European Journal of Pharmacology 815 (2017) 10–17

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and analgesia

Inhibition of morphine tolerance by MrgC receptor via modulation of interleukin-1β and matrix metalloproteinase 9 in dorsal root ganglia in rats

Yue Zhang, Kai Wang, Minyan Lin, Qi Li, Yanguo Hong*

Provincial Key Laboratory of Developmental Biology and Neuroscience, Fujian Normal University, Fuzhou, Fujian 350117, China

ARTICLE INFO

Keywords:
Chronic morphine
Dorsal root ganglia
Interleukin-1β (IL-1β)
Mas-related gene (Mrg) receptor
Matrix metalloproteinase-9 (MMP-9)

ABSTRACT

Opiate tolerance is a critical issue in pain management. Previous studies show that activation of Mas-related gene (Mrg) C receptor can modulate the development of morphine tolerance. This study was designed to investigate the underlying mechanism(s). Intrathecal (i.t.) administration of morphine (20 μg) increased the expression of interleukin-1β (IL-1β) and matrix metalloproteinase-9 (MMP-9) in small- and medium-sized neurons in dorsal root ganglia (DRG). Co-administration of bovine adrenal medulla 8–22 (BAM8-22), a selective MrgC receptor agonist, via i.t. route inhibited the increase of IL-1β and MMP-9 in the DRG. Exposure of DRG cultures to morphine (3.3 μM) for 3 or 5 days, but not for 1 day, induced an increase in MMP-9 mRNA expression. The treatment with BAM8-22 (10 nM) for 20, 40 or 60 min abolished chronic (5 days) morphine-induced increase of MMP-9 mRNA in the cultured DRG. The treatment with BAM8-22 for 1 h inhibited chronic morphine-induced increase of MMP-9 and IL-1β mRNA in DRG but these effects were abolished by MrgC receptor antibody. The treatment with BAM8-22 for 24 and 72 h respectively inhibited and enhanced morphine-induced expression of MMP-9 and IL-1β mRNA in the cultured DRG. The BAM8-22-induced inhibition and enhancement were abolished by MrgC receptor antibody. The results suggest that the inhibition of IL-1β and MMP-9 expressions in DRG underlain the modulation of morphine tolerance by the acute activation of MrgC receptors. The chronic activation of MrgC receptors can facilitate morphine-induced increase of MMP-9 and IL-1β expressions in DRG.

1. Introduction

Opioids, typified by morphine, are the most effective analgesics for severe pain to date (Kalso et al., 2004). However, repeated exposure to morphine results in a decrease or loss of morphine analgesia, called morphine tolerance. Interleukin-1β (IL-1β) plays a pivotal role in the pathogenesis of morphine tolerance (Johnston et al., 2004; Shavit et al., 2005). Chronic exposure to morphine increases expression of IL-1β (Johnston et al., 2004) while genetic depletion (Bessler et al., 2006) or pharmacological blockade (Johnston et al., 2004; Shavit et al., 2005) of IL-1β receptor prevents the development of morphine tolerance. The expression of IL-1β in dorsal root ganglia (DRG) is also increased following chronic exposure to morphine (Sun et al., 2012; Zeng et al., 2014). The level of IL-1β in DRG actually affects the potency of morphine analgesia. Acute administration of morphine induces an increase in IL-1β in DRG, but not in the spinal cord, in naïve animals leading to a decrease in morphine potency as inhibition of IL-1β in DRG increases the potency of morphine analgesia (Berta et al., 2012). IL-1β is activated via cleavage from its precursor by matrix metalloproteinase-9 (MMP-9) (Schonbeck et al., 1998) and this can occur in DRG (Kawasaki et al., 2008). Increase of MMP-9 in DRG also opposes the potency of morphine analgesia as evidenced by the findings that acute morphine increases MMP-9 in DRG, but not in the spinal cord, and inhibition of MMP-9 potentiates morphine analgesia (Liu et al., 2012).

It is not known whether Mrg C receptor inhibits chronic morphine-induced expression of IL-1β and MMP-9 in DRG. Mrg receptor is a family of G protein-coupled receptors (GPCR). Mrg receptor is divided into eight groups (MrgA-MrgH) in rodents (Dong et al., 2001; Zylka et al., 2003) and seven groups (MrgX1-7) in humans (Dong et al., 2001; Choi and Lahn, 2003). Rodent MrgC receptor, an orthologue of human MrgX1, is uniquely distributed in DRG and trigeminal ganglia (Dong et al., 2001; Lembo et al., 2002). It has been demonstrated that MrgC receptor modulates inflammatory (Chen et al., 2006; Guan et al., 2010; Jiang et al., 2013), neuropathic (He et al., 2014a, 2014b; Wang et al., 2016a, 2016b) and bone cancer (Sun et al., 2016) pain. These studies indicate that MrgC receptor is an important molecule modulating the activity of primary sensory neurons. Interestingly, MrgC receptor can prevent and reverse morphine tolerance (Cai et al., 2007; Chen et al., 2010). This finding suggests that MrgC receptor agonist could be a promising adjunct for sustained use of opiates. However, the

* Correspondence to: College of Life Sciences, Fujian Normal University, Fuzhou, Fujian 350108, China.
E-mail address: yhong@fjnu.edu.cn (Y. Hong).

http://dx.doi.org/10.1016/j.ejphar.2017.10.011
Received 21 April 2017; Received in revised form 30 September 2017; Accepted 5 October 2017
Available online 06 October 2017

mechanism(s) underlying the modulation of morphine tolerance by MrgC receptor is not clear. We have demonstrated that MrgC receptor inhibits morphine-induced increase of nNOS and CGRP expressions in DRG (Chen et al., 2010). This study was designed to further investigate the mechanisms by determining whether MrgC receptor could modulate morphine-induced expression of IL-1β and MMP-9 in DRG.

