Activation of Mas Oncogene-Related G Protein–Coupled Receptors Inhibits Neurochemical Alterations in the Spinal Dorsal Horn and Dorsal Root Ganglia Associated with Inflammatory Pain in Rats

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ABSTRACT

Mas oncogene-related G protein–coupled receptor C (MrgC) is unequally expressed in sensory ganglia and has been shown to modulate pathologic pain. This study investigated the mechanism underlying the effect of MrgC receptors on inflammatory pain. Intrathecal administration of the selective MrgC receptor agonist bovine adrenal medulla 8-22 (BAM8-22) (30 nmol) inhibited complete Freund’s adjuvant–evoked hyperalgesia. This was associated with the inhibition of protein kinase C-γ and phosphorylated extracellular signal-regulated protein kinase in the spinal cord and/or dorsal root ganglia (DRG). The complete Freund’s adjuvant injection in the hindpaw induced an increase in Gq, but not Gs and Gq, protein in the spinal dorsal horn. This increase was inhibited by the intrathecal administration of BAM8-22. The exposure of DRG cultures to bradykinin (10 μM) and prostaglandin E2 (1 μM) increased the expression of calcitonin gene-related peptide (CGRP) and neuronal nitric oxide synthase in small- and medium-sized neurons as well as the levels of CGRP, aspartate, and glutamate in the cultured medium. The bradykinin/prostaglandin E2–induced alterations were absent in the presence of BAM8-22 (10 nM). These results suggest that the activation of MrgC receptors can modulate the increase in the expression of CGRP and neuronal nitric oxide synthase as well as the release of CGRP and excitatory amino acids in DRG associated with inflammatory pain. This modulation results in the inhibition of pain hypersensitivity by suppressing the expression of Gq protein and protein kinase C-γ and extracellular signal-regulated protein kinase signaling pathways in the spinal cord and/or DRG. The present study suggests that MrgC receptors may be a novel target for relieving inflammatory pain.

Introduction

Inflammatory pain differs from acute or physiologic pain in that the response to noxious stimuli is enhanced (hyperalgesia) or innocuous stimulus produces pain (allodynia). Exaggerated sensitivity to painful and nonpainful stimuli is closely associated with high excitability of spinal nociceptive neurons, referred to as central sensitization, which is induced by the ongoing inputs conveyed from the primary sensory neurons in peripheral inflammation (Latremoliere and Woolf, 2009). On the other hand, the processing of pain hypersensitivity is also controlled by endogenous pain modulatory systems, such as the brainstem inhibitory descending pathway and spinal inhibitory interneurons. Noxious inputs can also be modulated in dorsal root ganglia (DRG) before traveling to the central nervous system. For example, the activation of opioid (Ji et al., 1995), acetylcholine, GABA (Li et al., 2002), galanin (Holmes et al., 2003), and cannabinoid (Bishay et al., 2010) receptors in the DRG can limit nociceptive information transferred from the periphery to the spinal cord and effectively relieve pain (Gold and Gebhart, 2010).

Mas oncogene-related G protein–coupled receptors (GPCRs) (Mrgr or Mrg) have been shown to modulate nociceptive processing in the DRG in pathologic pain states. Mrgr receptors, also known as sensory neuron–specific receptors (Lembo et al., 2002), are synthesized in DRG and trigeminal ganglia (Dong et al., 2001) and also found in the central terminals of primary afferents (He et al., 2014a; Huang et al., 2014). Mrgr receptors are divided into eight

