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$GABA_B$ receptors inhibit low-voltage activated and high-voltage activated Ca^{2+} channels in sensory neurons via distinct mechanisms

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Abbreviations: DRG, dorsal root ganglion; DTT, dithiothreitol; GABA, gamma-aminobutyric acid; GFP, green fluorescent protein; HEK293, human embryonic kidney 293 cells; HVA, high voltage activated Ca^{2+} channels; LVA, low voltage activated Ca^{2+} channels; NK1, neurokinin receptor isoform 1; ROS, reactive oxygen species; PTX, pertussis toxin; VGCC, voltage-gated Ca^{2+} channels

Abstract

Growing evidence suggests that mammalian peripheral somatosensory neurons express functional receptors for gamma-aminobutyric acid, $GABA_A$ and $GABA_B$. Moreover, local release of GABA by pain-sensing (nociceptive) nerve fibres has also been suggested. Yet, the functional significance of GABA receptor triggering in nociceptive neurons is not fully understood. Here we used patch-clamp recordings from small-diameter cultured DRG neurons to investigate effects of $GABA_B$ receptor agonist baclofen on voltage-gated Ca^{2+} currents. We found that baclofen inhibited both low-voltage activated (LVA, T-type) and high-voltage activated (HVA) Ca^{2+} currents in a proportion of DRG neurons by 22% and 32% respectively; both effects were sensitive to $G_{i/o}$ inhibitor pertussis toxin. Inhibitory effect of baclofen on both current types was about twice less efficacious as compared to that of the μ-opioid receptor agonist DAMGO. Surprisingly, only HVA but not LVA current modulation by baclofen was partially prevented by G protein inhibitor GDP-β-S. In contrast, only LVA but not HVA current modulation was reversed by the application of a reducing agent dithiothreitol (DTT). Inhibition of T-type Ca^{2+} current by baclofen and the recovery of such inhibition by DTT were successfully reconstituted in the expression system. Our data suggest that inhibition of LVA current in DRG neurons by baclofen is partially mediated by an unconventional signaling pathway that involves a redox mechanism. These findings reinforce the idea of targeting peripheral GABA receptors for pain relief.

Keywords: T-type Ca^{2+} channels/ GABAB receptors/ Opioid receptor/ Baclofen/ Nociceptor/ Redox mechanisms

Introduction

Mammalian peripheral somatosensory neurons detect and transmit to CNS versatile information about body's environment; a large population of these neurons ("pain" or nociceptive neurons) responds specifically to tissue damage. Cell bodies of these neurons reside in peripheral ganglia (e.g. dorsal root ganglia, DRG) while their long, T-shaped axons form afferent sensory fibres innervating skin, muscles, joints and other bodily organs. Excitation of peripheral terminals of somatosensory fibres initiate physiological somatosensory transmission, in addition, action potentials produced elsewhere along the fibre (i.e. ectopically) can result in pathological sensations (e.g. chronic pain or 'phantom' itch) [1]. Although main somatosensory integration and processing is performed by CNS, growing evidence suggest that peripheral neurons themselves can communicate with each other, both electrically and chemically [2,3,4]. Accordingly, cell bodies of sensory neurons express various receptors for classical neurotransmitters such as acetylcholine, glutamate and GABA [5]. Particularly, both $GABA_A$ [6,7,8,9] and $GABA_B$ [10,11] receptors are abundantly expressed in somatosensory neurons, including nociceptors. A recent study demonstrated that peripheral nociceptive terminals are capable of releasing GABA, which acts in an autocrine fashion at the endogenous $GABA_{B1}$ receptors to attenuate sensitization of heat- and inflammation-activated TRPV1 channels thus limiting hyperalgesia in rats [10]. Hence, emerging data demonstrate functional significance of peripheral GABA receptors in nociceptive neurons. Yet, the mechanisms of inhibitory action of $GABA_B$ receptors in the periphery are only beginning to emerge.

$GABA_B$ receptors belong to $G_{i/o}$ type of G-protein coupled receptors (GPCR) [12] which are known to exert inhibitory action in CNS via the inhibition of voltage-gated Ca^{2+} channels (VGCC) and activation of GIRK K^+ channels [13]. Therefore, we investigated effects of $GABA_B$ agonist baclofen on voltage-gated Ca^{2+} currents in cultured small-diameter DRG neurons. DRG neurons express variety of VGCC subtypes, including high-voltage activated (HVA) N, P/Q and L-type channels [14,15,16,17,18] as well as low–voltage activated (LVA) T-type Ca^{2+} channels [19,20,21].

Using patch-clamp recordings we analysed effects of baclofen on both LVA and HVA currents; we found that both currents are inhibited by baclofen in subpopulations of DRG neurons. Interestingly, we found that inhibition of LVA and HVA currents was mediated by distinct mechanisms. These findings extend our understanding of $GABA_B$ receptor action in peripheral somatosensory system and suggest a possibility of targeting these receptors for analgesia.

Materials and Methods

Cell cultures and transfection. DRG neurons were cultured as described previously [22,23,24]. Briefly, adult Sprague Dawley rats (170 g-180 g) were humanly euthanized by isoflurane overdose. DRGs from all spinal levels were extracted and dissociated using collagenase/dispase method as described [22,23,24]. Dissociated cells we cultured in DMEN supplemented with GlutaMax I, 10 % fetal calf serum, penicillin (50 U/ml) and streptomycin (50 µg/ml) on glass coverslips coated with poly-D-lysine for 2-5 days in a humidified incubator (37°C, 5% CO_2). No growth factors were added to the culture media.

Hunan embrionic kidney cells 293 (HEK293) cells were cultured in the same DMEM supplemented with GlutaMax I, 10 % fetal calf serum, penicillin (50 U/ml) and streptomycin (50 µg/ml) and passaged every 2-3 days. Human $GABA_{B2}$ receptor (GenBank accession number NM_005458) cDNA was purchased from the Missouri Science and Technology cDNA Resource Center. Human Cav3.2 (GenBank accession number AF051946) cDNA was kindly provided by Dr E. Perez-Reyes, (University of Virginia, USA). HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Electrophysiology. All recordings were made using Multiclamp 700B amplifier in combination with pCLAMP 10.4 software (Axon Instruments, Union City, CA, USA). Voltage-clamp recordings were sampled at 4 kHz. A Whole-cell configuration of the patch clamp technique was used throughout. The standard bath solution contained (in mM): 150 TEA-Cl; 2 $CaCl_2$; 10 HEPES; 10 glucose (pH 7.4 adjusted with CsOH). The standard pipette solution contained (in mM): 135 CsCl; 3 $MgCl_2$; 10 EGTA; 10 HEPES; 3 Mg-ATP; 0.6 GTP (PH 7.4 adjusted with CsOH). LVA currents were measured by 50 ms square voltage pulses to -40 mV from a holding potential of -90 mV. HVA currents were measured by 100 ms square voltage pulses to 0 mV from a holding potential of -90 mV. A 3% agar-salt (3 M KCl) bridges were used in order to avoid possible redox-induced offset potentials.

