Alteration of the mu opioid receptor: Ca\(^{2+}\) channel signaling pathway in a subset of rat sensory neurons following chronic femoral artery occlusion

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ALTERATION OF THE MU OPIOID RECEPTOR: CA\(^{2+}\) CHANNEL SIGNALING PATHWAY IN A SUBSET OF RAT SENSORY NEURONS FOLLOWING CHRONIC FEMORAL ARTERY OCCLUSION

Hassan B, Kim JS, Farrag M, Kaufman MP, Ruiz-Velasco V. Alteration of the mu opioid receptor: Ca\(^{2+}\) channel signaling pathway in a subset of rat sensory neurons following chronic femoral artery occlusion. J Neurophysiol 112: 3104–3115, 2014. First published September 17, 2014; doi:10.1152/jn.00630.2014.—The exercise pressor reflex, a crucial component of the cardiovascular response under physiological and pathophysiological states, is activated via metabolic and mechanical mediators that originate from contracting muscles and stimulate group III and IV afferents. We reported previously that stimulation of mu opioid receptors (MOR), expressed in both afferents, led to a significant attenuation of the reflex in rats whose femoral arteries had been occluded for 72 h. The present study examined the effect of arterial occlusion on the signaling components involved in the opioid-mediated modulation of Ca\(^{2+}\) channels in rat dorsal root ganglion neurons innervating the triceps surae muscles. We focused on neurons that were transfected with cDNA coding for enhanced green fluorescent protein (EGFP) whose expression was driven by the voltage-gated Na\(^{+}\) channel 1.8 (Na\(_{\text{v}}\)1.8) promoter region, a channel expressed primarily in nociceptive neurons. With the use of a small interference RNA approach, our results show that the pertussis toxin-sensitive Go\(_{i3}\) subunit couples MOR with Ca\(^{2+}\) channels. We observed a significant leftward shift of the MOR agonist [d-Ala2-N-Me-Phe4-Glyol5]-enkephalin concentration-response relationship in neurons isolated from rats with occluded arteries compared with those that were perfused freely. Femoral occlusion did not affect Ca\(^{2+}\) channel density or the fraction of the main Ca\(^{2+}\) channel subtype. Furthermore, Western blotting analysis indicated that the leftward shift did not result from either increased Go\(_{i3}\) or MOR expression. Finally, all neurons from both groups exhibited an inward current following exposure of the transient potential receptor vanilloid 1 (TRPV1) agonist, 8-methyl-N-vanillyl-6-nonenamide. These findings suggest that sensory neurons mediating the exercise pressor reflex express Na\(_{\text{v}}\)1.8 and TRPV1 channels, and femoral occlusion alters the MOR pharmacological profile.

exercise pressor reflex; Na\(_{\text{v}}\)1.8 promoter region; whole-cell patch clamp


OPIOID RECEPTORS ARE KNOWN TO SHARE OVERLAPPING DISTRIBUTION IN BOTH CENTRAL AND PERIPHERAL NERVOUS SYSTEMS (Goldstein and Naidi 1989; Williams et al. 2001). STIMULATION OF PERIPHERAL MU OPIOID RECEPTORS (MOR), EXPRESSED IN GROUP III AND IV AFFERENTS (Coggeshall et al. 1997), HAS BEEN REPORTED TO ATTENUATE THE EXERCISE PRESSOR REFLEX IN RATS WHOSE FEMORAL ARTERIES HAD BEEN OCCLUDED FOR 72 H (Tsuchimochi et al. 2010). ACTIVATION OF MOR LEADS TO INHIBITION OF VOLTAGE-GATED CA\(^{2+}\) CHANNELS (Ca\(_{\text{v}}\)), ACTIVATION OF G PROTEIN INWARDLY RECTIFYING K\(^{+}\) CHANNELS, AND NEGATIVE COUPLING TO ADENYL CYCLASE (Fig. 1). OPIOID PEPTIDES MEDIATE THEIR EFFECTS BY COUPLING MOR TO MEMBERS OF THE PERTUSSI TOXIN (PTX)-SENSITIVE G\(_{\text{i}}\)(i3) FAMILY OF HETEROTRIMERIC G PROTEINS (Williams et al. 2001).


