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Involvement of kappa opioid receptors in the inhibition of receptor desensitization and PKC activation induced by repeated morphine treatment

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Abstract

Analgesic tolerance to morphine can develop from long-term use of this drug for the treatment of pain. Many reports have shown that stimulation of the kappa opioid receptor (KOR) suppresses development of analgesic tolerance to morphine. Here, we studied the KOR-mediated inhibition of morphine tolerance during repeated morphine treatment, with a focus on desensitization of the receptor. The development of analgesic tolerance to morphine during repeated morphine administration (10 mg kg^{-1} s.c.) was completely suppressed by U-50488H (2 mg kg^{-1} i.p.), a KOR agonist. The decrease in [^{35}S] GTPγS binding activity stimulated by the mu opioid receptor (MOR) agonist [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) was also significantly inhibited by U-50488H. These results indicate that stimulation of KOR caused by repeated morphine treatment either inhibits MOR desensitization or accelerates recycling of MOR on the cell surface, thereby suppressing morphine tolerance. Furthermore, we found that activity of protein kinase C (PKC) was significantly decreased in mice treated with both U-50488H and morphine. These results suggest that the mechanisms underlying KOR-mediated inhibition of analgesic tolerance to morphine may be partly due to suppression of PKC activation and prevention of receptor desensitization.

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Introduction

Morphine has a major role in the treatment of cancer pain. Broad clinical evidence has shown that tolerance and dependence do not readily develop in patients receiving morphine for pain conditions (Suzuki 2001). Furthermore, we recently found that the inhibitory effect of chronic pain on development of analgesic tolerance to morphine seems to be mediated, at least in part, by kappa opioid receptors (KOR) (Tokuyama et al 2007). In response to analgesic tolerance caused by repeated administration of morphine, KOR agonists such as U-50488H (Tao et al 2000; Tokuyama et al 2007) and dynorphin (Brugos et al 2004) inhibit the development of tolerance at a dose at which no analgesic effect is observed. Moreover, this inhibitory effect was antagonized by nor-binaltorphimine (nor-BNI), a KOR antagonist, suggesting that KOR plays an important role in the mechanism of inhibition of morphine tolerance (Su et al 1998). Our previous data have also shown that inhibition of morphine tolerance under chronic pain conditions is blocked by antisense oligodeoxynucleotide directed against KOR (Tokuyama et al 2007). In addition, a condition of chronic pain caused the disappearance of the morphine-induced rewarding effect, which is an indicator of psychological dependence; this disappearance was prevented by KOR antagonists (Narita et al 2005). However, much is still unknown about the details of this mechanism.

Opioid receptors were first reported in 1992 (Evans et al 1992; Kieffer et al 1992). Three opioid receptor subtypes have been cloned: mu, delta and kappa. Each of these receptors has a molecular structure containing seven transmembrane domains and they are therefore classified as G-protein-coupled receptors (GPCR) (Ueda 1994). Recently, the desensitization (specifically, the loss of responsiveness) of opioid receptors has been considered to be one of the mechanisms by which tolerance to morphine develops. Some serine/threonine kinases such as GPCR kinase (GRK), protein kinase A (PKA) and protein kinase C (PKC) have been shown to play important roles in this mechanism. Specifically, PKC activity in the

brain has been observed to increase when tolerance has developed as a result of repeated morphine administration (Narita et al 1994; Mao et al 1995). Furthermore, various protein kinase inhibitors, including PKC inhibitors, are known to block the development of morphine tolerance (Ueda et al 2001; Smith et al 2006). In this study, we aimed to clarify the role of PKC in the mechanism of KOR-mediated inhibition of morphine tolerance and receptor desensitization.

Materials and Methods

Experimental animals

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

Drugs

Morphine HCl was from Takeda (Osaka, Japan); U-50488H (trans-3, 4-dichloro-*N*-methyl-*N*-(2-(1-pyrrolidinyl) cyclohexyl)-benzeneacetamide methanesulfonate hydrate, nor-BNI, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) and GTPγS were from Sigma (St Louis, MO, USA); [^{35}S] GTPγS was from Muromachi kagaku (Tokyo, Japan).

