ-OR cells does not include data in the presence of naloxone.

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**Fig. 2.** Agonist-dependent and agonist-independent OR activity defines total modulation of Ca_{2.3} and Ca_{2.2} channels. (A) DAMGO (1 μM), SNC80 (1 μM), and Sal-A (1 μM) inhibit I_{Ba} whereas BNTX (100 nM) and GNTI (100 nM) potentiate it; maximum potentiation occurred at 10 nM BNTX or GNTI (n = 4 for each; not shown). I_{Ba} was evoked by 150-ms step depolarizations (0.1 Hz) to +10 mV (Ca_{2.3}) or +15 mV (Ca_{2.2}), from a holding potential of ~80 mV. Note that only 20 ms of the 150-ms traces is shown. Horizontal scale, 10 ms; vertical scale, 1 nA; c, control; w, washout; dashed lines indicate zero-current level. Note the pronounced agonist-independent modulation of Ca_{2.2} channels via κ-ORs, consistent with the slow time course of control I_{Ba} (arrow) and the weak I_{Ba} inhibitory effect of Sal-A. (B) Average data (± S.E.M.) of percentage inhibition and potentiation of I_{Ba} through Ca_{2.3} or Ca_{2.2} channels in the presence of various ORs. In Ca_{2.3}/μ-, δ-, or κ-OR cells, DAMGO, SNC80, and Sal-A inhibit I_{Ba} by 35.2 ± 1.1%, 35.9 ± 1.8%, and 31.2 ± 2.7%, respectively. Naloxone weakly inhibits I_{Ba} by 7 ± 3%, whereas BNTX and GNTI potentiate I_{Ba} by 18.2 ± 4.7% and 22.8 ± 5.4%, respectively. In Ca_{2.2}/μ-, δ-, or κ-OR cells, DAMGO, SNC80, and Sal-A inhibit I_{Ba} by 52.8 ± 3.8%, 60.9 ± 4.9%, and 22.6 ± 4.9%, whereas BNTX and GNTI potentiate I_{Ba} by 77.3 ± 7.4% and 72.0 ± 8.9%, respectively. The number of experiments, n, is in parentheses.
channel inhibition can be revealed by voltage-dependent signaling and modulation of both Cav2.2 and Cav2.3 channels. These findings suggest Gbg of both Cav2.3 and Cav2.2 channels by OR pathways.

mediated event in G protein activation, preventing inhibition of both Cav2.2 and Cav2.3 channels by OR pathways.

3-kinase (LY294002 hydrochloride) (Fig. 4A) produced only subtle effects, suggesting phosphorylation plays a small or negligible role in this OR signaling pathway.

We tested the Gbg dependence of OR signaling by coexpressing the GRK-CT protein corresponding to the C-terminal domain of G protein–coupled receptor kinase 2 (GRK2 or β-adrenergic receptor kinase 1, βARK1). This domain is known to bind and specifically inactivate free G protein Gbg subunits (Koch et al., 1993; Ghahremani et al., 2000). Figure 4A shows GRK-CT efficiently prevented the Ibₐ inhibition produced by DAMGO and SNC80 (57 and 75%, respectively). GRK-CT also eliminated Ibₐ inhibition by Sal-A. Next, we coexpressed m-Phos to increase the likelihood of targeting the plasma membrane–associated Gbg fraction (Rishal et al., 2005). In this case, Cav2.2/µ, δ, or κ-OR cells served as positive control because Gbg-mediated Cav2.2 channel inhibition can be revealed by voltage-dependent facilitation. Compared with GRK-CT, m-Phos more effectively reduced Cav2.3 channel modulation via µ- or δ-ORs and abolished modulation via κ-ORs (Fig. 4, A and B). Similar results were obtained in Cav2.2/µ, δ, and κ-OR cells, where Gbg governs Ibₐ inhibition. Notably, m-Phos also eliminated prepulse facilitation of Ibₐ through Cav2.2, resulting in P2/P1 ratios <1 (Fig. 4C; Supplemental Table 1). Taken together, these findings suggest Gbg is directly required for OR signaling and modulation of both Cav2.2 and Cav2.3 channels.