2. Materials and methods

2.1. Animals

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

2.2. Intrathecal catheter implantation

Animals were implanted with chronic indwelling catheters (Pogatzki et al., 2000). Briefly, rat was injected with i.p. pentobarbital (50 mg/kg) and shaved along the occiput and neck. The dura mater overlying the atlanto-occipital junction was exposed by blunt dissection and an incision was made in the dura. A polyethylene catheter (PE-10, Stoelting, Wood Dale, IL, USA), 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 and the knot was immobilized by suturing to the musculature. The rostral tip of the catheter was exteriorized at 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 neurological deficits after catheter placement were used for experiment.

The chronic morphine protocol consisted of intrathecal (i.t.) administration of morphine hydrochloride (20 μg) to rat once per day in the morning for 6 consecutive days. This protocol has been proven to generate tolerance to morphine antinociception (Johnston et al., 2004; Cai et al., 2007). Drug or vehicle (saline) was delivered in a volume of 10 μl via i.t. catheter under conscious condition.

2.3. Assessment of nociceptive behavior

Tail flick latency (TFL) was determined by the radiant heat tail flick assay (n = 6 in each group) using a Tail Flick Meter (IITC Life Science Inc, CA, USA). A radiant heat was focused on the underside of the tail 3 cm from its distal end and tail flick latency automated by equipment with 0.01 s precision. Radiant heat intensity was adjusted to produce on average a baseline of 2–4 s in naive rats. The cutoff latency was established at 10 s to prevent possible tissue damage. TFL at any test time point was measured three times at 2 min intervals and the mean value of these measurements was taken. The investigator was blind to the test drug conditions.

TFL in the pain test was converted to a percentage of maximum possible effect (%MPE) using following formula:

$$\%MPE = ([post\text{−}drug\ latency − baseline\ latency] / [cut\text{−}off\ time − baseline\ latency]) \times 100\%$$

2.4. Ganglion explant cultures

Animals were killed by decapitation. DRG (C2–L6, n = 5 in each group) were dissected under sterile technique and collected in Hank's solution. Following a wash in Hank's solution, ganglia were transferred to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. The ganglia were grown at 37 °C in 5% CO_2. Media were replaced every other day. The cultures of ganglion explants were exposed to morphine, BAM8-22 or MrgC receptor antibody for various time as indicated. Then the ganglion explants were harvested, frozen on dry ice and stored at −80 °C until further processing for measurements.

2.5. Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p., n = 6 in each group). The animals were perfused intracardially with cold 0.01 M phosphate buffered saline (PBS) and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). DRG at L4-6 were removed and post-fixed in the same fixative overnight. Tissues were then transferred into 30% sucrose in PB for cryoprotection. DRG at a 10 μm were cut on a cryostat. For immunofluorescence staining, DRG sections were incubated overnight at 4 °C with rabbit anti-IL-1β (1:200, Santa Cruz Biotechnology Inc., Shanghai, China) or anti-MMP-9 (1:100, Santa Cruz) antibody followed by red rhodamine-conjugated (l:200, Abcam, Shanghai, China) secondary anti-rabbit antibody for 1 h at room temperature. Nonspecific staining was determined by excluding the primary antibodies using different sections (n = 2). This procedure resulted in the absence of staining.

The sections were examined with a fluorescence digital microscope (BX51, Olympus, Japan), and images were captured with a Q-Fire cooled camera (DP70, Olympus, Japan). Quantification of IL-1β or MMP-9 immunoreactivity-positive and negative DRG neurons was performed using image analysis software Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). A field of 210 μm × 210 μm was randomly selected from each of 7 DRG sections per animal. To determine the percentage of positive neurons in each DRG, a threshold of average cytoplasmic optical intensity of IL-1β or MMP-9 immunofluorescence was set using the software. The optical density threshold was then applied to whole section of DRG. All neurons sectioned through their nucleus with mean optical density exceeding the threshold were counted as IL-1β or MMP-9-positive. Otherwise, the cells were considered as negative neurons. The number of positive cells was expressed as a percentage of total neurons.

2.6. Quantitative real-time-PCR

The cultured ganglion explants were collected and frozen immediately in liquid nitrogen, then stored at −80 °C for total RNA isolation. Total RNA was extracted using the RNAprep pure Tissue Kit (Tiangen) according to the manufacturer's instructions and treated with Deoxyribonuclease I (Tiangen) to remove DNA contamination. RNA concentrations were measured using an Ultraviolet-visible spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). The quality and quantity of the RNA were assessed at 260/280 A, and all samples showed absorbency ratios ranging from 1.8 to 2.0. A total of 1 μg of RNA was reverse-transcripted using Quantscript Reverse Transcription Kit according to the protocol of the manufacturer (Tiangen). All agents were purchased from Tiangen Biochemical Technology (Beijing, China).

Primers for MMP-9, IL-1β and GAPDH were designed using Primer 3 software (Table 1). After being placed at room temperature, 20 ng of cDNA from the same cDNA batch was subjected to real-time PCR to amplify all genes in triplicate in a total reaction volume of 20 μl using SYBR Premix Ex Taq, ROX as internal reference dye (Takara Biotechnology Technologies, Dalian, China), and the required amount of forward and reverse primers (Jierui Biotechnology Company, Shanghai, China, Table 1). Reactions were conducted on a 7500 fast Sequence Detection System (Applied Biosystems, Foster, CA, USA) using following cycling conditions: one cycle at 95 °C for 30 s, followed by 40

Table 1
Primer sequences used for real-time PCR.