MATERIALS AND METHODS

DRG NEURON LABELING, ISOLATION, AND CDNA TRANSFECTION. THE PENN STATE COLLEGE OF MEDICINE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVED THE EXPERIMENTS PERFORMED IN THIS STUDY. THE LUMBAR (L\(_{4}\)–L\(_{6}\)) DRG NEURONS WERE ISOLATED, AS DESCRIBED PREVIOUSLY (Hassan and Ruiz-Velasco 2013). BRIEFLY, ADULT MALE SPRAGUE-DAWLEY RATS...
were anesthetized initially with CO₂ and then decapitated quickly with a laboratory guillotine. The lumbar (L₄–L₆) DRG were next removed and placed in ice-cold HBSS (Sigma-Aldrich, St. Louis, MO). The dissociated neurons were next plated onto polystyrene culture dishes, coated with poly-L-lysine, and stored in a humidified atmosphere containing 5% CO₂/95% air at 35°C. Following a 3-h incubation period, the DRG neurons were microinjected with a cDNA plasmid coding for EGFP, whose expression is driven by the putative Naᵥ1.8 promoter region [a kind gift from Dr. Henry L. Puhl III, National Institute on Alcohol Abuse and Alcoholism (NIAAA), U.S. National Institutes of Health (NIH), Bethesda, MD]. The final concentration of the microinjected clone was 0.4 µg/µL. Afterward, the neurons were stored in MEM containing 10% FBS, 1% glutamine, 1% penicillin-streptomycin, ciliary-derived growth factor (15 ng/ml), nerve growth factor (15 ng/ml), and glial-derived neurotrophic factor (6 ng/ml) and incubated overnight at 35°C. Whole-cell Ca²⁺ currents were acquired from EGFP-expressing DRG neurons.

In one set of experiments, the left femoral arteries of seven rats were ligated under anesthesia, and the DRG neurons innervating the triceps surae muscle were retrogradely labeled as follows. Five days before neuron isolation, the rats were anesthetized with isoflurane (3–5%), and thereafter, 15–20 µL of 1,1’-diododecyl-3,3,3',3'-tetramethyldiocarboxylic perchlorate (DiI; 3% in DMSO) was injected into the triceps surae muscles. Three days (72 h) before neuron isolation, the rats were re-anesthetized and had their left femoral arteries ligated with 5-0 silk sutures distal to the inguinal ligament, as described previously (Prior et al. 2004; Yang et al. 2000). This procedure reduces blood flow reserve capacity lower than normal, but there is sufficient blood flow to meet metabolic needs at rest (Prior et al. 2004; Waters et al. 2004). Afterward, the wounds were closed with skin clips, and the animals were allowed to recover for 72 h before DRG isolation. Femoral artery occlusion has been reported to have no effect on normal cage activity (Taylor et al. 2008). In this set of experiments, the freely perfused hindlimbs (right) served as controls. The rats were killed 72 h later, and the DRG (L₄–L₆) were collected from the freely perfused (right; seven samples) and ligated (left; seven samples) sides. The DRG neurons were dispersed and transfected with the cDNA construct, as described above. Following overnight incubation, whole-cell Ca²⁺ currents were recorded from DiI-labeled and EGFP-expressing DRG neurons (Fig. 2, A–D).

**Ca²⁺ CHANNEL MODULATION AND THE EXERCISE PRESSOR REFLEX**

![Diagram of G protein-mediated modulation of Ca²⁺ channels following mu opioid receptor (MOR) activation](image-url)

**Fig. 1.** Model depicting the G protein-mediated modulation of Ca²⁺ channels following mu opioid receptor (MOR) activation. The binding of the receptor agonist [i.e., [µ-Ala²-N-Me-Phe⁴-Gly⁵]-enkephalin (DAMGO)] leads to MOR activation and allosteric changes in the conformation of the heterotrimeric G protein. The Ga subunit releases GDP and binds GTP. The GTP-bound Ga thereafter dissociates from the Gβγ dimer, and the latter moiety binds to Ca²⁺ channels, leading to voltage-dependent inhibition of Ca²⁺ currents [N-type Ca²⁺ channel (CaV2.2)]. The cycle is completed following the hydrolysis of GTP and reassociation of the Ga and Gβγ subunits.

**DRG transfection with small interference RNA.** The DRG tissue was transfected with small interference RNA (siRNA) using electroporation and lipofection, as described previously (Mahmoud et al. 2012). Briefly, once L₄–L₆ DRG tissue was removed, it was first electroporated with the Neon electroporator (Life Technologies, Carlsbad, CA) with three 1,000-V pulses for 20 ms duration in an electroporation solution, which consisted of R solution (Life Technologies), scrambled or Ga-targeted siRNA (3,000 nM; Life Technologies), and 2 mM 2,3-butanediole monoxide (BDM). Following the electroporation procedure, the DRG were next placed in a 22-mm dish containing Opti-MEM and Lipofectamine 2000 (both from Life Technologies), 2 mM BDM, and scrambled or Ga-targeted siRNA for 5 h and kept in a humidified incubator (same as above). After the incubation period in the lipofection solution, the DRG was rinsed three times with supplemented MEM (same as above) and also stored in MEM in a humidified incubator. This double-transfection protocol was performed a second time, 48 h after the initial transfection. The siRNA sequences used to silence Ga subunits were: Ga₉₂, 5'-UCA CUG ACG UCA UCA UAU-3'; Ga₉₅, 5'-UCA AGG AGC AUU AUC AUU-3'; and Ga₉₆, 5'-CCA UCU GCU UUC CUG AAU AUU-3'. Control DRG groups were transfected with scrambled siRNA.