Chemicals. PTX was from Enzo Life Science; lipofectamine 2000 was from Life Technologies. Baclofen, DAMGO as well as all other chemicals were from Sigma.

Statistics. All data are given as mean ± S.E.M. Differences between groups were assessed by Student's t test (paired or unpaired, as appropriate). The differences were considered significant at $P \leq 0.05$. Statistical analyses were performed using Origin 8.6 (OriginLab Corporation, Northampton, CA, USA).

Results.

In order to analyse possible effects of $GABA_B$ receptor triggering on voltage-gated Ca^{2+} currents in DRG neurons we evaluated effects of baclofen (10 μM) on LVA and HVA Ca^{2+} currents from cultured small-diameter (soma diameter ~20 μm, input resistance ~25 pA/pF) DRG neurons using whole-cell patch clamp recording. LVA and HVA currents were evoked by a double-pulse voltage protocol with a 50 ms pulse to -40 mV followed by a 100 ms pulse to 0 mV to activate LVA and HVA currents accordingly; holding potential of -90 mV was maintained throughout (Fig. 1A). All neurons tested displayed large HVA currents (-840.92 ± 85.8 pA, n=65) but only 21/65 (32%) neurons displayed LVA currents at -40 mV (-166.67 ± 24.7 pA, n=21). Baclofen reversibly inhibited LVA and HVA calcium currents in DRG neurons by 22.53 ± 2.05% (n = 15) and 31.90 ± 3.11% (n = 27), respectively (Fig. 1A, B). Inhibition of HVA currents was significantly larger as compared to LVA current inhibition ($P < 0.05$). There was no significant effect on channel kinetics. Both LVA and HVA channel inhibition was significantly blocked by pre-treatment with the $G_{i/o}$ receptor inhibitor Pertussis toxin (PTX; 400 ng/ml PTX; Fig. 1C, D), confirming that inhibition is mediated by a $G_{i/o}$ type of GPCR. Surprisingly, only the HVA current inhibition by baclofen was sensitive to the non-hydrollyzable GDP analog GDP-β-s, which inhibits G protein activation [25]. Thus, inclusion of GDP-β-s (2 mM) into the intracellular pipette solution reduced baclofen-induced inhibition of HVA Ca^{2+} currents ($P < 0.05$, n = 16) but had no effect on the inhibition of LVA calcium currents ($P > 0.05$, n = 6) (Fig. 1E, F). This differential sensitivity of signaling cascades linking $GABA_B$ receptors with LVA and HVA Ca^{2+} channels was unexpected and suggested involvement of unconventional signaling cascade in the inhibition of LVA Ca^{2+} channels.

We have recently identified one such unconventional signaling cascade linking neurokinin NK1 receptors with the activation of M-type (Kv7) K^+ channels in DRG neurons [26]. Thus, we have shown, that acting via a PTX-sensitive mechanism, NK1 receptors stimulated release of reactive oxygen species (ROS) from mitochondria of DRG neurons and these ROS induced oxidative modification of the Kv7 channels thus potentiating their activity [26]. Indeed, Kv7

channels are highly sensitive to oxidation by ROS due to the specific cysteine pocket in the intracellular S2-S3 linker of the channel [27,28]. Interestingly, T-type Ca^{2+} channels are also sensitive to redox modulation, albeit in contrast to Kv7 channels, oxidizing compounds and ROS do not activate but inhibit T-type channel activity [21,29,30]. Since ROS generation by NK1 receptor triggering was sensitive to PTX [26] we hypothesized that unconventional signaling cascade causing inhibition of LVA current in DRG neurons by baclofen may also be mediated by a ROS-dependent mechanism. Reducing agent DTT has been used to reverse oxidative modification of Kv7 channels [26,27]. Therefore we tested if DTT can also reverse baclofen-induced inhibition of LVA VGCC in DRG neurons. In accordance with our hypothesis, inhibition of LVA Ca^{2+} current by baclofen was partially recovered by the reducing agent DTT (1 mM). DTT recovered baclofen-inhibited LVA current amplitude to 89.20 ± 2.5 % of control value (Fig. 2A, C; $P < 0.01$, $n = 6$), suggesting that a part of the baclofen effect is indeed mediated by a redox mechanism. In contrast, DTT had no effect on the inhibition of HVA Ca^{2+} currents by baclofen (Fig. 2B, D; $P > 0.05$, $n = 14$), further supporting our finding that inhibition of LVA and HVA Ca^{2+} currents by baclofen in DRG neurons is, at least in part, mediated by distinct mechanisms.

Next we tested if inhibition of LVA current by baclofen can be reconstituted in the expression system. Cav3.2 is a predominant T-type Ca^{2+} channel isoform in DRG neurons [19,20,31,32]. Thus, we co-expressed in HEK293 cells human $GABA_{B2}$ receptors, human Cav3.2 and GFP and tested the effect of baclofen on the recombinant Cav3.2 current. Similarly to DRG neurons, baclofen (10 μM) reversibly inhibited $Ca_V3.2$ currents by 24.73 ± 1.11% (Fig. 3A, B; $n = 12$; $P < 0.001$); the inhibition was very similar in amplitude to that observed in DRG neurons (22.53 ± 2.05%). Likewise, there was no significant effect on channel kinetics. Importantly (and similar to the effect observed in DRG neurons), the inhibition of the recombinant Cav3.2 channel currents in HEK293 cells could also be partly recovered by the reducing agent DTT (1 mM). Like in DRG neurons, DTT recovered baclofen-inhibited Cav3.2 current to 87.7 ± 0.9 % of control value (Fig. 3C, D; P <

0.001, n = 7). These experiments suggest that redox-mediated inhibition of T-type Ca^{2+} channels by baclofen is a general property of $GABA_B$ receptors.

Finally, for comparison, we tested the effect of another prominent $G_{i/o}$-coupled receptor with analgesic properties expressed in DRG neurons, μ-opioid receptor (MOR) on both LVA and HVA currents. The MOR agonist DAMGO (250 nM) reversibly inhibited HVA currents in 8/9 neurons by 65.8 ± 6.7% (Fig. 4A, B, D; $P < 0.001$; n = 8) with an IC_{50} of 17.72 ± 6.9 nM (not shown). In agreement with previous study [33], in the majority (11/14) of DRG neurons that displayed LVA current DAMGO had no effect on both LVA and HVA channels, suggesting lack of co-expression between T-type channels and MOR. Yet, in 3/14 LVA-positive neurons tested DAMGO did inhibit LVA current by 41.8 ± 10.4% (Fig. 4A-C; $P < 0.001$; n = 3). This set of experiments suggests that i) in small sub-population of DRG neurons T-type Ca^{2+} channels co-express with MOR; ii) MOR is approximately twice more efficacious in inhibiting both LVA and HVA currents in DRG neurons as compared to $GABA_B$ receptors.