Gene	Forward (5′–3′)	Reversed (5′–3′)
MMP – 9	CCACCGAGCTATCCACTCAT	GTCCGGTTTCAGCATGTTTT
IL – 1β	CATTGTGGCTGTGGAGAAG	ATCATCCCACGAGTCACAGA
GAPDH	GGCAAGTTCAACGGCACAG	CGCCAGTAGACTCCACGACAT

cycles at 95 °C for 5 s and 60 °C for 30 s. For each experiment, a non-template reaction served as negative control. Melting curve analysis of products as well as amplicon size verification on a 3% agarose gel confirmed the specificity of the PCR. The raw expression level for each gene was calculated using the same external standard curve made with a mixture of cDNA samples. The target gene expression data were then normalized to GAPDH gene expression to obtain relative concentrations and presented as relative expression units. The experiment in each condition was repeated five times.

2.7. Drugs and chemicals

Sodium pentobarbital was obtained from Shenwgong (Shenwgong Co., Shanghai, China). Morphine hydrochloride was purchased from Northeast (The first pharmaceutical industry of Shenyang, Shenyang, China). MrgC antibody was purchased from Phoenix (Phoenix Biotech, Shanghai, China). Morphine and MrgC antibody were diluted in 0.9% sterile physiological saline.

2.8. Statistical analysis

Data are expressed as mean ± standard error of mean (S.E.M.). Statistical significance between groups was examined using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Intermittent administration of BAM8-22 prevented morphine-induced analgesia tolerance

Morphine (20 μg, i.t.) was administered daily for 6 days. As shown in Fig. 1, morphine produced maximal analgesia on day 1. There was a great reduction in percentage of maximal possible antinociceptive effect (% MPE) on day 6, which was just slightly higher than the saline group, indicating antinociceptive tolerance. In accordance with previous observations (Cai et al., 2007; Chen et al., 2010), BAM8-22 (1 nmol) co-administered on days 1, 3 and 5 (defined as an intermittent injection) maintained morphine-induced analgesia on day 6 as morphine produced similar MPE on day 6 compared with the morphine alone group ($P > 0.05$, Fig. 1). Daily i.t. BAM8-22 (1 nmol (Chen et al., 2010)) or saline for 6 days did not change tail flick latency. These results confirmed the modulation of morphine tolerance by intermittent injection of BAM8-22.

3.2. Intermittent administration of BAM8-22 attenuated IL-1β and MMP-9 expressions in DRG in vivo

Saline, morphine (20 μg) or morphine plus BAM8-22 (1 nmol, intermittently) were administered i.t. for 6 days. The experiment in each condition was repeated six times. DRG at L4-6 were harvested on day 6 and processed with immunocytochemistry staining. Fig. 2 showed that chronic administration of morphine increased IL-1β expression in small- and medium-sized neurons ($P < 0.05$, Fig. 2B and D) compared with saline control (Fig. 2A and D). After intermittent BAM8-22 treatment, morphine-induced increase of IL-1β expression in small- and medium-sized neurons was greatly attenuated (Fig. 2C and D). Statistical analysis revealed that there was a significant difference between the morphine and morphine/BAM8-22 groups ($P < 0.05$).

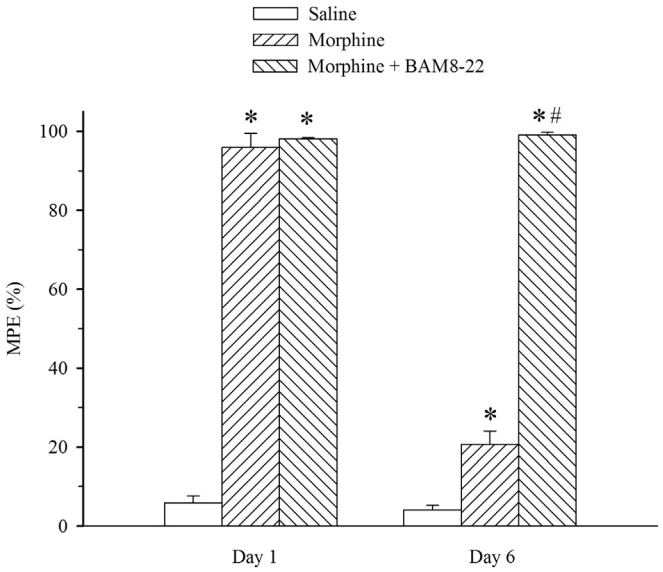

Fig. 1. Effect of BAM8-22 on the development of morphine tolerance. Morphine (20 μg) or saline was administered i.t. once daily. BAM8-22 (1 nmol) was given on days 1, 3, and 5. The tail-flick latency was assessed 20 min after each morphine injection. The data are presented as mean ± S.E.M.. *$P < 0.05$ compared with saline group. #$P < 0.05$ compared with morphine group. N = 6 each.

Similarly, chronic morphine-treated rats showed a significant increase of MMP-9 expression in small- and medium-sized neurons of DRG ($P < 0.05$, Fig. 3B and D) compared with the saline group (Fig. 3A and D). The increase disappeared when morphine was co-administered with the intermittent BAM8-22. The expression of MMP-9 in this group was significantly lower than that in the morphine alone group ($P < 0.05$, Fig. 3C and D). These results suggested that the chronic morphine increased the expression of MMP-9 in DRG and the activation MrgC receptor inhibited morphine-evoked upregulation of IL-1β and MMP-9 expressions.