Morphine HCl, U-50488H and nor-BNI were dissolved in physiological saline, and the drug solutions were administered either s.c. or i.p. in a volume equivalent to 0.1 mL (10 g body weight)$^{-1}$.

Measurement of analgesic effect

The analgesic effect was measured using 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 after morphine administration, for 90 min. With a single administration of morphine and U-50488H, measurements were taken every 30 min for 90 min. To test the development of analgesic tolerance, the analgesic effect of morphine (10 mg kg^{-1}, s.c.) was measured once daily for 5 days as reported previously (Tokuyama et al 2007). The difference in tail-flick time on each day compared with the first day was plotted against time (day of treatment) and the area under the curve calculated.

Preparation of cell membrane fractions for the measurement of [^{35}S] GTPγS binding

Brains were removed, and midbrain sections were immediately prepared on ice according to the method described by Glowinski and Iversen (1996). The midbrain sections were homogenized in 1 mL Tris-EDTA-sucrose buffer (25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.32 M sucrose) and then centrifuged (14500 *g*, 5 min, 4°C). The resulting supernatant was diluted with Tris-EDTA-DTT buffer (25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2.5 mM DTT) followed by centrifugation (17610 *g*, 20 min, 4°C). The precipitate was suspended in incubation buffer (0.5 M HEPES-KOH, pH 7.5 containing 50 mM EGTA, 5 M NaCl, 1 M $MgCl_2$). These samples were considered as the membrane fractions. The protein content in these preparations was determined using a commercial kit (Bio-Rad protein assay kit), adjusted to a concentration of 0.05–0.1 mg mL^{-1}.

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

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 by Traynor and Nahorski (1995). Samples were passed through nitrocellulose filters (Advantec, Fremont, CA, USA). Each filter was counted using an image analyser (BAS 3000, Fuji Film, Kanagawa, Japan).

Preparation of cytoplasmic fractions and cell membrane fractions for the measurement of protein kinase C activity

Brains were removed, and midbrain sections were immediately prepared on ice according to the method described by Glowinski and Iversen (1996). 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-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)) using a Teflon homogenizer, and then centrifuged (100000 *g*, 30 min, 4°C). The resulting supernatant was the cytosolic fraction. The precipitate was suspended in buffer B (i.e. buffer A containing Triton X-100), agitated (30 min at 4°C), then centrifuged (20000 *g*, 10 min, 4°C). The resulting supernatant was the membrane fraction. The protein content in these preparations was determined using the Bio-Rad protein assay kit and samples were adjusted to a protein concentration of 0.5 mg mL^{-1}.

Measurement of protein kinase C activity

Measurements were performed using the Mesacup protein kinase assay kit (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions. 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^{-1} phosphatidylserine (PS), 0.5 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol) at 25°C for 10 min in microwell strips coated with PS peptide, after which the reaction was stopped by addition of 20% H_3PO_4. After incubation with biotinylated antibody 2B9 (25°C, 60 min) and peroxidase-conjugated streptavidin (25°C, 60 min), samples were reacted with H_2O_2 and O-phenylenediamine at 25°C for 5 min. Finally, reactions were stopped by addition of 20% H_3PO_4, and the absorbance at 492 nm measured.

Isolation of RNA

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 (Chomczynski & Sacchi 1987) with some modifications. The midbrain was homogenized in GTC solution (4.23 M guanidine thiocyanate, 26.4 mM sodium citrate, pH 7.0, 0.5% sarkosyl and 0.72% 2-mercaptoethanol). After extraction with phenol/chloroform, chromosomal DNA contaminants were removed using RNase-free DNase I (Wako, Osaka, Japan). mRNA was extracted by the TaKaRa Oligotex-dT30 mRNA purification kit (TaKaRa; Shiga, Japan) according to the manufacturer's protocol.