**Quantitative real-time PCR assays.** To verify the efficiency of G protein knockdown, quantitative real-time PCR (QRT-PCR) analysis of Ga expression in DRG tissues was carried out. Total RNA and protein from DRG tissue were isolated with the NucleoSpin RNA/Protein Kit (Macherey-Nagel, Bethlehem, PA) and the standard protocol, according to the manufacturer. Thereafter, equal cDNA synthesis was performed using the High Capacity cDNA RT Kit (Life Technologies). For QRT-PCR experiments, the TaqMan Gene Expression assays (Life Technologies) specific for rat Ga₂, Ga₅, and GAPDH were carried out, according to the manufacturer’s instructions. The assays were run on a 7900HT PCR system (Life Technologies) and analyzed using the comparative threshold method. The results were normalized to internal GAPDH mRNA controls.

**Neuron imaging.** Phase contrast and fluorescence images were obtained with a Nikon TE2000 microscope using a 20x objective, the Photo Fluor II (89 North, Burlington, VT) for illumination, an Orca-ER-1394 digital charge-coupled device (CCD) camera (Hamamatsu Photonics, Bridgewater, NJ), and iVision software (BioVision Technologies, Exton, PA) for acquisition. The acquired images were processed and pseudocolored with iVision software.

**Electrophysiology and data analysis.** The whole-cell patch-clamp technique was used to record Ca²⁺ channel currents with an Axopatch 200B Amplifier (Molecular Devices, Sunnyvale, CA). Data acquisition was performed with custom-designed software (SS) on a Macintosh G4 computer (Apple, Cupertino, CA), written by Dr. Stephen R. Ikeda (NIAAA, NIH). Ca²⁺ currents were evoked every 10 s with the “double-pulse” voltage protocol, which consists of a holding potential of −80 mV, a test pulse to +10 mV (the prepulse), followed by a strong depolarization step to +80 mV, a brief return to −80 mV, and finally, another test pulse to +10 mV (the postpulse). The pipette solution consisted of (in mM): N-methyl-D-glucamine 80, tetraethyl ammonium hydroxide (TEA-OH) 20, CsCl 20, CsOH 40, creatine phosphate 14, HEPES 10, Mg-ATP 4, Na₂GTP 0.3, and EGTA 11. The pH was adjusted to 7.2 with CH₃SO₃H, and the osmolality was 293–302 mosmol/kgH₂O. The external solution consisted of (in mM): CH₃SO₃H 140, TEA-OH 145, HEPES 10, glucose 15, CaCl₂ 10, and TTX 0.0003. The pH was adjusted to 7.4 with TEA-OH, and the osmolality ranged from 320 to 330 mosmol/kgH₂O. The MOR agonist [µ-Ala²-N-Me-Phe⁴-Gly⁵]-enkephalin (DAMGO) and transient potential receptor vanilloid 1 (TRPV1) agonist 8-methyl-N-vanillyl-6-nonenamide (capsaicin; both from Sigma-Aldrich) were added in the external solution to their final concentrations on the day of the experiment. Stock solutions of α-conotoxin GVIA and α-agatoxin IVA (both from Alomone Labs, Jerusalem, Israel) were prepared in water and then diluted in the external solu-
tion. However, when using peptide toxins, 0.1 mg/ml cytochrome c (Sigma-Aldrich) was added to all external solutions to minimize the potential binding of the toxins to the capillary columns used for drug delivery. The external solution used to record capsaicin-induced currents consisted of (in mM): NaCl 140, KCl 5.4, HEPES 10, MgCl₂ 1, CaCl₂ 10, and glucose 10. The pH was adjusted to 7.4 with NaOH. For data and statistical analysis, IGOR Pro (WaveMetrics, Lake Oswego, OR) and Prism (GraphPad Software, San Diego, CA) were used, respectively. P < 0.05 was considered statistically significant.

Western blot assays. Protein concentrations were measured with the Qubit 2 fluorometer (Life Technologies). Protein samples (15–25 µg) were electrophoretically separated on Novex 10% Tris-glycine precast gels (Life Technologies) using 125 V at 4°C for 90 min and then transferred to polyvinylidene difluoride (PVDF; Life Technologies) or nitrocellulose (GE Healthcare, Piscataway, NJ) membranes. For MOR detection, the membranes were incubated with anti-MOR (1:8,000) rabbit MAB (Cat. No. ab134054; Abcam, Cambridge, MA) overnight (~12 h) at 4°C. For Gα subunit detection, the membranes were blocked with 7% milk in Tris-buffered saline-Tween 20 buffer overnight at 4°C. Afterward, they were incubated with anti-Gα1,-Gα2,-Gα3,-Go, and -actin. Each lane was loaded with 20–25 µg protein. Gα1 and Go were not detected in DRG tissue but were present in stellate ganglion (SG) and brain tissue, respectively. The lines/numbers to the left of the blots indicate the approximate molecular masses (kDa).
(UVP, Upland, CA), equipped with a 16-bit CCD camera and processed with VisionWorks LS software (UVP). To normalize for protein loading, the PVDF or nitrocellulose membranes were stripped with the Restore Western Blot Stripping Buffer (Thermo Scientific) and then retested with anti-actin (1:2,500–1:4,000) mouse MAB (Cat. No. ab11003; Abcam). The membranes were rinsed and incubated in HRP-conjugated anti-mouse IgG antibody (described above). The actin bands were also visualized and quantified with VisionWorks LS software (UVP). In one set of experiments (see Fig. 2E), the brain and stellate ganglion (SG) tissue were isolated (Margas et al. 2008) and used as a positive control for $\gamma_\alpha_5$ and $\gamma_\alpha_3$ protein detection, respectively.