3.3. Acute BAM8-22 reduced morphine-evoked expression of MMP-9 and IL-1β mRNA in a MrgC-dependent manner in DRG ex vivo

DRG explants were cultured and morphine (3.3 μM) was applied to the cultures for 1, 3 or 5 days. In other groups, DRG cultures were treated with morphine (3.3 μM) for 5 days. Then morphine (3.3 μM), BAM8-22 (10 nM) or BAM8-22 plus MrgC receptor antibody (10 ng/ml) were applied for various times on day 5. As illustrated in Fig. 4A, the treatment with morphine for 3 or 5 days, but not for 1 day, increased the level of MMP-9 mRNA which was significantly higher than that in the saline group ($P < 0.05$), confirming the association of MMP-9 increase with chronic use of morphine. The treatment with BAM8-22 for 20, 40 or 60 min significantly reduced chronic (5 days) morphine-induced increase of MMP-9 mRNA. Analysis showed that the level of MMP-9 mRNA in the morphine/BAM8-22 groups was similar to the saline group ($P > 0.05$, Fig. 4B) but significantly lower that the morphine alone group ($P < 0.05$). Fig. 4C showed that the one hour-treatment with BAM8-22 reduced morphine-induced increase of IL-1β mRNA. In the presence of MrgC antibody plus BAM8-22, chronic morphine still greatly increased the level of MMP-9 mRNA which was significantly higher than the morphine/BAM8-22 and saline groups ($P < 0.05$).

In agreement with previous report (Sun et al., 2012; Zeng et al., 2014), chronic morphine exposure for 5 days significantly increased the expression of IL-1β mRNA compared with the saline group ($P < 0.05$,

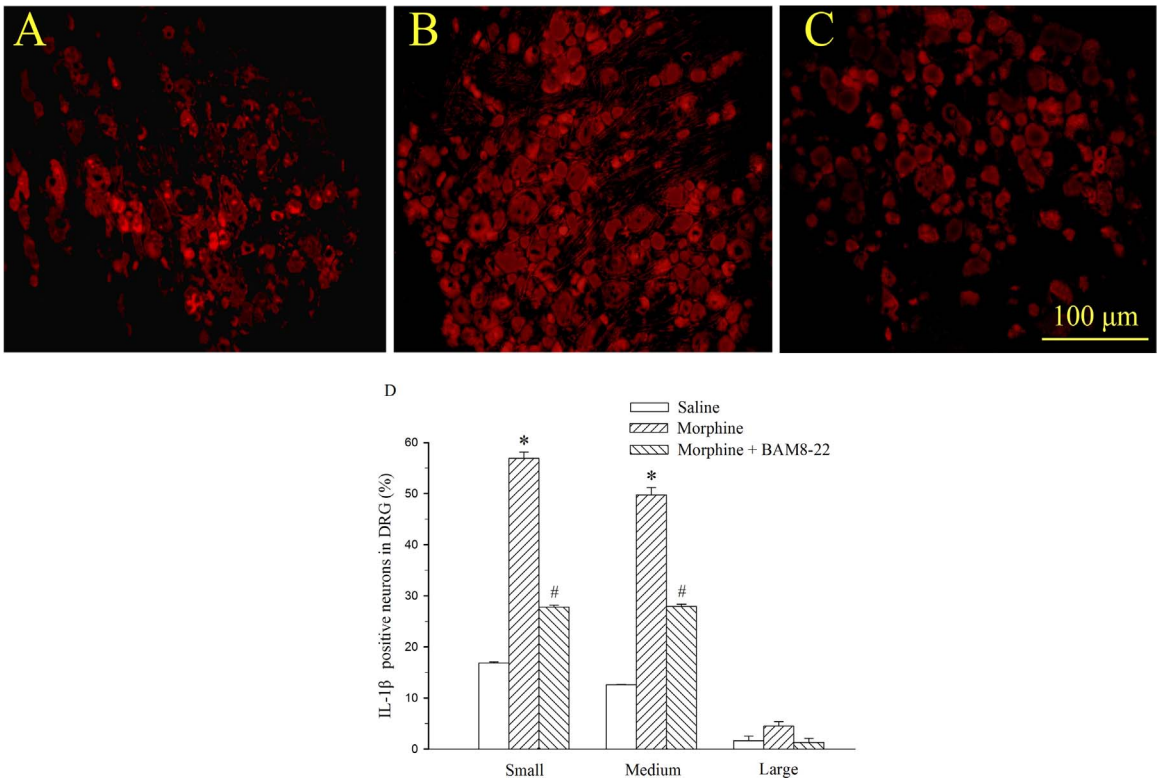

Fig. 2. Effect of BAM8-22 on morphine-evoked IL-1β expression in DRG. Saline (A) or morphine (20 μg, B) was administered i.t. once daily. BAM8-22 (1 nmol) was given on days 1, 3, and 5 (C). DRG at L4-6 were harvested on day 6. D. Histograms (mean ± S.E.M.) show the proportion of IL-1β-positive neurons over the total neurons in corresponding size subpopulation. *$P < 0.05$ compared with the saline group. #$P < 0.05$ compared with the morphine group. N = 6 each.

Fig. 5). However, chronic morphine-induced increase of IL-1β mRNA was abolished by the acute (1 h) administration of BAM8-22 ($P < 0.05$ *vs* morphine). This inhibition was significantly attenuated by MrgC receptor antibody ($P < 0.05$ *vs* morphine/BAM8-22 group). These results suggested that acute activation of MrgC receptor suppressed the morphine-induced increase of MMP-9 and IL-1β mRNA in DRG.