Discussion

$GABA_B$ receptors are abundantly expressed in DRG neurons [11], particularly in the cell bodies and peripheral terminals of nociceptive neurons expressing polymodal noxious sensory channel TRPV1 [10]. While at the moment there is no widely accepted theory for the potential source of GABA in the periphery, study by Hanack and colleagues suggested that nociceptive neurones themselves can produce GABA and release it at the level of peripheral terminals in response to noxious stimulation; these authors further suggested that such peripherally-released GABA acts in an autocrine way to counteract sensitization of TRPV1 channels and reduce hyperalgesia. Here we investigated additional ways by which peripheral $GABA_B$ receptors might influence nociceptive transmission. $GABA_B$, similar to other $G_{i/o}$-coupled receptors, inhibit HVA currents using a 'fast' pathway that involves direct interaction of G protein with the channels [34,35,36,37,38] resulting in a conformational change of the channels complex that makes it harder to open by voltage [39,40]. There are also number of other pathways by which GPCR can inhibit VGCC, including phosphatidylinositol 4,5-bisphosphate (PIP_2 depletion) [41,42], arachidonic acid [43] and Src kinase [15]. Inhibition of T-type channels by $GABA_B$ receptors in DRG neurons has been reported [44] but the mechanism of inhibition (and particularly if it is the same as for the inhibition of HVA currents) has not been investigated. We report that in cultured small-diameter rat DRG neurons $GABA_B$ receptor agonist baclofen inhibits both HVA and LVA currents (by ~32% and ~22% respectively) but the mechanisms of inhibition are at least partially different. In both cases inhibition was sensitive to the $G_{i/o}$ inhibitor PTX, however, only the HVA current inhibition was sensitive to the non-hydrollyzable GDP analog GDP-β-s, which inhibits G protein activation. In contrast, only the LVA current inhibition was sensitive to the reducing agent DTT suggesting that a non-canonical, redox-mediated mechanism contributes to the $GABA_B$ inhibition of LVA but not HVA Ca^{2+} currents. Quantitatively very similar results were obtained in reconstituted system (HEK293 cells transiently overexpressing $GABA_{B2}$ receptors and Cav3.2) where, again, baclofen-induced inhibition of Cav3.2 was partially recovered by DTT. Recently we have described a novel

PTX-sensitive signaling cascade in nociceptive DRG neurons in which endogenous NK1 receptors produced intracellular effects by stimulating release of ROS (i.e. superoxide anion) from mitochondria. Acting as intracellular second messengers, such ROS augmented the activity of inhibitory Kv7 channels providing a mechanism for substance P mediated peripheral endogenous analgesia [26]. Because i) T-type channels are inhibited by oxidizing agents and ROS [21,29,30]; and ii) baclofen-induced inhibition of T-type channels is sensitive to DTT and PTX but not to GDP-β-s (Fig. 1 & 2), we hypothesize that similar ROS-mediated mechanism might be at play in the case of $GABA_B$-mediated inhibition of LVA channels described here. It will be interesting to test in the future if baclofen, similar to substance P, also augments Kv7 channel activity in DRG neurons; such an effect would be predicted. Finally, our data suggest that $GABA_B$ receptor triggering produces similar (although less robust) effect on LVA and HVA currents in DRG neurons as that of MOR stimulation.

In sum, we report that $GABA_B$ receptors inhibit both HVA and LVA currents in peripheral sensory neurons albeit via distinct mechanisms. These effects reinforce the idea of targeting peripheral GABA receptors for pain relief.

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References

[1] A.I. Basbaum, D.M. Bautista, G. Scherrer, D. Julius, Cellular and molecular mechanisms of pain, Cell 139 (2009) 267-284.

[2] R. Amir, M. Devor, Chemically mediated cross-excitation in rat dorsal root ganglia, J Neurosci 16 (1996) 4733-4741.

[3] R. Amir, M. Devor, Electrical excitability of the soma of sensory neurons is required for spike invasion of the soma, but not for through-conduction, Biophys J 84 (2003) 2181-2191.

[4] G.M. Rozanski, A.R. Nath, M.E. Adams, E.F. Stanley, Low voltage-activated calcium channels gate transmitter release at the dorsal root ganglion sandwich synapse, J Physiol 591 (2013) 5575-5583.

[5] M.S. Gold, Molecular biology of sensory transduction, in: S.B. McMahon, Koltzenburg, M., Tracey, I., Turk, D (Ed.), Wall & Melzack's Textbook of Pain, Saunders, 2013, pp. 31-48.

[6] N. Akaike, M. Inoue, O.A. Krishtal, 'Concentration-clamp' study of gamma-aminobutyric-acid-induced chloride current kinetics in frog sensory neurones, J Physiol 379 (1986) 171-185.

[7] J.P. Gallagher, H. Higashi, S. Nishi, Characterization and ionic basis of GABA-induced depolarizations recorded in vitro from cat primary afferent neurones, J Physiol 275 (1978) 263-282.

[8] B. Robertson, Characteristics of GABA-activated chloride channels in mammalian dorsal root ganglion neurones, J Physiol 411 (1989) 285-300.

[9] E. Persohn, P. Malherbe, J.G. Richards, In situ hybridization histochemistry reveals a diversity of GABAA receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia, Neuroscience 42 (1991) 497-507.

[10] C. Hanack, M. Moroni, W.C. Lima, H. Wende, M. Kirchner, L. Adelfinger, K. Schrenk-Siemens, A. Tappe-Theodor, C. Wetzel, P.H. Kuich, M. Gassmann, D. Roggenkamp, B. Bettler, G.R. Lewin, M. Selbach, J. Siemens, GABA blocks pathological but not acute TRPV1 pain signals, Cell 160 (2015) 759-770.

[11] S. Towers, A. Princivalle, A. Billinton, M. Edmunds, B. Bettler, L. Urban, J. Castro-Lopes, N.G. Bowery, GABAB receptor protein and mRNA distribution in rat spinal cord and dorsal root ganglia, Eur J Neurosci 12 (2000) 3201-3210.

[12] N.G. Bowery, B. Bettler, W. Froestl, J.P. Gallagher, F. Marshall, M. Raiteri, T.I. Bonner, S.J. Enna, International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function, Pharmacol Rev 54 (2002) 247-264.

[13] B. Hille, Ion channels of excitable membranes, 3rd ed., Sinauer, Sunderland, Mass., 2001.

[14] C.G. Acosta, H.S. Lopez, delta opioid receptor modulation of several voltage-dependent Ca^{2+} currents in rat sensory neurons, J Neurosci 19 (1999) 8337-8348.