RT-PCR

Reverse transcription was performed using SuperScript II RNase H reverse transcriptase (Gibco BRL, Rockville, MD, USA). The amplification reaction was carried out in a final volume of 100 μL with 2.5U thermostable *Taq* DNA polymerase (TaKaRa LA Taq; Takara). The temperature steps were 1 min at 94°C, 1 min at 54°C and 1 min at 72°C. Twenty-seven cycles were performed and one additional cycle was added with a final elongation time of 15 min at 72°C. The primers used for the PCR were the 5′-primer 5′-GCAGAGCTTCTTCCAGTC-3′ and the 3′-primer 5′-CACCACAGAGTAGACAGC-3′ (Chien et al 1994; Kolesnikov et al 1996).

Southern blot analysis

PCR samples (20 μL) were run in 2% agarose gels and then transferred to Nytran N (Schleicher & Schuell; Keene, NH, USA). Hybridizations were performed overnight. Filters were washed twice in saline sodium citrate/0.1% sodium dodecyl sulfate at 60°C for 20 min. 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.

Statistical analysis

Data are presented as mean ± s.e.m. In behavioural tests, significant differences were evaluated by one-way analysis of variance followed by Dunnett's multiple comparison test. When measuring PKC activity, differences were evaluated by Student's *t*-test. A *P* value below 0.05 was regarded as significant.

Results

Effect of repeated morphine treatment on KOR gene expression in the midbrain

As shown in Figure 1A, repeated morphine treatments (10 mg kg^{-1} s.c. for 5 days) significantly increased the expression of KOR mRNA in the midbrain. Quantitative analysis revealed that mRNA levels were 1.7-fold greater in the mice treated repeatedly with morphine than in saline-treated mice (Figure 1B).

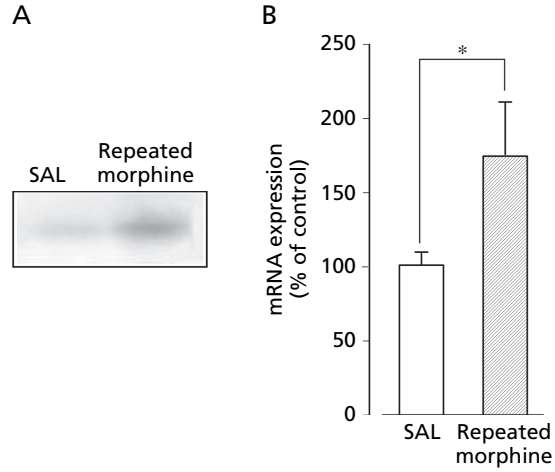

Figure 1 Effect of repeated morphine treatment on expression of KOR mRNA in the midbrain. Mice were given morphine (10 mg kg^{-1}) or saline (SAL) s.c. daily for 5 days. Mice were decapitated and the midbrain removed 24 h after the last administration of morphine. (A) Representative photographs of KOR gene expression determined with Southern blot analysis. (B) Quantitative analysis of KOR gene expression. Ratios of Image Hyper II analysis are percentages of control. Each point is the mean ± s.e.m. (n = 6); *$P < 0.05$.

Effect of U-50488H on the development of analgesic tolerance to morphine

In the control mice treated daily with morphine (10 mg kg^{-1} s.c. for 5 days), a significant decrease in morphine analgesia (morphine tolerance) was observed on days 3–5. However, the administration of U-50488H (2 mg kg^{-1} i.p.) 5 min after morphine administration inhibited the onset of tolerance. This inhibitory effect was completely antagonized by the KOR antagonist nor-BNI (1 mg kg^{-1} i.p.) (Figure 2). We confirmed that nor-BNI did not effect morphine analgesia or the development of morphine tolerance (data not shown).

