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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EXERCISE LEADS TO THE STIMULATION of both cardiovascular and respiratory functions, a response that is mediated by a reflex arising from contracting muscles. The afferent arm of the reflex, named the exercise pressor reflex (Mitchell et al. 1983), is comprised of group III and IV muscle afferents (Coote et al. 1971; Kaufman et al. 1983; McCloskey and Mitchell 1972). Under certain pathological conditions, such as peripheral arterial disease (PAD), the skeletal muscle has a limited blood supply. In patients with PAD, blood flow meets the metabolic needs under resting conditions but fails to do so during exercise and results in reports of pain, which in turn, have been termed intermittent claudication. Furthermore, in patients with PAD, the exercise pressor reflex is greater than that evoked in their healthy counterparts (Bakke et al. 2007).

Opioid receptors are known to share overlapping distribution in both central and peripheral nervous systems (Goldstein and Naidu 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 adenylyl cyclase (Fig. 1). Opioid peptides mediate their effects by coupling MOR to members of the pertussis toxin (PTX)-sensitive Go\(_{\alpha}\)i3 family of heterotrimeric G proteins (Williams et al. 2001).

Voltage-gated Na\(^{+}\) channels (Na\(_{\text{v}}\)) also mediate the action potential in group III and IV fibers, which can be subdivided into TTX sensitive and TTX resistant (Rush et al. 2007). Group III afferents express only TTX-sensitive Na\(^{+}\) channels, whereas group IV afferents express TTX-resistant and possibly TTX-sensitive Na\(^{+}\) channels. The TTX-resistant Na\(_{\text{v}}\) channels are involved in pain transmission and are expressed primarily in small and medium neurons within dorsal root ganglia (DRG) and cranial sensory ganglia (Akopian et al. 1996; Novakovic et al. 1998; Sangamaswaran et al. 1996). A recent report identified the putative promoter region of the Na\(_{\text{v}}\) channel in DRG neurons (Puhl and Ikeda 2008). In that study, the enhanced green fluorescent protein (EGFP) was used as the reporter construct for this region (~4 kb), making it a valuable tool to study sensory neurons that transmit pain signals. Thus the purpose of the present study was twofold: first, we wanted to identify the specific PTX-sensitive Go\(_{\alpha}\)i3 subunit that mediates the functional coupling of MOR and Ca\(^{2+}\) channels in acutely isolated DRG neurons expressing EGFP (whose expression was driven by the Na\(_{\text{v}}\) promoter region); second, we examined the effect of 72-h femoral ligation on the signaling elements involved in the MOR-mediated modulation of Ca\(^{2+}\) channel currents of EGFP-expressing DRG neurons. This animal model simulates the blood flow patterns found in the legs of patients with PAD (Waters et al. 2004).
Ca²⁺ CHANNEL MODULATION AND THE EXERCISE PRESSOR REFLEX

Fig. 1. Model depicting the G protein-mediated modulation of Ca²⁺ channels following mu opioid receptor (MOR) activation. The binding of the receptor agonist \(\text{[\text{D-Ala}2-\text{N-Me-Phe}4-\text{Gly}5\text{-ol}5]}\)-enkephalin (DAMGO) leads to MOR activation and allosteric changes in the conformation of the heterotrimeric G protein. The Go subunit releases GDP and binds GTP. The GTP-bound Go thereafter dissociates from the Gβγ dimer, and the latter moieties 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.

were anesthetized initially with CO₂ and then decapitated quickly with a laboratory guillotine. The lumbar (L₁–L₆) DRG were then removed and placed in ice-cold HBSS (Sigma-Aldrich, St. Louis, MO). The dissociated neurons were then 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 Naᵥα₁.8 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% agonist \(\text{[\text{D-Ala}2-\text{N-Me-Phe}4-\text{Gly}5\text{-ol}5]}\)-enkephalin (DAMGO) leads to following mu opioid receptor (MOR) activation. The binding of the receptor

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, CaCl₂ 1, Mg-ATP 4, Na₃GTP 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.