[15] J. Raingo, A.J. Castiglioni, D. Lipscombe, Alternative splicing controls G protein-dependent inhibition of N-type calcium channels in nociceptors, Nat Neurosci 10 (2007) 285-292.

[16] T.J. Bell, C. Thaler, A.J. Castiglioni, T.D. Helton, D. Lipscombe, Cell-specific alternative splicing increases calcium channel current density in the pain pathway, Neuron 41 (2004) 127-138.

[17] B. Fioretti, L. Catacuzzeno, L. Sforna, M.B. Gerke-Duncan, A.M. van den Maagdenberg, F. Franciolini, M. Connor, D. Pietrobon, Trigeminal ganglion neuron subtype-specific alterations of $Ca_V2.1$ calcium current and excitability in a Cacna1a mouse model of migraine, J Physiol 589 (2011) 5879-5895.

[18] D.J. Mogul, A.P. Fox, Evidence for multiple types of Ca^{2+} channels in acutely isolated hippocampal CA3 neurones of the guinea-pig, J Physiol 433 (1991) 259-281.

[19] J. Elies, J.L. Scragg, S. Huang, M.L. Dallas, D. Huang, D. MacDougall, J.P. Boyle, N. Gamper, C. Peers, Hydrogen sulfide inhibits Cav3.2 T-type Ca^{2+} channels, FASEB J 28 (2014) 5376-5387.

[20] K.E. Rose, N. Lunardi, A. Boscolo, X. Dong, A. Erisir, V. Jevtovic-Todorovic, S.M. Todorovic, Immunohistological demonstration of $Ca_V3.2$ T-type voltage-gated calcium

channel expression in soma of dorsal root ganglion neurons and peripheral axons of rat and mouse, Neuroscience 250 (2013) 263-274.

[21] S.M. Todorovic, V. Jevtovic-Todorovic, A. Meyenburg, S. Mennerick, E. Perez-Reyes, C. Romano, J.W. Olney, C.F. Zorumski, Redox modulation of T-type calcium channels in rat peripheral nociceptors, Neuron 31 (2001) 75-85.

[22] X. Du, H. Hao, S. Gigout, D. Huang, Y. Yang, L. Li, C. Wang, D. Sundt, D.B. Jaffe, H. Zhang, N. Gamper, Control of somatic membrane potential in nociceptive neurons and its implications for peripheral nociceptive transmission, Pain 155 (2014) 2306-2322.

[23] H.M. Kirton, L. Pettinger, N. Gamper, Transient overexpression of genes in neurons using nucleofection, Methods Mol Biol 998 (2013) 55-64.

[24] J.E. Linley, K. Rose, M. Patil, B. Robertson, A.N. Akopian, N. Gamper, Inhibition of M current in sensory neurons by exogenous proteases: a signaling pathway mediating inflammatory nociception, J Neurosci 28 (2008) 11240-11249.

[25] D.A. Peterson, D.C. Peterson, H.L. Reeve, S.L. Archer, E.K. Weir, GTP (gammaS) and GDP (betaS) as electron donors: new wine in old bottles, Life Sci 65 (1999) 1135-1140.

[26] J.E. Linley, L. Ooi, L. Pettinger, H. Kirton, J.P. Boyle, C. Peers, N. Gamper, Reactive oxygen species are second messengers of neurokinin signaling in peripheral sensory neurons, Proc Natl Acad Sci U S A 109 (2012) E1578-E1586.

[27] N. Gamper, O. Zaika, Y. Li, P. Martin, C.C. Hernandez, M.R. Perez, A.Y. Wang, D.B. Jaffe, M.S. Shapiro, Oxidative modification of M-type K^+ channels as a mechanism of cytoprotective neuronal silencing, EMBO J 25 (2006) 4996-5004.

[28] L. Ooi, S. Gigout, L. Pettinger, N. Gamper, Triple cysteine module within M-type K^+ channels mediates reciprocal channel modulation by nitric oxide and reactive oxygen species, J Neurosci 33 (2013) 6041-6046.

[29] M.T. Nelson, P.M. Joksovic, P. Su, H.W. Kang, A. Van Deusen, J.P. Baumgart, L.S. David, T.P. Snutch, P.Q. Barrett, J.H. Lee, C.F. Zorumski, E. Perez-Reyes, S.M. Todorovic,

Molecular mechanisms of subtype-specific inhibition of neuronal T-type calcium channels by ascorbate, J Neurosci 27 (2007) 12577-12583.

[30] P. Orestes, D. Bojadzic, J. Lee, E. Leach, R. Salajegheh, M.R. Digruccio, M.T. Nelson, S.M. Todorovic, Free radical signalling underlies inhibition of $Ca_V3.2$ T-type calcium channels by nitrous oxide in the pain pathway, J Physiol 589 (2011) 135-148.

[31] J.B. Shin, C. Martinez-Salgado, P.A. Heppenstall, G.R. Lewin, A T-type calcium channel required for normal function of a mammalian mechanoreceptor, Nat Neurosci 6 (2003) 724-730.

[32] S.M. Todorovic, V. Jevtovic-Todorovic, T-type voltage-gated calcium channels as targets for the development of novel pain therapies, Br J Pharmacol 163 (2011) 484-495.

[33] Z.Z. Wu, Y.Q. Cai, H.L. Pan, A functional link between T-type calcium channels and mu-opioid receptor expression in adult primary sensory neurons, J Neurochem 109 (2009) 867-878.

[34] K. Dunlap, G.D. Fischbach, Neurotransmitters decrease the calcium ocmponent of sensory neurone action potentials, Nature 276 (1978) 837-839.

[35] D. Lipscombe, S. Kongsamut, R.W. Tsien, Alpha-adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium-channel gating, Nature 340 (1989) 639-642.

[36] L. Bernheim, D.J. Beech, B. Hille, A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons, Neuron 6 (1991) 859-867.

[37] M.S. Shapiro, B. Hille, Substance P and somatostatin inhibit calcium channels in rat sympathetic neurons via different G protein pathways, Neuron 10 (1993) 11-20.

[38] P. Forscher, G.S. Oxford, D. Schulz, Noradrenaline modulates calcium channels in avian dorsal root ganglion cells through tight receptor-channel coupling, J Physiol 379 (1986) 131-144.

[39] S.R. Ikeda, G.G. Schofield, F.F. Weight, Somatostatin blocks a calcium current in acutely isolated adult rat superior cervical ganglion neurons, Neurosci Lett 81 (1987) 123-128.

[40] B.P. Bean, Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence, Nature 340 (1989) 153-156.

[41] N. Gamper, V. Reznikov, Y. Yamada, J. Yang, M.S. Shapiro, Phosphatidylinositol 4,5-bisphosphate signals underlie receptor-specific $G_{q/11}$-mediated modulation of N-type Ca^{2+} channels, J Neurosci 24 (2004) 10980-10992.