ABBR EV IATIONS: BAM8-22, bovine adrenal medulla 8-22; BK, bradykinin; CFA, complete Freund’s adjuvant; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; ERK, extracellular signal-regulated protein kinase; GPCR, G protein–coupled receptors; IR, immunoreactivity; Mrg, Mas oncogene-related G protein–coupled receptor; NMDA, N-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; PBS, phosphate-buffered saline; PErK, phosphorylated extracellular signal-regulated protein kinase; PGE2, prostaglandin E2; PKC, protein kinase C; PWL, paw withdrawal latency.
subtypes (MrgA–MrgH) in rodents (Dong et al., 2001) and seven subtypes (MrgX1–MrgX7) in humans (Dong et al., 2001; Choi and Lahn, 2003). The members of the Mrg receptor subtype are not identical in the mouse and rat. A mouse has 14 MrgC genes, whereas a rat has just a single MrgC gene (Zylka et al., 2003). The characteristics of rat MrgC and human MrgX1 are very similar as both receptors are uniquely expressed in small-sized neurons of DRG and trigeminal ganglia (Han et al., 2002; Lembo et al., 2002), share genetic homogeneity as well as structural homology (Zylka et al., 2003), and are selectively activated by bovine adrenal medulla 8-22 (BAMS-22) (Lembo et al., 2002; Guan et al., 2010), an analog of the endogenous opioid peptide BAM22 (Lembo et al., 2002). MrgC receptors exert protective actions in the inflammatory pain state, as evidenced by the studies showing that MrgC cluster knock-out animals display an enhanced windup responding to C fiber inputs and/or increased pain behavior following an intraplantar injection of formalin (Guan et al., 2010), complete Freund's adjuvant (CF), and carrageenan (Liu et al., 2009). Moreover, MrgC receptor mRNA in DRG is upregulated and the activation of these receptors recruits μ-opioid bioactivity in the spinal cord and DRG in inflammatory pain (Jiang et al., 2013). Therefore, the investigation of MrgC receptors has important clinical implications. We have shown that an intrathecal administration of BAMS-22 produces immediate short and delayed long-lasting attenuations of CFA-induced hyperalgesia. The delayed inhibition by MrgC receptors is attributed to the recruitment of opioid receptor activity as it can be abolished by the μ-opioid receptor antagonist CTAP (Jiang et al., 2013). However, the mechanism underlying the immediate effect of MrgC receptors on inflammatory pain is not clear. The present study examined the hypothesis that acute activation of MrgC receptors modulated CFA-induced neurochemical changes in spinal dorsal horn and DRG neurons, leading to the inhibition of inflammatory pain.

Materials and Methods

Animals. Male Sprague-Dawley rats (250–320 g) were housed individually at 22°C with 50% humidity under a 12-hour light/dark cycle and given free access to food and water. The care and treatment of animals were according to the guidelines for the investigation of experimental pain in a conscious animal (Zimmermann, 1983) and were approved by the Animal Care Committee of Fujian Normal University (Fuzhou, China). All efforts were made to minimize animal suffering and the number of animals used in our experiments.

Treatments. Inflammation was induced by subcutaneous injection of 200 μl of 1 mg/ml CFA (Sigma-Aldrich, St. Louis, MO) into the hindpaw. Control rats received the intraplantar injection of saline (200 μl). The MrgC receptor agonist BAMS-22 (10 μl, pH 6.1) or saline (vehicle) was injected intrathecally at 24 hours following the intraplantar CFA or saline injection. Twenty minutes later, the animals were sacrificed and the spinal cord and DRG were harvested. This protocol will allow us to determine the effects of acute MrgC activation on CFA-induced cellular alterations.

Intrathecal Catheter Implantation. Animals were implanted with chronic indwelling catheters, with some modification of the previously described technique (Pogatzki et al., 2000). Briefly, a rat was injected with intraperitoneal pentobarbital (50 mg/kg) (Shenwongyong, Shanghai, China) and shaved along the occiput and neck. An incision was made overlying the atlanto-occipital junction, and the dura mater was exposed by blunt dissection. An incision was made in the dura, and a polyethylene catheter (PE-10; Stoelting, Wood Dale, IL), with a loose knot at 8.0 cm from the end, was threaded caudally to position its tip at the L4–L5 segments of the spinal cord. The other end of the catheter was anchored in the back of the neck. The catheter was then flushed with 10 μl of saline and plugged. The rats were housed individually after surgery and allowed to recover for approximately 7 days before being used for behavioral testing. Only the animals with no evidence of neurologic deficits after catheter placement were used for the experiment.

Assessment of Noxious Behavior. Radiant heat from a Plantar test meter (ITC Life Sciences Inc., Woodland Hills, CA) was applied by aiming a light beam through a hole in the light box through the glass plate to the middle of the plantar surface of the rat hindpaw. The heat intensity was adjusted to obtain an average paw withdrawal latency (PWL) of 7–9 seconds. The cut-off time was set at 20 seconds to prevent tissue damage. PWL at any test time point was measured twice at a 2-minute interval, and the mean value of these measurements was taken. Average values were taken from seven rats per group. The investigator was blind to the test drug conditions.

