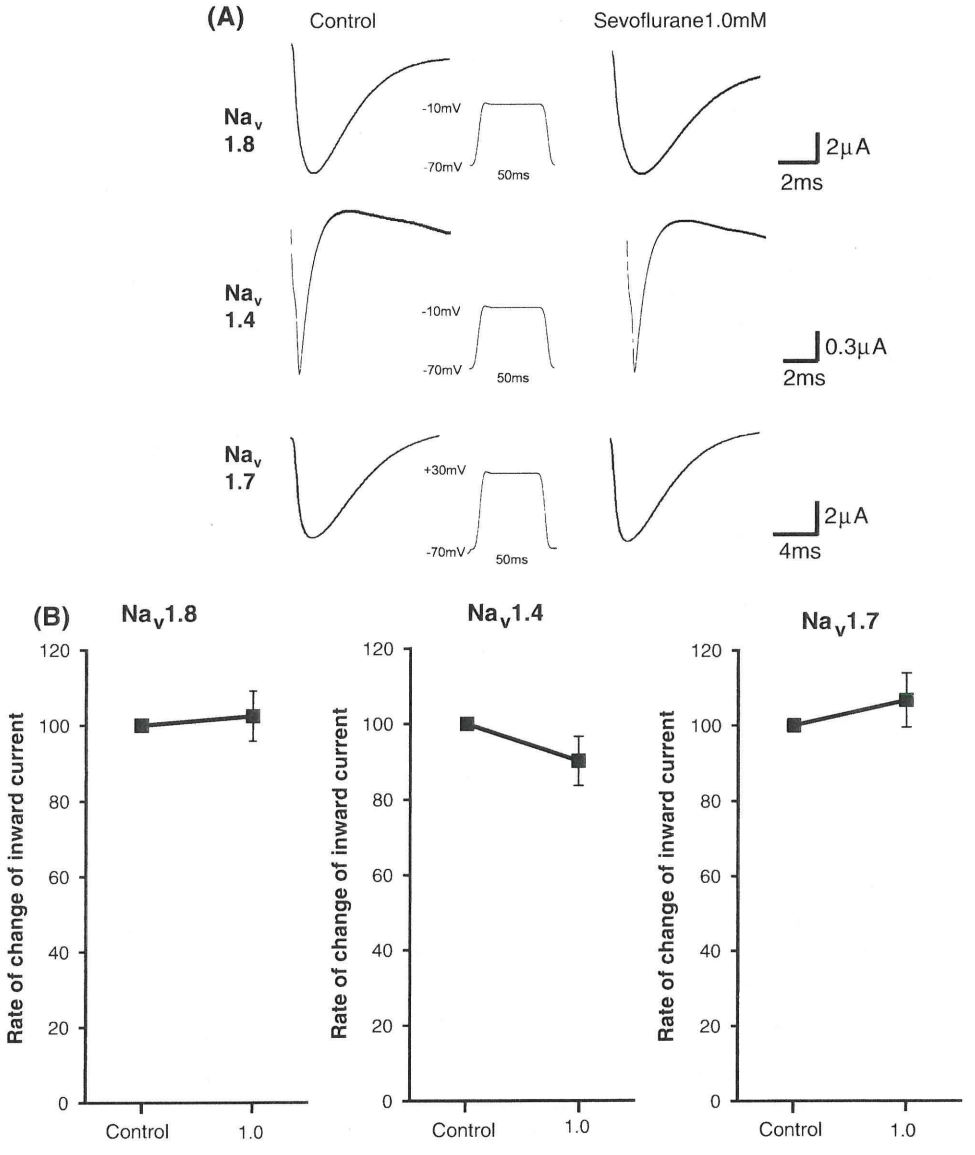

Fig. 3 Sevoflurane modulates voltage-gated sodium channels through the protein kinase C pathway. **a** Representative examples of the effect of bisindolylmaleimide I (GF109203X) on Na_v 1.8, Na_v 1.4 and Na_v 1.7. **b** Summary data for the effects of GF109203X on sevoflurane on peak inward current of voltage-gated sodium channels (Na_v 1.8, Na_v 1.4, and Na_v 1.7.). The effects were expressed as rate of change (± SEM)

1.4, and Na_v 1.7. In the control condition, the PKC inhibitor did not affect the voltage-gated inward currents. Pretreatment with GF109203X (200 nM) for 120 min abolished the sevoflurane-induced inhibition of voltage-evoked inward currents in *Xenopus* oocytes expressing Na_v1.4, Na_v1.8, and Na_v 1.7 (Na_v1.4, 90.2% ± 6.5% of control, $P > 0.05$, $n = 9$; Na_v1.8, 102% ± 6.6% of control, $P > 0.05$, $n = 11$; Na_v 1.7, 106% ± 7.2% of control, $P > 0.05$, $n = 9$) (Fig. 3a,b).