### Discussion

Cav2.3 channels are localized to somatodendritic and presynaptic regions of various central and peripheral neurons and are also expressed in endocrine cells (Schneider et al., 2013). Although they are recognized as potential drug targets for treating chronic pain, their mode of regulation is poorly understood (Schneider et al., 2013; Rittenhouse, 2014). Here, we demonstrate that Cav2.3 channels are efficiently modulated via µ-, δ-, or κ-OR activation. The underlying signaling is voltage-independent, in contrast to the primarily voltage-dependent modulation of Cav2.2. We provide evidence that Gbg scavengers, in particular m-Phos, disrupt the Gbg-mediated event in G protein activation, preventing inhibition of both Cav2.3 and Cav2.2 channels by OR pathways.

In heterologous expression systems, Cav2.3 channels appear relatively insensitive to modulation via µ- and κ-ORs, whereas modulation via δ-ORs is not yet reported. We reveal that, depending on the pre-existing state of δ- or κ-OR activity, Ibₐ through Cav2.3 are efficiently inhibited by OR agonists and potentiated by OR antagonists (Figs. 1 and 2). A previous investigation demonstrated constitutive κ-OR activity in the presence of the κ-OR antagonist norbinaltrophimine (Simen and Miller, 1998). We did not investigate the mechanisms controlling agonist-independent OR activity. However, a similar mechanism leading to tonic opioid receptor-like (ORL1) activity is regulated by ORL1 expression levels and by Gbg interacting with the ORL1–N-type channel signaling complex in rat dorsal root ganglion (DRG) neurons (Beedle et al., 2004). It is likely that Cav2.3 channels are also embedded in a specialized protein complex required for the coordinated interaction between ORs and Cav2.3. Notably, Ibₐ cannot be potentiated in Cav2.2 or Cav2.3/µ-OR cells (Figs. 1 and 2). This is consistent with previous data showing µ-ORs do not exert tonic modulation of N-type channels in DRG neurons (Beedle et al., 2004).

Previous studies reported the absence or relatively weak (≤20%) inhibition of human Cav2.3 channels via murine µ-ORs (Bourinet et al., 1996; Ottolia et al., 1998) or murine κ-ORs (Simen and Miller, 1998). It is conceivable that, in the absence of agonist, Cav2.3 channels reside in a state of intermediate/partial inhibition. On the other hand, modulation of Cav2.2 channels via ORs is more pronounced. This is likely because Cav2.2 is more susceptible to direct Gbg modulation than Cav2.3 (Simen and Miller, 1998), and OR activation alters the voltage dependence of the Cav2.2 channel (Bourinet et al., 1996). Nevertheless, it is also evident that the inhibitory effects of OR agonists have a ~20–30% voltage-independent component in Cav2.2/OR cells (Fig. 3). This is consistent with previous studies showing similar contributions of the voltage-independent components to the OR-mediated total N-type current inhibition in rat DRG neurons and bovine chromaffin cells (Womack and McCleskey, 1995; Albillos et al., 1996). This modulation is generally less well characterized than the voltage-dependent component and can include phosphorylation and/or lipid signaling (Zamponi and Currie, 2013).

In our experiments, activation and steady-state inactivation characteristics of Cav2.3 channels remained unchanged in the presence of OR activation compared with control (Table 2), and Cav2.3 did not exhibit voltage-dependent changes in the P2/P1 ratio (Fig. 3). In contrast, Simen and Miller (1998) reported a relatively small (≤20%) depolarizing prepulse-induced Cav2.3 channel facilitation in the presence of the κ-OR agonist U69593. Interestingly, the Cav2.3 channel splice variant studied included an insert in the C terminus (insert 3, consisting of 43 amino acids), which was absent in the Cav2.3 channel used in our study. It is likely that splice variation in Cav2.3 channels may also contribute to the divergence of Cav2.3 modulation by ORs. Alternative splicing controls G protein–dependent inhibition of Cav2.2 channels (Raingo et al., 2007) and endows Cav2.2 with differential roles in pain pathways (Altier et al., 2007).

