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TITLE PAGE

R-type ($Ca_v2.3$) calcium channel inhibition via human μ- δ- and κ-opioid receptors is voltage-independently mediated by Gβγ protein subunits

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RUNNING TITLE PAGE

Running title: $Ca_v2.3$ channel inhibition via opioid receptor activation

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Abbreviations:

ATP, adenosine 5′-triphosphate; $Ca_v2.x$, neuronal voltage-gated calcium channel; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid 3,12-bis(carboxymethyl)-6,9-dioxa-3,12-diazatetradecanedioic acid; GPCR, G protein-coupled receptor; GRK-CT, C-terminal fragment of G protein receptor kinase 2; GTP, guanosine 5′-triphosphate; HEK293, human embryonic kidney 293 cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (systematic) N-2-hydroxyethylpiperazine-N[prime]-2-ethanesulfonic acid; HP, holding potential; m-Phos, myristoylated-Phosducin; ORs, opioid receptors; TEA, tetraethylammonium

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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 of how opioids modulate pain. Neuronal voltage-gated N-type ($Ca_v2.2$) calcium channels are inhibited by activation of G protein-coupled opioid receptors (ORs). However, inhibition of R-type ($Ca_v2.3$) channels by μ- or κ-ORs is poorly defined and has not been reported for δ-ORs. To investigate such interactions, we co-expressed human μ-, δ- or κ-ORs with human $Ca_v2.3$ or $Ca_v2.2$ in human embryonic kidney (HEK293) cells and measured depolarization-activated Ba^{2+} currents (I_{Ba}). Selective agonists of μ-, δ- and κ-ORs inhibited I_{Ba} through $Ca_v2.3$ channels by 35%. $Ca_v2.2$ channels were inhibited to a similar extent by κ-ORs, but more potently (60%) via μ- and δ-ORs. Antagonists of δ- and κ-ORs potentiated I_{Ba} amplitude mediated by $Ca_v2.3$ and $Ca_v2.2$ channels. Consistent with G protein βγ (Gβγ) 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 GDP-β-S and GTP-γ-S. Co-expression of Gβγ 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 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 Gβγ subunits in a voltage-independent manner.

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INTRODUCTION

In presynaptic nerve terminals, Ca^{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 μ-, δ-, and κ- 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 Gβγ with the channel pore formed by the α_{1A} or α_{1B} subunit, respectively (Zamponi and Currie, 2013). Previous studies suggest there are considerable differences in the ability of Gβγ 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_v 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 to neuronal excitability, memory and learning (Breustedt 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 $Ca_v2.3$ knockout mice exhibiting reduced pain perception (Saegusa et al., 2000; Yang and Stephens, 2009). In pharmacological studies, intrathecal delivery of the $Ca_v2.3$ channel blocker, SNX-482, produces analgesia in animal models of neuropathic pain (Matthews et al., 2007; Terashima et al., 2013). Despite the clear functional importance of $Ca_v2.3$ channels in pain pathways, the mechanisms by which they are modulated by GPCRs

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has not yet been adequately examined (Rittenhouse, 2014). It is known that the $Ca_v2.3$ channel is regulated by muscarinic (Bannister et al., 2004), dopamine (Page et al., 1998) and $GABA_B$ (Berecki et al., 2014) receptors. From reconstitution in heterologous expression systems, it is also known that $Ca_v2.3$ activity is weakly modulated via μ- and κ-ORs (Ottolia et al., 1998; Simen and Miller, 1998) but not δ-ORs; lack of $Ca_v2.3$ modulation via μ-OR has also been reported (Bourinet et al., 1996). The current consensus is that, compared to $Ca_v2.2$, $Ca_v2.3$ 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 α_{1B} compared to α_{1E} 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 $Ca_v2.3$ and $Ca_v2.2$ calcium channels via human μ-, δ-, or κ-OR activation. Our data suggests membrane-delimited Gβγ subunits not only mediate signal transduction between ORs and $Ca_v2.2$ channels, but also between ORs and $Ca_v2.3$. However, in contrast with the primarily voltage-dependent modulation of $Ca_v2.2$ channels, $Ca_v2.3$ modulation is voltage-independent. We also reveal that δ- or κ-OR but not μ-OR activation directly potentiates I_{Ba} through $Ca_v2.2$ and $Ca_v2.3$ channels in the absence of δ- or κ-OR agonists. Using Gβγ protein specific scavengers, we demonstrate that membrane-delimited Gβγ subunits not only mediate signal transduction between ORs and $Ca_v2.2$ channels, but also between ORs and $Ca_v2.3$ in a voltage-independent manner. Delineating the diversity of OR-mediated signaling at Ca_v2 channels will enhance our understanding of pain perception and of how opioids modulate pain.

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MATERIALS AND METHODS

Cells and clones. Human embryonic kidney (HEK)-293 cells stably co-expressing human $Ca_v2.3$ (R-type) channel (α_{1E-3}; major neuronal splice variant; GenBank accession no. L29385) or human Cav2.2 (N-type) channel (α_{1B-1} splice variant; M94172.1) and human $\alpha_{2b}\delta$-1 (M76559), human β_{3a} (NM_000725) auxiliary subunits and human KCNJ4 channel (Kir2.3; U07364), were obtained from Merck and cultured according to procedures described previously (Dai et al., 2008). Cells were transiently co-transfected using calcium phosphate precipitation as described previously (Berecki et al., 2014). Transfection with 1.5 μg of plasmids carrying cDNAs of human μ-, δ- or κ-ORs (NM_001145279.1, NM_000911.3 and NM_000912.2, respectively; OriGene Technologies, Inc.) and eGFP (0.5 μg) resulted in $Ca_v2.3$ or $Ca_v2.2$/μ-, δ-, or κ-OR expressing cells 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 (AF087455) (GRK-CT, 3 μg; kindly provided by Dr Paul R Albert, University of Ottawa, Canada) or a myristoylated bovine phosducin (m-Phos, 5 μg) (GB M33529) (kindly provided by Dr Nathan Dascal, Tel Aviv University, Israel) were included in transfections. In separate experiments, HEK-293 cells stably expressing the SV40 large T antigen (HEK293T), were transiently co-transfected with human α_{1E-3} (3 μg), human $\alpha_{2b}\delta$-1 (M76559; 3 μg), human β_2 (NM_000724; 3 μg) or human β_{3a}, and human μ-, δ- or κ-opioid receptors and eGFP cDNAs as described above. After transfections, cells were plated on glass coverslips and cultured as described previously (Berecki et al., 2014).

Electrophysiology. 3–5 d after transfection, depolarization-activated I_{Ba} were recorded in the whole-cell patch clamp configuration, using superfused (~600 μl/min) extracellular bath solution containing 10 mM $BaCl_2$, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM CsCl, 30 mM TEA-

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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-gluconate, 2 mM $MgCl_2$, 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 signalling pathways when the whole-cell recording configuration was established (Raingo et al., 2007). Data were recorded with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) controlled by a Clampex 9.2/DigiData 1332 acquisition system (Molecular Devices). Fire-polished borosilicate patch pipettes typically exhibited resistance values of 1.5–2.5 MΩ and series resistances were compensated > 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-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO), naloxonazine dihydrochloride (Naloxone), D-Phe-*cyc*[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH2 (CTAP), (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC80), 7-Benzylidenenaltrexone maleate (BNTX), (2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Acetyloxy)-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic acid methyl ester (Salvinorin-A), and 5'-Guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-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 (PTX) was purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). In a series of experiments, Guanosine 5′-[β-thio]diphosphate trilithium salt (GDP-β-S) (500 nM; Sigma-Aldrich), Guanosine 5′-[γ-

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thio]triphosphate tetralithium salt (GTP-γ-S) (200 nM; Sigma-Aldrich), or pp60c-Src peptide (50 μM; Tocris) were included in the pipette solution.