Western Blot. Animals were decapitated, and the dorsal half of the lumbar spinal cord and L4–L6 DRG on the side ipsilateral to CFA injection were harvested. Tissue samples were homogenized and sonicated in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM NaVO₃, 1% Igepal CA-630, and 0.1% SDS) supplemented with protease inhibitors (5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotenin, all from Sigma-Aldrich). Homogenates were shaken at 4°C, followed by centrifugation at 15,000g for 30 minutes each. The cell membranes of the spinal dorsal horn were fractionated using a membrane protein extraction kit (Beyotime Institute of Biotechnology, Shanghai, China), as recommended by the manufacturer. All samples were stored at −80°C until assay. Proteins were quantitated using the BCA protein assay (Pierce Chemical, Rockford, IL) and 20 μg of protein in SDS loading buffer was resolved on 7.5% SDS polyacrylamide gels. After protein transfer, a polyvinylidene difluoride membrane was blocked in 5% skim milk in Tween-20/phosphate-buffered saline (PBS) for 1 hour at room temperature. The membrane was then blotted with polyclonal rabbit anti-Gαi (1:2000) (abcam, Cambridge, UK), Goq/11, Goq (1:300) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), protein kinase C (PKC) μ (1:300), and phosphorylated extracellular signal-regulated protein kinase (pERK) (1:1000) (Cell Signaling Technology Inc., Boston, MA) overnight at 4°C in 5% milk. Membranes were then incubated with horseradish peroxidase–conjugated goat anti-rabbit antibody (1:5000) (Zhongshan Co., Beijing, China) in 5% milk for 2 hours at room temperature. A band was detected using an enhanced chemiluminescence detection kit (Amersham Biosciences, Little Chalfont, UK). Membranes were then incubated with a mouse β-actin antibody (1:300; Santa Cruz) for 2 hours at room temperature overnight at 4°C and then with horseradish peroxidase–conjugated goat anti-mouse antibody (1:3000; Santa Cruz Biotechnology) for 2 hours. Densitometry was performed using the Image J program, and the density of the target protein band was normalized to the β-actin loading band. The results were expressed as a percentage over the saline-treated control (n = 5 for each group).

Drgng Explant Culture. Animals were sacrificed by decapitation. Whole Drgng (C2–L6) were dissected under a sterile technique and collected in Hanks' balanced salt solution. Following a wash in Hanks' balanced salt solution, ganglia were transferred to Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The ganglia were grown at 37°C in 95% O₂/5% CO₂. The ganglion explant cultures were maintained for 24 hours. Then, the cultures were exposed to bradykinin (BK) (10 μM) and prostaglandin E₂ (PGE₂) (1 μM) (Sigma-Aldrich) as previously described (Patwardhan et al., 2005; Ceruti et al., 2011). BAMS-22 (10 nM) (Huadatianyuan Co., Shanghai, China) was applied in some cultures (15 minutes in advance to BK/PGE₂). One hour later, the ganglion explants and medium were then harvested, frozen on dry ice, and stored at −80°C until further processing.

Immunohistochemistry. DRG explants were fixed in 4% paraformaldehyde overnight and then transferred into 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for cryoprotection. DRG at 10 μm were cut on a
cryostat (HM550; Microm, Wallldorf, Germany) and mounted on slides. Immunocytochemistry was performed at room temperature on slides using an avidin-biotin complex technique as described previously (Cai et al., 2007). To permit comparisons across treatment groups, sections from different groups were processed simultaneously. After pretreatment with 0.3% H2O2 and 10% normal goat serum, sections were incubated at 4°C with polyclonal rabbit anti–calcitonin gene-related peptide (CGRP) (1:10,000) (Santa Cruz Biotechnology) or anti–neuronal nitric oxide synthase (nNOS) (1:2500) (Biosciences Pharmingen, San Jose, CA) for 24 hours. The sections were then transferred to a biotinylated secondary IgG complex (1:200 in 10% goat serum in PBS) for 2 hours, followed by exposure to avidin-biotin horseradish peroxidase complex (1:100) (Vector Laboratories, Burlingame, CA) for 1 hour. The chromogen was developed with 0.01% H2O2 and 0.05% diaminobenzidine. After being thoroughly rinsed with PBS, the sections were air dried, dehydrated in a series of graded ethanol, cleared in xylene, and coverslipped. Using different sections (n = 2), the primary antibody against either CGRP or nNOS was omitted in the immunocytochemical process, which resulted in the absence of staining.

Quantification of the CGRP- and nNOS-immunoreactivity (IR) positive and negative DRG neurons was performed using image-analysis software. Seven sections (870 × 640 μm2/section) per animal were randomly selected. The optical density of the IR product was quantified using a 255-level grayscale. To determine positive CGRP- and nNOS-IR neurons in each animal, a threshold of the average cytoplasmic density level of CGRP- and nNOS-IR products was set using an image of a control DRG. The optical density threshold was then applied to all other DRG sections. All neurons sectioned through their nucleus, for which the mean optical density exceeded the threshold, were counted as positive. Approximately 150 cells from each section and seven DRG sections per rat (n = 3–5 for each group) were measured. The number of positive cells was expressed as a percentage of total DRG neurons in the small- or medium-sized subpopulation. For neuron-size measurement, the neuronal soma area was calculated using the imaging software Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Small neurons were <600 μm2, medium neurons were 600–1200 μm2, and large neurons were >1200 μm2 (Gendron et al., 2006). The investigators were blind to the drug treatments.

