Introduction

Morphine is a μ-opioid receptor agonist and is commonly used in the clinic for the management of various types of pain. Although morphine is very effective against most types of pain, specifically acute pain, there are several pain types that are difficult to control using morphine. These types of pain include neuropathic pain, cancer pain, or a component of inflammatory pain known as morphine-resistant intractable pains. In these pain states, an increased dose of morphine can sometimes retain good analgesia; however, elevated side effects by increased doses of morphine, such as drowsiness, respiratory depression, nausea, and constipation, can seriously affect patient quality of life. Thus, it is important to understand the mechanism of morphine-resistant intractable pain and develop new treatments for such pain.

Among the types of morphine-resistant intractable pain, inflammatory pain exhibits distinct characteristics. Unlike other types of intractable pain, whose expression is restricted to the ipsilateral side, inflammatory pain is observed on both the ipsilateral and contralateral sides (1). Moreover, mechanical allodynia, thermal hyperalgesia, and mechanical hyperalgesia are prominent features of inflammatory pain in addition to edema. Several types of inflammatory pain induced by formalin, carrageenan, and complete Freund’s adjuvant (CFA), among others, have been developed in rodents and used as experimental inflammatory pain models. In particular, the CFA-induced inflammatory pain model is widely.

Abstract. The antinociceptive effect of morphine in the inflammatory pain state was described in the von Frey filament test using the complete Freund’s adjuvant (CFA)-induced mouse inflammatory pain model. After an i.pl. injection of CFA, mechanical allodynia was observed in the ipsilateral paw. The antinociceptive effect of morphine injected s.c. and i.t. against mechanical allodynia was reduced bilaterally at 1 day and 4 days after the CFA pretreatment. The expression level of mRNA for μ-opioid receptors at 1 day after the CFA pretreatment was reduced bilaterally in the lumbar spinal cord and dorsal root ganglion (DRG). In contrast, the protein level of μ-opioid receptors at 1 day after CFA pretreatment was decreased in the ipsilateral side in the DRG but not the lumbar spinal cord. Single or repeated i.t. pretreatment with the protein kinase Ca (PKCa) inhibitor Ro-32-0432 completely restored the reduced morphine antinociception in the contralateral paw but only partially restored it in the ipsilateral paw in the inflammatory pain state. In conclusion, reduced morphine antinociception against mechanical allodynia in the inflammatory pain state is mainly mediated via a decrease in μ-opioid receptors in the ipsilateral side and via the desensitization of μ-opioid receptors in the contralateral side by PKCa-induced phosphorylation.

Keywords: antinociception, morphine, inflammatory pain, μ-opioid receptor, protein kinase C
used to describe the mechanisms of the inflammatory pain state and the effectiveness of analgesics against inflammatory pain.

It has been well established that the antinociceptive effect of μ-opioid receptor agonists, including morphine, is enhanced or retained against mechanical hyperalgesia (2, 3) and thermal hyperalgesia (2) in the CFA-induced inflammatory pain rodent model. However, most studies describing the antinociceptive effect of μ-opioid receptor agonists in this inflammatory pain model have focused on their effectiveness against hyperalgesia but not against allodynia. We recently found that the antinociceptive effect of morphine was markedly suppressed against mechanical allodynia in this inflammatory pain model (4). In the present study, using the CFA-induced inflammatory pain model in mice, we characterized the antinociceptive effect of morphine against mechanical allodynia and investigated the mechanism underlying the reduction of its antinociceptive effect on the inflammatory pain state.

Materials and Methods

All experiments were performed with the approval of the Ethics Committee for Animal Experiments at Tohoku Pharmaceutical University and according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals and animal suffering in the following experiments.

Animals

Male ddY mice (Japan SLC, Hamamatsu) weighing 20 – 24 g were used. The mice were housed in a room with controlled temperature (22°C – 23°C) and humidity (50% – 60%), with an alternating 12-h light/dark (lights on at 7:00 and off at 19:00) cycle. Food and water were provided ad libitum.