In our results, sevoflurane had little effects on the current–voltage relationship. However, sevoflurane (1.0 mM) significantly inhibited the peak component of the transient inward currents of Na_v 1.8, Na_v 1.4, and Na_v 1.7; 0.5 mM sevoflurane did not affect the peak component of the transient inward currents inward current of these three channels. In clinical situations, the free plasma concentration of sevoflurane is approximately 0.5 mM in humans [21, 22]. Ouyang et al. [12] reported that the function of Na_v1.4 was inhibited slightly by equipotent concentrations of sevoflurane (0.46 mM), consistent with our present results. From this evidence and our results, sevoflurane would have little effect on these channels, at least in a clinical situation.

In our present results, 1 mM sevoflurane inhibited the peak component of the transient inward currents of Na_v 1.8, Na_v 1.4, and Na_v 1.7. This finding raises the question of how sevoflurane inhibits these channel functions. Sodium channels are also rapidly phosphorylated by PKC [23], and recent reports have shown that the functions of Na_v 1.7 expressed in *Xenopus* oocytes are modulated by PKC [24]. Moreover, there are several lines of evidence revealing that sevoflurane activated PKC [2, 3]. Inhibition by sevoflurane on Na_v 1.8, Na_v 1.7, and Na_v 1.4 functions was abolished by pretreatment with the PKC inhibitor,

suggesting that sevoflurane would inhibit Na_v 1.8, Na_v 1.7, and Na_v 1.4 functions by PKC-mediated pathways.

In conclusion, we demonstrated inhibition by sevoflurane on the functions of Na_v 1.8, Na_v 1.7, and Na_v 1.4, and that the inhibition would be mediated by the PKC pathway. However, these sodium channels might not be related to the clinical anesthetic effects of sevoflurane.

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Sevoflurane Inhibits the μ-Opioid Receptor Function Expressed in *Xenopus* Oocytes

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Key Words
μ-Opioid receptor · $G_{i/o}$-protein-coupled receptors · Sevoflurane · *Xenopus* oocyte

Abstract
Sevoflurane is widely used for anesthesia, and is commonly used together with opioids in clinical practice. However, the effects of sevoflurane on μ-opioid receptor (μOR) functions is still unclear. In this study, the effects of sevoflurane on μOR functions were analyzed by using *Xenopus* oocytes expressing a μOR fused to chimeric Gα protein G_{qi5} (μOR-G_{qi5}). Sevoflurane by itself did not elicit any currents in oocytes expressing μOR-G_{qi5}, whereas sevoflurane inhibited the [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO)-induced Cl^- currents at clinically used concentrations. Sevoflurane did not affect the Cl^- currents induced by AlF_4^-, which directly led to activation of G proteins. The inhibitory effects of sevoflurane on the DAMGO-induced currents were not observed in oocytes pretreated with the protein kinase C (PKC) inhibitor GF109203X. These findings suggest that sevoflurane would inhibit μOR function. Further, the mechanism of inhibition by sevoflurane would be mediated by PKC.

Introduction

Sevoflurane is widely used as an inhalation general anesthetic agent due to its low solubility in blood. Opioids are commonly used with sevoflurane at the same time. There have been some reports that have pointed out interactions between sevoflurane and opioid receptors (ORs) in animal experiments; the potency of sevoflurane was modulated by the endogenous μ-opioid system, but not by the κ- and δ-opioid systems in experiments using mice lacking the ORs [1]. In animals, morphine decreases the minimal alveolar concentration (MAC) of sevoflurane [2, 3]. However, it has been reported that morphine does not affect MAC for sevoflurane in humans. More recently, it has been reported that μOR-knockout mice have no different MAC of sevoflurane [4]. The interaction between sevoflurane and OR function is still controversial.

The ORs belong to the G-protein-coupled receptor family and three types of receptors, μ, δ and κ, have been identified by molecular cloning [5]. Within the three subtypes of these receptors, μORs are the major receptors that mediate the analgesic effects of opioids [5]. On the basis of second-messenger signaling, μOR couples to $G\alpha_{i/o}$ protein to cause inhibition of adenylate cyclase, in-

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hibition of voltage-dependent Ca^{2+} channels, and activation of G-protein-coupled inwardly rectifying K^+ channels [5]. There have been many reports that revealed direct effects of general anesthetics on G_q-coupled receptors [6–11]. As far as the functions of $G_{i/o}$-coupled receptors including μOR are concerned, much less is known about the direct effects of volatile anesthetics. Moreover, we recently reported that the volatile anesthetic halothane inhibited μOR function at clinical concentrations [12]. It would be interesting to study whether sevoflurane affects μOR functions.