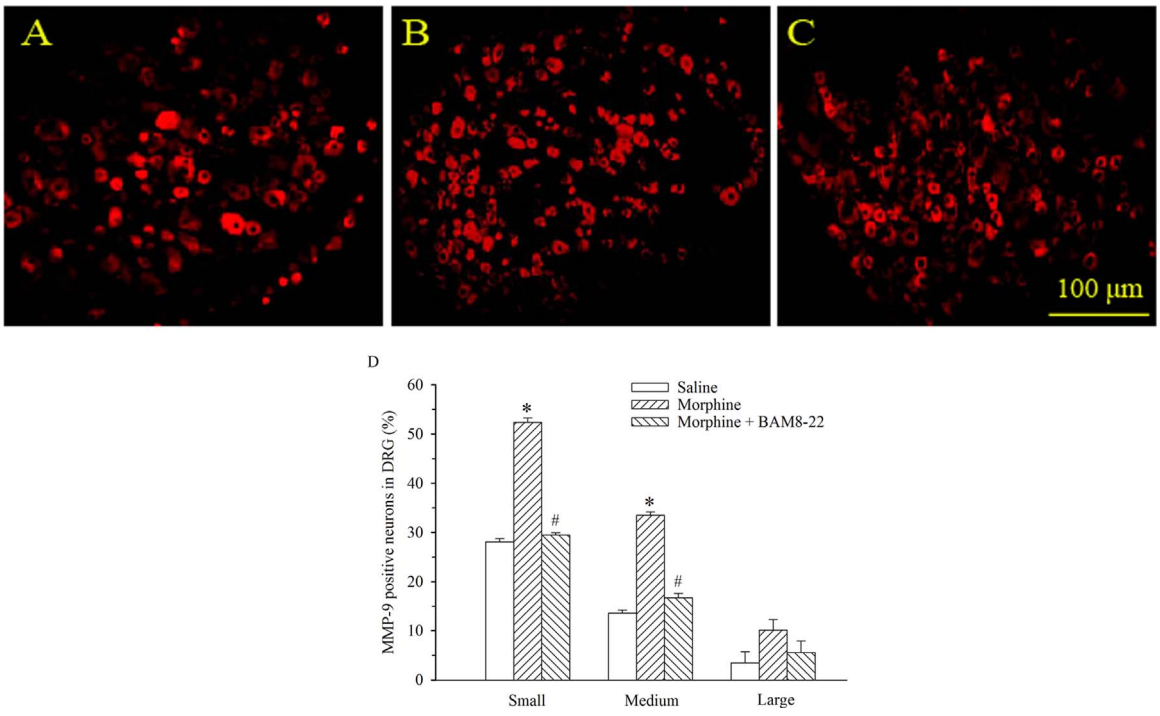

Fig. 3. Effect of BAM8-22 on morphine-evoked MMP-9 expression in DRG. Saline (A) or morphine (20 μg, B) was administered i.t. once daily. BAM8-22 (1 nmol, i.t.) was given on days 1, 3, and 5 (C). DRG at L4-6 were harvested on day 6. D. Histograms (mean ± S.E.M.) show the proportion of MMP-9-positive neurons over the total neurons in corresponding size subpopulation. *$P < 0.05$ compared with the saline group. #$P < 0.05$ compared with the morphine group. N = 6 each.

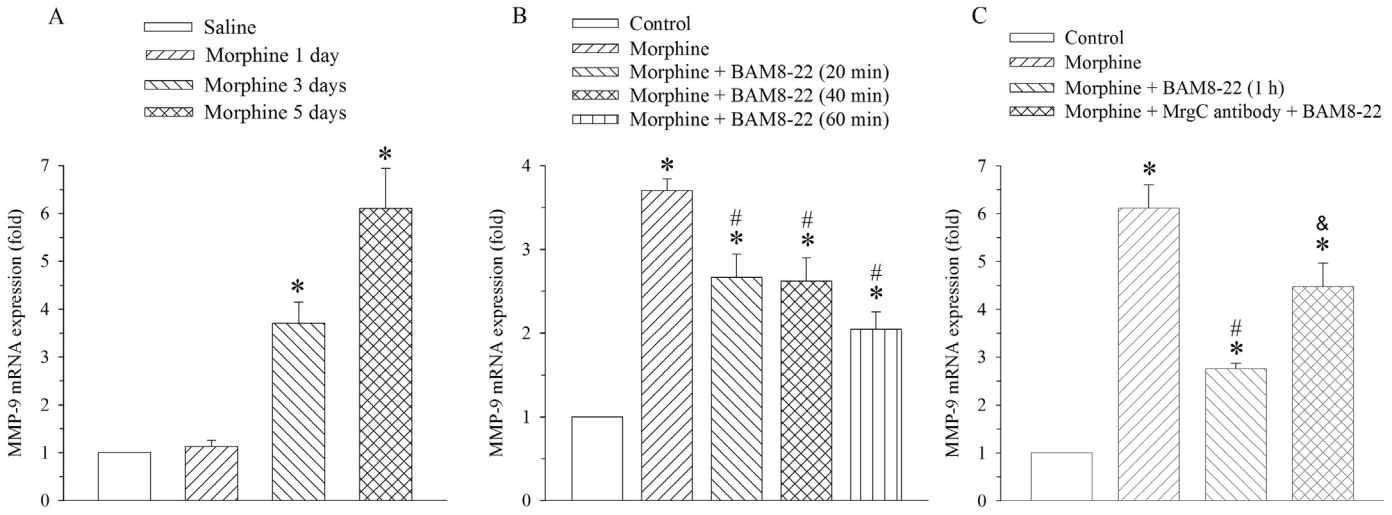

Fig. 4. Effect of BAM8-22 on morphine-evoked MMP-9 mRNA expression in cultured DRG. A. DRG cultures were treated with morphine (3.3 μM) for 1, 3 or 5 days. B. DRG cultures were treated with morphine (3.3 μM) for 5 days. BAM8-22 (10 nM) was applied for 20, 40 or 60 min on day 5. C. DRG cultures were treated with morphine (3.3 μM) for 5 days and BAM8-22 was applied for 1 h in the absence or presence of MrgC receptor antibody (10 ng/ml) on day 5. *$P < 0.05$ compared with saline. #$P < 0.05$ compared with morphine/BAM8-22 group. N = 5 each.

