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Involvement of kappa opioid receptors in the formalin-induced inhibition of analgesic tolerance to morphine via suppression of conventional protein kinase C activation

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Abstract

Objectives Repeated morphine treatment results in a decreased analgesic effect or the development of analgesic tolerance. However, we reported that some inflammatory chronic pain may inhibit morphine tolerance via kappa opioid receptor (KOR) activation. In this study, we further investigated the role of KOR in the inhibition of morphine tolerance in a chronic pain condition with a focus on the regulation of protein kinase C (PKC) activity.

Methods Chronic pain was induced by formalin treatment into the dorsal part of the left hind paws of mice. The analgesic effect of morphine was measured by the tail flick method. We analysed the protein expression of PKC and its activity, and G-protein activity of mu opioid receptor (MOR) under repeated morphine treatment with or without formalin treatment.

Key findings We found that conventional subtypes of PKC (cPKC) were up-regulated by repeated morphine treatment. Also, antisense oligonucleotide (AS-ODN) targeting cPKC completely suppressed the development of morphine tolerance. The disappearance of the repeated morphine-induced up-regulation of cPKC was completely reversed by treatment with AS-ODN targeting KOR. In addition, AS-ODN targeting KOR significantly reversed the chronic pain-induced down-regulation of PKC activity or up-regulation of MOR [^{35}S]GTPγS binding activity after repeated morphine treatment.

Conclusions These results indicate that KOR plays an important role in the inhibition of repeated morphine-induced cPKC up-regulation under chronic pain condition. Furthermore, this may result in the increase of MOR activity and in the inhibition of morphine tolerance under chronic pain condition.

Keywords chronic pain; kappa opioid receptor; morphine tolerance; protein kinase C; receptor desensitization

Introduction

Opioids are one of the major treatments for cancer pain in palliative care. However, it has been shown that chronic opioid treatment weakens the anti-nociceptive effect of opioids (i.e. the development of analgesic tolerance to morphine),[1,2] which may be one of the reasons for the discontinuance of opioid therapy. There have been several putative mechanisms that could lead to the development of analgesic tolerance to morphine, including the bimodal opioid regulation system (i.e. preferential activation of excitatory pathways), counter-regulatory mechanisms with release of anti-opioid neuropeptides and glial cell activation that produces pain augmentation. Furthermore, the possible pathways may include NMDA activation,[3] increase in substance P,[4] dynorphin synthesis,[5] neuropeptide FF,[6] cholecystokinin,[7] nociceptin/orphanine up-regulation[8] and iNOS,[9] while details have yet to be determined.[10]

We, as well as others, have found that the analgesic tolerance could be suppressed under chronic pain conditions.[1,11] Specifically, we found that the inhibitory effect of chronic pain on analgesic tolerance to morphine is mediated by kappa opioid receptors (KOR).[1] Some reports suggesting that chronic pain up-regulates the expression of dynorphin, an endogenous KOR agonist within the spinal cord, might support our study, while contradictions still

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exist regarding the role of KOR under stress conditions, indicating that further research must be conducted.[12,13] Recently, we found that the mechanisms underlying KOR-mediated preservation of chronic morphine analgesia under chronic pain conditions may be partly due to the suppression of protein kinase C (PKC) activation and prevention of mu opioid receptor (MOR) desensitization.[2]

Here, we investigated the role of KOR-mediated PKC regulatory pathway in the inhibition of analgesic tolerance to morphine under chronic pain condition with clearer analysis using specific gene targeted antisense oligodeoxynucleotides. Specifically, we tried to identify specific PKC subtypes responsible for the development of analgesic tolerance to morphine or KOR-mediated inhibition of morphine tolerance.

Materials and Methods

Animals

Male ddY mice, 18–20 g (purchased from Saitama Experimental Animals, Saitama, Japan), were housed eight per group in plastic bracket cages maintained at a constant temperature of 22 ± 1°C. They had free access to food and tap water and were used in experiments at a body weight of 23–28 g. All experimental procedures conformed to the Guiding Principles for the Care and Use of Laboratory Animals adopted by the Japanese Pharmacological Society. In addition, all experiments were approved by the ethical committee for animals of Kobe Gakuin University.