MOR agonist \(\text{[\text{D-Ala}2-\text{N-Me-Phe}4-\text{Gly}5\text{-ol}5]}\)-enkephalin (DAMGO) and transient potential receptor vanilloid 1 (TRPV1) agonist 8-methyl-N-vanillyl-6-nonenamide (capsaicin; 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 \( \mu \)g) 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-\( \alpha \),-Gα1, -Gα2, -Gα3, -GαO, and -actin. Each lane was loaded with 20–25 \( \mu \)g protein. Gα1 and GαO 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).

E PTX-sensitive Gα Expression Profile

Fig. 2. Fluorescence imaging of retrograde-labeled and enhanced green fluorescent protein (EGFP) reporter-microinjected cDNA in rat dorsal root ganglia (DRG) neurons and detection of pertussis toxin (PTX)-sensitive Gα subunit expression in DRG tissue. Phase (A) and fluorescence (B–D) images of acutely isolated DRG neurons, 5 days post-1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) injection in the triceps surae muscles and ~18 h post-cDNA transfection. The neurons were imaged at 20×, with a filter set containing an excitation filter at 480 nm and an emission filter at 535 nm (for EGFP, B) and an excitation filter at 540 nm and an emission filter at 585 nm (for DiI, C). D: images represent color-joined images from B and C. The images were pseudocolored; scale bars represent 60 μm. The arrows (A) point to DiI-labeled and EGFP-expressing neurons. E: expression of Gα subunits in DRG tissue. Western blot assays showing the natively expressed Gα subunits. The blots used anti-Gα1, -Gα2, -Gα3, -GαO, and -actin. Each lane was loaded with 20–25 \( \mu \)g protein. Gα1 and GαO 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 Gαi1 and Gαo protein detection, respectively.

RESULTS

Gαi3 proteins couple MOR to Ca2+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 (Tsuchimochi et al. 2010). Thus in the present study, we began to probe further the signaling elements that specifically couple MOR with Ca2+ channels (Fig. 1) and whether a 72-h femoral occlusion would alter the MOR-mediated modulation of Ca2+ 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. 2, B–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 neurons from a rat in which DiI was injected into the triceps, shows fluorescence images of acutely dissociated DRG in MATERIALS AND METHODS). Figure 2 illustrates that Gαi2 and Gαi3 (~40 kDa) are expressed in DRG tissue. On the other hand, Gαi1 and Gαi3 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 Gα subunit mediates the coupling of Ca2+ channels with nociceptin/orphanin FQ receptors (Margas et al. 2008). The plots show that under our experimental conditions, both Gαi1 and Gαi3 expression was not detected in DRG tissue and brain tissue.

We next used sRNA nucleotides designed to silence Gαi2 and Gαi3 proteins detected in DRG tissue and thereafter, examined the coupling specificity of MOR with Ca2+ channels. Figure 3A summarizes the QRT-PCR results, 96 h post-sRNA transfection. It can be observed that mRNA levels for each targeted Gα subunit were lower compared with DRG tissue transfected with scrambled sRNA. Furthermore, Fig. 3B shows Western blots used to measure Gα protein levels in DRG tissue transfected with scrambled and Gα-targeted sRNA. Both Gαi3 and Gαi3 protein levels were lower than those in DRG tissue transfected with scrambled sRNA.