The *Xenopus* oocyte expression system has widely been employed to study the functions of a number of G-protein-coupled receptors [13, 14]. In the case of G_q-coupled receptors, receptor stimulation results in activation of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes by G_q-mediated phospholipase C activation and subsequent formation of IP_3 and diacylglycerol [14]. The IP_3 formed causes release of Ca^{2+} from the endoplasmic reticulum, which in turn triggers the opening of Ca^{2+}-activated Cl^- channels endogenously expressed in the oocytes [14]. However, in case of $G_{i/o}$-coupled receptors, analysis has been difficult, due to lack of appropriate analytical output in oocytes. We have established an assay method for $G_{i/o}$ PCRs by using a μOR fused to G_{qi5} (μOR-G_{qi5}) in *Xenopus* oocytes [12, 15].

We examined the effects of sevoflurane on the function of μOR using this assay system. Moreover, we investigated the mechanisms of the effects of sevoflurane on μOR.

Materials and Methods

Materials

Adult *Xenopus laevis* female frogs were purchased from Kato Kagaku (Tokyo, Japan). The Ultracomp *E. coli* Transformation Kit was from Invitrogen (San Diego, Calif., USA). Sevoflurane was purchased from Maruishi Pharmaceutical (Osaka, Japan). Purification of cDNAs was performed with a Qiagen purification kit (Qiagen, Chatworth, Calif., USA). Gentamicin, sodium pyruvate, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) and other chemicals were from Nacalai Tesque (Kyoto, Japan). The rat μOR was provided by Dr. N. Dascal (Tel Aviv University, Ramat Aviv, Israel). The chimeric $G\alpha_{qi5}$ was a kind gift from Dr. B.R. Conklin (UCSF, San Fransico, Calif., USA). Each of the cRNAs was prepared using a mCAP mRNA Capping Kit, and transcribed with a T7 RNA polymerase in vitro transcription kit (Stratagene, La Jolla, Calif., USA).

Preparation of Chimeric μOR-G_{qi5}

The tandem cDNAs of chimeric μOR-G_{qi5} were created by ligating the receptor cDNA sequences into the *Nhe*I site of G_{qi5} cDNAs. The sequences of all PCR products were confirmed by sequencing with ABI3100 (Applied BioSystems, Tokyo, Japan). All cDNAs for the synthesis of cRNAs were subcloned into the pGEMHJ vector, which provides the 5′- and 3′-untranslated regions of the *Xenopus* β-globin RNA [16], ensuring a high level of protein expression in the oocytes. Each of the cRNAs was synthesized using the mCAP mRNA Capping Kit, with the T7 RNA polymerase in vitro transcription kit (Ambion, Austin, Tex., USA) from the respective linearized cDNAs.

Recording and Data Analysis

Isolation and microinjection of *Xenopus* oocytes were performed as previously described [7, 9–12, 17–19]. *Xenopus* oocytes were injected with appropriate amounts of cRNAs (in 50 ng; μOR-G_{qi5}) and incubated with ND 96 medium composed of (in mmol/l); NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.4, adjusted with NaOH), supplemented with 2.5 mmol/l sodium pyruvate and 50 μg/ml gentamicin for 3–7 days until recording. Oocytes were placed in a 100-μl recording chamber and perfused with modified Barth's saline (MBS) composed of (in mmol/l): NaCl 88, KCl 1, $NaHCO_3$ 2.4, HEPES 10, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, and $CaCl_2$ 0.91, (pH 7.4 adjusted with NaOH) at a rate of 1.8 ml/min at room temperature. Recording and clamping electrodes (1–2 MΩ) were pulled from 1.2-mm outside-diameter capillary tubing and filled with 3 mol/l KCl. A recording electrode was imbedded in the animal's pole of oocytes, and once the resting membrane potential stabilized, a clamping electrode was inserted and the resting membrane potential was allowed to restabilize. A Warner OC 725-B oocyte clamp (Hampden, Conn., USA) was used to voltage clamp each oocyte at –70 mV. We analyzed the peak component of the transient inward currents induced by receptor agonists because this component is dependent on the concentrations of the receptor agonist applied, and is quite reproducible, as described by Minami et al. [10]. Sevoflurane was applied for 2 min before and during the application of DAMGO (1 μmol/l) to allow complete equilibration in the bath. The solutions of sevoflurane were freshly prepared immediately before use. We calculated the final concentration of sevoflurane in the recording chamber using a gas chromatography method, and accordingly the concentrations of sevoflurane represent the bath concentrations.