[42] B.C. Suh, K. Leal, B. Hille, Modulation of high-voltage activated Ca^{2+} channels by membrane phosphatidylinositol 4,5-bisphosphate, Neuron 67 (2010) 224-238.

[43] L. Liu, A.R. Rittenhouse, Arachidonic acid mediates muscarinic inhibition and enhancement of N-type Ca^{2+} current in sympathetic neurons, Proc Natl Acad Sci U S A 100 (2003) 295-300.

[44] K.A. Green, G.A. Cottrell, Actions of baclofen on components of the Ca-current in rat and mouse DRG neurones in culture, Br J Pharmacol 94 (1988) 235-245.

Figure legends.

Figure 1. Baclofen inhibits LVA and HVA calcium currents in DRG neurons. (A) Voltage protocol (top) and exemplary current traces showing inhibition of LVA and HVA Ca^{2+} currents in DRG neurons by 10 μM baclofen recorded using whole-cell patch clamp. Panel (B) shows summary of the effects of baclofen on peak LVA and HVA current amplitudes, number of recordings is indicated within the bars. (C, D) Pre-treatment of DRG cultures with $G_{i/o}$ inhibitor Pertussis toxin (PTX, 400 ng/ml) reduced baclofen-induced inhibition of LVA (C) and HVA (D) currents in DRG neurons. (E, F) Inclusion of the non-hydrollyzable GDP analog GDP-β-s (2 mM) into the intracellular pipette solution reduced baclofen-induced inhibition of HVA (F) but not LVA (E) Ca^{2+} currents. The number of experiments is shown within the bars. *, *** Significantly different from the group indicated by the line connector with $P<0.05$ or $P<0.001$ (paired or unpaired t-test, as appropriate).

Figure 2. Baclofen inhibition of LVA but not HVA currents involves a redox-sensitive mechanism. (A, B) Example time courses of the effects of 10 μM baclofen, 1 mM DTT and the VGCC inhibitor selective for T-type channels, mibefradil (MIB; 3 μM) on the LVA (A) and HVA (B) Ca^{2+} currents. Plotted are peak Ca^{2+} current amplitudes recorded using voltage protocol shown in Fig. 1A; periods of drug application are indicated by the vertical grey bars. Panels (C, D) summarize these effects for LVA (C) and HVA (D) currents respectively. The number of experiments is shown within the bars. *** Significantly different from the group indicated by the line connector with $P<0.001$ (paired t-test).

Figure 3. Modulation of T-type Ca^{2+} current by baclofen can be recapitulated in the expression system. (A) Example time course of the effect of baclofen (10 μM) on Cav3.2 Ca^{2+} currents in HEK293 cells overexpressing hCav3.2, $hGABA_{B2}$ and GFP. Plotted are peak Ca^{2+} current amplitudes; periods of drug application are indicated by the vertical grey bars. Exemplary current traces and the voltage protocol are shown in the inset. Panel (B) summarizes the effect. (C)

Example time courses demonstrating the recovery of baclofen-induced Cav3.2 inhibition with DTT (1 mM). Panel (D) summarizes the effect. The number of experiments is shown within the bars. *** Significantly different from the group indicated by the line connector with P<0.001 (paired t-test).

Figure 4. Inhibition of LVA and HVA currents by μ-opioid receptor agonist DAMGO in small-diameter DRG neurons. (A) Voltage protocol (top) and exemplary current traces showing inhibition of LVA and HVA Ca^{2+} currents in DRG neurons by 250 nM DAMGO recorded using whole-cell patch clamp. Panel (B) shows summary of the effects of DAMGO on peak LVA and HVA current amplitudes, number of recordings is indicated within the bars. *, *** Significantly different from the control group with P<0.05 or P<0.001 (paired t-test). (C, D) Example time courses of the effects of DAMGO on the LVA (C) and HVA (D) Ca^{2+} currents. Plotted are peak Ca^{2+} current amplitudes; periods of drug application are indicated by the vertical grey bars.

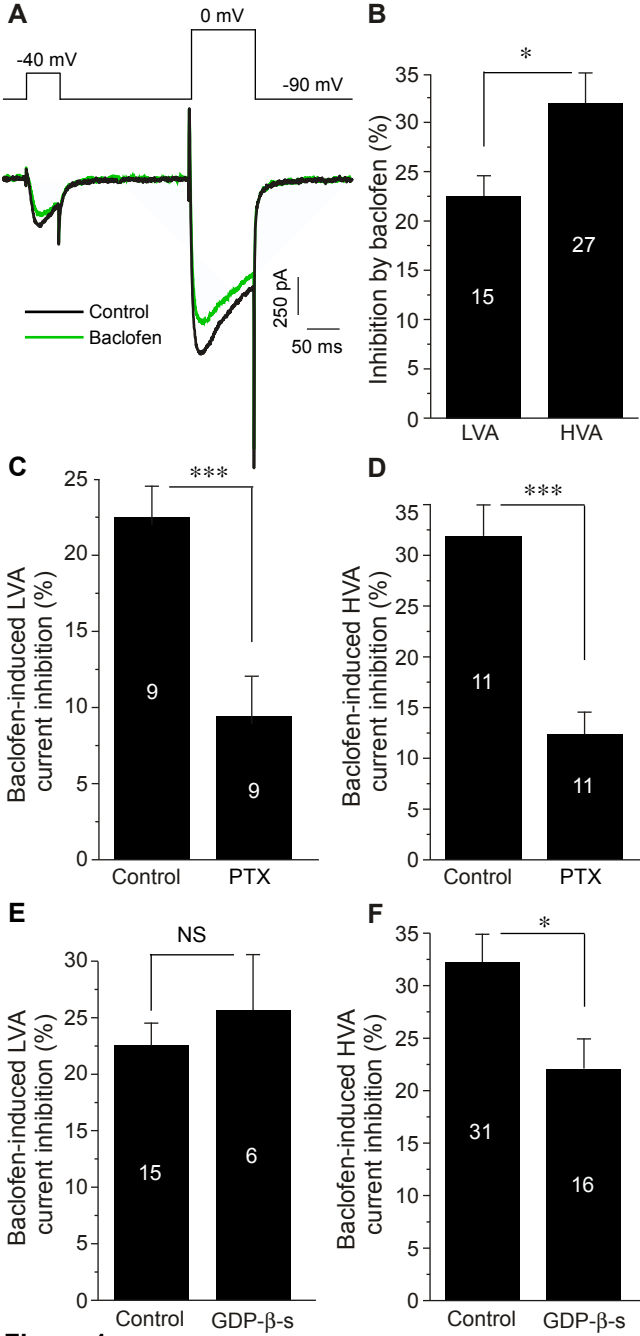

A
0 mV
-40 mV
-90 mV
250 pA
50 ms
Control
Baclofen
B
Inhibition by baclofen (%)
*
15
27
LVA
HVA
C
Baclofen-induced LVA current inhibition (%)
***
9
9
Control
PTX
D
Baclofen-induced HVA current inhibition (%)
***
11
11
Control
PTX
E
Baclofen-induced LVA current inhibition (%)
NS
15
6
Control
GDP-β-s
F
Baclofen-induced HVA current inhibition (%)
*
31
16
Control
GDP-β-s