RESULTS

$\gamma_\alpha_3$ proteins couple MOR to $\gamma_\alpha_4$,2,2 channels in EGFP-expressing DRG neurons. Our group has previously shown that MOR stimulation attenuated the exercise pressor reflex in rats whose femoral arteries were ligated for 72 h but had minimal effect on the reflex in their freely perfused counterparts (Tsushima et al. 2010). Thus in the present study, we began to probe further the signaling elements that specifically couple MOR with $Ca^{2+}$ channels (Fig. 1) and whether a 72-h femoral occlusion would alter the MOR-mediated modulation of $Ca^{2+}$ channel currents in sensory DRG neurons innervating the triceps surae muscles. The DRG neurons under study were transfected with a cDNA construct coding for EGFP (described in MATERIALS AND METHODS). Figure 2A shows phase, and Fig. 2B–D, shows fluorescence images of acutely dissociated DRG neurons from a rat in which DiI was injected into the triceps surae muscle, 5 days before cell dispersion, and microinjected with the cDNA construct, 3–5 h post-dispersion. The images in Fig. 2B and C, illustrate, respectively, four neurons expressing EGFP and five neurons effectively labeled with DiI.

In the first set of experiments, Western blotting assays were performed to determine the expression profile of PTX-sensitive $\gamma_\alpha$ proteins in $L_{4,5}$-DRG neurons. Figure 2E illustrates that $\gamma_\alpha_2$ and $\gamma_\alpha_3$ (40 kDa) are expressed in DRG tissue. On the other hand, $\gamma_\alpha_1$ and $\gamma_\alpha_5$ expression was not detected in DRG tissue. However, as positive controls, we used SG and brain tissue, since we have shown previously that the former $\gamma_\alpha$ subunit mediates the coupling of $Ca^{2+}$-2 channels with nociceptin/orphanin FQ receptors (Margas et al. 2008). The blots show that under our experimental conditions, both $\gamma_\alpha_1$ and $\gamma_\alpha_5$ were detected in SG and brain tissue.

We next used siRNA nucleotides designed to silence $\gamma_\alpha_2$ and $\gamma_\alpha_3$ proteins detected in DRG tissue and thereafter, examined the coupling specificity of MOR with $Ca^{2+}$ channels. Figure 3A summarizes the QRT-PCR results, 96 h post-siRNA transfection. It can be observed that mRNA levels for each targeted $\gamma_\alpha$ subunit were lower compared with DRG tissue transfected with scrambled siRNA. Furthermore, Fig. 3B shows Western blots used to measure $\gamma_\alpha$ protein levels in DRG tissue transfected with scrambled and $\gamma_{\alpha}$-targeted siRNA. Both $\gamma_\alpha_3$ and $\gamma_\alpha_5$ protein levels were lower than those in DRG tissue transfected with scrambled siRNA.

The functional coupling of MOR and $Ca^{2+}$ channels was examined next in DRG neurons isolated from DRG tissue that had been transfected with specific $\gamma_\alpha_2$ or $\gamma_\alpha_3$ siRNA and also transfected with the cDNA construct containing the $Na_{\alpha}_{1,8}$ promoter region (described in MATERIALS AND METHODS). It should be noted that in all control electrophysiological experiments performed, coupling of MOR with $Ca^{2+}$ channels was observed in ~90% of neurons tested. Figure 4A shows the time course of the peak $Ca^{2+}$ current amplitude of pre- and postpulse currents acquired before (traces a and b and e and f; Fig. 4B) and during (traces c and d and g and h; Fig. 4B) application of the high-affinity MOR agonist DAMGO (10 $\mu$M) and ATP (10 $\mu$M) in a DRG neuron transfected with scrambled siRNA. The $Ca^{2+}$ channel currents were evoked every 10 s with the double-pulse voltage protocol (shown in Fig. 4B), and the peak current amplitude was measured isochronally, 10 ms after the start of the pre- and postpulse. The superimposed $Ca^{2+}$ currents shown in Fig. 4B correspond to those plotted in Fig. 4A. Application of DAMGO resulted in a 70% inhibition of the prepulse current (trace c). After a recovery period, exposure of the neuron to ATP led to a 66% inhibition of the prepulse current (trace e). It can be seen that the current inhibition was greater during the prepulse (traces c and g) than the postpulse (traces d and h) for both DAMGO and ATP. This is indicative of a voltage-dependent inhibition of the current and also characterized by a “kinetic slowing” of the prepulse current and an enhanced postpulse current (Ikeda 1991). The time course shown in Fig. 4C was recorded from a DRG neuron transfected with $\gamma_{\alpha}3$ siRNA, and the corresponding numbered current traces are shown in Fig. 4D. The neuron was exposed initially to DAMGO, and the $Ca^{2+}$ current was blocked by ~17% (trace c). As a positive control, ATP was applied next (trace g), and $Ca^{2+}$ currents were inhibited by ~55%. For both agonists, the currents were also inhibited in a voltage-dependent manner, although to a lesser extent in the presence of DAMGO. Figure 4E is a summary scatter plot showing the mean (±SE) DAMGO- and ATP-mediated $Ca^{2+}$ current inhibition in DRG neurons transfected with scrambled or $\gamma_\alpha_3$ siRNA. The plot indicates that the silencing of $\gamma_\alpha_3$ subunits led to a significant ($P < 0.05$) decrease in coupling between MOR and $Ca^{2+}$ channels, whereas the ATP (i.e., purinergic/P2Y) modulation of $Ca^{2+}$ channels was not overtly affected ($P = 0.54$). Thus in this set of DRG neurons, P2Y G protein-coupled receptors do not use $\gamma_\alpha_2$ proteins. The ATP-mediated modulation of $Ca^{2+}$ currents is similar to that reported previously in this subset of DRG neurons (Ramachandra et al. 2013).