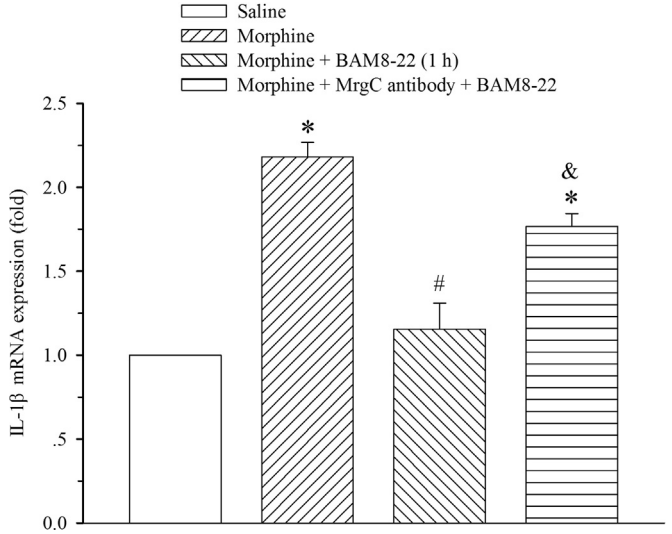

Fig. 5. Effect of BAM8-22 on morphine-evoked IL-1β mRNA expression in cultured DRG. DRG cultures were exposed to morphine (3.3 μM) for 5 days. Morphine (3.3 μM) plus BAM8-22 (10 nM) were applied for additional 24 h in the absence or presence of MrgC receptor antibody (10 ng/ml). *$P < 0.05$ compared with saline. #$P < 0.05$ compared with morphine group. &$P < 0.05$ compared with morphine/BAM8-22 group. N = 5 each.

3.4. Chronic BAM8-22 facilitated morphine-evoked increase of MMP-9 and IL-1β mRNA in a MrgC-dependent manner in DRG ex vivo

Previous study demonstrates that the development of morphine tolerance can be inhibited by intermittent (every other day), but not consecutive (every day for 6 days), administration of BAM8-22 (Chen et al., 2010). To characterize the pharmacological property of MrgC receptor, DRG cultures were treated with morphine (3.3 μM) for 5 days. Then, morphine and BAM8-22 (10 nM) or BAM8-22 plus MrgC receptor antibody (10 ng/ml) were applied to the cultures for additional 24 or 72 h.

Similar to the one-hour treatment, application of BAM8-22 for 24 h still inhibited chronic morphine-induced increase of MMP-9 mRNA level which was significantly lower than that in the morphine group ($P < 0.05$, Fig. 6A). BAM8-22 did not reduce the morphine-induced increase of MMP-9 mRNA expression in the presence of MrgC receptor antibody as the level of MMP-9 mRNA was similar to that in the morphine group but significantly higher than that in the morphine/BAM8-22 group ($P < 0.05$). In contrast, the 72 h-treatment with BAM8-22 enhanced the increase of MMP-9 mRNA level which was significantly higher than the morphine alone group ($P < 0.05$, Fig. 6B). The BAM8-22-induced enhancement of MMP-9 mRNA disappeared when MrgC receptor antibody was present. The level of MMP-9 mRNA in this group was significantly lower than the morphine/BAM8-22 group ($P < 0.05$).

In agreement with the changes of MMP-9, chronic morphine-induced IL-1β mRNA expression was significantly inhibited by the 24 h-treatment of BAM8-22 but increased by the 72 h-treatment of BAM8-22 compared with the morphine group ($P < 0.05$, Fig. 7A and B). Both the BAM8-22-induced decrease and increase were greatly attenuated by the application of MrgC receptor antibody ($P < 0.05$ *vs* morphine/BAM8-22). There results suggested that the chronic activation of MrgC receptor facilitated, but not inhibited, the morphine-induced change of MMP-9 and IL-1β mRNA in DRG.

4. Discussion

We have observed that intermittent activation of MrgC receptors by i.t. administration of the selective receptor agonist BAM8-22 (Cai et al., 2007; Chen et al., 2010) or (Tyr^6)-2-MSH-6–12 (MSH) (Chen et al., 2010) prevents the development of morphine tolerance. The present study showed that intermittent administration of BAM8-22 inhibited chronic morphine-induced increase of MMP-9 and IL-1β expressions in small- and medium-sized DRG neurons. Application of BAM8-22 for 20, 40 or 60 min inhibited chronic morphine-induced increase of MMP-9 or IL-1β mRNA in the cultured DRG. Morphine-induced increase of MMP-9 or IL-1β mRNA in the cultured DRG was also abolished by the 24 h-treatment of BAM8-22 but was facilitated following the 72 h-treatment of BAM8-22. The BAM8-22-induced inhibition or facilitation of MMP-9 and IL-1β mRNA was attenuated in the presence of MrgC receptor antibody. These results suggest that inhibition of morphine tolerance by MrgC receptors can be attributed to the suppression of MMP-9 and IL-1β expressions in DRG. We first tested the experimental protocol that was done before (Cai et al., 2007; Chen et al., 2010) and confirmed that bolus injection BAM8-22 at days 1, 3 and 5 maintained the potency of morphine analgesia. Then we examined the effect of intermittent BAM8-22 on morphine-induced IL-1β expression in DRG. Previous studies showed increase of IL-1β in DRG following chronic exposure of morphine (Sun et al., 2012; Zeng et al., 2014). IL-1β is expressed in