***Curve fitting and statistical analysis*.** Data were analysed off-line using Clampfit 9.2 (Molecular Devices) and Origin 9.0 (Microcal Software Inc.). I_{Ba} amplitude, voltage dependence of I_{Ba} activation, the concentration–response curves, and the 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 $I/I_0 \times 100$, where I represents the peak I_{Ba} amplitude obtained in the presence of a compound and I_0 is the peak I_{Ba} amplitude under control condition. Total I_{Ba} modulation was estimated as the sum of % inhibition (agonist response) and % potentiation (antagonist response). Steady-state inactivation was determined by analysing 10 mV step-elicited I_{Ba} amplitude values from a holding potential (HP) of −80 mV and a 2 s 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 fitted using the Boltzmann equation, $I/I_{max} = 1/[1 + \exp((V-V_{0.5,inact})/k))$, where V and $V_{0.5,inact}$ 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 (Berecki et al., 2014) and defined as the P2/P1 ratio, where P1 and P2 represent peak I_{Ba} amplitude in the absence and presence of a depolarizing pre-pulse, respectively. Statistical analyses were performed in Sigma Plot 11.0 (Systat Software, Inc.). Data are shown as mean ± SEM; n, number of experiments. Student's *t*-test for two groups or one-way ANOVA with Bonferroni post-hoc testing for multiple comparisons was used. Differences were considered statistically significant at $P < 0.05$.

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Online supplemental material. Supplemental Figure 1 shows the time course of potentiation of current densities in the presence of κ-OR antagonist GNTI after κ-OR agonist Sal-A washout in $Ca_v2.3$/κ-OR and $Ca_v2.2$/κ-OR cells. Supplemental Figure 2 demonstrates that in the absence of opioid receptors OR agonists and antagonists do not modulate $Ca_v2.3$ or $Ca_v2.2$ channels in HEK293 cells. Supplemental Figure 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 co-expressing μ-ORs. Supplemental Table 1 shows average P2/P1 values in HEK293 cells co-expressing $Ca_v2.2$ or $Ca_v2.3$ channels and various ORs. The online supplemental material is available at molpharm.aspetjournals.org.

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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 co-expressing 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 I_{Ba} amplitude via μ- and δ-ORs, respectively. In comparison, Salvinorin-A (Sal-A) rapidly and irreversibly reduced I_{Ba} by ~30% via κ-OR activation (Fig. 1*A*). 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 nM, 76.1 ± 19 nM, and 4.2 ± 0.6 nM for DAMGO, SNC80 and Sal-A, respectively (Fig. 1*A*). I_{Ba} 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-ORs antagonists, BNTX (100 nM) and GNTI (100 nM), respectively. Remarkably, BNTX and GNTI but not naloxone potentiated I_{Ba} through $Ca_v2.3$ channels without any previous agonist exposure. Similarly, application of the antagonist after washout of the agonist also potentiated peak I_{Ba} amplitude in $Ca_v2.3$ or $Ca_v2.2$/κ-OR cells (Supplemental Figure 1) and in $Ca_v2.3$ or $Ca_v2.2$/δ-OR cells (not shown). This suggests that poor reversibility of the κ agonist-mediated inhibition of $Ca_v2.3$ or $Ca_v2.2$ channels is not due to endocytosis of the receptor/channel complex but is more likely due to poor washout of the agonist. Overall, the results with δ- and κ-OR antagonists suggested a pre-existing, intermediate degree of I_{Ba} inhibition (Fig. 1*B*). Similar to naloxone, the potent and selective μ-OR antagonist, CTAP (1 μM), blocked the effect of DAMGO but was unable to potentiate I_{Ba} in the absence of the agonist (n = 4; data not shown). It should also be noted that neither opioid receptor agonists nor antagonists modulated $Ca_v2.3$ or $Ca_v2.2$ channels in the absence

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of opioid receptors (Supplemental Figure 2), indicating that these compounds do not have direct effects on the channels or other off-target actions.

In a previous study, norbinaltrophimine (norBNI) enhanced I_{Ba} through various α_{1B} and/or α_{1E} chimeric Ca^{2+} channels heterologously co-expressed with murine κ-OR, indicating that intrinsic κ-OR activity significantly contributes to Ca^{2+} channel modulation and total modulation can be estimated by taking into account both I_{Ba} inhibition in the presence of a κ-OR agonist and I_{Ba} potentiation induced by a κ-OR antagonist (Simen and Miller, 1998). Based on these findings, we proceeded to quantitatively and separately study the effect of agonists and antagonists in $Ca_v2.3$ or $Ca_v2.2$/μ-, δ- or κ-OR cells (Fig. 2). $Ca_v2.2$ channels were inhibited via κ-ORs by ~25%, but more potently (~60%) via μ- or δ-ORs. Similar to $Ca_v2.3$ channels (Fig. 1 and Fig. 2*A*), selective δ- and κ-OR, but not μ-OR antagonists (naloxone or CTAP), markedly potentiated I_{Ba} through $Ca_v2.2$ channels (Fig. 2*A*). We did not evaluate either BNTX or GNTI concentration-dependence of I_{Ba} potentiation. However, the I_{Ba} potentiating effects of 10 nM and 100 nM of BNTX or GNTI were similar in $Ca_v2.3$ or $Ca_v2.2$/δ- or κ-OR cells ($n \geq 3$ for each cell type; not shown). Overall, these results suggest BNTX and GNTI act as inverse agonists and relieve I_{Ba} inhibition through either $Ca_v2.3$ or $Ca_v2.2$ channels. In these cells, the sum of the inhibited and potentiated I_{Ba} fraction represents total modulation (Fig. 2*B*).

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).