Mouse model for inflammatory pain

To generate the inflammatory pain model, mice were injected intraplantarly (i.pl.) with 50 μl of CFA (Sigma-Aldrich, St. Louis, MO, USA) in the left hind paw using a syringe with a 27-gauge needle. Control mice were injected i.pl. with sterile saline.

Measurement of mechanical allodynia and morphine analgesia

Mechanical allodynia and the antinociceptive effect of morphine were measured using the von Frey filament test. Prior to the experiment, mice were placed in a clear plastic compartment (10 cm × 10 cm × 14 cm) for 1 h to habituate to the experimental environment. The presence of the withdrawal response (biting, licking, or flinching) due to stimulation of the plantar surface of the hind paw by the variable strength of the von Frey filament (applied pressure by each filament: 0.041, 0.073, 0.163, 0.400, 0.610, 1.000, 1.430, 2.100, and 4.200 g) within 3 s after the filament application was determined. The up-down method was used to evaluate the withdrawal threshold from the filament. The intensity of the application of the filament was adjusted so that the pre-drug threshold was 0.400 or 0.610 g. Mice demonstrating a threshold of 0.073 or 0.163 g after the CFA injection were used in the inflammatory pain model.

Intrathecal (i.t.) administration

Intrathecal administration was performed according to the procedure described by Hylden and Wilcox (5) using a 50-μl Hamilton microsyringe with a 29-gauge needle. For i.t. injection, drugs were dissolved in sterile artificial cerebrospinal fluid (aCSF) containing 126.6 mM NaCl, 2.5 mM KCl, 2.0 mM MgCl₂, and 1.3 mM CaCl₂. The injection volume of the drugs was 5 μl.

Drugs

The drugs used included morphine hydrochloride (Takeda, Osaka) and the protein kinase Ca (PKCa) inhibitor Ro-32-0432 (Merck KGaA, Darmstadt, Germany). Morphine hydrochloride and Ro-32-0432 were dissolved in sterile saline and dimethyl sulfoxide (DMSO), respectively.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA in the mouse dorsal root ganglion (DRG: L4 – L6) and lumbar spinal cord was extracted using the RNasy Lipid Tissue Mini Kit (QIAGEN K.K., Tokyo). The purified total RNA was quantified using a spectrophotometer at A260. RT-PCR amplification was performed using the SuperScript One Step RT-PCR system, Platinum version (Life Technologies, Carlsbad, CA, USA). The synthetic forward primer and reverse primer for the μ-opioid receptor were 5’-CAG CCA TTC AGA ACC ATG G-3’ and 5’-ATG GTG CAG AGG GTG AAG ATA CTG G-3’, respectively. The synthetic forward primer and reverse primer for β-actin were 5’-GCT CGT CGT CGA CAA CGG CTC-3’ and 5’-ACA TGA TCT GGG TCA TCT TCT T-3’, respectively. The samples were heated to 50°C for 30 min for cDNA synthesis and to 94°C for 2 min for pre-denaturation and then cycled 30 times at 94°C for 30 s for denaturation, and 55°C for 30 s for annealing, and 72°C for 30 s for extension; the samples were finally heated to 72°C for 7 min for the final extension. The resulting PCR product was electrophoresed on a 0.8%
agarose gel containing ethidium bromide. The agarose gel was imaged using a UV Imaging Analyzer FAS-III (Toyobo, Osaka), and the PCR product for the \( \mu \)-opioid receptor in agarose gel was semi-quantified against \( \beta \)-actin using Lumino Imaging Analyzer FAS-1000 (Toyobo).