**Enzyme Immunoassay.** The levels of CGRP (Peninsula Laboratories, Shanghai, China) were assayed using a commercially available enzyme immunoassay kit. All procedures were performed according to the manufacturer’s instructions. Samples were read using a microplate reader (Molecular Devices, Sunnyvale, CA). The amount of CGRP was calculated with reference to 1 ml of solution (pg/ml culture medium). Average values from four or five rats were taken to express the level of peptide in each group.

**Measurement of Excitatory Amino Acids.** The levels of aspartate and glutamate were measured by high-performance liquid chromatography using a fluorescence detector (Vale et al., 2006). Derivatization was performed by adding 70 μl of AccQ Tag Fluor borate buffer and 20 μl of AccQ Tag derivative reagent to 10 μl of sample or standard solution, shaking the mixture, and then allowing it to react for 10 minutes at 55°C. The derivatized sample (20 μl) was then injected onto a C18 reversed phase column. An analytic system was performed using Waters high-performance liquid chromatography with fluorescence detector (model 2475; Milford, MA), with a Waters Pico-Tag column (150 × 3.9 mm, 4 μm), at an excitation wavelength of 250 nm, emission wavelength of 395 nm, and column temperature of 40°C. The mobile phase was composed of 0.1 M sodium acetate buffer (pH 6.8) (A) and acetonitrile (B). The flow rate was 1.0 ml/min. All solvents were vacuum filtered through a 0.22-μm membrane (Millipore, Billerica, MA) and degassed by sonication for 30 minutes before use. External standard solutions were run before and after each sample group.

**Statistical Analyses.** Data are expressed as mean ± S.E.M. The statistical significance between groups was examined using one-way analysis of variance followed by Tukey’s test for multiple comparisons by the software SigmaStat (Systat Software, San Jose, CA). A two-tailed P value of less than 0.05 was considered statistically significant.

**Results**

**Intrathecal Administration of BAM8-22 Attenuates Heat Hyperalgesia.** Our previous study showed that after the treatment with intrathecal BAM8-22 on day 1, the intrathecal BAM8-22 administered on day 2 produced instant and delayed (24 hours later) inhibitions of CFA-induced hyperalgesia in a dose-dependent manner (Jiang et al., 2013). A slightly different protocol was used in the present study. CFA was injected in the unilateral hindpaw on day 1. BAM8-22 (30 nmol) or saline was administered intrathecally on day 2, which is the protective phase of inflammation. In accordance with the literature, CFA injection produced a remarkable decrease in PWL at 24 hours (from 8.3 ± 0.1 to 2.2 ± 0.1 seconds; P < 0.001; n = 7), indicating the induction of hyperalgesia (Fig. 1). Similar to our previous observation (Jiang et al., 2013), intrathecal BAM8-22 significantly attenuated CFA-induced hyperalgesia at 20 minutes after administration (4.5 ± 0.3 seconds; P < 0.001; n = 7).

**BAM8-22 Attenuates CFA-Induced Increase of PKCγ in the Spinal Dorsal Horn.** BAM8-22 (30 nmol) or saline was administered intrathecally 24 hours after CFA or saline injection in the hindpaw. Twenty minutes later, the dorsal part of the lumbar spinal cord on the inflamed side was harvested. This protocol was similar to that performed in the behavioral study and allowed us to determine the mechanism underlying the immediate effect of MrgC receptors on inflammatory pain. As illustrated in Fig. 2, the expression of PKCγ in the cell membrane of the spinal dorsal horn was significantly increased following the injection of CFA (139 ± 3%; P < 0.001 versus saline group; n = 5). However, the treatment with BAM8-22 reduced CFA-induced PKCγ expression (90 ± 3%; n = 5), which was significantly lower than the CFA group (P < 0.001). BAM8-22 administration by itself did not alter the level of PKCγ (103 ± 4%; n = 5).

![Fig. 1. Acute effect of BAM8-22 on CFA-induced hyperalgesia. Animals were treated with intraplantar saline or CFA (200 μl) on day 1 and intrathecal BAM8-22 (30 nmol) on day 2. Paw withdrawal latency in response to radiant heat was measured at 20 minutes after intrathecal administration. ***P < 0.001 compared with the saline group; ###P < 0.001 compared with the CFA-alone group. N = 7 each.](image)
expression, which was significantly lower than the CFA-alone mission. As illustrated in Fig. 4, the CFA injection increased the membrane of the spinal dorsal horn, which is associated with neurotrans- 

Intrathecal BAM8-22.