Figure 1

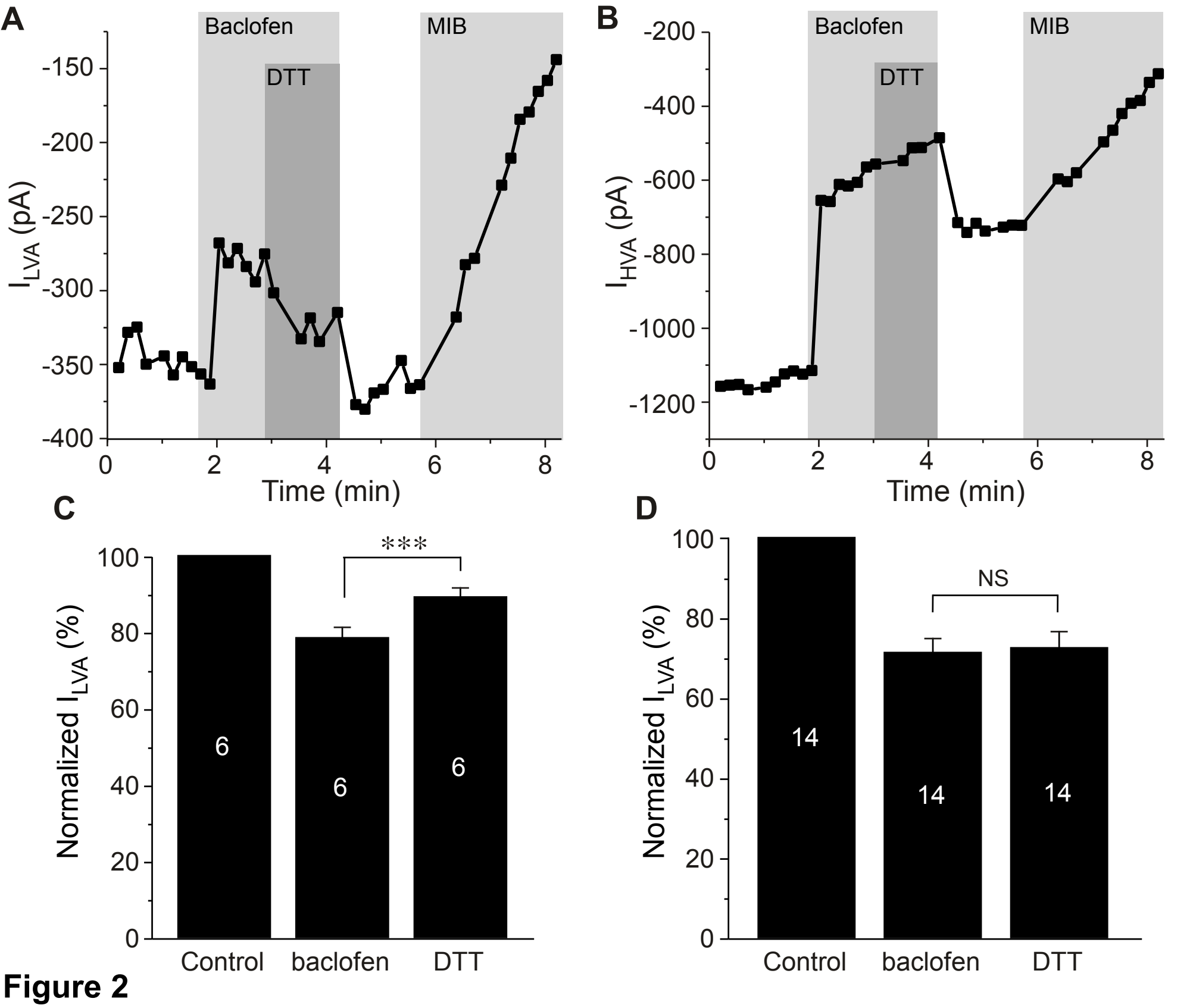
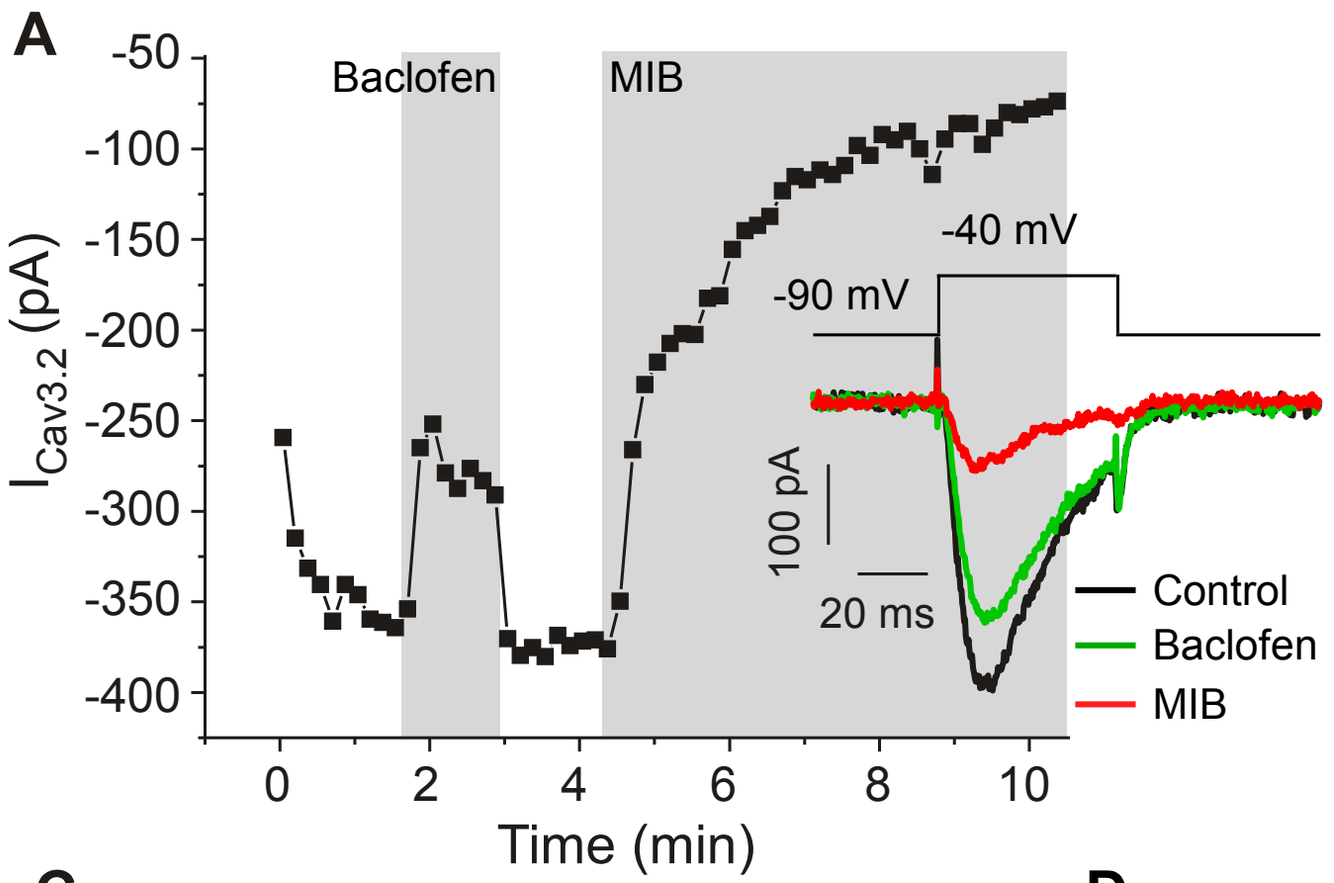