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$Ca_v2.2$ channel inhibition via various ORs involves direct and voltage-dependent Gβγ binding to the channel pore-forming subunit and inhibition can be transiently relieved by strong depolarizing pre-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 pre-pulses. In the absence of agonists (control), pre-pulses resulted in P2/P1 ratios > 1, suggesting facilitation of I_{Ba} may be associated with constitutive or tonic OR activity (Figs. 3A 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 $Ca_v2.2$/μ- or δ-OR cells, respectively (Fig. 3B and Supplemental Table 1). Remarkably, OR-mediated $Ca_v2.3$ channel inhibition was voltage-independent and was not affected by depolarizing pre-pulses. This is indicated by the P2/P1 < 1 ratios that remained unchanged in the absence or presence of OR agonists (Figs. 3A and B and Supplemental Table 1). Previous studies indicated that inactivation typically obscures facilitation of $Ca_v2.3$ current amplitudes (Meza and Adams, 1998; Toth et al., 1996). Consequently, the smaller I_{Ba} amplitudes during P2 compared to those during P1 are attributable to voltage-dependent inactivation that occurs during the pre-pulse in $Ca_v2.3$-OR cells. To minimize I_{Ba} inactivation during a pre-pulse and to unmask any pre-pulse-induced facilitation, we transiently co-expressed $Ca_v2.3$ ($\alpha_{1E\text{-}3}$), $\alpha_{2b}\delta$-1, and $Ca_v\beta_2$ subunits and μ-, δ-, or κ-ORs in HEK293T cells ($^{tr}Ca_v2.3/\alpha_2b\text{-}\delta1\text{-}\beta_2$ + OR cells) and studied the voltage-dependence of I_{Ba} in the absence or presence of OR agonists (Figs. 3C and D and Supplemental Table 1). The results suggest that depolarizing pre-pulses have negligible effect on I_{Ba} facilitation through $Ca_v2.3$ 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 pre-pulse and P2 (Δt1) or the pre-pulse duration (Δt2), to determine I_{Ba} facilitation in the absence and presence of DAMGO while altering these parameters (Supplemental Figure 3). In $Ca_v2.2$/μ-OR cells, increasing

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Δt1 progressively reduced the degree of I_{Ba} facilitation, whereas increasing Δt2 initially resulted in an increase in I_{Ba} facilitation that peaked at a Δt2 = 20 ms and then decreased. In $Ca_v2.3$/μ-OR cells, altering Δt1 had negligible effects on the P2/P1 ratio, whereas increasing Δt2 progressively reduced the P2 ratio, confirming that I_{Ba} inactivation during pre-pulse obscures I_{Ba} facilitation through $Ca_v2.3$ channels. For both channels, P2/P1 values followed a similar trend when using a +120 mV depolarizing pre-pulse instead of +80 mV (not shown). $Ca_v2.3$ channel inhibition via ORs evidently does not follow the conventional Gβγ signalling paradigm associated with the change in the voltage-dependence that opens $Ca_v2.2$ channels (Bean, 1989).

Gβγ scavengers disrupt signal transduction between ORs and $Ca_v2.3$ channels

Functional interaction between $Ca_v\beta$ subunits and Gβγ is known to limit GPCR-mediated inhibition of Ca_v2 channels (Dolphin, 2012). We next investigated the effects of $Ca_v\beta$ subunits on inhibition of I_{Ba} in HEK293T cells transiently co-expressing μ-, δ- or κ-ORs, α_{1E-3}, and $\alpha_{2b}\delta$-1, and in the absence or presence of the $Ca_v\beta_2$ or $Ca_v\beta_3$ subunits. Specific OR agonists inhibited I_{Ba} to a similar extent as control indicating no role for $Ca_v\beta$ (Fig. 4*A*). In $Ca_v2.3$/μ-, δ- or κ-OR cells, I_{Ba} inhibition was sensitive to intracellular application of hydrolysis-resistant GDP-β-S, GTP-γ-S and was abolished by PTX. These results suggest activation of the $G\alpha_{i/o}$ subunit is also required for I_{Ba} inhibition. Various protein kinase inhibitors, known to modify the activities of c-Src (pp60-c-Src, staurosporine), protein kinase A, protein kinase C, and Ca^{2+}/calmodulin-dependent protein kinase II (staurosporine), or PI3-kinase (LY294002) (Fig. 4*A*) produced only subtle effects, suggesting phosphorylation plays little or negligible role in this OR signalling pathway.

We tested the Gβγ dependence of OR signalling by co-expressing the GRK-CT protein corresponding to the C-terminal domain of G protein-coupled receptor kinase 2

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(GRK2 or βARK1). This domain is known to bind and specifically inactivate free G protein Gβγ subunits (Ghahremani et al., 2000; Koch et al., 1993). Figure 4*A* shows GRK-CT efficiently prevented the I_{Ba} inhibition produced by DAMGO and SNC80 (57% and 75%, respectively). GRK-CT also eliminated I_{Ba} inhibition by Sal-A. Next, we co-expressed m-Phos to increase the likelihood of targeting the plasma membrane-associated Gβγ fraction (Rishal et al., 2005). In this case, $Ca_v2.2$/μ-, δ-, or κ-OR cells served as positive control because Gβγ-mediated $Ca_v2.2$ channel inhibition can be revealed by voltage-dependent facilitation. Compared to GRK-CT, m-Phos more effectively reduced $Ca_v2.3$ channel modulation via μ- or δ-ORs and abolished modulation via κ-ORs (Fig. 4*A* and *B*). Similar results were obtained in $Ca_v2.2$/μ-, δ-, or κ-OR cells, where Gβγ governs I_{Ba} inhibition. Notably, m-Phos also eliminated pre-pulse facilitation of I_{Ba} through $Ca_v2.2$, resulting in P2/P1 ratios of < 1 (Fig. 4*C* and Supplemental Table 1). Taken together, these findings suggest Gβγ is directly required for OR signaling and modulation of both $Ca_v2.2$ and $Ca_v2.3$ channels.

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DISCUSSION

$Ca_v2.3$ channels are localized to somatodendritic and presynaptic regions of various central and peripheral neurons and are also expressed endocrine cells (Schneider et al., 2013). Although they are recognised as potential drug targets for treating chronic pain, their mode of regulation is poorly understood (Rittenhouse, 2014; Schneider et al., 2013). Here, we demonstrate that $Ca_v2.3$ 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 $Ca_v2.2$. We provide evidence that Gβγ scavengers, in particular m-Phos, disrupt the Gβγ-mediated event in G protein activation, preventing inhibition of both $Ca_v2.3$ and $Ca_v2.2$ channels by OR pathways.

In heterologous expression systems, $Ca_v2.3$ 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 a pre-existing state of δ- or κ-OR activity, I_{Ba} through $Ca_v2.3$ are efficiently inhibited by OR agonists and potentiated by OR antagonists (Fig. 1 and Fig. 2). A previous investigation demonstrated constitutive κ-OR activity in the presence of the κ-OR antagonist, norBNI (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) receptor activity is regulated by ORL1 expression levels and by Gβγ interacting with the ORL1– N-type channel signaling complex in rat dorsal root ganglion (DRG) neurons (Beedle et al., 2004). It is likely that $Ca_v2.3$ channels are also embedded in a specialized protein complex required for the coordinated interaction between ORs and $Ca_v2.3$. Notably, I_{Ba} cannot be potentiated in $Ca_v2.2$ or $Ca_v2.3$/μ-OR cells (Fig. 1 and Fig. 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).

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Previous studies reported the absence or relatively weak (≤20 %) inhibition of human $Ca_v2.3$ 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, $Ca_v2.3$ channels reside in a state of intermediate/partial inhibition. On the other hand, modulation of $Ca_v2.2$ channels via ORs is more pronounced. This is likely because $Ca_v2.2$ is more susceptible to direct Gβγ modulation than $Ca_v2.3$ (Simen and Miller, 1998) and OR activation alters the voltage-dependence of the $Ca_v2.2$ channel (Bourinet et al., 1996). Nevertheless, it is also evident that the inhibitory effects of OR agonists are associated with ~20-30% voltage-independent component in $Ca_v2.2$/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 (Albillos et al., 1996; Womack and McCleskey, 1995). This modulation is generally less well characterized than the voltage-dependent component and can include phosphorylation and/or lipid signalling (Zamponi and Currie, 2013).