Absence of the Cav2.3 subunit does not prevent inhibition of Cav2.2 channels via GPCRs. However, it does abolish the voltage-dependent facilitation typical of Cav2.2 modulation via GPCRs (Dolphin, 2003). Interestingly, the functional
Fig. 3. Inhibition of Ca,2.3 channels via μ-, δ-, or κ-ORs is voltage-independent, whereas that of Ca,2.2 is voltage-dependent. (A) Representative I_{Ba} traces in the absence (control) and presence of DAMGO (1 μM), SNC80 (1 μM), or Sal-A (1 μM) in Ca,2.3/μ-, δ-, or κ-OR and Ca,2.2/μ-, δ-, or κ-OR cells. Dotted lines indicate zero-current level. Open and filled circles reflect I_{Ba} elicited by a single depolarizing test pulse (P1) or a test pulse preceded by a prepulse to +80 mV (P2) (top: voltage protocol). Horizontal scale, 20 ms; vertical scale, 500 pA. (B) Average P2/P1 values (± S.E.M.) representing I_{Ba} relief of inhibition. In Ca,2.3/μ-, δ-, and κ-OR, the P2/P1 ratios were ∼0.8 (see P2/P1 values in Supplemental Table 1), whereas in Ca,2.2/μ-, δ-, and κ-OR cells, the ratios were 1.16 ± 0.02, 1.23 ± 0.03, and 2.2 ± 0.06, respectively (control) and 2.4 ± 0.04, 2.7 ± 0.05, and 2.1 ± 0.07, respectively (OR agonist). See Supplemental Table 1 for statistical analyses and P values. Numbers above bars indicate the number of experiments. (C) Ca,2.3 channel inhibition via μ-, δ-, or κ-ORs is voltage-independent in the presence of Ca,β2 subunit. Representative I_{Ba} traces in the absence (control) and presence of DAMGO (1 μM), SNC80 (1 μM), or Sal-A (1 μM) in Ca,2.3/μ-, δ-, or κ-OR cells coexpressing Ca,β2 (A). Dotted lines indicate zero-current level; horizontal scale, 20 ms; vertical scale, 1000 pA. The voltage protocol (top inset) and the interpretation of symbols are similar to that shown in (A). (D) Average P2/P1 ratios in Ca,2.3/α2δ1-1/β2 + OR cells (see P2/P1 values in Supplemental Table 1). Data are the mean ± S.E.M.; the numbers of experiments are in parentheses.
Fig. 4. Gβγ has a central role in CaV2.3 channel modulation via μ-, δ-, and κ-ORs. (A) Average data (± S.E.M.) of percentage inhibition by DAMGO (1 μM), SNC80 (1 μM), or Sal-A (1 μM) during several co-expression conditions: absence of ORs, control (CaV2.3/μ-, δ-, or κ-OR cells), absence of the CaVβ subunit, presence of the CaVβ2 subunit, GRK-CT, or m-Phos; and treatment with pertussis toxin (500 ng/ml), GDP-β-S (500 nM), GTP-γ-S (200 nM), phosphorylated pp60c-Src peptide (50 μM), staurosporine (1 μM), or LY294002 (3 μM). In all cases, asterisks denote statistically significant differences; P < 0.001 versus corresponding controls labeled by different colors, in the presence of pertussis toxin, GDP-β-S, GTP-γ-S, GRK-CT, and m-Phos; P < 0.05 versus control, with pp60c-Src (one-way analysis of variance). (B) m-Phos coexpression abolishes I_{Ba} modulation by OR agonists and antagonists in CaV2.3 or CaV2.2/μ-, δ-, or κ-OR cells and eliminates prepulse facilitation in CaV2.2/μ-, δ-, or κ-OR cells (C). Note that in (B) and (C), the percentage of inhibition and potentiation values and P2/P1 values are compared with control values determined in the absence of m-Phos (white bars with dashed borders; same data as shown in Figs. 2 and 3). Data are the mean ± S.E.M. The number of experiments is in parentheses.
coupling between murine μ-ORs and human CaV2.3 was reduced or abolished when the CaVβ3 or CaVβ2 subunit, respectively, was coexpressed in Xenopus oocytes (Ottolia et al., 1998). However, our results suggest the CaVβ subunit has negligible effects on inhibition of CaV2.3 channels (Fig. 4), and that CaVβ and Gβγ do not compete for a binding site in Ca2.3/μ-, δ-, or κ-OR cells. The Gβγ requirement for direct and voltage-dependent modulation of CaV channels has been well studied (Herlitze et al., 1996; Ikeda, 1996). In many cell types, Gβγ is an active component of signal transduction systems and interacts with numerous effectors. Not surprisingly, coexpression of Gβγ scavenger proteins can alter direct Gβγ signaling to effectors such as CaV2.2 and G protein–coupled inwardly rectifying potassium channels (Dolphin, 2003; Beedle et al., 2004; Rishal et al., 2005). GRK-CT and phosducin have been used as tools to distinguish between signaling via Gα or Gβγ pathways (Koch et al., 1993; Rishal et al., 2005). They also modulate signal amplification at the G protein level. Phosducin and phosducin-like proteins specifically chelate Gβγ and preclude association of Gβγ with Gα (Gaudet et al., 1996). Our data suggest Gβγ directly modulates INa through CaV2.3 channels. However, further experiments are required to dissect the precise regions of the CaV2.3 channel involved in this interaction. As domain I and linker I-II of α1E are likely not involved in Gβγ binding, this interaction cannot be revealed by depolarization-induced facilitation. Interestingly, there is evidence for a direct Gβγ requirement in both voltage-dependent and voltage-independent N-type CaV2+ current inhibitory pathways activated by muscarinic or α2-adrnergic agonists in rat superior cervical ganglion neurons (Delmas et al., 1999; Kammermeier et al., 2000). The proposed voltage-independent action of Gβγ consists of Gαq-GTP binding to the Gβγ-bound N-type calcium channel in superior cervical ganglion neurons. This interaction prevents Gβγ release by strong depolarization (Kammermeier et al., 2000). Conversely, it is plausible that Gαq-GTP might obstruct the prepulse-induced recovery from Gβγ inhibition of CaV2.3 channels.