The effect of silencing $\gamma_\alpha_2$ in DRG neurons was examined next. Figure 5A shows the time course of peak current amplitude of an EGFP-expressing DRG neuron, transfected with scrambled siRNA, before and during DAMGO (10 $\mu$M) application. The superimposed current traces (traces a–d) are also shown (Fig. 5B). Application of DAMGO resulted in a 50% block of the $Ca^{2+}$ currents that was also voltage dependent. Similarly, the time course in Fig. 5C illustrates that exposure to DAMGO of a $\gamma_\alpha_3$-silenced and EGFP-expressing neuron led to voltage-dependent inhibition of $Ca^{2+}$ currents. The superimposed traces (a–d) are shown in Fig. 5D. The scatter plot shown in Fig. 5E is a summary of the DAMGO-mediated $Ca^{2+}$ current inhibition in EGFP-expressing neurons transfected with scrambled or $\gamma_\alpha_2$ siRNA. No significant difference in current inhibition was found between both groups of neurons. These results indicate that unlike $\gamma_\alpha_2$ subunits, $\gamma_\alpha_3$ proteins are essential for coupling of MOR with $Ca^{2+}$ channels in this neuron subtype.
**Fig. 3.** Detection of Goα2 and Goα3 subunits by quantitative real-time PCR (QRT-PCR) and Western blot analysis in DRG, 96 h post-small-interference RNA (siRNA) transfection. A: a plot of the quantitative assessment of Goα2 and Goα3 mRNA expression by QRT-PCR in DRG tissue, 96 h post-siRNA transfection. The changes in comparative threshold values were determined for all groups, and the specific Go mRNA levels were measured relative to GAPDH mRNA levels. The mRNA levels are expressed as fold change compared with DRG tissue that was transfected with scrambled (Scr) siRNA. The numbers in parentheses indicate the number of rats used for each Go subunit knockdown experiment.

**Fig. 6D.** The neuron was exposed initially to 0.03 μM DAMGO, and the Ca2+ current was blocked by ~28% (trace c). Similarly, application of 3 μM DAMGO resulted in a 60% inhibition (trace g). For both DAMGO concentrations, the currents were also inhibited in a voltage-dependent manner.

**Femoral occlusion alters the MOR pharmacological profile of DRG neurons.** Given that the exercise pressor reflex was attenuated following MOR stimulation in rats with ligated femoral arteries (Tsuchimochi et al. 2010), we next examined the effect of 72-h femoral arterial ligation on the concentration-response relationship for DAMGO in DiI-labeled and EGFP-expressing DRG neurons. Figure 5A shows the time course of the peak Ca2+ current amplitude of pre- and postpulse currents acquired before (traces a and b and e and f; Fig. 6B) and during (traces c and d and g and h; Fig. 6B) application of 0.03 and 3 μM DAMGO on a DRG neuron from a rat with a freely perfused leg. The Ca2+ channel currents were evoked every 10 s with the double-pulse voltage protocol (shown in Fig. 4B), and the peak current amplitude was measured as described above. The superimposed Ca2+ currents shown in Fig. 6B correspond to those plotted in Fig. 6A. Application of 0.03 μM DAMGO resulted in a 5% inhibition of the prepulse current (trace c). After a recovery period, exposure of the neuron to 3 μM DAMGO led to a 25% inhibition of the prepulse current (trace g). The time course shown in Fig. 6C was recorded from a DRG neuron isolated from a rat with a ligated femoral artery, and the corresponding numbered current traces are shown in Fig. 6D.
neurons at the end of each experiment for both groups. Thereafter, capsaicin-induced currents were recorded while maintaining the holding potential at −80 mV. From a total of 52 DRG neurons tested, capsaicin-induced currents were observed in all neurons tested. Figure 6A shows a capsaicin (1 μM)-activated inward current of a DRG neuron isolated from a rat with a freely perfused artery. This finding suggests that DRG neurons innervating the triceps surae muscle coexpress TRPV1 channels, MOR, and presumably NaV1.8 channels.