Drugs

The following drugs and substances were used: morphine HCl (Takeda, Osaka, Japan), U-50,488H (trans-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate hydrate (Sigma, St Louis, USA), formalin (Wako, Osaka, Japan), calphostin C (Wako), antisense oligodeoxynucleotide (AS-ODN) targeting PKCα (5′-GAAA ACGTCAGCCATGGTCC-3′), PKCβ (5′-CGCAGCCGGG TCAGCATC-3′), PKCγ (5′-AGGGCCCAGACCCGCCAT-3′), PKCδ (5′-GAAGGAGATGCGCTGGAA-3′), PKCε (5′-GCCATTGAACACTACCAT-3′), PKCι (5′-TCCTGCTGG GCAT-3′) and KOR (5′-GGTGCCTCCAAGGACTATCGC-3′), primary antibodies for PKCα, β, γ (anti-PKCα rabbit: Calbiochem, USA, anti-PKCβ, γ rat: BD Transduction Laboratories, NJ, USA), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Chemicon, Temecula, USA). Twenty microlitres of a 2% formalin solution was administered subcutaneously into the dorsal part of the left hind paws of the mice. Morphine HCl (10 mg/kg) and U-50,488H (2 mg/kg) were dissolved in physiological saline, and subcutaneously and intraperitoneally administered. Calphostin C was dissolved in 50% dimethyl sulfoxide (DMSO) and diluted with physiological saline at the time of use. According to the method described by Haley and McCormick,[14] 10 μl of calphostin C (3 pmol/mouse) was intracerebroventricularly (i.c.v.) administered 10 min after administration of morphine. [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) was purchased from Sigma and [^{35}S]GTPγS was from Muromachi Kagaku (Tokyo, Japan).

Measurement of analgesic effect

The analgesic effect was measured by a tail-flick analgesic meter (MK-330B; Muromachi Kikai, Tokyo, Japan). To avoid tissue damage, the response time was measured up to a maximum of 10 s. Measurements were taken every 30 min for 90 min. To test the development of analgesic tolerance, the analgesic effect of morphine (10 mg/kg, s.c.) was measured once daily for five days, as we reported previously.[1,2] The difference in tail-flick time on each day compared with the first day was plotted against time (days of treatment), and the area under the curve (AUC) was calculated.

Antisense oligodeoxynucleotide treatment

AS-ODN targeting KOR and PKCα, β, γ, δ, ε, ι were administered (10 μg per mouse, i.c.v.) daily for three days before the start of measurements and for 1–3 days after measurements were initiated.

Western blotting

Unanaesthetized mice were decapitated, then midbrain sections were immediately prepared under ice cooling according to the method described by Glowinski and Iversen.[15] Midbrain sections were homogenized in 1 ml of sucrose-Tris buffer (STB) (1 M Tris-HCl, 0.5 M EDTA, 0.5 M EGTA, 250 mM sucrose, 0.3% β-mercaptoethanol (β-ME), 10 mg/ml leupeptin, 10 mg/ml pepstatin and 1 mg/ml aprotinin), centrifuged (100 000*g*, 30 min, 4°C), and the supernatant was used as the cytoplasmic fraction. The samples were applied to 10% acrylamide gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Blocking buffer (PBS with 0.2% Aurora blocking reagent, 0.1% Tween 20) was applied to the membrane for 90 min at room temperature. Membranes were then incubated with mouse anti-GAPDH monoclonal antibody (1 : 200) and with anti-PKCα (1 : 100), anti-PKCβ (1 : 250) or anti-PKCγ(1 : 1000) primary antibodies in blocking buffer for 1 h at room temperature. After washing with the wash buffer (PBS with 0.1% Tween 20), membranes were incubated with secondary antibodies (1 : 1000) for 1 h in blocking buffer. After washing, all immunoreactive bands were detected with ECL system (Amersham biosciences, Little Chalfont, UK).

Measurement of protein kinase C activity

The midbrain sections were homogenized in 1 ml buffer A (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% β-ME, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and cytoplasmic and membrane fraction were obtained as described previously.[2] Measurements were performed using Mesacup protein kinase assay kit (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions.[2] Briefly, the cytosolic or membrane fractions were incubated in the phosphorylation solution (25 mM Tris-HCl, pH 7.0, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM ATP, 50 μg/ml phosphatidylserine (PS), 0.5 mM EDTA, 1 mM EGTA, 5 mM β-ME) at 25°C for 10 min in Microwell strips coated with PS peptide. After incubation with the biotinylated antibody 2B9 and peroxidase (POD)-conjugated streptavidin, reactions were stopped and the absorbance at 492 nm was measured.

Southern blot analysis

Midbrain sections were prepared as described above, and then immediately freeze-dried using liquid nitrogen. The total RNA was then extracted using the acid–guanidium thiocyanate–phenol–chloroform method, as described previously.[2,16] Reverse transcription was performed using Super-Script II RNase H reverse transcriptase (Gibco BRL; Rockville, USA). The 5′-primer used for PCR was 5′-GCAGAGCTTCTTCCAGTC-3′ and the 3′-primer used was 5′-CACCACAGAGTAGACAGC-3′.[2,17,18] PCR samples (20 μl) were run in 2% agarose gels and then transferred to Nytran N (Schleicher & Schuell, Keene, USA). Probes used in hybridization experiments were directed against KOR (cDNA 400 bp; 30–429) and were labelled with [^{32}P]dCTP. Radioactivity was quantified using an image analyser (BAS 3000; Fuji Film, Kanagawa, Japan) and an autoradiogram prepared on X-ray film.