Although we did not analyze the time course of INa inhibition through CaV2.3 channels, our data suggest regulation via rapid and membrane-delimited OR-mediated pathways. In addition, similar to the rapid pathway responsible for N-type CaV2+ channel inhibition via μ-ORs in DRG neurons, phosphorylation is not required (Wildeing et al., 1995). Nevertheless, signals mediating effects of pp60c-Src in CaV2.3/δ-OR cells need to be characterized in more detail. Our previous work shows c-Src phosphorylation of specific tyrosine residues in the C terminus of the CaV2.3 channel is sufficient to abolish α-conotoxin Vc1.1 and reduce baclofen inhibition via GABAβ receptor activation (Berecki et al., 2014).

Further elucidation of the underlying mechanisms involved will increase our understanding of the diversity of signaling at ORs. A demonstration of R-type channel modulation via ORs in native cells involved in affective pain pathways is necessary to confirm the physiologic significance of opioid modulation of CaV2.3 channels and their roles as potential targets for opioid analgesics in chronic pain management. By all accounts, the selective targeting of R-type channels via ORs is currently not feasible in somatosensory neurons, and members of the CaV2.3 channel family jointly account for the effects of opioids in these cells.

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CaV2.3 Channel Inhibition via Opioid Receptor Activation

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Authorship Contributions

Participated in research design: Berecki, Motin, Adams.
Conducted experiments: Berecki, Motin.
Contributed new reagents or analytic tools: Berecki, Motin, Adams.
Performed data analysis: Berecki, Motin.
Wrote or contributed to the writing of the manuscript: Berecki, Motin, Adams.

References


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Schneider T, Dibus M, and Hescheler J (2013) How “Pharmacoresistant” is Ca\(_{v2.3}\), the major component of voltage-gated R-type Ca\(^{2+}\) channels? Pharmaceuticals (Basel) 6:759–776.