In our experiments, activation and steady-state inactivation characteristics of $Ca_v2.3$ channels remained unchanged in the presence of OR activation compared with control (Table 2) and $Ca_v2.3$ 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 pre-pulse-induced $Ca_v2.3$ channel facilitation in the presence of the κ-OR agonist U69593. Interestingly, the $Ca_v2.3$ channel splice variant studied included an insert in the C-terminus (insert 3, consisting of 43 amino acids), which was absent in the $Ca_v2.3$ channel used in our study. It is likely that splice variation in $Ca_v2.3$ channels may also contribute to the divergence of $Ca_v2.3$ modulation by ORs. Alternative splicing controls G protein-dependent inhibition of $Ca_v2.2$ channels (Raingo et al., 2007) and endows $Ca_v2.2$ with differential roles in pain pathways (Altier et al., 2007).

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Absence of the $Ca_v\beta$ subunit does not prevent inhibition of $Ca_v2.2$ channels via GPCRs. However, it does abolish the voltage-dependent facilitation typical of $Ca_v2.2$ modulation via GPCRs (Dolphin, 2003). Interestingly, the functional coupling between murine μ-ORs and human $Ca_v2.3$ was reduced or abolished when the $Ca_v\beta3$ or $Ca_v\beta2$ subunit, respectively, was co-expressed in *Xenopus* oocytes (Ottolia et al., 1998). However our results suggest the $Ca_v\beta$ beta subunit has negligible effects on inhibition of $Ca_v2.3$ channels (Fig. 4), and that $Ca_v\beta$ and Gβγ do not compete for a binding site in $Ca_v2.3$/μ-, δ-, or κ-OR cells. The Gβγ requirement for direct and voltage-dependent modulation of Ca_v 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, co-expression of Gβγ scavenger proteins can alter direct Gβγ signalling to effectors such as $Ca_v2.2$ and G protein-coupled inwardly-rectifying potassium (GIRK) channels (Beedle et al., 2004; Dolphin, 2003; Rishal et al., 2005). GRK-CT and Phosducin have been used as tools to distinguish between signalling via Gα or Gβγ pathways (Koch et al., 1993; Rishal et al., 2005). They also modulate signal amplification at the G protein level. Phosducin (Phos) and Phos-like proteins specifically chelate Gβγ and preclude association of Gβγ with Gα (Gaudet et al., 1996). Our data suggest Gβγ directly modulates I_{Ba} through $Ca_v2.3$ channels. However, further experiments are required to dissect the precise regions of the $Ca_v2.3$ channel involved in this interaction. As domain I and linker I-II of α_{1E} are likely not involved in Gβγ binding, this interaction cannot be revealed by depolarization-induced facilitation. Interestingly, there is evidence for direct Gβγ requirement in both voltage-dependent and voltage-independent N-type Ca^{2+} current inhibitory pathways activated by muscarinic or α_2-adrenergic agonists in rat superior cervical ganglion (SCG) neurons (Delmas et al., 1999; Kammermeier et al., 2000). The proposed voltage-independent action of Gβγ consist of $G\alpha_q$–GTP binding to the Gβγ-bound N-type calcium channel in SCG neurons. This interaction prevents Gβγ release by

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strong depolarization (Kammermeier et al., 2000). Conversely, it is plausible that $G\alpha_{i/o}$–GTP might obstruct the pre-pulse-induced recovery from $G\beta\gamma$ inhibition of $Ca_v2.3$ channels.

Although we did not analyse the time course of I_{Ba} inhibition through $Ca_v2.3$ channels, our data suggest regulation via rapid and membrane-delimited OR-mediated pathways. In addition, similar to the rapid pathway responsible for N-type Ca^{2+} channel inhibition via μ-ORs in DRG neurons, phosphorylation is not required (Wilding et al., 1995). Nevertheless, signals mediating effects of pp60c-Src in $Ca_v2.3$/δ-OR cells need to be characterised in more detail. Our previous work shows c-Src phosphorylation of specific tyrosine residues in the C terminus of the $Ca_v2.3$ channel is sufficient to abolish α-conotoxin Vc1.1 and reduce baclofen inhibition via $GABA_B$ 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 afferent pain pathways is necessary to confirm the physiological significance of opioid modulation of $Ca_v2.3$ 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 Ca_v2 channel family jointly account for the effects of opioids in these cells.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Berecki, Motin, Adams

Conducted experiments: Berecki, Motin

Contributed new reagents: Berecki, Motin, Adams

Performed data analysis: Berecki, Motin

Wrote and contributed to the writing of the manuscript: Berecki, Motin, Adams

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Modulation of $Ca_v2.3$ channels via μ-, δ-, or κ-OR activation. (*A*) μ-OR agonist DAMGO (1 μM) and δ-OR agonist SNC80 (1 μM) reversibly inhibit I_{Ba}, whereas the effect of the κ-OR agonist Sal-A (1 μM) is largely irreversible; in these experiments, the inhibited I_{Ba} fraction exhibited ~7% recovery after ~5 min washout. Peak I_{Ba} amplitudes were evoked at 0.1 Hz by 150 ms depolarizations to +10 mV; current density values, determined by dividing current amplitudes by the cell capacitance, are plotted as a function of time. Bars indicate the duration of agonist application. Representative current traces (insets) are shown at the time points indicated by lowercase letters; dashed lines indicate zero-current level. Bottom: concentration-dependence of I_{Ba} inhibition by DAMGO, SNC80 and Sal-A. Data are mean ± SEM. See text for IC_{50} values ($n \geq 5$, for each data point). (*B*) Time course of current densities in the presence of μ-, δ-, or κ-OR antagonists naloxone (1 μM), BNTX (100 nM) or GNTI (100 nM), added before DAMGO (1 μM), SNC80 (1 μM), or Sal-A (1 μM), respectively. In each case, co-application of the antagonist precluded the full agonist effects. Bars indicate the duration of agonist or antagonist application with the same voltage protocol as in *A*. Representative current traces (insets) are shown at the time points indicated by lowercase letters (only 25 ms of the 150 ms traces are shown).

Figure 2. Agonist-dependent and agonist-independent OR activity defines total modulation of $Ca_v2.3$ and $Ca_v2.2$ 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 at10 nM BNTX or GNTI ($n \geq 4$ for each, not shown). I_{Ba} was evoked by 150 ms step depolarizations (0.1 Hz) to +10 mV ($Ca_v2.3$) or +15 mV ($Ca_v2.2$), from a HP of –80 mV. Note that only 20 ms of the 150 ms traces are shown. Horizontal scale, 10 ms;

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vertical scale, 1 nA; c, control; w, washout; dashed lines indicate zero-current level. Note the pronounced agonist-independent modulation of $Ca_v2.2$ 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 (± SEM) of percentage inhibition and potentiation of I_{Ba} through $Ca_v2.3$ or $Ca_v2.2$ channels in the presence of various ORs. In $Ca_v2.3$/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 23.8 ± 5.4%, respectively. In $Ca_v2.2$/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.