Membrane of the Spinal Dorsal Horn Is Inhibited by Intrathecal BAM8-22. Animals were treated with intraplantar saline or CFA (200 μl) on day 1 and intrathecal BAM8-22 (30 nmol) or saline on day 2. The dorsal half of the lumbar spinal dorsal horn on the ipsilateral side was harvested 20 minutes after intrathecal administration. Western blot assay of PKCγ levels in the cell membrane of the spinal cord was performed. Histograms (mean ± S.E.M.) show the results of a densitometric analysis of the immunoreactive bands. ***P < 0.001 compared with the saline group; ###P < 0.001 compared with the CFA-alone group. N = 5 each.

CFA-Induced Increase of pERK in the Spinal Dorsal Horn and DRG Is Inhibited by Intrathecal BAM8-22. We next investigated whether MrgC receptor activation exerted a modulation on the extracellular signal-regulated protein kinase (ERK) signaling pathway in the spinal dorsal horn and DRG as it links to central (Ji et al., 2002) and peripheral (Dai et al., 2002) sensitization, respectively. Compared with saline treatment, the CFA injection produced an increase in pERK in the spinal dorsal horn (138 ± 1%; P < 0.001; n = 5; Fig. 3A) and DRG (165 ± 11%; P < 0.001; n = 5; Fig. 3B) on the inflamed side. The increase in both sites was significantly inhibited by the 20-minute treatment of BAM8-22 (101 ± 2% or 112 ± 12%; P < 0.001 versus CFA-alone group; n = 5 each). The administration of BAM8-22 by itself did not change the pERK levels (97 ± 4% or 123 ± 10%; P > 0.05 versus saline group; n = 5 each).

CFA-Induced Increase of G1α Protein in the Cell Membrane of the Spinal Dorsal Horn Is Inhibited by Intrathecal BAM8-22. We further determined the effect of intrathecal BAM8-22 on CFA-induced G protein expression in the spinal dorsal horn, which is associated with neurotransmission. As illustrated in Fig. 4, the CFA injection increased the expression of G1α (135 ± 1%; P < 0.001; n = 5; Fig. 4B), but not G1β (Fig. 4A; n = 5) or G1δ (Fig. 4C; n = 5) protein in the cell membrane of the spinal dorsal horn compared with saline treatment. However, after intrathecal BAM8-22, the CFA injection only produced 115 ± 2% (n = 5) of G1α protein expression, which was significantly lower than the CFA-alone group (P < 0.01). G1α protein was 103 ± 7% in the group of intrathecal BAM8-22 plus intraplantar saline, which was not significantly different from the saline group (P > 0.05; n = 5).

BAM8-22 Attenuates BK/PGE2-Induced CGRP and nNOS Expressions in Cultured DRG Explants. To explore the mechanisms underlying the activity of MrgC receptors, the effects of BAM8-22 on BK/PGE2-induced response in DRG cultures were examined. The reason for choosing BK and PGE2 was based on the notion that exposure of nociceptors to inflammatory mediators results in neuronal molecular changes contributing to the development of a chronic pathologic pain state (Watkins and Maier, 2003). DRG explants were cultured with saline (control) or 10 μM BK plus 1 μM PGE2 (BK/PGE2) in the absence or presence of BAM8-22 (10 nM, added 15 minutes before BK/PGE2). The DRG explants were harvested 1 hour later. In agreement with those shown in an in vivo study (Hong et al., 2009), a number of small- (35 ± 1%) to medium- (31 ± 1%) sized neurons exhibited staining for CGRP-IR in the cultured DRG (control group, n = 3; Fig. 5, A and D). The treatment with BK/PGE2 induced an increase in the proportion of CGRP-IR positive neurons over the total small and medium subpopulations (69 ± 2% or 57 ± 1%; P < 0.001; n = 3; Fig. 5, B and D) compared with the control group. The application of BAM8-22 greatly inhibited the BK/PGE2-induced increase in the proportion of CGRP-IR–positive neurons of those subpopulations (50 ± 1% or 42 ± 2%; P < 0.001 versus BK/PGE2 group; n = 4; Fig. 5, C and D).

nNOS-IR was moderately expressed in the cytoplasm of DRG neurons. The nNOS-IR–positive neurons consisted of small- (30 ± 2%), medium- (14 ± 1%; n = 4) and large-sized subpopulations in the control group (Fig. 6, A and D). Similar to those induced by CFA injection in an in vivo study (Wang et al., 2013), the percentage of small (70 ± 3%) and medium (30 ± 3%) neurons with nNOS-IR staining over the total neurons of corresponding subpopulations significantly increased following treatment with BK/PGE2 (P < 0.01–0.001; n = 4; Fig. 6, B and D) compared with the control group. However, the BK/PGE2-induced increase in nNOS-IR expression in small-sized neurons was significantly suppressed in the presence of BAM8-22 (51 ± 4%; P < 0.05; n = 5; Fig. 6, C and D). BAM8-22 also inhibited the increase in nNOS-IR expression in medium-sized neurons, but the inhibition did not reach the significant level (24 ± 3%).