**Western blotting analysis**

Mouse lumbar spinal cord or DRG (L4 – L6) were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2.5 mM EGTA, 1 mM Na,VO_4, 0.5% TritonX-100, 0.5% NP-40) containing 1% Protease Inhibitor Cocktail (Sigma-Aldrich) and centrifuged at 20,000 \( \times \) g for 10 min at 4°C. The supernatants were collected, and total protein was measured using the Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). The proteins (12 \( \mu \)g) were boiled in sample buffer (31.25 mM Tris-HCl, 1% sodium dodecyl sulfate (SDS), 5% glycerol, 0.00625% bromophenol blue, and 2.5% \( \beta \)-mercaptoethanol: pH 6.8), electrophoresed in a 10% SDS-PAGE gel, and then transferred onto a Hybond-P membrane (GE Healthcare UK Ltd., Buckinghamshire, UK). The blotted membrane was incubated in Blocking One (Nacalai Tesque, Osaka) for 1 h at room temperature to inhibit non-specific binding reactions. After washing, the membranes were incubated for 16 h at 4°C with a rabbit antiserum for the \( \mu \)-opioid receptor (Abcam plc, Cambridge, UK) at 1:3000 dilution in Can Get Signal solution 1 (Toyobo) or with a rabbit antiserum for \( \beta \)-actin (IMGENEX Corp., San Diego, CA, USA) at 1:1000 dilution in Can Get Signal solution 1. The membranes were extensively washed and then incubated for 1 h with a goat HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 1:5000 dilution in Can Get Signal solution 2 (Toyobo). After washing, the proteins were detected using the ECL-Plus Western blotting detection system (GE Healthcare UK, Ltd.) and visualized with the FAS-1000 Lumino Imaging Analyzer (Toyobo).

**Statistical analyses**

The data are expressed as means ± S.E.Ms for at least 10 mice. The statistical significance of the differences between groups was assessed using one-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni’s test.

**Results**

**CFA-induced mechanical allodynia**

Groups of mice were injected i.pl. with 50 \( \mu \)l of saline or CFA in the left hind paw, and the withdrawal threshold against mechanical stimulation by a von Frey filament applied to the plantar surface of the each hind paw was measured for 40 days. After the CFA injection, the decrease in mechanical threshold (mechanical allodynia) was observed in the ipsilateral paw but not in the contralateral paw (Fig. 1). The mechanical allodynia in the ipsilateral paw in the CFA-treated mice peaked at 1 day after the CFA treatment and was prolonged to 34 days after CFA treatment. In contrast, the mechanical threshold was not altered in mice treated i.pl. with saline.

**Antinociceptive effect of morphine injected subcutaneously (s.c.) or i.t. in the inflammatory pain state**

Groups of mice pretreated i.pl. with 50 \( \mu \)l of saline or CFA in the left hind paw were injected s.c. with morphine (7.1 mg/kg) at 1 day and 4 days after i.pl. pretreatment, and the withdrawal threshold against mechanical stimulation by a von Frey filament applied to the plantar surface of the each hind paw was measured for 180 min. The potent increase of the mechanical threshold (antinociceptive effect) by morphine was observed bilaterally in saline-pretreated mice (Fig. 2: A and B). However, the antinociceptive effect of morphine was significantly suppressed bilaterally at 1 day and 4 days after CFA pretreatment. More potent suppression of morphine antinociception was observed in the ipsilateral paw (Fig. 2B) compared to the contralateral paw (Fig. 2A). Moreover, in the contralateral paw, the suppression of morphine antinociception tended to increase its intensity at 4 days after the pretreatment...
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compared to 1 day after pretreatment (Fig. 2A).

Other groups of mice pretreated i.pl. with 50 μl of saline or CFA in the left hind paw were injected i.t. with morphine (5.0 nmol) at 1 day and 4 days after i.pl. pretreatment, and the withdrawal threshold against mechanical stimulation by a von Frey filament applied to the plantar surface of each hind paw was measured for 180 min. The potent antinociceptive effect of morphine was observed bilaterally in saline-pretreated mice (Fig. 2: C and D). However, the antinociceptive effect of morphine was significantly suppressed bilaterally at 1 day and 4 days after CFA pretreatment. More potent suppression of morphine antinociception was observed in the ipsilateral paw (Fig. 2D) compared to the contralateral paw (Fig. 2C). Moreover, in the contralateral paw, the magnitude of suppression for morphine antinociception was significantly increased at 4 days after pretreatment compared to 1 day after pretreatment (Fig. 2C).