The functional coupling of MOR and Ca2+ channels was examined next in DRG neurons isolated from DRG tissue that had been transfected with specific Gαi2 or Gαi3 sRNA and also transfected with the cDNA construct containing the NaV1.8 promoter region (described in MATERIALS AND METHODS). It should be noted that in all control electrophysiological experiments performed, coupling of MOR with Ca2+ channels was observed in ~90% of neurons tested. Figure 4A 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. 4B) and during (traces c and d and g and h; Fig. 4B) application of the high-affinity MOR agonist DAMGO (10 μM) and ATP (10 μM) in a DRG neuron transfected with scrambled sRNA. 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 isochronally, 10 ms after the start of the pre- and postpulse. The superimposed Ca2+ 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 c). 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 Gαi3 sRNA, and the corresponding numbered current traces are shown in Fig. 4D. The neuron was exposed initially to DAMGO, and the Ca2+ current was blocked by ~17% (trace c). As a positive control, ATP was applied next (trace g), and Ca2+ 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 Ca2+ current inhibition in DRG neurons transfected with scrambled or Gαi3 sRNA. The plot indicates that the silencing of Gαi3 subunits led to a significant (P < 0.05) decrease in coupling between MOR and Ca2+ channels, whereas the ATP (i.e., purinergic/P2Y) modulation of Ca2+ channels was not overtly affected (P = 0.54). Thus in this set of DRG neurons, P2Y G protein-coupled receptors do not use Gαi2 proteins. The ATP-mediated modulation of Ca2+ currents is similar to that reported previously in this subset of DRG neurons (Ramachandra et al. 2013).

The effect of silencing Gαi3 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 sRNA, before and during DAMGO (10 μM) application. The superimposed current traces (traces a–d) are also shown (Fig. 5B). Application of DAMGO resulted in a 50% block of the Ca2+ currents that was also voltage dependent. Similarly, the time course in Fig. 5C illustrates that exposure to DAMGO of a Gαi2-silenced and EGFP-expressing neuron led to voltage-dependent inhibition of Ca2+ currents. The superimposed traces (a–d) are shown in Fig. 5D. The scatter plot shown in Fig. 5E is a summary of the DAMG-mediated Ca2+ current inhibition in EGFP-expressing neurons transfected with scrambled or Gαi2 sRNA. No significant difference in current inhibition was found between both groups of neurons. These results indicate that unlike Gαi2 subunits, Gαi3 proteins are essential for coupling of MOR with Ca2+ channels in this neuron subtype.
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 Dil-labeled and EGFP-expressing DRG neurons. Figure 5A 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. 6B) and during (traces c and d and g and h; Fig. 6B) application of 0.03 and 3 \(\mu\)M DAMGO on a DRG neuron from a rat with a freely perfused leg. 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 as described above. The superimposed Ca\(^{2+}\) currents shown in Fig. 6B correspond to those plotted in Fig. 6A. Application of 0.03 \(\mu\)M DAMGO resulted in a 5% inhibition of the prepulse current (trace c). After a recovery period, exposure of the neuron to 3 \(\mu\)M 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. The neuron was exposed initially to 0.03 \(\mu\)M DAMGO, and the Ca\(^{2+}\) current was blocked by \(\sim28\%\) (trace c). Similarly, application of 3 \(\mu\)M DAMGO resulted in a 60% inhibition (trace g). For both DAMGO concentrations, the currents were also inhibited in a voltage-dependent manner.

The DAMGO concentration-response curves for freely perfused and ligated groups are illustrated in Fig. 7. The pooled data points for both groups were fit to the Hill equation: 

\[
I = \frac{I_{\text{MAX}}}{1 + (\text{IC}_{50}/[\text{ligand}])^{nH}},
\]

where \(I\) is the percent inhibition, \(I_{\text{MAX}}\) is the maximum current inhibition, \(\text{IC}_{50}\) is the half-inhibition concentration, [ligand] is the agonist concentration, and \(nH\) is the Hill coefficient. The mean (±SE) \(I_{\text{MAX}}\) and \(\text{IC}_{50}\) (nM) values for the freely perfused and occluded groups were 47.4 ± 3.7 and 66.2 ± 3.9 and 283 and 146, respectively. The fits for both groups showed that they were significantly (\(P = 0.0007\)) different from each other. These results indicate that the 72-h femoral occlusion altered morphine’s pharmacological profile, leading to a greater potency and efficacy of Dil-labeled and EGFP-expressing neurons.