Figure 3. Inhibition of $Ca_v2.3$ channels via μ-, δ-, or κ-ORs is voltage-independent, whereas that of $Ca_v2.2$ 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 $Ca_v2.3$/μ-, δ-, or κ-OR and $Ca_v2.2$/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 pre-pulse to +80 mV (P2) (top: voltage protocol). Horizontal scale, 20 ms; vertical scale, 500 pA. (*B*) Average P2/P1 values (± SEM) representing I_{Ba} relief of inhibition. In $Ca_v2.3$/μ-, δ, or κ-OR cells, the P2/P1 ratios were ~0.8 (see P2/P1 values in Supplemental Table 1), whereas in $Ca_v2.2$/μ-, δ, or κ-OR cells were 1.16 ± 0.02, 1.23 ± 0.03, or 2.2 ± 0.06, respectively (control) and 2.4 ± 0.04, 2.7 ± 0.05, or 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*) $Ca_v2.3$ channel inhibition via μ-, δ-, or κ-opioid receptors (ORs) is voltage-

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independent in the presence of $Ca_v\beta_2$ subunit. Representative I_{Ba} traces in the absence (control) and presence of DAMGO (1 μM), SNC80 (1 μM) or Sal-A (1 μM). OR agonists inhibited ~35% of peak I_{Ba} amplitude. Note the slower time course of I_{Ba} inactivation compared with I_{Ba} recorded in $Ca_v2.3$/OR cells co-expressing $Ca_v\beta_3$ (Fig. 3A). 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 $^{tr}Ca_v2.3/\alpha_2b$-δ1-β_2 + OR cells (see P2/P1 values in Supplemental Table 1). Data are mean ± SEM; the numbers of experiments are in parentheses.

Figure 4. Gβγ has a central role of in $Ca_v2.3$ channel modulation via μ-, δ-, and κ-ORs. (*A*) Average data (± SEM) of percentage inhibition by DAMGO (1 μM), SNC80 (1 μM), or Sal-A (1 μM) during several co-expression conditions: absence of ORs, control ($Ca_v2.3$/μ-, δ-, or κ-OR cells, absence of $Ca_v\beta$ subunit, presence of $Ca_v\beta_2$ subunit, GRK-CT, or m-Phos; and treatment with PTX (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). *P < 0.001 versus corresponding controls in the presence of PTX, GDP-β-S, GTP-γ-S, GRK-CT, and m-Phos; *P < 0.05 versus control with pp60c-Src (one-way ANOVA). (*B*) m-Phos co-expression abolishes I_{Ba} modulation by OR agonists and antagonists in $Ca_v2.3$ or $Ca_v2.2$/μ-, δ-, or κ-OR cells and eliminates pre-pulse-facilitation in $Ca_v2.2$/μ-, δ-, or κ-OR cells (*C*). Note that in *B* and *C*, the % 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 Fig. 2 and Fig. 3. Data are mean ± SEM. The number of experiments is in parentheses.

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TABLES

Table 1. Percentages of I_{Ba} modulation by opioid receptor agonists and antagonists in $Ca_v2.3$/μ-, δ- or κ-OR cells and $Ca_v2.2$/μ-, δ- or κ-OR cells.

Cell type	Agonist (% inhibition)	Antagonist (% potentiation)	Total modulation (%)
$Ca_v2.3$/μ-OR	35.2 ± 1.1 (34)	−7.3 ± 3.0 (8)[§]	35.2[#]
$Ca_v2.3$/δ-OR	35.9 ± 1.6 (22)	18.2 ± 4.7 (7)	54.1
$Ca_v2.3$/κ-OR	31.2 ± 2.7 (27)	23.8 ± 5.4 (6)	55
$Ca_v2.2$/μ-OR	52.8 ± 3.8 (11)	−1.3 ± 2.4 (5)	52.8[#]
$Ca_v2.2$/δ-OR	60.9 ± 4.9 (9)	77.3 ± 7.4 (6)	138.2
$Ca_v2.2$/κ-OR	22.6 ± 4.9 (12)	72.0 ± 8.9 (5)	94.0

DAMGO (1 μM), SNC80 (1 μM), and Sal-A (1 μM) were used as agonists, whereas naloxone (1 μM), BNTX (100 nM), and GNTI (100 nM) were used as antagonists of μ-, δ-, or κ-ORs, respectively. Inhibition and potentiation data are shown as mean ± SEM; n, number of experiments in parentheses. Total modulation (mean) is estimated as the sum of inhibition and potentiation. [§]Naloxone slightly inhibited I_{Ba} in $Ca_v2.3$/μ-OR cells (see also Figure 2). [#] Total modulation in $Ca_v2.3$ or $Ca_v2.2$/μ-OR cells does not include data in the presence of naloxone.

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Table 2. Activation and steady-state inactivation characteristics of $Ca_v2.3$ channels in $Ca_v2.3$/μ-, δ- or κ-OR cells.

Cell type	Condition (n)	$V_{0.5,\ act}$ (mV)	k (mV)	$V_{0.5,\ inact}$ (mV)	k (mV)
$Ca_v2.3$/μ-OR	control (8)	1.2 ± 1.4	4.8 ± 0.3	–56.8 ± 1.7	9.6 ± 0.5
	DAMGO (8)	1.6 ± 1.4	5.4 ± 0.2	–57.1 ± 1.3	9.9 ± 0.6
	naloxone (5)	0.5 ± 2.1	4.4 ± 0.9	–56.9 ± 1.4	11.1 ± 0.7
$Ca_v2.3$/δ-OR	control (8)	3.7 ± 1.2	5.5 ± 0.3	–54.3 ± 1.6	9.6 ± 0.5
	SNC80 (8)	3.3 ± 1.0	5.7 ± 0.3	–54.5 ± 1.5	9.9 ± 0.8
	BNTX (6)	2.2 ± 1.4	5.2 ± 0.9	–55.4 ± 1.9	10.9 ± 0.9
$Ca_v2.3$/κ-OR	control (7)	2.9 ± 1.5	5.4 ± 0.1	–56.7 ± 2.1	9.1 ± 0.6
	Sal-A (7)	3.5 ± 1.6	5.5 ± 0.1	–57.0 ± 2.8	9.6 ± 0.5
	GNTI (4)	2.6 ± 2.0	4.9 ± 0.8	–59.9 ± 1.8	11.4 ± 1.5

Parameters derived from G–V and steady-state inactivation curves fitted with the Boltzmann equation. In all cases, the concentration of the OR agonist and OR antagonist was 1 μM and 100 nM, respectively. Values represent mean ± SEM; n, number of experiments in parentheses.

Figure 1.