The arterial occlusion-induced leftward shift of the DAMGO concentration-response curve suggested that upregulation of one or more of the signaling proteins involved in the MOR: Ca²⁺ channel signaling pathway occurred. The enhanced coupling of MOR with Ca²⁺ channels could have resulted from an overexpression of these channels. Figure 8A shows a scatter plot of the Ca²⁺ current density of all DRG neurons tested for both groups. A statistical comparison of both groups revealed no significant difference (P = 0.60). In addition, the scatter plot of the cell capacitance (Fig. 8B) indicates that Ca²⁺ currents were obtained from DRG neurons that were comparable in size and not significantly (P = 0.58) different. Another possibility for the leftward shift may have been a change in the fraction of Ca²⁺ channel subtypes in DRG neurons from ligated rats. In a previous report (Ramachandra et al. 2013), we showed that N- and P/Q-type Ca²⁺ channels account for ∼50% and 20%, respectively, of the total Ca²⁺ current in this neuron subtype. Thus in the next set of experiments, peak Ca²⁺ currents were recorded from DiI-labeled and EGFP-expressing DRG neurons before and after application of ω-conotoxin GVIA (10 μM; N-type channel blocker) and ω-agatoxin IVA (0.2 μM; P/Q-type channel blocker). Figure 8C is a summary plot illustrating the mean (±SE) peak Ca²⁺ current block (%) mediated by both toxins in both groups of neurons. Similar to our previous observations, both N- and P/Q-type Ca²⁺ channel subtypes contributed >60% of the total Ca²⁺ current. In addition, the results show that there was no significant change.
in either contribution of N-type (P = 0.74) or P/Q-type (P = 0.33) channel subtypes between both sets of DRG neurons. Therefore, the change of the DAMGO pharmacological profile following arterial occlusion did not result in either a higher expression of Ca\textsuperscript{2+} channels or alteration in the proportion of N- and P/Q-type Ca\textsuperscript{2+} channels.

In the final set of experiments, Western blotting assays were used to determine whether MOR or G\textsubscript{i3} expression was altered in DRG neurons from occluded arteries and could explain the leftward shift of the DAMGO concentration curve. Thus seven rats had their left femoral arteries ligated for 72 h, and then, the DRG tissue was isolated. The DRG from freely perfused legs served as controls. We have shown recently that the pressor and cardioaccelerator responses to static contraction in sham-operated rats are not significantly different than those from rats with freely perfused sides (Copp et al. 2014). Figure 9A shows the Western blots used for detecting MOR and G\textsubscript{i3} expression. Numbers in parentheses indicate the number of neurons tested.

DISCUSSION

The afferent limb of the exercise pressor reflex is comprised of group III and IV muscle afferents that are activated by metabolic and mechanical stimuli originating from the contracting muscle. In the present study, we began to identify the signal transduction elements that couple MOR and Ca\textsuperscript{2+} channels in a defined subpopulation of DRG neurons involved in this reflex. We focused our attention on acutely isolated, DiI-labeled DRG neurons innervating the triceps surae muscle and microinjected with cDNA coding for EGFP whose expression is driven by the TTX-resistant Na\textsubscript{v1.8} promoter region. Our results indicate that in DRG tissue, the G\textsubscript{i3} PTX-sensitive Ga\textsubscript{i3} and Ga\textsubscript{i3} proteins are natively expressed, noted, however, that the relative levels of MOR to actin were lower than the relative levels of Ga\textsubscript{i3} to actin. Therefore, the enhanced responses of the DRG from animals with occluded arteries are likely a result of changes in other signaling proteins (discussed below).
whereas both $\gamma_i1$ and $\gamma_i2$ were not detected. Unlike our results, one report has shown that in rat DRG, all PTX-sensitive $\gamma_o$ proteins are natively expressed (Hall et al. 2001). In regard to that study, it should be noted that an antibody common to both $\gamma_i1$ and $\gamma_i2$ was used. It is possible that only detection of $\gamma_i2$ by the antibody occurred. Alternatively, the Western blot assays were performed with both lumbar and thoracic DRG tissue, whereas in the present study, we focused on L3-L6 DRG. Therefore, it is possible that the use of different neuronal tissue may help explain this discrepancy.