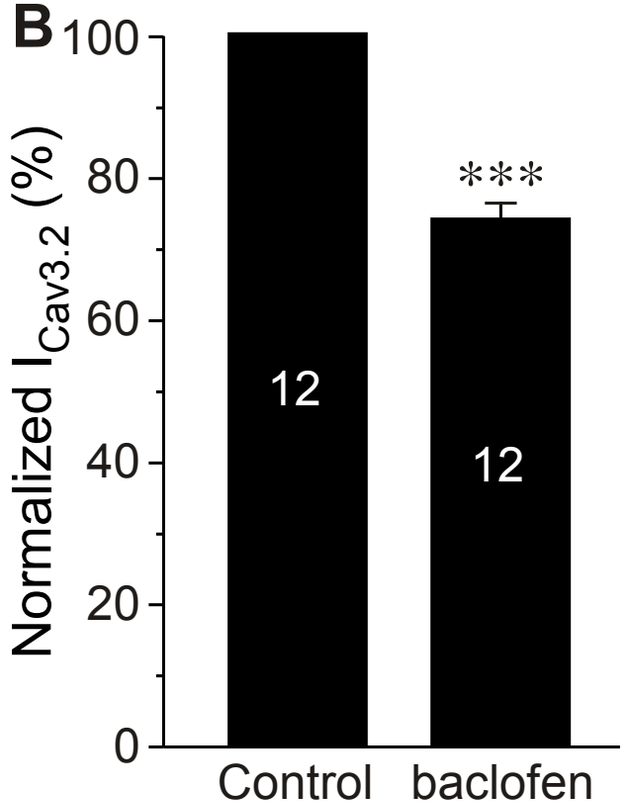
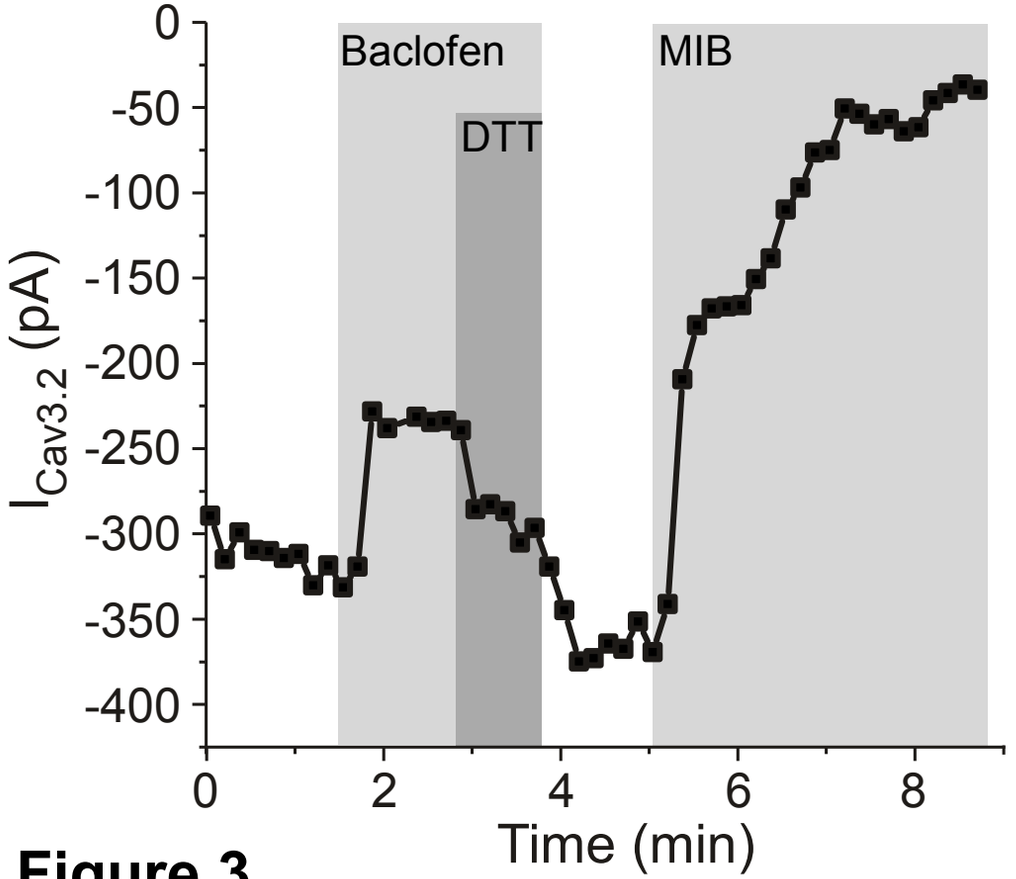