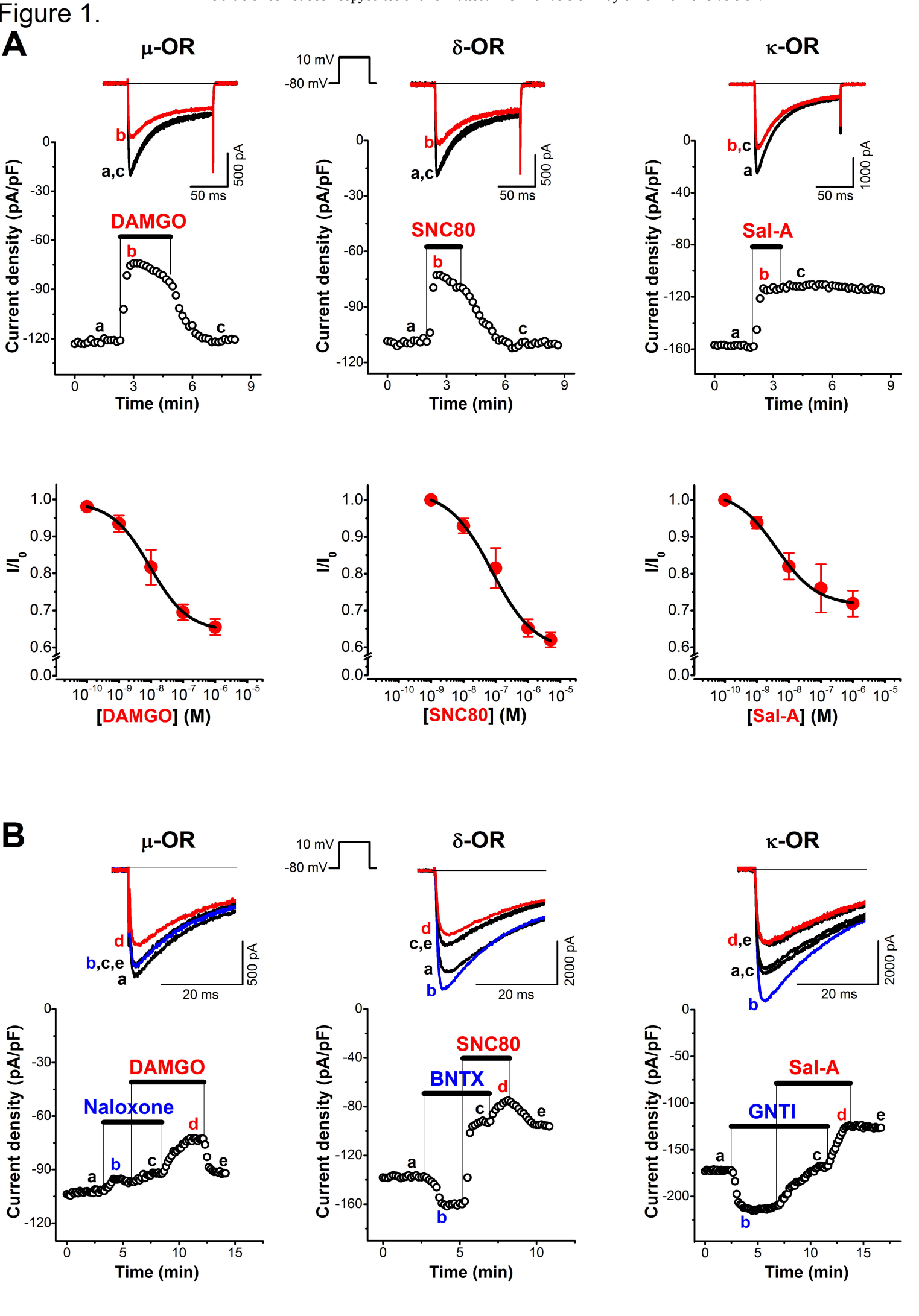
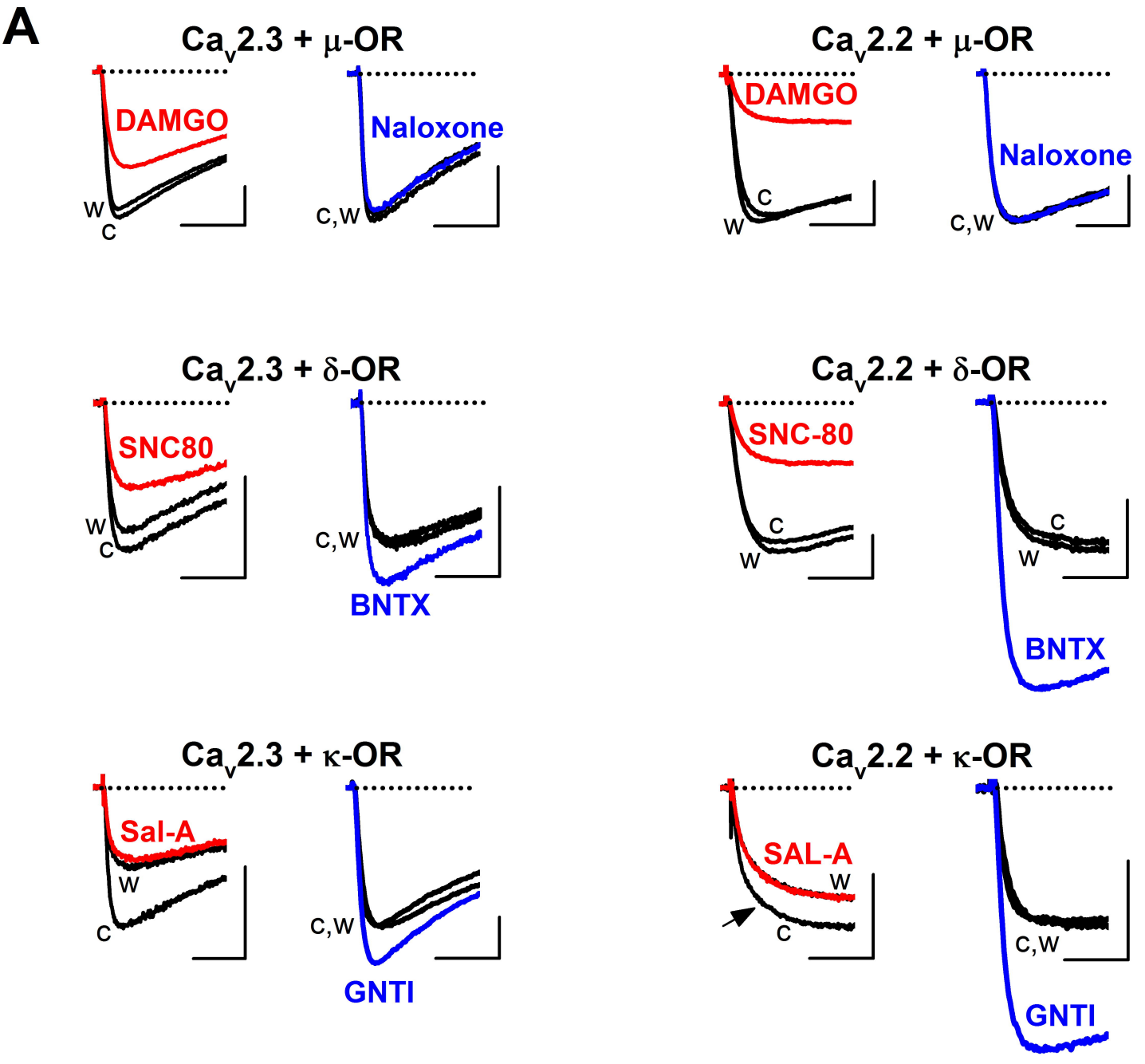