Measurement of [^{35}S]GTPγS binding activity

The midbrain sections were homogenized in 1 ml Tris-EDTA-sucrose (TES) buffer (25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.32 M sucrose) and membrane fractions were obtained as described previously.[2] Membranes were incubated for 60 min in a shaking water bath at 30°C in 0.5 M HEPES-KOH, pH 7.5, containing 50 mM EGTA, 5 M NaCl, 1 M $MgCl_2$, 3 mM GDP, 100 nM [^{35}S]GTPγS, and 0.01–10 μM DAMGO or distilled water, as described previously.[2,19] Samples were passed through nitrocellulose filters (Advantec, Fremont, USA). Each filter was counted using an image analyser (BAS 3000; Fuji Film).

Statistical analysis

Data are presented as mean ± SEM. Significant differences were evaluated by one-way analysis of variance followed by Dunnett's multiple comparison tests for the comparison between more than three groups or by Student's *t*-test for the comparison between two groups. $P < 0.05$ was regarded as significant.

Results

Effect of protein kinase C inhibitor on the development of morphine tolerance

As shown in Figure 1a, the analgesic effect of morphine was gradually attenuated and then completely eliminated by repeated administration (open circles, 10 mg/kg of morphine,

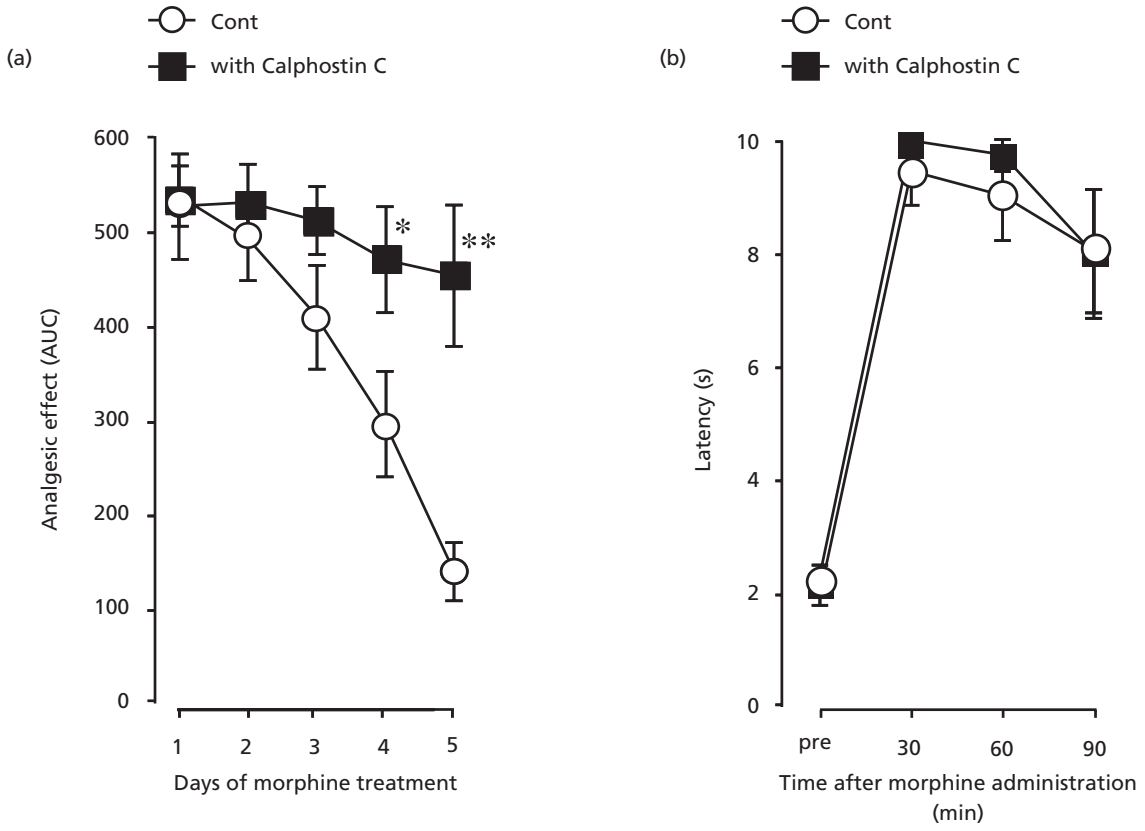

Figure 1 Effect of calphostin C on the development of analgesic tolerance to morphine. Calphostin C (3 pmol/mouse, i.c.v. 0 was administered daily 10 min after the morphine (10 mg/kg, s.c.) for five days. Control mice (Cont) were treated with 50% DMSO instead of calphostin C for five days. (a) Change in the analgesic effect of morphine with repeated daily morphine administration (expressed as AUC). Each point indicates mean ± SEM, $n = 8$. (b) The analgesic effect of morphine on day 1. Each point indicates mean ± SEM, $n = 8$; $^*P < 0.05$, $^{**}P < 0.01$ vs corresponding value with morphine alone (Cont).