Our finding that $\gamma_o$ subunits are not natively expressed in DRG neurons should be noted. In the present study, we used two different antibodies. The first was a mouse monoclonal raised against both $\gamma_o$ splice variants, and the second was a rabbit polyclonal raised against residues surrounding arginine 15 of human $\gamma_o$. The latter antibody produced inconsistent results—from absent to scarcely visible. Since this G protein subunit is highly expressed in the brain (Sternweis and Robishaw 1984), we used brain tissue as a positive control and obtained successful detection (Fig. 2). Nevertheless, some reports have shown that $\gamma_o$ subunits are used by MOR and couple to $\cal{C}a^{2+}$ channels. For example, two studies reported that dialyzing antibodies specific for $\gamma_o$ and $\gamma_i1-3$ in the patch pipette removed the MOR (Moises et al. 1994) and kappa opioid receptor (Wiley et al. 1997)-mediated $\cal{C}a^{2+}$ current inhibition in rat DRG neurons that were only exposed to $\gamma_o$ antibodies. The latter report showed expression of $\gamma_o$ subunits in thoracic DRG. To eliminate the possibility of $\gamma_o$ involvement in this pathway, we compared coupling of MOR with $\cal{C}a^{2+}$ channels in scrambled- and $\gamma_o$ siRNA-transfected DRG neurons. The DAMGO-mediated $\cal{C}a^{2+}$ current inhibition was not significantly different ($P = 0.72$) for both groups of neurons ($59.9 \pm 3.8\%$, $n = 13$, scrambled vs. $57.7 \pm 5.1\%$, $n = 9$, $\gamma_o$ siRNA; data not shown). Thus it appears that the discrepant results may result from the DRG population studied (i.e., thoracic) and/or the assays used.

The results of our experiments reveal that following $\gamma_i3$ subunit knockdown, the coupling efficiency between DAMGO-stimulated MOR and $\cal{C}a^{2+}$ channels was significantly attenuated, whereas $\gamma_i2$ silencing was without effect. However, studies in the Garzón laboratory (Sánchez-Blázquez et al. 1999, 2001) found that the silencing of individual $\gamma_i$ subunits via intracerebroventricular injection of antisense oligodeoxynucleotides led to a selective loss of opioid agonist-mediated supraspinal antinociception. For example, $\gamma_i2$, $\gamma_i3$, or $\gamma_{i/1}$ knockdown significantly attenuated the DAMGO-mediated antinociception in the tail-flick assay. On the other
hand, morphine’s antinociception was diminished following Go13 and Go23 knockdown. The observations with the tail-flick assays by Garzón and colleagues (Sánchez-Blázquez et al. 1999, 2001) suggest that the Go proteins play a number of key roles that modulate neurotransmission along nociceptive signaling pathways. The differences observed between opioid agonists and signaling moieties used have given rise to the concept of “functional selectivity” or “biased agonism” [for review, see Raehal et al. (2011) and Williams et al. (2013)].

The term is used to explain how different agonists are capable of activating different (“preferential”) signaling pathways over others to produce a different response. Nevertheless, our results indicate that DRG neurons, which innervate triceps surae muscles and express EGFP (under control of the putative NaV1.8 promoter), primarily use Go13 proteins to couple MOR with Ca2+ channels.

Intermittent claudication is a typical symptom associated with PAD that encompasses leg pain during exercise. After identifying Go13 as a key component involved in opioid modulation of Ca2+ currents, we examined the effects of arterial occlusion on this transduction pathway. Our results indicate that arterial occlusion led to an alteration of the DAMGO pharmacological profile (i.e., potency and efficacy; Fig. 7). The IC50 value that we determined for DRG neurons from the freely perfused group was comparable with those reported previously in DRG (Walwyn et al. 2009) and trigeminal ganglion neurons (Borgland et al. 2001). We previously observed in rats that DAMGO exerted a significantly greater inhibitory effect on the exercise pressor reflex in limbs with ligated arteries than freely perfused limbs (Tsuchimoto et al. 2010). Thus coupled to the findings of the present study, the leftward shift of the DAMGO concentration-response curve (greater potency) suggests that it is possible that patients with PAD would require a lower effective opiate dose to reduce the exercise-induced sympathetic response than matched controls without claudication.

We investigated whether the shift of the DAMGO potency and efficacy following occlusion was a result from changes in Ca2+ channel, Go13, or MOR expression levels. Arterial occlusion has been shown to lead to an augmented, sympathetic response of the ischemic leg, which appears to result from an upregulation of acid-sensing ion channel 3 (Liu et al. 2010). Therefore, we hypothesized that the shift in DAMGO pharmacology could be mediated by an upregulation in Ca2+ channel expression. In this DRG neuron subpopulation, we reported recently that N-type Ca2+ channels (CaV2.2) carry the majority of the Ca2+ current (Ramachandra et al. 2013). Based on the current density measurements, no difference in Ca2+ current density between freely perfused and ligated animals was observed. We also explored the possibility that changes in Ca2+ channel subtype (i.e., a switch in preferential coupling of MOR with N- and P/Q-type) would explain the pharmacological shift. However, when both groups of DRG neurons were exposed to specific Ca2+ channel blockers, the contribution of N- and P/Q-type Ca2+ channels to the entire Ca2+ current was unchanged. Therefore, the absence of an increase in Ca2+ channel expression or shift in channel subtype expression could not explain the augmented DAMGO response.