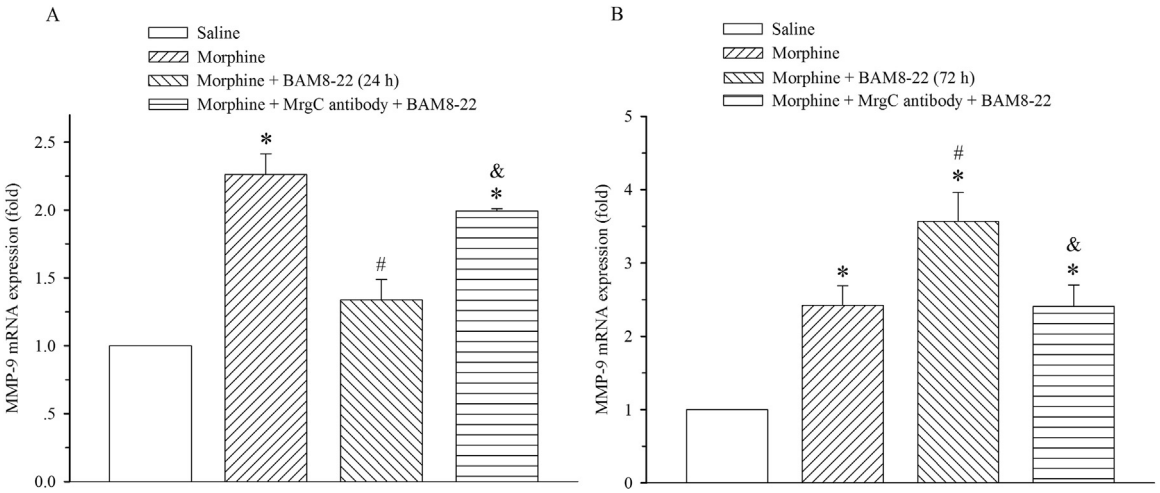

Fig. 6. Effect of chronic treatment with BAM8-22 on morphine-evoked expression of MMP-9 mRNA in cultured DRG. Saline or morphine was applied to DRG cultures for 5 days. Morphine (3.3 μM)/BAM8-22 (10 nM) or morphine/BAM8-22 + MrgC receptor antibody (10 ng/ml) were applied for additional 24 (A) or 72 (B) h. *$P < 0.05$ compared with saline. #$P < 0.05$ compared with morphine group. &$P < 0.05$ compared with morphine/BAM8-22 group. N = 5 each.

both neurons (Copray et al., 2001; Kawasaki et al., 2008) and satellite cells (Takeda et al., 2007; Kawasaki et al., 2008). The increase of IL-1β occurs in satellite cells following acute morphine (Berta et al., 2012). The present study demonstrated that chronic morphine exposure increased the expression of IL-1β in DRG neurons. Importantly, the morphine-induced increase of IL-1β was abolished by the intermittent administration of BAM8-22, a highly selective MrgC receptor agonist (Lembo et al., 2002; Guan et al., 2010). Moreover, the one hour-treatment with BAM8-22 inhibited chronic morphine-induced increase of IL-1β mRNA in DRG *ex vivo* and this inhibition disappeared following neutralization of MrgC receptor. IL-1β has been documented to increase sodium currents and suppress potassium currents, causing hyperexcitability of primary sensory neurons (Takeda et al., 2007, 2008; Binshtok et al., 2008). The increase of IL-1β in DRG can counteract the potency of morphine analgesia (Berta et al., 2012). Therefore, the present study suggests that the inhibition of IL-1β expression in DRG can be ascribed for the modulation of morphine tolerance by MrgC receptor.MMP-9 belongs to the metalloproteinase superfamily. This molecule can remodel the protein constituents of the extracellular matrix and process a variety of nonmatrix protein (Page-McCaw et al., 2007). MMP-9 is involved in many biological processes including long-term potentiation, inflammation (Parks et al., 2004) and peripheral nerve injury (Chattopadhyay et al., 2007; Kawasaki et al., 2008). MMP-9 is expressed in the superficial laminae of the spinal cord (Liu et al., 2010) and DRG (Kawasaki et al., 2008) and have been documented to contribute to the development of neuropathic pain (Kawasaki et al., 2008). MMP-9 is also involved in morphine tolerance as evidenced by the findings that chronic morphine exposure induces an increase in MMP-9 activity in the spinal cord leading to neuronal hyperactivity via phosphorylation of NMDA receptor, CaMKII, ERK and CREB (Liu et al., 2010). MMP-9 is located in neurons in DRG (Kawasaki et al., 2008; Liu et al., 2012) and has been suggested to result in ongoing nociceptive sensitization (Bali et al., 2013). Acute morphine can induce an increase in MMP-9 expression in DRG (Liu et al., 2012). The present study demonstrated that the chronic morphine also induced an increase in MMP-9 expression in DRG and this change occurred in small- and medium-sized neurons. The morphine-induced increase of MMP-9 was time-dependent as morphine application for 3 or 5 days, but not for 1 day, increased MMP-9 mRNA expression in DRG *ex vivo*. As MMP-9 is expressed primarily in DRG neurons co-expressing μ-opioid receptors (Liu et al., 2017), our results suggeted the association of MMP-9 increase in DRG with chronic morphine. We showed that the increase of