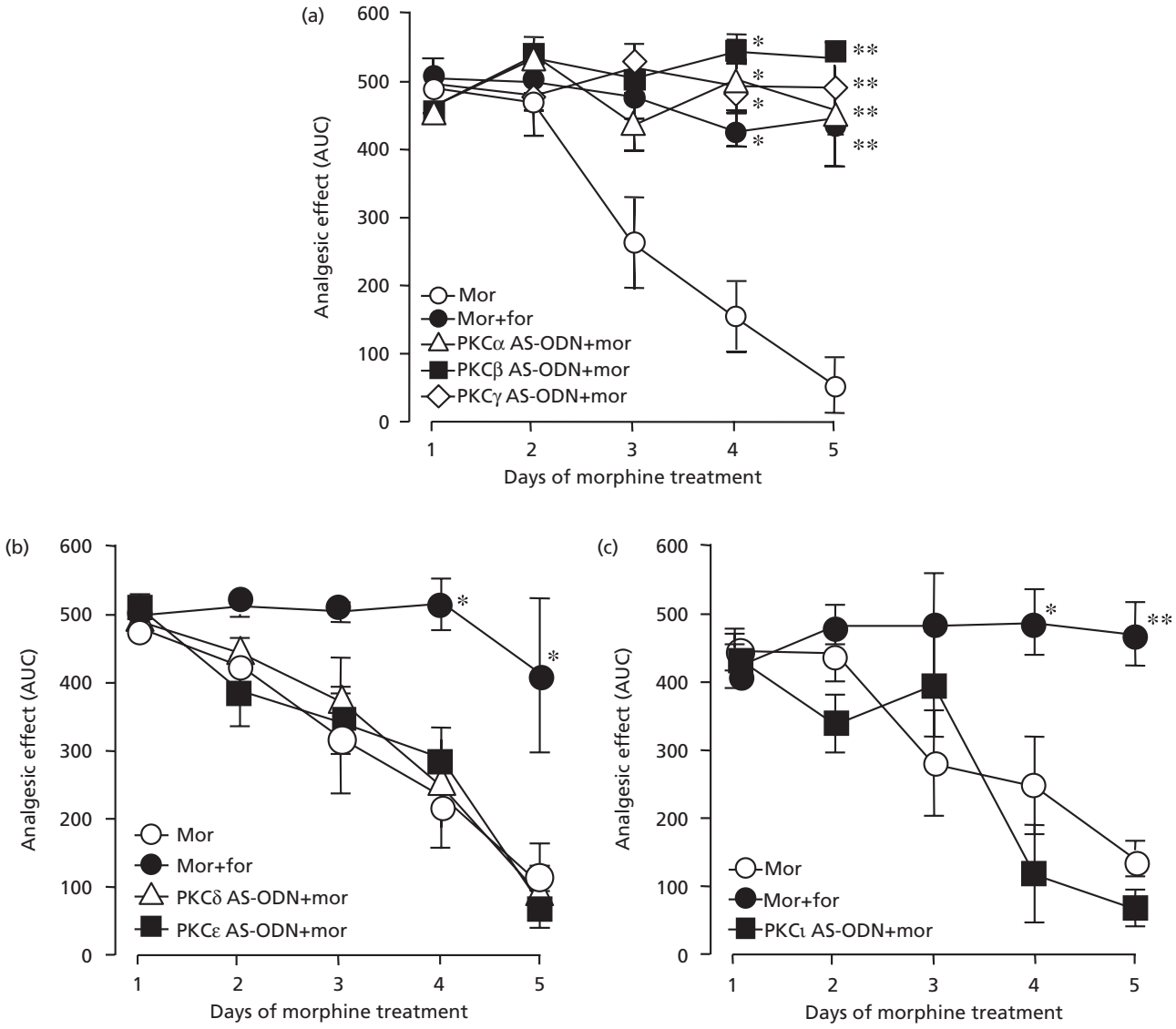

Figure 2 Effect of formalin pretreatment and AS-ODN against (a) conventional, (b) novel and (c) atypical PKC on the development of morphine tolerance. AS-ODN (10 μg, i.c.v.) was administered daily for three days before the start of the measurement of analgesic effects, and it was also administered on days 1–3 of the repeated morphine treatment (mor; 10 mg/kg, s.c.). Formalin (for; 2%, s.c.) was administered into the dorsal part of left hind-paw 24 h before the start of experiments. Each point indicates mean ± SEM, n = 8. PKC, protein kinase C. *$P < 0.05$, **$P < 0.01$ vs corresponding value with morphine alone.

s.c., for five days). Calphostin C, a PKC inhibitor, had no effect on the initial analgesic effect of morphine (Figure 1b). On the other hand, compared with the control group, concomitant daily administration of calphostin C (closed squares) significantly inhibited the development of morphine tolerance.