During the acquisition of the DAMGO concentration-response curves, the TRPV1 agonist, capsaicin (1 or 10 \(\mu\)M), was applied to some (52 of 73 total neurons) of the DRG
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: Ca\(^{2+}\) channel signaling pathway occurred. The enhanced coupling of MOR with Ca\(^{2+}\) channels could have resulted from an overexpression of these channels. Figure 8A shows a scatter plot of the Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) channel subtypes in DRG neurons from ligated rats. In a previous report (Ramachandra et al. 2013), we showed that N- and P/Q-type Ca\(^{2+}\) channels account for 50% and 20%, respectively, of the total Ca\(^{2+}\) current in this neuron subtype. Thus in the next set of experiments, peak Ca\(^{2+}\) currents were recorded from DiI-labeled and EGFP-expressing DRG neurons before and after application of \(-\text{conotoxin GVIA} (10 \mu\text{M}; \text{N-type channel blocker}) and \text{agatoxin IVA} (0.2 \mu\text{M}; \text{P/Q-type channel blocker}). Figure 8C is a summary plot illustrating the mean (±SE) peak Ca\(^{2+}\) current block (%) mediated by both toxins in both groups of neurons. Similar to our previous observations, both N- and P/Q-type Ca\(^{2+}\) channel subtypes contributed >60% of the total Ca\(^{2+}\) 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$^{2+}$/H11001 channels or alteration in the proportion of N- and P/Q-type Ca$^{2+}$/H11001 channels.

In the final set of experiments, Western blotting assays were used to determine whether MOR or G$i_i$/H9251 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. 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$i_i$/H9251 levels from four rats. The results summarized in Fig. 9B indicated that occlusion of the femoral arteries did not lead to significant changes in MOR or G$i_i$/H9251 levels. It should be noted, however, that the relative levels of MOR to actin were lower than the relative levels of G$i_i$/H9251 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).

**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$^{2+}$/H11001 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$^+$ channel promoter region. Our results indicate that in DRG tissue, the G$i_i$/H9251 PTX-sensitive G$i_i$/H9251 and G$i_i$/H9251 proteins are natively expressed.
Fig. 7. DAMGO concentration-response relationships of DRG neurons from rats with freely perfused (A) and 72 h-ligated (B) femoral arteries (see Fig. 6 legend for details). Each data point represents the mean (SEM) prepulse Ca2+ current inhibition; the numbers in parentheses indicate the number of neurons that was tested. The smooth curves were obtained by fitting the data points to the Hill equation, and the calculated IC50 values are shown in the figure key.

Fig. 6. Time courses of Ca2+ current amplitude for prepulse and postpulse acquired from the sequential application of 0.03 and 3 μM DAMGO in acutely isolated DRG neurons from rats with freely perfused (A) and 72 h-ligated femoral arteries (C). The currents were obtained from DiI-labeled and EGFP-expressing neurons and were evoked every 10 s with the double-pulse voltage paradigm (4B, top). A and C: the lowercase letters represent current traces shown to the right (B and D, respectively). A, inset: an 8-methyl-N-vanillyl-6-nonenamide [capsaicin (cap); 1 μM]-induced current, where the neuron was held at −80 mV, and capsaicin was applied for 30 s (denoted by filled bar).

whereas both Goαi and Goαi were not detected. Unlike our results, one report has shown that in rat DRG, all PTX-sensitive Go proteins are natively expressed (Hall et al. 2001). In regard to that study, it should be noted that an antibody raised against both Goαi and Goαi was used. It is possible that only detection of Goαi 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 L4–L6 DRG. Therefore, it is possible that the use of different neuronal tissue may help explain this discrepancy.