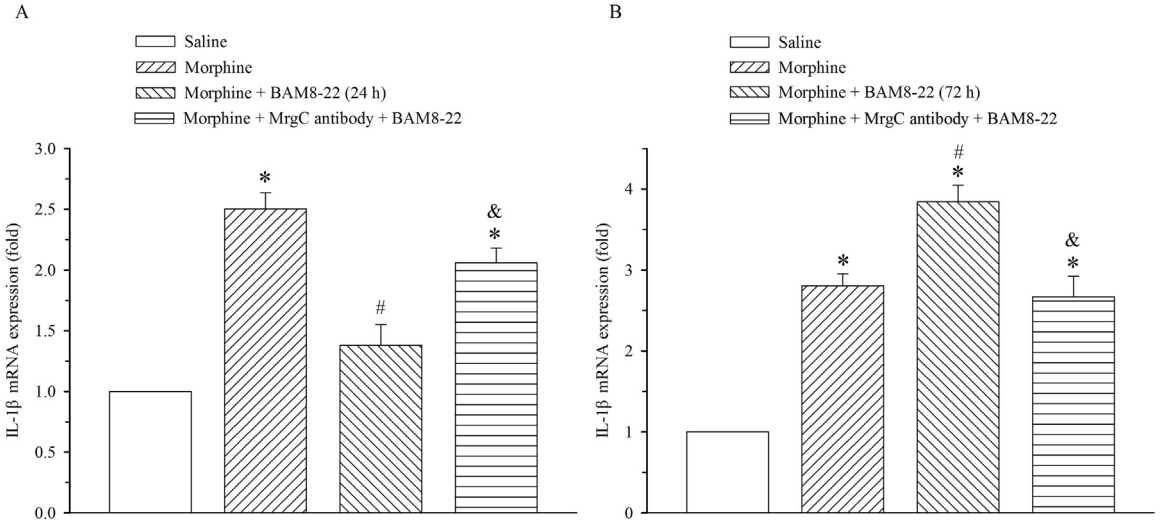

Fig. 7. Effect of chronic treatment with BAM8-22 on morphine-evoked expression of IL-1β mRNA in cultured DRG. Saline or morphine was applied to DRG cultures for 5 days. Morphine (3.3 μM)/BAM8-22 (10 nM) or morphine/BAM8-22 + MrgC receptor antibody (10 ng/ml) were applied for additional 24 (A) or 72 (B) h. *$P < 0.05$ compared with saline. #$P < 0.05$ compared with morphine group. &$P < 0.05$ compared with morphine/BAM8-22 group. N = 5 each.

MMP-9 protein or mRNA in DRG was abolished by the intermittent (every other day, in vivo) or short (20–60 min, *ex vivo*) application of BAM8-22. The inhibition of morphine-induced increase of MMP-9 mRNA by BAM8-22 was abolished by MrgC receptor antibody. These results were very similar with those observed for IL-1β and consistent with the finding that enhanced MMP-9 expression in DRG induces active cleavage of IL-1β (Kawasaki et al., 2008). Increase of MMP-9 in DRG has been shown to evoke pain hypersensitivity (Kawasaki et al., 2008; Bali et al., 2013) and oppose the potency of morphine analgesia (Liu et al., 2012). We, therefore, suggest that the suppression of MMP-9 expression in DRG underlay the inhibition of morphine tolerance by MrgC receptor.

We have noticed that consecutive administration of BAM8-22 fails to inhibit the development of morphine tolerance (Chen et al., 2010). To find out the possible mechanism, we determined the effect of chronic activation of MrgC receptor on IL-1β and MMP-9 expressions in DRG. The results showed that the 72, but not 24, hour-treatment with BAM8-22 actually enhanced morphine-induced increase of IL-1β and MMP-9 mRNA. This effect was MrgC receptor-dependent as the enhancement disappeared in the presence of MrgC receptor antibody. As the expression of MMP-9 (Liu et al., 2012) and IL-1β (Berta et al., 2012) in DRG remarkably impacts the potency of morphine analgesia, the increase of MMP-9 and IL-1β following chronic BAM8-22 exposure may underlie the failure of modulation of morphine tolerance by MrgC receptor (Chen et al., 2010). The similar dual effects are seen for opioid receptor. For example, acute administration of morphine inhibits expression of CGRP (Friese et al., 1997) and TRPV1 (Endres-Becker et al., 2007) in DRG while repeated exposure of morphine enhances activity of these receptors (Belanger et al., 2002; Rowan et al., 2014). The dual effects of MrgC receptor on expressions of IL-1β and MMP-9 in DRG may be attributed to its association with both Gi/o and Gq proteins (Chen et al., 2012). Studies to understand mechanisms of dual effects of MrgC receptor on the expressions of IL-1β and MMP-9 are warranted.

Tolerance to pain-relieving effect of opioids is a problem in a clinical setting in patients receiving these drugs. The prevention of morphine tolerance is important for the treatment of chronic pain. The intriguing feature of MrgC receptor is their unique distribution in primary sensory neurons (Dong et al., 2001; Lembo et al., 2002). Targeting this receptor would be without central nervous system side effects. Therefore, MrgC receptor agonist, such as BAM8-22, could be a promising adjunct for sustained use of opiates. The underlying mechanisms involve the inhibition of expression of nociceptive molecules in DRG including nNOS, CGRP (Chen et al., 2010), IL-1β and MMP-9. However, MrgC receptor should be activated intermittently for the modulation. Chronic or consecutive activation of MrgC receptor could increase IL-1β and MMP-9 expressions in DRG, leading to the failure of modulation of morphine tolerance.

Funding sources

This study was supported by the grants from the Natural Science Foundation of China (31371124 and 81571084).

Conflict of interest

The authors declare no conflict of interest.

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