The involvement of conventional protein kinase C in the development of morphine tolerance

The development of analgesic tolerance to morphine was completely suppressed by treatment of AS-ODN against PKCα, β and γ (Figure 2a), while AS-ODN against PKCδ, ε and ι did not have any effect (Figure 2b, c). In the formalin-pretreated mice, the development of morphine tolerance was not observed (Figure 2).

Effect of chronic pain on the up-regulation of conventional protein kinase C after repeated morphine treatment

As shown in Figure 3, the protein expression levels of PKCα, β and γ subtypes increased after repeated morphine treatment, while it was completely suppressed by the formalin pretreatment in the cytoplasmic fraction of midbrain. On the other hand, formalin *per se* did not affect expression levels at all.

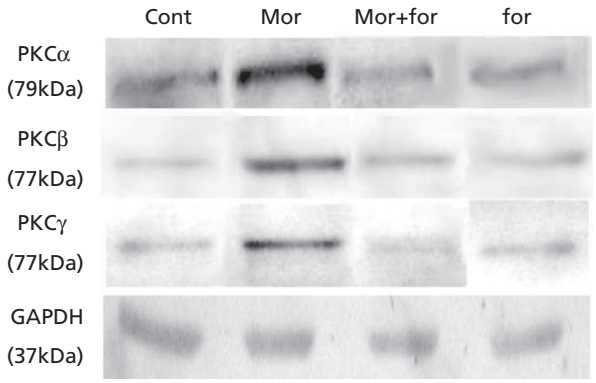

Figure 3 Effect of formalin pretreatment on the repeated morphine induction of conventional PKC. Mice were treated with morphine (Mor; 10 mg/kg, s.c.) for five days. Control mice (Cont) were treated with saline instead of morphine. Formalin (for; 2%, s.c.) was administered into the dorsal part of the left hind-paw 24 h before the start of experiments. Mice were decapitated and the midbrain was dissected 24 h after the last morphine administration. The cytosolic fraction was used for Western blot analysis. Image shows the representative photograph.

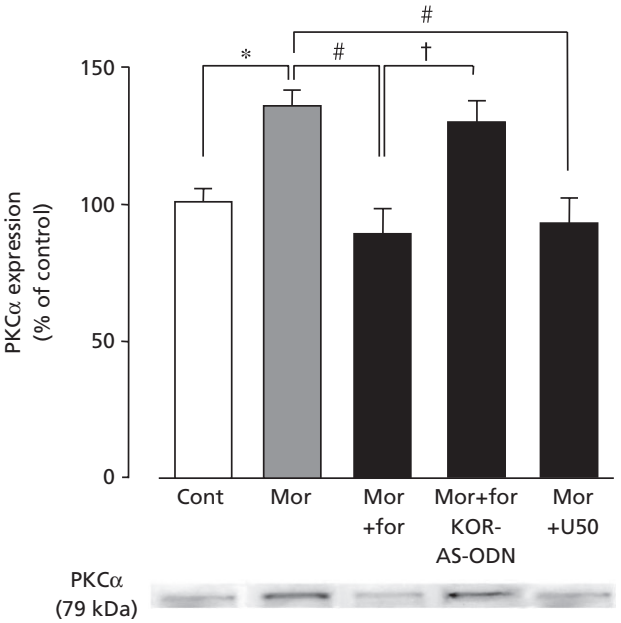

Figure 4 Effect of AS-ODN against KOR on the inhibiting effect of formalin pretreatment on up-regulation of PKCα after repeated morphine treatment. Mice were treated with morphine (Mor; 10 mg/kg, s.c.) daily for five days. Control mice (Cont) were treated with saline instead of morphine. Formalin (for; 2%, s.c.) was administered into the dorsal part of the left hind-paw 24 h before the start of experiments. AS-ODN against KOR was administered as described in Materials and Methods. U-50,488H (U50; 2 mg/kg, i.p.) was administered 5 min after the morphine treatment. Mice were decapitated and the midbrain was dissected 24 h after the last morphine administration. The cytosolic fraction was used for Western blot analysis. Each column indicates mean ± SEM, $n = 3$; $^{\dagger}P < 0.05$, $^{*}P < 0.05$, $^{\#}P < 0.05$.

The involvement of kappa opioid receptors in the inhibitory effect of chronic pain on up-regulation of protein kinase Cα after repeated morphine treatment

The significant up-regulation of PKCα by repeated morphine treatment was completely suppressed by formalin pretreatment, while it was reversed by AS-ODN against KOR (Figure 4). Furthermore, U-50,488H, a typical KOR agonist, significantly inhibited the up-regulation of PKCα induced by repeated morphine treatment (Figure 4).