Previous studies that have examined Ca2+ channel modulation by DAMGO in sensory neurons have sorted the cells based on size, lectin binding, and presence or absence of T-type Ca2+ channels. The capacitance values, a measure of cell size, measured in the present study for both group of neurons, were not significantly different. Furthermore, a previous study suggested that there was no clear correlation between cell body size and afferent fiber type (i.e., groups III and IV) (Hoheisel and Mense 1987). Our findings in the present study are also consistent with this report and suggest that occlusion did not affect the neurons’ size. However, in guinea pig DRG neurons, substance P immunoreactivity was observed primarily in
small-diameter neurons (Lawson et al. 1997). On the other hand, not all nociceptive neurons were substance P immuno-reactive.

Our Western blotting analysis of DRG tissue provided evidence that neither MOR nor G\(_{\alpha_3}\) expression levels increased significantly as a result of arterial occlusion. Limited information is available regarding the effects of femoral occlusion on MOR or G protein function. However, one recent study demonstrated that the DAMGO-mediated stimulation of MOR, expressed in group III and IV afferents, attenuated the femoral occlusion-mediated, augmented exercise pressor reflex (Tsuchimochi et al. 2010). Additionally, in cats, MOR activation lessened the increases in heart rate and blood pressure during static muscle contraction and passive stretch (Hill and Kaufman 1990; Meintjes et al. 1995). These reports emphasize a critical role played by MOR in regulating exercise pressor reflex. Nevertheless, the small changes in MOR and G\(_{\alpha_3}\) expression levels that we obtained are difficult to reconcile with the significant shift with DAMGO. One possible explanation is that the Western blotting assays were performed with the entire tissue, which includes both glia and a mixture of sensory neurons. The electrophysiological experiments were conducted on a defined subset of neurons. Thus the contribution of each neuron type to the Western blot assay cannot be determined. An alternative explanation is that another signaling protein may have changed as a result of occlusion. One likely candidate(s) is known as regulators of G protein signaling (RGS), a family with 20 members (Sjogren 2011). For instance, RGS4 protein overexpression has been recently reported to suppress the efficacy of the muscarinic receptor agonist pilocarpine in an electrophysiological assay (Chen et al. 2014). Furthermore, expression of RGS2 has been reported to be upregulated in a model of ischemia (Endale et al. 2010). Further studies are needed to determine which RGS protein family member is affected by femoral occlusion.

The TRPV1 is a ligand-gated, nonselective cation channel. TRPV1 channels are mainly expressed in small nociceptive DRG neurons (Greffrath et al. 2003) and have been demon-

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Fig. 9. A: detection of MOR and G\(_{\alpha_3}\) expression by Western blot analysis in DRG tissue isolated from rats with freely perfused (FP) and ligated (Lig) femoral arteries. The MOR and G\(_{\alpha_3}\) lanes were loaded with 25 and 15 \(\mu\)g protein, respectively. The blots show the results for anti-MOR, -G\(_{\alpha_3}\), and -actin (loading control) from 4 rats. The lines/numbers to the left of the blots indicate the approximate molecular weight (kDa), and the arrow to the right of the MOR blot points to the approximate molecular weight of MOR (see text for details). B: summary plot illustrates the analysis of the blots for MOR, G\(_{\alpha_3}\), and actin, which was performed by measuring the area density (i.e., intensity) and then plotting the ratio of either the MOR or G\(_{\alpha_3}\) subunit to its respective actin value. The values represent the means (±SE).
strated to mark group IV afferent fibers (Michael and Priestley 1999). In this study, we also observed that all DRG neurons exposed to capsaicin (1–10 μM) exhibited an inward current. This suggests that the Dil-labeled and EGFP-expressing neurons studied are likely to be involved in pain perception. In summary, we have shown that we can successfully isolate and study signaling events of a defined subpopulation of DRG neurons that innervate the triceps surae and likely express the Na\(_{v}\)1.8 channel. With the use of an siRNA approach, we determined that MOR and Ca\(^{2+}\) channels are coupled specifically by PTX-sensitive Go\(_{i3}\) proteins. Furthermore, hindlimb muscle ischemia significantly enhanced the DAMGO-mediated Ca\(^{2+}\) current inhibition in this neuron subset. The altered DAMGO pharmacology did not result from increases in either MOR or Go\(_{i3}\) expression levels or in significant changes in Ca\(^{2+}\) current density or channel subtype expression.

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DISCLOSURES

The authors declare no potential conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

Author contributions: M.F., M.P.K., and V.R-V. conceived and design of research; B.H., J.S.K., M.F., and V.R-V. performed experiments; B.H., J.S.K., M.F., and V.R-V. analyzed data; M.F. and V.R-V. interpreted results of research; B.H., J.S.K., M.F., and V.R-V. performed experiments; B.H., J.S.K., M.F., and V.R-V. drafted manuscript; B.H., J.S.K., M.F., M.P.K., and V.R-V. revised and approved manuscript.

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