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SUPPLEMENTAL DATA

MOLECULAR PHARMACOLOGY

R-type (Ca\textsubscript{v}2.3) Calcium Channel Inhibition via Human \(\mu\)-\(\delta\)- and \(\kappa\)-Opioid Receptors is Voltage-Independently Mediated by G\(\beta\gamma\) Protein Subunits

G. Berecki, L. Motin, and D. J. Adams

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

Supplemental Figure 1. Modulation of Ca\textsubscript{v}2.3 and Ca\textsubscript{v}2.2 channels by the \(\kappa\)-opioid receptor (OR) agonist Sal-A and the \(\kappa\)-OR antagonist GNTI in HEK cells stably expressing Ca\textsubscript{v}2.3 or Ca\textsubscript{v}2.2 channels and transiently co-expressing \(\kappa\)-ORs (Ca\textsubscript{v}2.3/\(\kappa\)-OR and Ca\textsubscript{v}2.2/\(\kappa\)-OR cells, respectively). Bottom: Time course of peak Ba\textsuperscript{2+} current (I\textsubscript{Ba}) densities in the presence of Sal-A (1 \(\mu\)M) and subsequently applied GNTI (100 nM) in Ca\textsubscript{v}2.3/\(\kappa\)-OR (A) and Ca\textsubscript{v}2.2/\(\kappa\)-OR (B) cells. In both cases, Sal-A inhibition of I\textsubscript{Ba} was largely irreversible, exhibiting 7.2 \(\pm\) 0.6% (\(n = 17\)) or 14.8 \(\pm\) 1.6% (\(n = 8\)) recovery after \(\sim\)5 min washout for Ca\textsubscript{v}2.3 or Ca\textsubscript{v}2.2 channels, respectively, whereas the subsequent GNTI potentiation of I\textsubscript{Ba} was reversible. Bars indicate the duration of Sal-A or GNTI application. Top: Representative current traces, shown at the time points indicated by lowercase letters (only 25 ms of the 150-ms traces are shown); dashed lines indicate zero-current level. Peak I\textsubscript{Ba} amplitudes were evoked by 150 ms depolarizations to +10 mV (A) or +15 mV (B) at 0.1 Hz. This experiment was repeated four and five times for Ca\textsubscript{v}2.3/\(\kappa\)-OR and Ca\textsubscript{v}2.2/\(\kappa\)-OR cells, respectively, with similar results.
Supplemental Figure 2. Opioid receptor agonists and antagonists do not modulate $I_{\text{Ba}}$ in HEK293 cells stably expressing Ca$_{v}$2.3 or Ca$_{v}$2.2 channels alone. A, The effects of µ-, δ-, or κ-OR agonists DAMGO (1 μM), SNC80 (1 μM), and Sal-A (1 μM), respectively, and µ-, δ-, or κ-OR antagonists naloxone (1 μM), BNTX (100 nM), and GNTI (100 nM), respectively, were tested in separate HEK293 cells stably expressing Ca$_{v}$2.3 or Ca$_{v}$2.2 channels. Representative whole-cell $I_{\text{Ba}}$ traces in the absence (C, control, black) and presence of OR agonists (red) or antagonists (blue); dashed lines indicate zero-current level. Peak $I_{\text{Ba}}$ amplitudes were evoked by depolarizations to +10 mV (Ca$_{v}$2.3) or +15 mV(Ca$_{v}$2.2) at 0.1 Hz (only 50 ms of the 110 ms traces are shown). Vertical bars represent 500 pA; horizontal bars represent 30 ms. B, Summary of experiments shown in A. Data are mean ± SEM; the number of experiments is in parentheses.
Supplemental Figure 3. Determination of the extent of pre-pulse facilitation in HEK cells stably expressing Ca\(_{\text{v}}\)2.2 or Ca\(_{\text{v}}\)2.3 channels and transiently co-expressing µ-ORs (Ca\(_{\text{v}}\)2.2/µ-OR and Ca\(_{\text{v}}\)2.3/µ-OR cells, respectively). A, In Ca\(_{\text{v}}\)2.2/µ-OR and Ca\(_{\text{v}}\)2.2/µ-OR cells, representative I\(_{\text{Ba}}\) traces were elicited by step depolarizations to +10 and +15 mV, respectively, (P1), in the absence (control) and presence of DAMGO (1 µM). A second depolarizing step (P2) was applied immediately subsequent to a strong depolarizing pre-pulse to +80 mV (top insets: voltage protocols). A progressive increase in duration between the pre-pulse and P2 (Δt1 = 1, 3, 5, 10, 20, 30, and 50 ms), while maintaining a pre-pulse duration of 20 ms, revealed the presence and absence of voltage-dependent re-inhibition by G\(\beta\)\(\gamma\) subunit in Ca\(_{\text{v}}\)2.2 and Ca\(_{\text{v}}\)2.3 channels, respectively. Varying the pre-pulse duration (Δt2 = 1, 3, 5, 10, 20, 30, 50, and 100 ms), while maintaining a duration of 5 ms between the pre-pulse and P2, served to estimate the dependence of facilitation on the duration of the depolarizing pre-pulse and/or recovery from G\(\beta\)\(\gamma\) inhibition. Note the absence of facilitation in Ca\(_{\text{v}}\)2.3/µ-OR cells. Dotted lines indicate zero-current level. Data in B and C represent average P2/P1 values (± SEM); the numbers of experiments are in parentheses. The shaded regions highlight the Δt1 and Δt2 values used also in experiments described in the Results section.
**Supplemental Table 1.** Average values of Ba$^{2+}$ current (I$_{Ba}$) facilitation expressed as the P2/P1 ratio.