BAM8-22 Attenuates BK/PGE2-Induced Levels of CGRP, Aspartate, and Glutamate in DRG Culture Medium. To determine if the treatment with BAM8-22 also inhibited the release of the neurotransmitters CGRP and excitatory amino acids from sensory neurons, DRG explants were cultured with 10 μM BK plus 1 μM PGE2 (BK/PGE2) in the absence or presence of BAM8-22 (10 nM, added 15 minutes before BK/PGE2). The cultured medium was collected 1 hour later. Figure 7A shows that there was a basal CGRP content (29.7 ± 4.3 pg/ml; n = 4) in the culture medium in the saline group. Exposure of ganglion cultures to BK/PGE2 (BK/PGE2) in the absence or presence of BAM8-22 (10 nM, added 15 minutes before BK/PGE2) induced the release of CGRP and excitatory amino acids from sensory neurons, DRG explants were cultured with saline (control) or 10 μM BK plus 1 μM PGE2 (BK/PGE2) in the absence or presence of BAM8-22 (10 nM, added 15 minutes before BK/PGE2). The DRG explants were harvested 1 hour later. In agreement with those shown in an in vivo study (Hong et al., 2009), a number of small- (35 ± 1%) to medium- (31 ± 1%) sized neurons exhibited staining for CGRP-IR in the cultured DRG (control group, n = 3; Fig. 5, A and D). The treatment with BK/PGE2 induced an increase in the proportion of CGRP-IR positive neurons over the total small and medium subpopulations (69 ± 2% or 57 ± 1%; P < 0.001; n = 3; Fig. 5, B and D) compared with the control group. The application of BAM8-22 greatly inhibited the BK/PGE2-induced increase in the proportion of CGRP-IR–positive neurons of those subpopulations (50 ± 1% or 42 ± 2%; P < 0.001 versus BK/PGE2 group; n = 4; Fig. 5, C and D).

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As shown in Fig. 7, B and C, the basal content of aspartate and glutamate in the medium was 0.11 ± 0.01 and 1.5 ± 0.06 μmol/ml (saline group, n = 3), respectively. Treatment with BK/PGE2 resulted in a significant increase in the content of aspartate and glutamate (0.17 ± 0.04 or 2.16 ± 0.07 μmol/ml; n = 5; P < 0.001 versus saline group). However, BK/PGE2 treatment only induced 0.12 ± 0.01 μmol/ml of aspartate and 1.59 ± 0.06 μmol/ml of glutamate in the presence of BAM8-22 (n = 3). These levels were
significantly lower than those for the BK/PGE₂-alone group ($P < 0.001$).

**Discussion**

Our previous study demonstrated that intrathecal administration of the highly selective MrgC receptor agonist BAM8-22 (Guan et al., 2010) at a dose of 30 nmol produced an immediate antihyperalgesic effect lasting approximately 40 minutes (Jiang et al., 2013). We first repeated a behavioral study and observed that intrathecal BAM8-22 inhibited CFA-induced hyperalgesia at 20 minutes. This result validated the protocol that was used to determine the acute effect of MrgC activation on CFA-induced biochemical alterations.

Fig. 3. pERK1/2 levels in the spinal dorsal horn and DRG. Animals were treated with intraplantar saline or CFA (200 μl) on day 1 and intrathecal BAM8-22 (30 nmol) or saline on day 2. The dorsal half of the lumbar spinal dorsal horn and DRG (L4–6) on the ipsilateral side were harvested 20 minutes after intrathecal administration. Western blot assay of pERK1/2 levels in the (A) spinal dorsal horn and (B) DRG was performed. Histograms (mean ± S.E.M.) show the results of a densitometric analysis of the immunoreactive bands. **$P < 0.01$ and ***$P < 0.001$ compared with the saline group; **$P < 0.01$ and ###$P < 0.001$ compared with the CFA-alone group. $N = 5$ each.