The involvement of kappa opioid receptors in the inhibitory effect of chronic pain on activation of PKCα after repeated morphine treatment

As shown in Figure 5, repeated morphine treatment significantly increased the PKC activity in the membrane fraction but not in the cytosolic fraction. The enhancement of membrane PKC activity induced by repeated morphine treatment was completely suppressed by formalin pretreatment (Figure 5b). Furthermore, cytosolic PKC activity was also significantly decreased by formalin pretreatment (Figure 5a). In addition, these effects of formalin pretreatment were significantly reversed by AS-ODN against KOR both in cytosolic and membrane fractions (Figure 5).

Effect of chronic pain on alteration of kappa opioid receptor gene expression after repeated morphine treatment

As shown in Figure 6, repeated morphine treatment (10 mg/kg, s.c. for five days) significantly increased the expression of KOR mRNA in the midbrain. On the other hand, the increase of KOR mRNA by repeated morphine treatment was completely inhibited by formalin pretreatment. In addition, formalin *per se* did not affect the expression levels of KOR mRNA (Figure 6).

The involvement of kappa opioid receptors in the chronic pain-induced increase of GTPγS activity of mu opioid receptors

As shown in Figure 7, the GTPγS activity of MOR was decreased after repeated morphine treatment, while it was significantly suppressed by formalin pretreatment. Rather, the GTPγS activity in formalin-pretreated mice was significantly increased (Figure 7, closed circles). On the other hand, the enhancement of GTPγS activity by formalin pretreatment was completely suppressed by AS-ODN against KOR (Figure 7, open triangles).

Discussion

Here, we observed that calphostin C, which has been generally used as a novel and potent inhibitor of PKC, could reversibly inhibit the development of morphine tolerance induced by repeated morphine administration (Figure 1). PKC has been shown to serve various functions in the nervous system, including modulation of ion channel/neurotransmitter receptor sen-

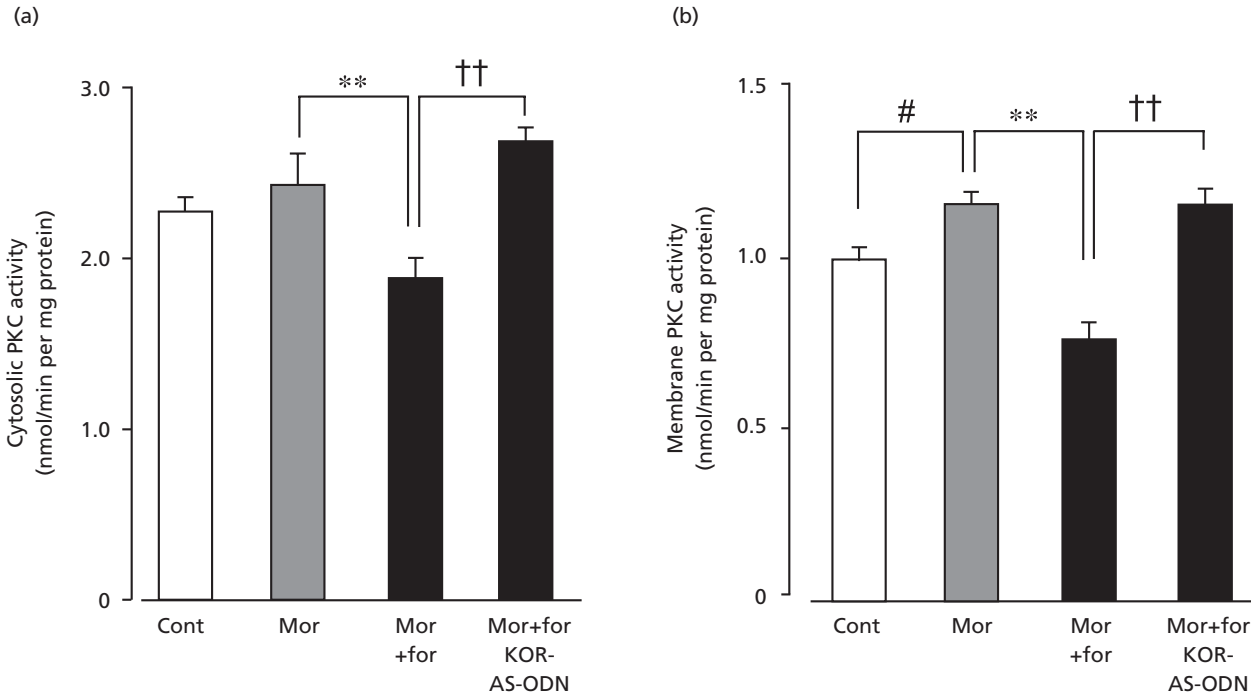

Figure 5 Effect of AS-ODN against KOR in the inhibitory effect of formalin pretreatment on the activation of PKC after repeated morphine treatment. Mice were treated with morphine (Mor; 10 mg/kg, s.c.) daily for five days. Control mice (Cont) were treated with saline instead of morphine. Formalin (for; 2%, s.c.) was administered into the dorsal part of the left hind-paw 24 h before the start of experiments. AS-ODN against KOR was administered as described in Materials and Methods. Mice in all groups were decapitated 15 min after morphine administration. PKC activity was measured in the (a) cytosolic and (b) membrane fractions of the midbrains. Each point indicates mean ± SEM, $n = 3$; $^{\#}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{**}P < 0.01$.