A
Baclofen
DTT
MIB
-150
-200
-250
-300
-350
-400
I_{LVA} (pA)
0
2
4
6
8
Time (min)
B
Baclofen
DTT
MIB
-200
-400
-600
-800
-1000
-1200
I_{HVA} (pA)
0
2
4
6
8
Time (min)
C
***
100
80
60
40
20
0
Normalized I_{LVA} (%)
6
6
6
Control
baclofen
DTT
D
NS
100
80
60
40
20
0
Normalized I_{LVA} (%)
14
14
14
Control
baclofen
DTT

Figure 2


A
Baclofen
MIB
-40 mV
-90 mV
100 pA
20 ms
Control
Baclofen
MIB
$I_{Cav3.2}$ (pA)
Time (min)


B
Normalized $I_{Cav3.2}$ (%)
***
12
12
Control
baclofen


C
Baclofen
DTT
MIB
$I_{Cav3.2}$ (pA)
Time (min)

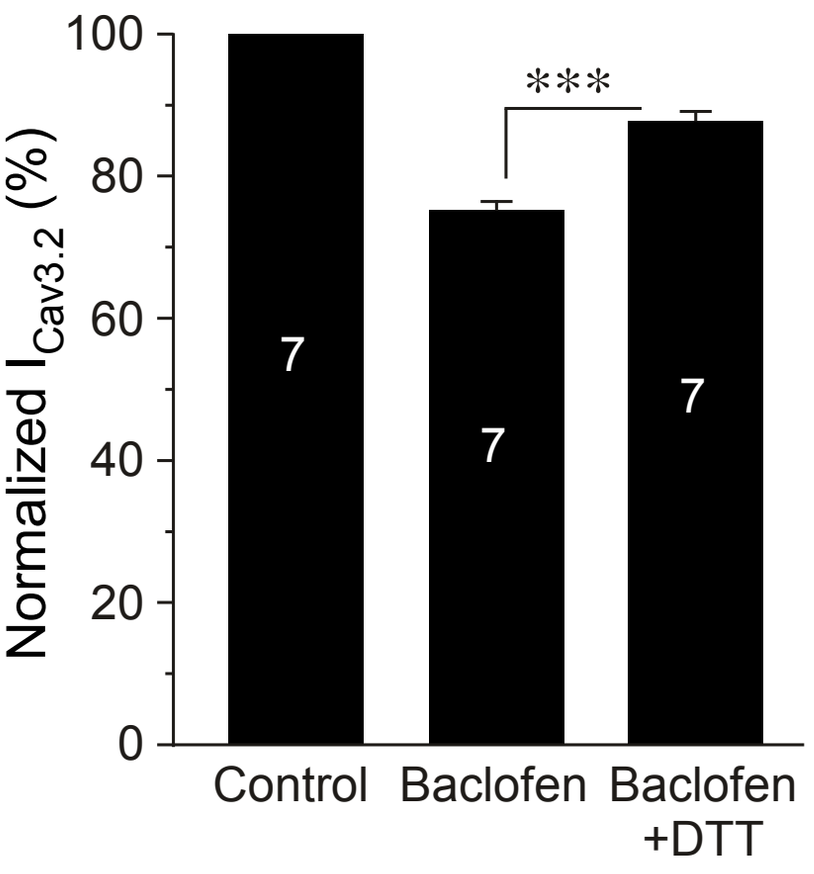

D
Normalized $I_{Cav3.2}$ (%)
***
7
7
7
Control
Baclofen
Baclofen
+DTT

Figure 3

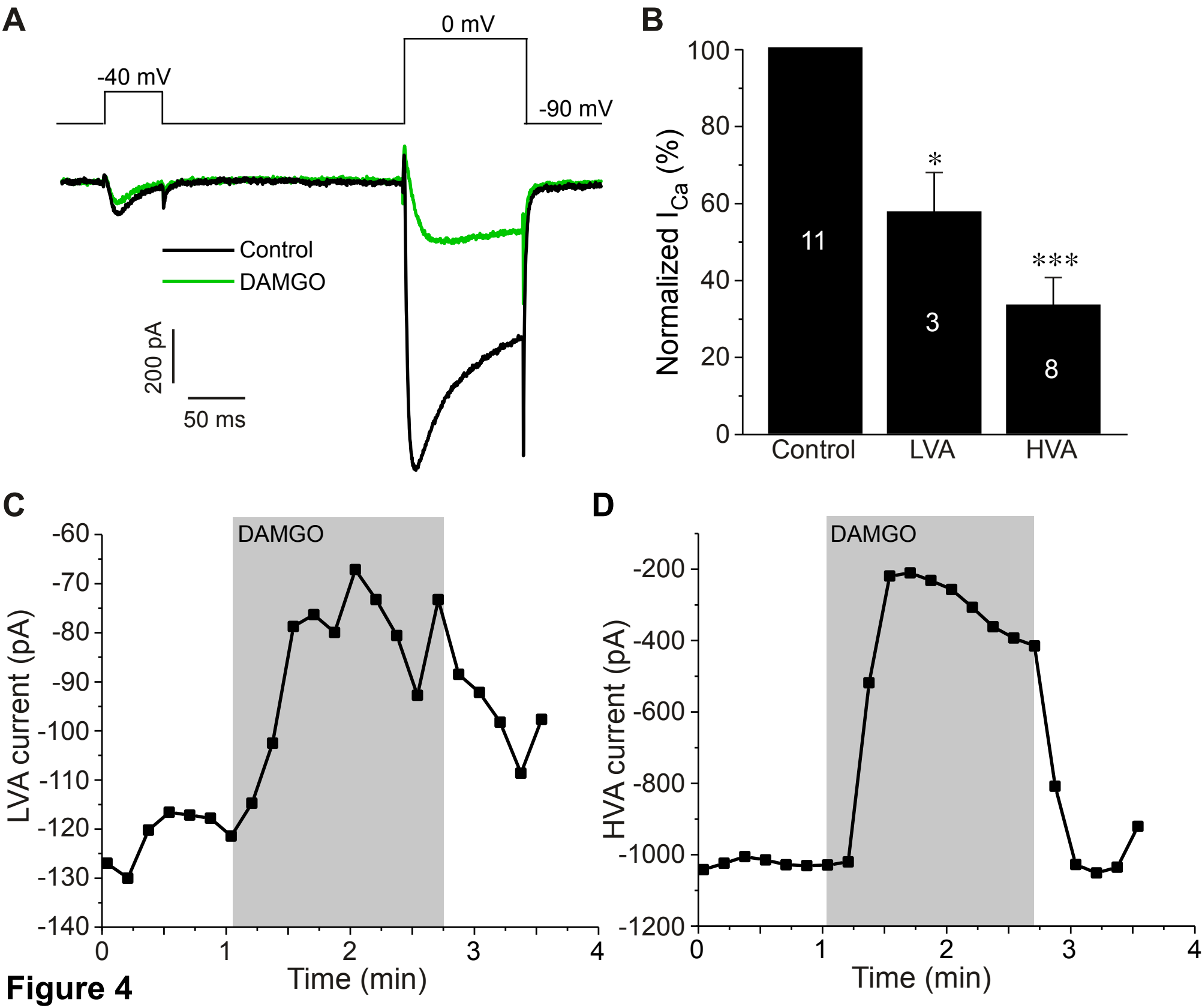

A
0 mV
-40 mV
-90 mV
Control
DAMGO
200 pA
50 ms
B
Normalized I_{Ca} (%)
100
80
60
40
20
0
11
3
8
*
***
Control
LVA
HVA
C
DAMGO
LVA current (pA)
-60
-70
-80
-90
-100
-110
-120
-130
-140
0
1
2
3
4
Time (min)
D
DAMGO
HVA current (pA)
-200
-400
-600
-800
-1000
-1200
0
1
2
3
4
Time (min)

Figure 4