Fig. 4. G protein levels in the membrane-bound fraction of the spinal dorsal horn. Animals were treated with intraplantar saline or CFA (200 μl) on day 1 and intrathecal BAM8-22 (30 nmol) or saline on day 2. The dorsal half of the lumbar spinal dorsal horn on the ipsilateral side was harvested 20 minutes after intrathecal administration. Western blot assay of (A) $G_i$, (B) $G_q$, and (C) $G_s$ proteins was performed on the membrane fraction of the spinal dorsal horn. Histograms (mean ± S.E.M.) show the results of a densitometric analysis of immunoreactive bands. *$P < 0.05$ compared with the CFA-alone group, **$P < 0.01$ compared with the saline group. $N = 5$ each.
in spinal dorsal horn and DRG neurons. We examined the expression of PKC\(_\gamma\) in the cell membrane of the spinal dorsal horn as it plays a pivotal role in spinal neuron sensitization in inflammatory pain (Velazquez et al., 2007). PKC\(_\gamma\) is the major isoform of PKC in the spinal dorsal horn and is expressed in nociceptive neurons in lamina II (Malmberg et al., 1997; Polgar et al., 1999). The injection of CFA in the periphery increases the expression of PKC in the spinal dorsal horn (Martin et al., 1999; Honore et al., 2000; Weyerbacher et al., 2010), whereas the inhibition of PKC reduces CFA-induced miniature excitatory postsynaptic currents and c-Fos expression in dorsal horn neurons (Giles et al., 2007). PKC\(_\gamma\) knockout mice display a reduced pain behavior and c-Fos expression in the spinal dorsal horn following formalin injection (Malmberg et al., 1997). The present study showed that the injection of CFA in the hindpaw increased PKC\(_\gamma\) expression in the spinal dorsal horn following formalin injection (Malmberg et al., 1997). The present study showed that the injection of CFA in the hindpaw increased PKC\(_\gamma\) expression in the plasma membranes of dorsal horn neurons. This is in accordance with the finding that a major feature of PKC\(_\gamma\) activation is its translocation to the cell membrane (Martin et al., 1999; Cui et al., 2009). Importantly, the treatment with intrathecal BAM8-22 inhibited the CFA-induced increase in PKC\(_\gamma\).

ERK phosphorylation in spinal dorsal horn neurons is considered as a marker for central sensitization evoked by peripheral inflammation (Ji et al., 1999; Karim et al., 2001). Particularly, the ERK signaling pathway involved in the hypersensitivity of dorsal horn neurons can be activated independently of PKC (Slack et al., 2004; Giles et al., 2007). ERK1/2 is activated in the spinal dorsal horn following CFA injection (Ji et al., 2002; Adwanikar et al., 2004), whereas the inhibition of the ERK signaling pathway by intrathecal administration of the inhibitor (Ji et al., 2002) or small interfering RNA (Xu et al., 2008) attenuates both the induction and maintenance of pain hypersensitivity. On the other hand, activation of ERK signaling in primary sensory neurons has been documented to contribute to peripheral sensitization in inflammatory pain (Dai et al., 2002; Obata et al., 2003). Similar to the previous reports, the present study showed that pERK was increased in the spinal dorsal horn and DRG 24 hours following the injection of CFA. These increases were inhibited by intrathecal BAM8-22. Given that ERK (Wei et al., 2006; Weyerbacher et al., 2010) and PKC (Malmberg et al., 1997; Polgar et al., 1999) transduction pathways play a critical role in the hypersensitivity of spinal dorsal horn and/or DRG neurons in peripheral inflammation, the above results may suggest that the activation of MrgC receptors inhibited central and peripheral sensitization.

It has been established that the excitability of spinal dorsal horn neurons is increased by glutamate released from the central terminals of sensitized DRG neurons via acting on the N-methyl-D-aspartate (NMDA) receptors during inflammation (Bennett, 2000). The activity of dorsal horn neurons is also modulated by GPCRs. For example, substance P, which is coreleased from the central terminals, facilitates the relief of the Mg\(^{2+}\) block in the NMDA receptor, leading to the enhancement of NMDA receptor signaling (Khasabov et al., 2002; Suzuki et al., 2003). Other postsynaptic GPCRs that modulate NMDA receptors include metabotropic glutamate (D’Mello...
and Dickenson, 2008), CGRP (Yan and Yu, 2004), and μ-opioid receptors (Gracy et al., 1997; Kline and Wiley, 2008). These receptors are known to couple to Gq, Gs, or Gi proteins. However, very few studies have investigated the changes of G proteins in the spinal dorsal horn in peripheral inflammation. The present study demonstrated that CFA injection in the hindpaw increased the expression of Gq protein but did not change Gs and Gi proteins in the lumbar spinal dorsal horn. This was in agreement with the increase of PKCγ, a downstream of Gq protein (Neves et al., 2002). Our data were comparable to the study showing that specific depletion of the Gq protein in the nociceptor using a conditional gene-targeting approach results in the reduced pain hypersensitivity following paw inflammation (Tappe-Theodor et al., 2012). Furthermore, the CFA-induced increase in spinal Gq protein was abolished by intrathecal administration of BAM8-22, indicating that the normalization of the inflammatory pain-associated upregulation of Gq protein expression in the spinal dorsal horn was ascribed to the inhibition of inflammatory pain by MrgC receptors.