Effect of U-50488H on DAMGO-stimulated [^{35}S] GTPγS binding activity in mice treated repeatedly with morphine

In control mice treated with saline, [^{35}S] GTPγS binding activity was dose-dependently increased by DAMGO, a mu opioid receptor (MOR) agonist. The DAMGO-induced increment of [^{35}S] GTPγS binding activity was significantly suppressed by repeated morphine (10 mg kg^{-1} s.c.) treatment compared with saline treatment (Figure 3A). The [^{35}S] GTPγS binding activity was significantly potentiated by concomitant treatment with U-50488H (2 mg kg^{-1} i.p.) and morphine (Figure 3B).

Effect of U-50488H on PKC activity in repeated-morphine-treated mice

In the control mice treated with a single dose of morphine (10 mg kg^{-1} s.c.), the cytoplasmic PKC activity in the midbrain was 2.27 ± 0.04 nmol min^{-1} (mg protein)$^{-1}$, and was not

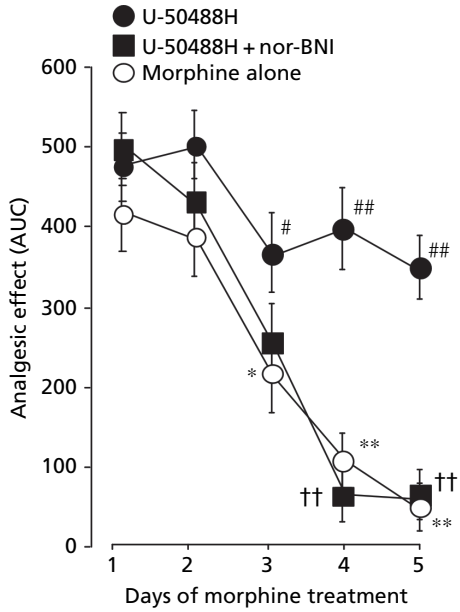

Figure 2 Effect of U-50488H on the development of analgesic tolerance to morphine. U-50488H (2 mg kg^{-1} i.p.; closed circles) was administered daily 5 min after the morphine (10 mg kg^{-1} s.c.) for 5 days. In mice treated with U-50488H + nor-BNI (closed squares), nor-BNI (1 mg kg^{-1} i.p.) was administered 10 min before morphine. Control mice (morphine alone; open circles) were treated with saline instead of U-50488H or nor-BNI. Each point indicates mean ± s.e.m. (n = 8); $^{*}P < 0.05$, $^{**}P < 0.01$ vs corresponding value on day 1. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs corresponding value of morphine alone on each day. $^{\dagger\dagger}P < 0.01$ vs value for U-50488H on each day. AUC, area under the curve.

affected by repeated morphine treatment. However, it was significantly decreased by the co-administration of U-50488H with morphine (Figure 4A). By contrast, in the cell membrane fraction, the PKC activity induced by a single dose of morphine (1.12 ± 0.02 mmol min^{-1} (mg protein)$^{-1}$) was significantly increased by repeated morphine treatment (Figure 4B). As with cytosolic PKC activity, the repeated-morphine-induced increment of cell membrane PKC activity was significantly inhibited by co-administration of U-50488H (Figure 4B).

Discussion

In this study, we confirmed that stimulation of KOR during repeated morphine treatment suppresses the development of analgesic tolerance to morphine (Figure 2), which is consistent with our previous report (Tokuyama et al 2007). Although we determined the inhibitory effect of U-50488H on the development of morphine tolerance using the tail-flick test, it is possible that the inhibitory effect of U-50488H on morphine tolerance could be observed using different paradigms for measuring analgesia other than the tail-flick test. Surprisingly, the mRNA for KOR in the midbrain increased significantly after repeated morphine treatment (Figure 1), suggesting a rationale for the increased sensitivity to KOR agonists under these conditions. As is well known, the midbrain periaqueductal grey matter and its descending projections to the rostral ventromedial medulla provide an essential neural circuit for the anti-nociceptive effects of opiates, and has been implicated in the development of tolerance to morphine. Since it is known that KORs have opposite effects to MORs (Ko et al 2003; Margolis et al