sitivity and regulation of gene expression leading to synaptic plasticity.[20] One possibility is that PKC activation may be related to receptor autophosphorylation in the mechanism of the development of morphine tolerance.[21] Here, in contrast to the significant induction of PKC in the cytosolic fraction, PKC activation by repeated morphine administration was observed significantly in the membrane fraction rather than in the cytosolic fraction, suggesting the possibility that PKC-mediated modulation of MOR sensitivity might be involved in the development of analgesic tolerance to morphine (Figure 5).

The PKC family of isoenzymes, known as serine/threonine kinases, is categorized into three subtypes: conventional (cPKC) (α, βI, βII and γ), novel (nPKC) (δ, ε, η, μ (also known as PKD) and θ), and atypical (aPKC) (ζ and ι/λ).[22] Each subtype of PKC functions as key proteins in a variety of cellular processes, including receptor desensitization/internalization, ion channel modulation, neurotransmitter release, synaptic plasticity and survival.[20] Furthermore, each process is reported to be involved in the mechanism of analgesic tolerance to morphine.[3] In this study, treatment with AS-ODN targeting PKCα, β and γ, but not with AS-ODN targeting PKCδ, ι and ε, completely inhibited the development of morphine tolerance. In addition, the expression levels of PKCα, PKCβ and PKCγ in midbrain, known as one of the essential brain regions responsible for the anti-nociceptive effects of opioids, were obviously increased by repeated morphine treatment. These findings suggest that among over 10 types of PKC, PKCα, β and γ, comprising the conventional subtype, but not novel PKC, could be responsible for the development of analgesic tolerance to morphine. The differences in the calcium requirement for activation for each of the PKC subtypes may contribute to the differences in their involvement in the development of morphine tolerance. Other reports demonstrating that NMDA receptors or intracellular calcium is closely linked to the development of morphine tolerance[23,24] might support our study. In addition, Belanger *et al*. reported that chronic exposure to opiates led to an up-regulation of PKCα followed by the induction of neuropeptides such as calcitonin gene-related peptide and substance P in dorsal root ganglion cells, which may be involved in the mechanism of morphine tolerance and may support our results.[25]

Although the involvement of PKC in the development of morphine tolerance had been demonstrated previously,[24,26] our study additionally implicated the involvement of KOR-mediated negative regulation of PKC in the inhibition of the development of morphine tolerance under a chronic pain condition. For our model of chronic pain, we used formalin pretreatment on mice before the repeated administration of morphine. It had already been confirmed that swelling persisted for at least 10 days and formalin-related mechanical hyperalgesia estimated by the paw pressure Randall-Selitto test was observed for 14 days after formalin administration.[1] Under this chronic pain condition, the development of analgesic tolerance to morphine and the induction of cPKC in mid-

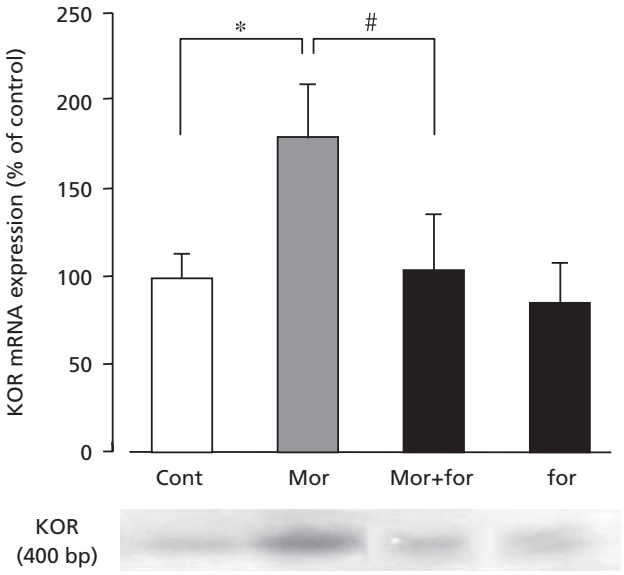

Figure 6 Effect of formalin pretreatment on the repeated morphine-induced alteration of KOR mRNA expression in the midbrain. Mice were given morphine (10 mg/kg) or saline (control; Cont) subcutaneously daily for five days. Formalin (for; 2%, s.c.) was administered into the dorsal part of the left hind-paw 24 h before the start of experiments. Mice were decapitated and the midbrain removed 24 h after the last administration of morphine. Quantitative analysis of KOR gene expression and representative photographs of KOR gene expression were determined with Southern blot analysis. Ratios of Image Hyper II analysis are percentages of control. Each point is the mean ± SEM, $n = 6$; $^{*}P < 0.05$, $^{\#}P < 0.05$.