**mRNA expression and protein levels of μ-opioid receptors in the inflammatory pain state**

Groups of mice were injected i.pl. with 50 μl of saline or CFA in the left hind paw, and the mRNA expression levels of μ-opioid receptors in the DRG (L4–L6) and lumbar spinal cord in the ipsilateral and contralateral sides were measured using semi-quantitative RT-PCR. In the lumbar spinal cord, the mRNA expression levels
of \(\mu\)-opioid receptors were significantly decreased bilaterally at 1 day and 4 days after CFA pretreatment (Fig. 3: A and B). Bilateral reduction of the mRNA expression levels of \(\mu\)-opioid receptor was also observed in the DRG at 1 day after CFA pretreatment (Fig. 3C) but disappeared at 4 days after CFA pretreatment (Fig. 3D).

Other groups of mice were injected i.pl. with 50 \(\mu\)l of saline or CFA in the left hind paw, and the protein levels of \(\mu\)-opioid receptors in the DRG (L4 – L6) and lumbar spinal cord in the ipsilateral and contralateral sides were measured using western blotting analysis. In the DRG, the protein levels of \(\mu\)-opioid receptors were significantly reduced only on the ipsilateral side at 1 day after CFA pretreatment (Fig. 4C), not at 4 days after CFA pretreatment (Fig. 4D). In contrast, the protein levels of \(\mu\)-opioid receptors in the lumbar spinal cord was not altered at either 1 day or 4 days after CFA pretreatment (Fig. 4: A and B).

**Involvement of the phosphorylation of \(\mu\)-opioid receptors in the reduction of morphine analgesia in the inflammatory pain state**

Groups of mice pretreated i.t. with the PKCa inhibitor Ro-32-0432 (1 nmol) or DMSO along with i.pl. pretreatment with CFA (50 \(\mu\)l) or saline in the left hind paw were injected s.c. with morphine (7.1 mg/kg) at 1 day after pretreatment. The increase in the withdrawal threshold (antinociception) induced by morphine against mechanical stimulation by a von Frey filament applied to the plantar surface of the each hind paw was then measured at 45 min after morphine treatment. The antinociceptive effect of morphine was bilaterally reduced
at 1 day after CFA pretreatment (Fig. 5A). However, the reduced antinociception of morphine was completely restored in the contralateral paw and was partially restored in the ipsilateral paw by pretreatment with Ro-32-0432. In mice pretreated i.pl. with saline, the morphine antinociception in both the ipsilateral and contralateral paws was not altered by the i.t. pretreatment with Ro-32-0432.

Other groups of mice repeatedly pretreated i.t. with Ro-32-0432 (1 nmol) or DMSO for 4 days along with a single i.pl. pretreatment with CFA (50 μl) or saline in the left hind paw were injected s.c. with morphine (7.1 mg/kg) at 4 days after the i.pl. pretreatment, and the increase in the withdrawal threshold (antinociception) induced by morphine against mechanical stimulation by a von Frey filament applied to the plantar surface of each hind paw was measured at 45 min after morphine treatment. The antinociceptive effect of morphine was bilaterally reduced at 4 days after CFA pretreatment (Fig. 5B). However, the reduced antinociception of morphine was completely restored in the contralateral paw and was partially restored in the ipsilateral paw by repeated pretreatment with Ro-32-0432. In mice pretreated i.pl. with saline, the morphine antinociception in both the ipsilateral and contralateral paws was not altered by the repeated pretreatment with Ro-32-0432.