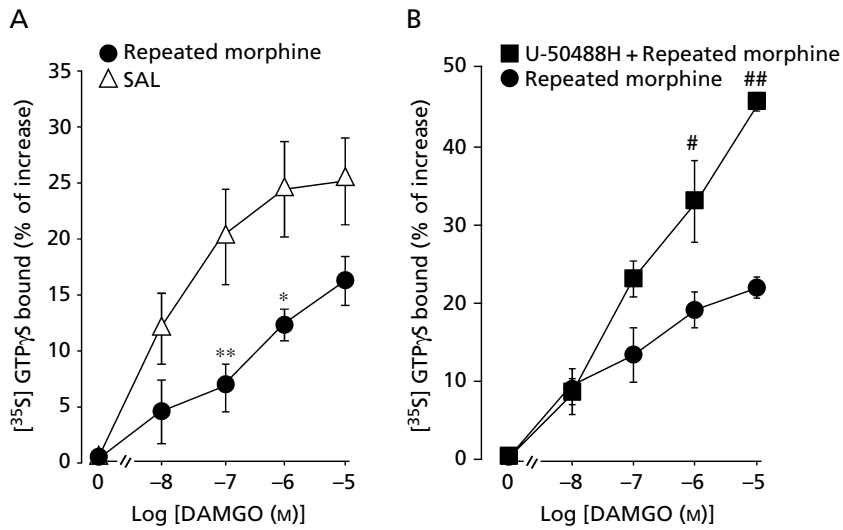

Figure 3 Change in DAMGO-stimulated [^{35}S] GTPγS binding activity in the midbrain region of mice treated repeatedly with morphine. (A) Effect of repeated morphine treatment on DAMGO-stimulated [^{35}S] GTPγS binding activity. Mice were given morphine (10 mg kg^{-1} s.c.) (closed circles) or saline (SAL; open triangles) daily. Membrane fractions were prepared 24 h after the last dose of morphine. (B) Inhibitory effect of U-50488H (2 mg kg^{-1} i.p.) on the repeated-morphine-induced decrease of DAMGO-stimulated [^{35}S] GTPγS binding activity. Mice were given morphine with or without U-50488H as described in Figure 2. Data represent the percentages of the basal activity without DAMGO and are mean ± s.e.m. (n = 5); $^{*}P < 0.05$, $^{**}P < 0.01$ vs corresponding value with SAL. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs corresponding value with repeated morphine treatment.