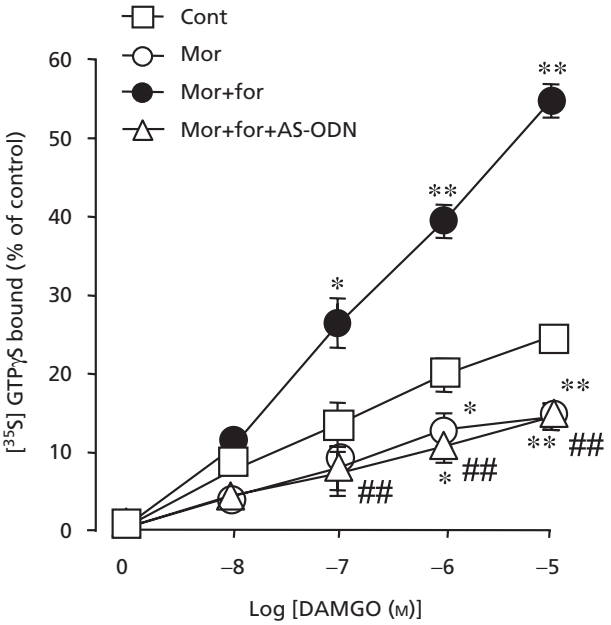

Figure 7 Effect of AS-ODN against KOR on the increase of GTPγS activity of MOR by formalin pre-treatment. Mice were given morphine (10 mg/kg, s.c.; Mor) or saline (Cont) daily. Membrane fractions were prepared 24 h after the last dose of morphine. Formalin (for; 2%, s.c.) was administered into the dorsal part of the left hind-paw 24 h before the start of experiments. AS-ODN against KOR was administered as described in Materials and Methods. Data represent the percentages of the basal activity without DAMGO and are mean ± SEM, $n = 5$; $^{*}P < 0.05$, $^{**}P < 0.01$ vs corresponding value with Cont. $^{\#\#}P < 0.01$ vs corresponding value with repeated morphine treatment under chronic pain condition (Mor + for).

brain by repeated morphine treatment were completely inhibited (Figures 2 and 3). In our findings, KOR activation seems to inhibit both the induction and activation of cPKC, especially PKCα in midbrain under a chronic pain condition (Figures 3–5). The observation that formalin pretreatment significantly suppressed the basal PKC activity might be due to the KOR-mediated suppression of protein expression levels of PKC. As we described above, predominantly, PKC has been considered to be involved in MOR desensitization as the mechanism of analgesic tolerance to morphine.[27] In addition, from our observation (Figure 7), KOR-mediated suppression of PKC activation may be important to maintain the MOR sensitivity.

Interestingly, as we have reported previously, after repeated morphine treatment, mRNA levels of KOR significantly increased.[2] This effect is supported by another report demonstrating that the binding number could be increased in a morphine-resistant condition.[28] Since the increase of KOR mRNA completely disappeared under chronic pain condition (Figure 6), it is likely that KOR activation under a chronic pain condition might be due to an increase of some endogenous KOR agonists rather than a quantitative increase of KOR, although this relationship remains to be determined.

Since chronic inflammation results in far greater changes to global signalling cascades than simply effects on PKC, the detailed mechanisms upstream of PKC inhibition need to be identified. From our consecutive studies, it is considered that some interaction between KOR and MOR may be involved in the mechanism. Interestingly, it is widely known that despite the high degree of homology among the opioid receptors, MOR and KOR seem to have opposing effects in central nervous systems or in inflammatory systems,[29–32] while details are unclear. It is important to elucidate downstream signalling of KOR relating to the inhibition of morphine-induced cPKC up-regulation in the future.

Again, it is hypothesized that under a chronic pain condition, the inhibition of the development of analgesic tolerance to morphine occurred by activation of KOR-mediated negative regulation of cPKC-related intracellular signalling pathways, such as induction of pro-nociceptive factors, without increasing the number of KOR.

Conclusions

In this study, we simply demonstrated that cPKC is involved in the mechanism of the development of analgesic tolerance to morphine. Furthermore, under chronic pain conditions, the increase in activity of cPKC is inhibited by activation of KOR, which eventually inhibits the development of morphine tolerance via inhibiting the decrement of MOR GTPγS activity. Although the detailed mechanism underlying KOR mediated PKC inhibition remains to be elucidated, in consideration of our latest series of study,[1,2] it is obvious that KOR activation

mediated cPKC inhibition would result in prevention of morphine tolerance. These findings may help the therapeutic advancement for patients with morphine tolerance.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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