Discussion

CFA, which is commonly used as an emulsified antigen solution to produce antibodies, consists of mineral oil with inactivated and dried mycobacteria (usually M. tuberculosis). It stimulates cell-mediated immunity and potentiates the production of immunoglobulins as
an immunopotentiator (6). These reactions induced by CFA result in tissue inflammation at the site of injection. The administration of CFA into the knee joint in rodents causes knee joint inflammation, which is used as an experimental model for knee arthritis (7). However, inflammation in the hind paw, which is caused by the i.pl. administration of CFA, is used as an experimental model for inflammatory pain (8). In the present study, the i.pl. administration of CFA induced strong edema (an approximately 50% increase in paw volume), thermal hyperalgesia, and mechanical allodynia on the ipsilateral paw, which were apparent at 1 day after CFA injection and retained for 97 days, 16 days, and 34 days, respectively (data not shown). It has been established in rats that CFA-induced inflammation expands from the ipsilateral paw to the contralateral paw and that mechanical allodynia is observed bilaterally, whereas thermal hyperalgesia is observed in only the ipsilateral paw (1). In the present study, mechanical allodynia in mice was only observed in the ipsilateral paw, not in the contralateral paw, after CFA injection. Differences in the animal species used and in the magnitude of the induced inflammation may be involved in this discrepancy.

It is well established that the antinociceptive effect of morphine against thermal hyperalgesia and mechanical hyperalgesia is enhanced or retained in the CFA-induced inflammatory pain state (2, 3). However, in the present study, the antinociceptive effect of morphine injected s.c. against mechanical allodynia in the CFA-induced inflammatory pain state was reduced in the ipsilateral paw. Allodynia is defined as pain perceived in response to non-noxious stimuli after tissue or nerve injury and is considered to be mainly mediated through the Aβ fibers of the primary afferent nerves (9). In contrast, hyperalgesia is a hypersensitivity to noxious stimuli after tissue or nerve injury and is considered to be mainly mediated through the C fibers of the primary afferent nerves (10). The discrepancy in morphine antinociception between hyperalgesia and allodynia in the CFA-induced inflammatory pain state may be correlated to the neurons mediating each pain type. Interestingly, in the present study, the antinociceptive effect of morphine given s.c. against mechanical allodynia in the CFA-induced inflammatory pain state was also reduced in the contralateral paw. It has been reported that mechanical allodynia in the CFA-induced inflammatory pain state is observed bilaterally (1). Although we did not observe mechanical allodynia in the contralateral paw after CFA injection in the present study, inflammation may bilaterally reveal plastic changes in the primary afferent nerves and

Fig. 5. Effects of single (A) and repeated (B) pretreatment with protein kinase Ca inhibitor on the antinociceptive effect of morphine in the inflammatory pain state in mice. A) Groups of mice pretreated i.t. with protein kinase Ca inhibitor Ro-32-0432 (Ro: 1 nmol) or DMSO (D) along with the i.pl. pretreatment with CFA (50 μl) or saline in the left hind paw were injected s.c. with morphine (7.1 mg/kg) at 1 day after the pretreatment. B) Groups of mice repeatedly pretreated i.t. with the protein kinase Ca inhibitor Ro-32-0432 (Ro: 1 nmol) or DMSO (D) for 4 days combined with the single i.pl. pretreatment with CFA (50 μl) or saline in the left hind paw were injected s.c. with morphine (7.1 mg/kg) at 4 days after the i.pl. pretreatment. The increase in the withdrawal threshold (antinociception) induced by morphine against mechanical stimulation by a von Frey filament applied to the plantar surface of each hind paw was measured at 45 min after the morphine treatment. Each value represents the mean ± S.E.M. for at least 10 mice. **p < 0.01, ***p < 0.001 vs. DMSO + CFA-pretreated mice.
may affect the antinociceptive effect of morphine given s.c. against mechanical alldynia in both paws. The bilateral reduction of morphine antinociception against mechanical alldynia in the CFA-induced inflammatory pain state was also observed after the i.t. injection of morphine. The neural plastic changes, which may relate to the bilateral reduction of morphine antinociception against mechanical alldynia after CFA injection, may be present at least in the spinal site.