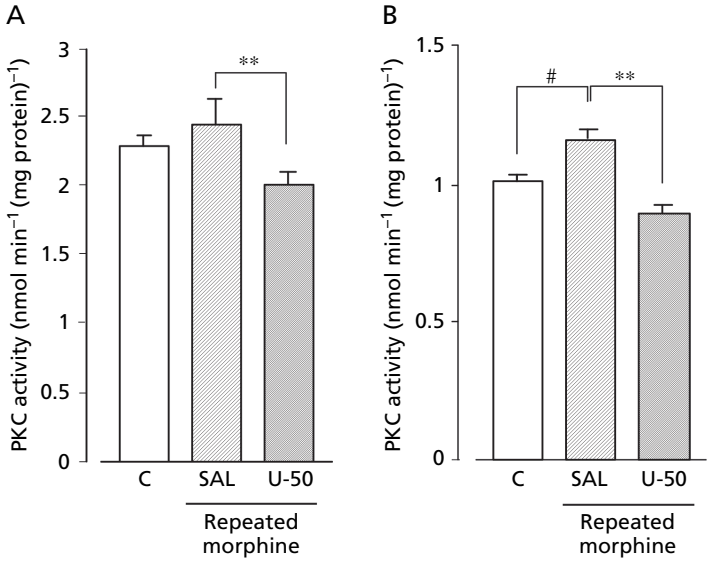

Figure 4 Effect of U-50488H on protein kinase C (PKC) activity in the midbrain region of mice treated repeatedly with morphine. All mice (repeated morphine) were given morphine (10 mg kg^{-1} s.c.) daily for 5 days. U-50488H (U-50; 2 mg kg^{-1} i.p.) was administered 5 min after morphine. SAL = treated with saline instead of U-50488H. Mice in all groups were decapitated 15 min after morphine administration. Control mice (C) were treated with a single dose of morphine (10 mg kg^{-1}). PKC activity was measured in the (A) cytosolic and (B) membrane fractions of the midbrains. Each point indicates mean ± s.e.m. (n = 4); $^{**}P < 0.01$; $^{\#}P < 0.05$.

2003), the increment of KOR mRNA observed in the present study might be the physiological compensatory response against the development of morphine tolerance. Furthermore, this phenomenon might help the minute dose of KOR agonist to activate KOR and exert the inhibiting effect on the development of morphine tolerance.

As shown in Figure 3A, the DAMGO-stimulated [^{35}S] GTPγS binding activity was significantly decreased in the membrane fractions of midbrain in mice repeatedly treated with morphine. G proteins are located at the top of the opioid signalling cascade and not only act as signalling mediators, but are also believed to directly affect ligand–receptor binding via mechanisms including receptor desensitization (Freedman & Lefkowitz 1996). Emerging data suggest that receptor desensitization, which causes a decrease in the stimulus response resulting from repeated drug administration, is the underlying molecular mechanism mediating analgesic tolerance to morphine (Waldhoer et al 2004). Other reports have shown that repeated morphine treatment potentiates receptor desensitization and reduces receptor resensitization (Dang & Williams 2004). In the present study, we found that the co-administration of U-50488H with morphine for 5 days significantly increased the sensitivity of [^{35}S] GTPγS binding activity stimulated by DAMGO (Figure 3B). These results suggest that KOR-mediated inhibition of MOR desensitization or acceleration of MOR recycling during repeated morphine treatment may prevent the development of analgesic tolerance to morphine.

It is well known that the structural changes in receptors caused by phosphorylation are a key process in the mechanism underlying desensitization of GPCRs (Yang & Xia 2006), and phosphorylation of many receptors is mediated by serine/threonine kinases such as GRK, PKA and PKC (Ferguson 2001). In this study, we investigated PKC activity in the midbrains of mice with or without repeated morphine treatments. PKC activity was increased significantly in membrane fractions from animals that developed tolerance through repeated morphine treatment compared with fractions from control mice given only a single dose of morphine (Figure 4B). Although this increment of PKC activity seems small, it has a significant meaning, as reported previously (Tokuyama et al 1995). This increase in PKC activity may have the potential to accelerate phosphorylation of MOR or MOR desensitization. By contrast, there was no significant increase in activation in the cytoplasmic fraction when morphine tolerance developed (Figure 4A), suggesting that the translocation of activated PKC to the cell membrane is important for phosphorylation and desensitization of MOR. These findings are consistent with recent reports suggesting the important roles of several types of PKC in the development of morphine tolerance (Lim et al 2005; Smith et al 2007). As shown in Figure 4, PKC activity in both cytoplasmic and cell membrane fractions was significantly attenuated by repeated co-administration of U-50488H and morphine. These findings suggest that KOR stimulation decreases PKC activity in both cytoplasmic and cell membrane fractions, causing MOR desensitization and morphine tolerance.

Conclusion

In this study we clarify part of the intracellular mechanisms governing KOR-mediated inhibition of morphine tolerance. Our findings suggest that the mechanisms underlying KOR-mediated inhibition of analgesic tolerance to morphine may be partly due to a decrease in PKC activation and prevention of MOR desensitization. Such knowledge may aid in clarifying the underlying mechanism behind the clinical observation that patients with chronic pain do not develop morphine tolerance.

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