As MrgC receptors are expressed in the DRG and trigeminal ganglia (Dong et al., 2001), the modulatory effect of pain

Fig. 6. BK/PGE2-evoked nNOS expression in cultured DRG. DRG explants were cultured with (A) saline (control), (B) 10 μM BK + 1 μM PGE2 (BK/PGE2), or (C) BK/PGE2 + BAM8-22 (10 nM, added 15 minutes in advance) for 1 hour. nNOS-IR was expressed in small (small arrow), medium (medium arrow), and large (large arrow) sized neurons in DRG. (D) Histograms (mean ± S.E.M.) show the proportion of nNOS-IR-positive small and medium neurons over the total corresponding subpopulations of DRG. **P < 0.01 and ***P < 0.001 compared with the control group; #P < 0.05 compared with the BK/PGE2-alone group. N = 4–5. Scale bar, 100 μm.

Fig. 7. BK/PGE2-evoked CGRP, aspartate, and glutamate contents in the medium of DRG cultures. DRG explants were cultured with saline, 10 μM BK + 1 μM PGE2 (BK/PGE2), or BK/PGE2 + BAM8-22 (10 nM, added 15 minutes in advance) for 1 hour. (A) CGRP, (B) aspartate, or (C) glutamate content in the cultured medium was assayed. ***P < 0.001 compared with the saline group; ###P < 0.001 compared with the BK/PGE2-alone group. N = 3–5.
transmission by MrgC receptors must occur in the ganglia. The effects of MrgC receptors on inflammation-associated changes in DRG were examined using ganglion explant cultures. Inflammatory pain is attributable to the upregulation of CGRP and excitatory amino acids signaling in DRG associated with inflammatory pain. As a result, the release of CGRP and excitatory amino acids signaling in DRG associated with inflammatory pain. As MrgC receptors are exclusively located in primary nociceptive neurons (Dong et al., 2001; Lembo et al., 2002), targeting MrgC receptors may be a promising therapy for relieving inflammatory pain, with limited unwanted effects.

Authorship Contributions
Participated in research design: Hong, D. Wang.
Conducted experiments: D. Wang, P. Wang, Jiang, Lv, Zeng.
Performed data analysis: P. Wang, Jiang, D. Wang.
Wrote or contributed to the writing of the manuscript: Hong.

References
Chen T, Cai Q, and Hong Y (2006) Intrathecal sensory neuron-specific receptor agonists induce intracellular cAMP and cGMP in small- and medium-sized neurons in DRG. These results were very similar to those seen in an in vivo study following CFA injection (Hong et al., 2009; Wang et al., 2013). BK/PGE2 exposure also increased the levels of CGRP, aspartate, and glutamate in the medium of DRG cultures, suggesting an increase in their release. However, all these changes were greatly attenuated in the presence of BAMS-22. These results can be interpreted as a modulation of the inflammation-associated increase in the expression and/or release of CGRP, nNOS, and excitatory amino acids by MrgC receptors. This suggestion was supported by the findings showing the colocalization of MrgC receptors with CGRP (He et al., 2014a) and nNOS (Jiang et al., 2013) in DRG neurons and the attenuation of a miniature excitatory postsynaptic current in spinal dorsal horn neurons by MrgC receptors (He et al., 2014b).

The MrgC receptors are upregulated in the conditions of inflammatory (Jiang et al., 2013) and neuropathic (He et al., 2014a) pain. Previous studies have shown that activation of MrgC receptors modulates inflammatory pain (Chen et al., 2006; Jiang et al., 2013) and does not influence physiologic pain (Cai et al., 2007; Guan et al., 2010). This is understandable as the mechanisms underlying different types of pain are not identical and are inflammatory but not physiologic, and pain involves peripheral and central sensitization (Schaible et al., 2005). The activation of MrgC receptors inhibited the expression of nNOS and CGRP and the activation of ERK signaling in DRG associated with inflammatory pain. As a result, the release of CGRP and excitatory amino acids was suppressed, leading to the normalization of Gq protein and the inhibition of PKCɛ and ERK pathways in the spinal dorsal horn. Therefore, the inhibition of biochemical alterations in spinal and DRG neurons associated with pain hypersensitivity underlies the effect of MrgC receptors on inflammatory pain. As MrgC receptors are exclusively located in primary nociceptive neurons (Dong et al., 2001; Lembo et al., 2002), targeting MrgC receptors may be a promising therapy for relieving inflammatory pain, with limited unwanted effects.
Huang H, Li Q, Hong YG, and Wang DM (2014) [MrG receptor activation reverses chronic morphine-evoked alterations of glutamate transporters and nNOS in rats].


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