<table>
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<th>Cell type</th>
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<th>antagonist</th>
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<td>$0.77 \pm 0.02$ (7)</td>
<td>NS$0.84 \pm 0.03$ (7)</td>
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<td>NS$0.79 \pm 0.03$ (5)</td>
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<td>$\text{Ca}_{2.3} + \mu$-OR (Ca$^{2+}$</td>
<td>$0.93 \pm 0.04$ (6)</td>
<td>NS$1.01 \pm 0.04$ (6)</td>
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<tr>
<td>$\text{Ca}_{2.3} + \delta$-OR (Ca$^{2+}$</td>
<td>$0.92 \pm 0.04$ (4)</td>
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<td>NS$0.73 \pm 0.04$ (3)</td>
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<td>$0.77 \pm 0.02$ (8)</td>
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<tr>
<td>$\text{Ca}_{2.2} + \mu$-OR</td>
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<td>$^b2.7 \pm 0.05$ (6)</td>
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<td>NS$0.92 \pm 0.03$ (4)</td>
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Voltage-dependent relief of inhibition was estimated from the P2/P1 ratio, where P1 and P2 represent peak I$_{Ba}$ amplitude in the absence and presence of a depolarizing pre-pulse, respectively (see Materials and Methods). Cell types: HEK293 stably expressing Ca$^{2+}$, or Ca$^{2+}$ and Methods) Cell types: HEK293 stably expressing Ca$^{2+}$ (Ca$^{2+}$ expressing Ca$^{2+}$ or Ca$^{2+}$ expressing Ca$^{2+}$ transiently co-expressing Ca$^{2+}$ or Ca$^{2+}$ transiently co-expressing Ca$^{2+}$ or Ca$^{2+}$ transiently co-expressing Ca$^{2+}$ or Ca$^{2+}$ transiently co-expressing m-Phos. Values represent mean ± SEM; n, number of experiments in parentheses. DAMGO (1 μM), SNC80 (1 μM), and Sal-A (1 μM) were used as agonists, whereas naltrexone (1 μM), BNTX (100 nM), and GNTI (100 nM) were used as antagonists of μ-, δ-, or κ-ORs, respectively. Student’s t-test for two groups, $^aP < 0.002$ versus Ca$^{2+}$/control, and one-way ANOVA with Bonferroni post-hoc testing, $^bP < 0.001$, $^cP = 0.003$ versus Ca$^{2+}$/control, $^dP < 0.001$, versus Ca$^{2+}$/control, $^eP < 0.001$ versus Ca$^{2+}$/control, $^fP < 0.001$, versus Ca$^{2+}$/control, and $^gP < 0.001$, versus Ca$^{2+}$/control, were used to test for statistically significant differences. NS, not significantly different from the corresponding control; ND, not determined.