A
μ-OR
δ-OR
κ-OR
10 mV
-80 mV
500 pA
1000 pA
50 ms
a,c
b
b,c
DAMGO
SNC80
Sal-A
Current density (pA/pF)
Time (min)
I/I0
[DAMGO] (M)
[SNC80] (M)
[Sal-A] (M)
B
20 ms
2000 pA
d
b,c,e
c,e
d,e
a,c
Naloxone
BNTX
GNTI

Figure 2


A
Ca$_v$2.3 + μ-OR
DAMGO
Naloxone
W
C
C,W
Ca$_v$2.2 + μ-OR
DAMGO
Naloxone
C
W
C,W
Ca$_v$2.3 + δ-OR
SNC80
W
C
C,W
BNTX
Ca$_v$2.2 + δ-OR
SNC-80
C
W
C
W
BNTX
Ca$_v$2.3 + κ-OR
Sal-A
W
C
C,W
GNTI
Ca$_v$2.2 + κ-OR
SAL-A
W
C
C,W
GNTI

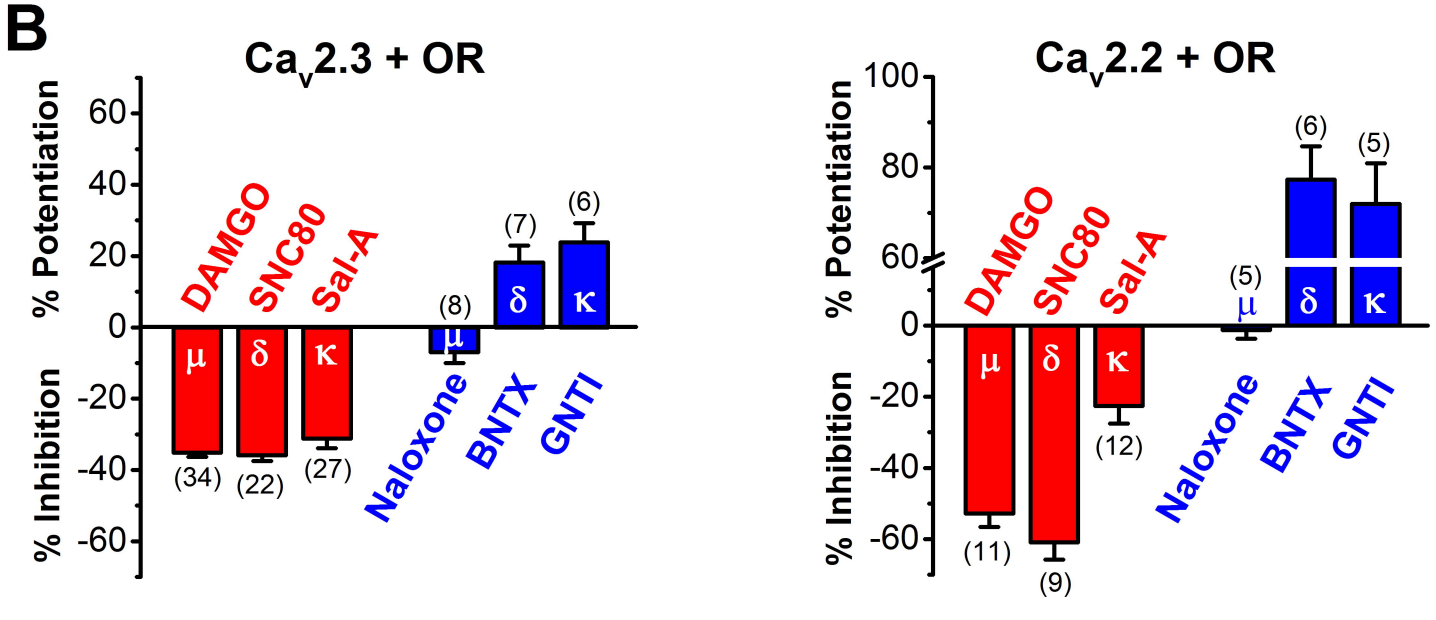

B
Ca$_v$2.3 + OR
% Potentiation
% Inhibition
60
40
20
0
-20
-40
-60
DAMGO
SNC80
Sal-A
Naloxone
BNTX
GNTI
μ
δ
κ
μ
δ
κ
(34)
(22)
(27)
(8)
(7)
(6)
Ca$_v$2.2 + OR
% Potentiation
% Inhibition
100
80
60
0
-20
-40
-60
DAMGO
SNC80
Sal-A
Naloxone
BNTX
GNTI
μ
δ
κ
μ
δ
κ
(11)
(9)
(12)
(5)
(6)
(5)