Our finding that Goαi 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 Goαi splice variants, and the second was a rabbit polyclonal raised against residues surrounding arginine 15 of human Goαi. 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 Goαi subunits are used by MOR and couple to Ca2+ channels. For example, two studies reported that dialyzing antibodies specific for Goαi and Goαi in the patch pipette removed the MOR (Moises et al. 1994) and kappa opioid receptor (Wiley et al. 1997)-mediated Ca2+ current inhibition in rat DRG neurons that were only exposed to Goαi antibodies. The latter report showed expression of Goαi subunits in thoracic DRG. To eliminate the possibility of Goαi involvement in this pathway, we compared coupling of MOR with Ca2+ channels in scrambled- and Goαi siRNA-transfected DRG neurons. The DAMGO-mediated Ca2+ current inhibition was not significantly different (P = 0.72) for both groups of neurons (59.9 ± 3.8%, n = 13, scrambled vs. 57.7 ± 5.1%, n = 9, Goαi 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 Goαi subunit knockdown, the coupling efficiency between DAMGO-stimulated MOR and Ca2+ channels was significantly attenuated, whereas Goαi silencing was without effect. However, studies in the Garzón laboratory (Sánchez-Blázquez et al. 1999, 2001) found that the silencing of individual Go subunits via intracerebroventricular injection of antisense oligodeoxynucleotides led to a selective loss of opioid agonist-mediated supraspinal antinociception. For example, Goαi, Goαi, or Goαi knockdown significantly attenuated the DAMGO-mediated antinociception in the tail-flick assay. On the other
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 Na\textsubscript{v}1.8 promoter), primarily use Go\textsubscript{a3} proteins to couple MOR with Ca\textsuperscript{2+} channels.

Intermittent claudication is a typical symptom associated with PAD that encompasses leg pain during exercise. After identifying Go\textsubscript{a3} as a key component involved in opioid modulation of Ca\textsuperscript{2+} 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 IC\textsubscript{50} 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 (Tsujimoto 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 Ca\textsuperscript{2+} channel, Go\textsubscript{a3}, 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 Ca\textsuperscript{2+} channel expression. In this DRG neuron subpopulation, we reported recently that N-type Ca\textsuperscript{2+} channels (Ca\textsubscript{v}2.2) carry the majority of the Ca\textsuperscript{2+} current (Ramachandra et al. 2013). Based on the current density measurements, no difference in Ca\textsuperscript{2+} current density between freely perfused and ligated animals was observed. We also explored the possibility that changes in Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} channel blockers, the contribution of N- and P/Q-type Ca\textsuperscript{2+} channels to the entire Ca\textsuperscript{2+} current was unchanged. Therefore, the absence of an increase in Ca\textsuperscript{2+} channel expression or shift in channel subtype expression could not explain the augmented DAMGO response.

Previous studies that have examined Ca\textsuperscript{2+} channel modulation by DAMGO in sensory neurons have sorted the cells based on size, lectin binding, and presence or absence of T-type Ca\textsuperscript{2+} 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 immunoreactive.

Our Western blotting analysis of DRG tissue provided evidence that neither MOR nor G\(_{\text{i3}}\) expression levels increased significantly as a result of arterial occlusion. Limited information is available regarding the effects of femoral occlusion on MOR or G\(_{\text{i3}}\) 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\(_{\text{i3}}\) 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 demonstrated...
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 NaV1.8 channel. With the use of an siRNA approach, we determined that MOR and Ca2+ channels are coupled specifically by PTX-sensitive G α3 proteins. Furthermore, hindlimb muscle ischemia significantly enhanced the DAMGO-mediated Ca2+ current inhibition in this neuron subset. The altered DAMGO pharmacology did not result from increases in either MOR or G α3 expression levels or in significant changes in Ca2+ 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. conception and design of experiments; V.R-V. prepared figures; B.H., M.F., and V.R-V. drafted manuscript; B.H., J.S.K., M.F., and V.R-V. edited and revised manuscript; M.F., M.P.K., and V.R-V. approved final version of manuscript.

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