The most likely neural plastic changes to occur, which result in the bilateral reduction of morphine antinociception against mechanical alldynia after CFA injection, may be the down-regulation of spinal \(\mu\)-opioid receptors, which is observed in morphine-resistant neuropathic pain (11). Thus, in the present study, the mRNA expression levels of \(\mu\)-opioid receptors and the protein levels of \(\mu\)-opioid receptors after CFA injection at the spinal site were quantified using RT-PCR and western blotting analysis, respectively. As a result, the mRNA expression levels of \(\mu\)-opioid receptors were decreased bilaterally in the DRG at 1 day after CFA injection and in the lumbar spinal cord at 1 day and 4 days after CFA injection. In contrast, the decrease in the protein levels of \(\mu\)-opioid receptors was only observed in the ipsilateral DRG at 1 day after CFA injection, not in the lumbar spinal cord. These results suggest that the downregulation of \(\mu\)-opioid receptors in the ipsilateral DRG is at least involved in the reduced morphine antinociception against mechanical alldynia in the ipsilateral paw in the CFA-induced inflammatory pain state. However, the mechanism underlying reduced morphine antinociception against mechanical alldynia in the contralateral paw in the CFA-induced inflammatory pain state remains uncertain because the protein levels of \(\mu\)-opioid receptors in the contralateral DRG or lumbar spinal cord were not altered after CFA injection. It has been suggested that the phosphorylation of \(\mu\)-opioid receptors by PKC enhances the desensitization of \(\mu\)-opioid receptors by retaining the desensitized \(\mu\)-opioid receptors at the membrane surface (12). Thus, in the present study, involvement of the phosphorylation of \(\mu\)-opioid receptors by PKC in the reduction of morphine antinociception against mechanical alldynia in the CFA-induced inflammatory pain state was investigated. PKC has several isoforms known as conventional PKC (\(\alpha, \beta I, \beta II, \) and \(\gamma\)), novel PKC (\(\delta, \epsilon, \eta, \) and \(\theta\)) and atypical PKC (\(\zeta, \iota, \) and \(\lambda\)) (13). Among these isoforms, PKCa has been reported to be distributed in both pre-synaptic and post-synaptic neurons and to play an important role in the desensitization of opioid receptors (14). In the present study, the reduced antinociception of morphine against mechanical alldynia in the CFA-induced inflammatory pain state was completely restored in the contralateral paw but was only partially restored in the ipsilateral paw by i.t. pretreatment with Ro-32-0432, a cell-permeable selective inhibitor for PKCa. These results clearly suggest that the desensitization of spinal \(\mu\)-opioid receptors by PKCa-induced phosphorylation is involved in the bilateral reduction of morphine antinociception against mechanical alldynia in the CFA-induced inflammatory pain state, particularly in the contralateral paw. In the present study, the phosphorylation levels of \(\mu\)-opioid receptors at CFA-induced inflammatory pain state were not examined. Therefore, the involvement of other PKC subtypes, as downstream signal transductions activated following the \(\mu\)-opioid receptor stimulation, in the bilateral reduction of morphine antinociception against mechanical alldynia in the CFA-induced inflammatory pain state can not be completely excluded at this point. To conform this hypothesis, extensive research may be required.

In conclusion, the antinociceptive effect of morphine was markedly reduced bilaterally against mechanical alldynia in the inflammatory pain state. The reduced morphine antinociception might be mainly mediated via a decrease in \(\mu\)-opioid receptors on the ipsilateral side and via the desensitization of \(\mu\)-opioid receptors on contralateral side as a result of PKCa-induced phosphorylation.

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