Figure 3

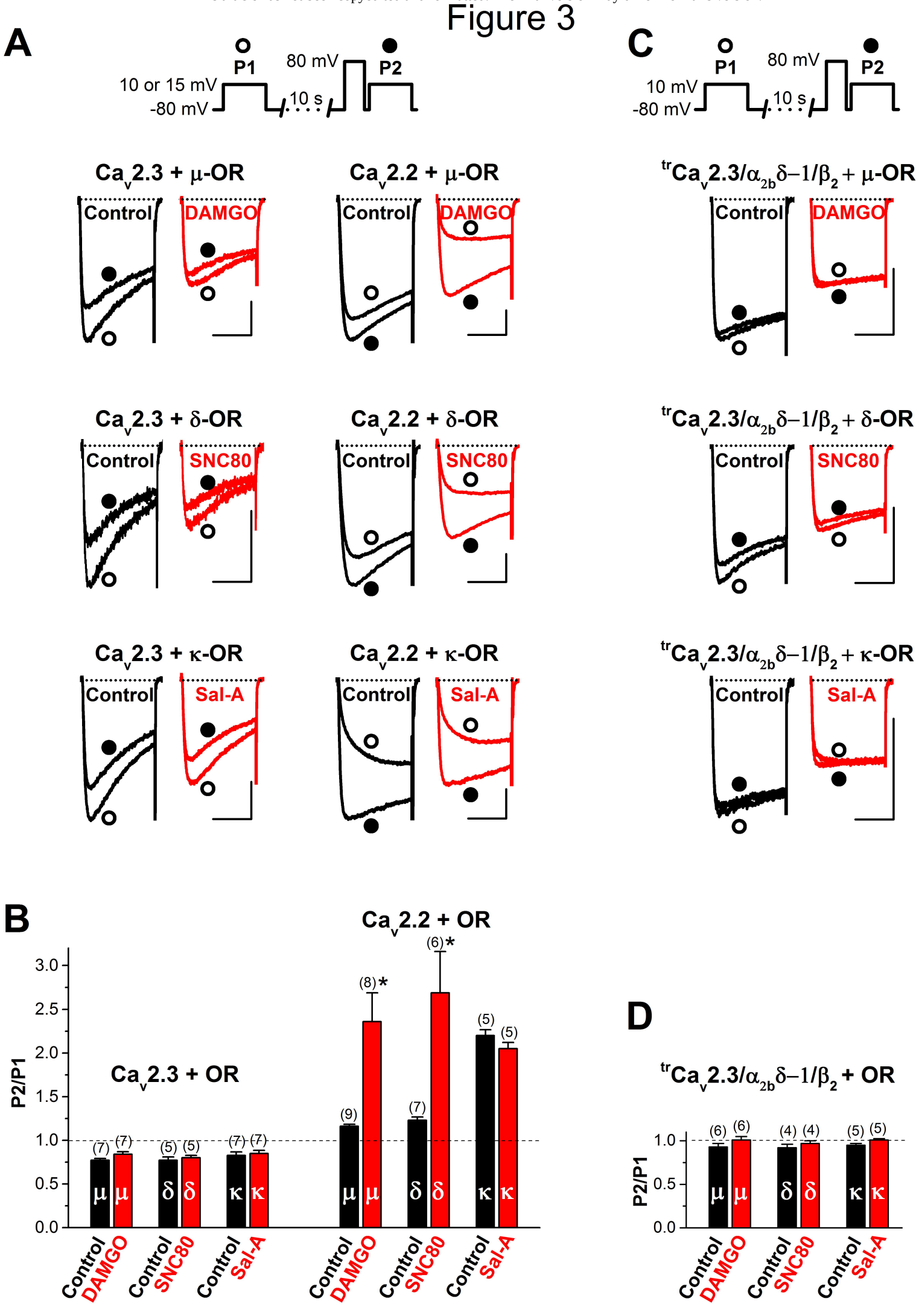
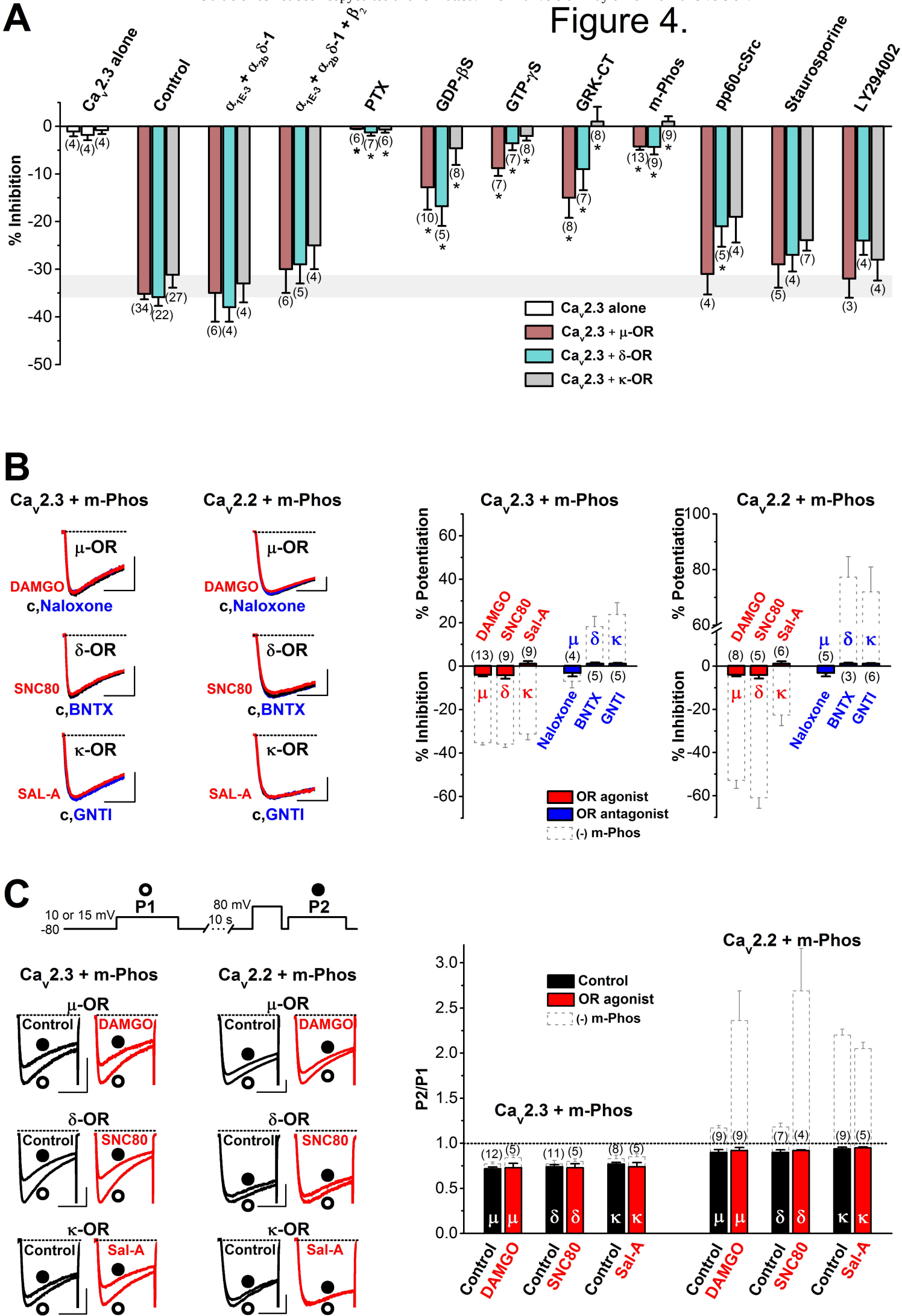

A
P1
P2
80 mV
10 or 15 mV
-80 mV
10 s
Ca$_v$2.3 + μ-OR
Ca$_v$2.2 + μ-OR
Control
DAMGO
Ca$_v$2.3 + δ-OR
Ca$_v$2.2 + δ-OR
SNC80
Ca$_v$2.3 + κ-OR
Ca$_v$2.2 + κ-OR
Sal-A
C
10 mV
^{tr}Ca$_v$2.3/α$_{2b}$δ–1/β$_2$ + μ-OR
^{tr}Ca$_v$2.3/α$_{2b}$δ–1/β$_2$ + δ-OR
^{tr}Ca$_v$2.3/α$_{2b}$δ–1/β$_2$ + κ-OR
B
Ca$_v$2.2 + OR
Ca$_v$2.3 + OR
P2/P1
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(7) (7)
(5) (5)
(7) (7)
(9)
(8)*
(7)
(6)*
(5)
(5)
μ μ δ δ κ κ
Control DAMGO
Control SNC80
Control Sal-A
D
^{tr}Ca$_v$2.3/α$_{2b}$δ–1/β$_2$ + OR
(6) (6)
(4) (4)
(5) (5)

Figure 4.


A
Ca$_V$2.3 alone
Control
α_{1E-3} + α_{2b} δ-1
α_{1E-3} + α_{2b} δ-1 + β_2
PTX
GDP-βS
GTP-γS
GRK-CT
m-Phos
pp60-cSrc
Staurosporine
LY294002
% Inhibition
0
-10
-20
-30
-40
-50
Ca$_V$2.3 alone
Ca$_V$2.3 + μ-OR
Ca$_V$2.3 + δ-OR
Ca$_V$2.3 + κ-OR
B
Ca$_V$2.3 + m-Phos
Ca$_V$2.2 + m-Phos
μ-OR
DAMGO
c,Naloxone
δ-OR
SNC80
c,BNTX
κ-OR
SAL-A
c,GNTI
% Potentiation
% Inhibition
DAMGO
SNC80
Sal-A
Naloxone
BNTX
GNTI
OR agonist
OR antagonist
(-) m-Phos
C
10 or 15 mV
-80
P1
80 mV
10 s
P2
Control
P2/P1
Control
DAMGO
Control
SNC80
Control
Sal-A