AlF_4^- was used as a direct activator of G proteins, and with this system we could bypass the signal to G proteins from activated receptors. Under a two-electrode voltage clamp, we injected 30 nl of solution containing NaF and $AlCl_3$ into the oocyte by using a pressure injector (PM2000B; MicroData Instruments, South Plainfield, N.J., USA). The concentrations of NaF and $AlCl_3$ used in this study were 20 mmol/l and 60 μmol/l, respectively.

To determine whether activation of protein kinase C (PKC) plays a role in anesthetic modulation of μOR-mediated events, oocytes were exposed to a PKC inhibitor, bisindolylmaleimide I (GF109203X; 200 nmol/l) [20], in MBS for 120 min. We compared the effects of anesthetics on DAMGO (1 μmol/l)-induced Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes expressing μOR-G_{qi5} before and after the exposure to GF109203X.

Statistical Analysis

Results are expressed as percentages of control responses. The control responses were measured before and after each drug ap-

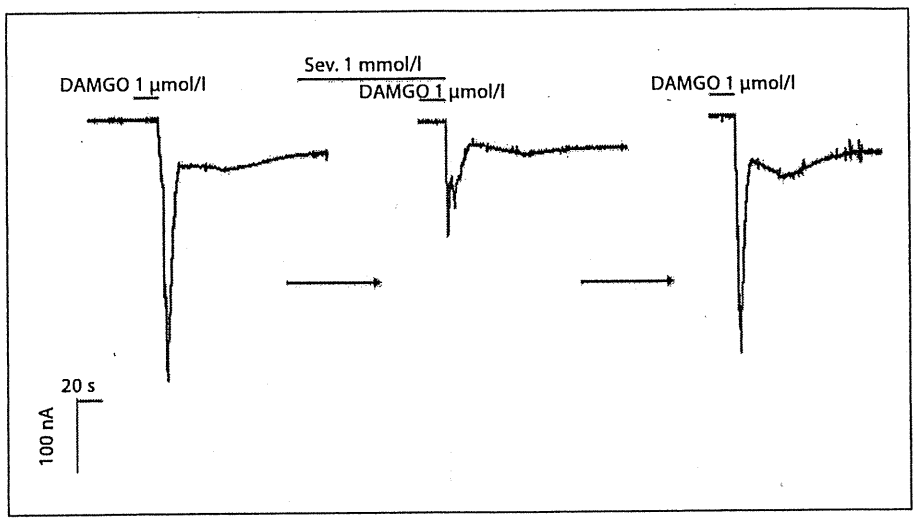


Fig. 1. Typical tracings of the effect of 1 mmol/l sevoflurane (Sev.) on the Cl^- current evoked by 1 µmol/l DAMGO in *Xenopus* oocytes expressing µOR-G_{qi5}.

plication, to take into account possible shifts in the control currents as recording proceeded. The 'n' values refer to the number of oocytes studied. Each experiment was carried out with oocytes from at least two different frogs. Statistical analyses were carried out by one-way ANOVA followed by Dunnett's correction and paired t test using GraphPad Prism 4 (GraphPad Softwear, Inc; La Jolla, Calif., USA). Values of $p < 0.05$ were considered to be significant.

Results

Pretreatment of sevoflurane by itself did not elicit any currents in oocytes expressing µOR-G_{qi5}, whereas the sevoflurane significantly inhibited DAMGO (1 µmol/l)-induced Ca^{2+}-activated Cl^- currents in a concentration-dependent manner (fig. 1); sevoflurane at 0.25, 0.5 and 1 mmol/l inhibited the DAMGO (1 µmol/l)-induced Cl^- currents to 84.7 ± 12.3, 50.6 ± 10.1 ($p < 0.01$), and 48.8 ± 8.2% ($p < 0.01$) of the control value, respectively (n = 8 each) (fig. 2).

AlF_4^- has been reported to bind to guanosine diphosphate on heterotrimeric G protein, and guanosine diphosphate-AlF_4^- complex promotes the dissociation of heterotrimeric G proteins into Gα and Gβγ subunits, which directly (without receptor stimulation) leads to the activation of G protein and subsequent G-protein-mediated pathways downstream [21]. The peak amplitude of AlF_4^--induced currents was 282 ± 121 nA (n = 6), and sevoflurane did not affect the AlF_4^--induced currents (418 ± 86.4 nA; n = 6) (fig. 3).

Treatment with a PKC inhibitor, GF109203X (200 nmol/l), which has a K_i value for inhibiting PKC activity

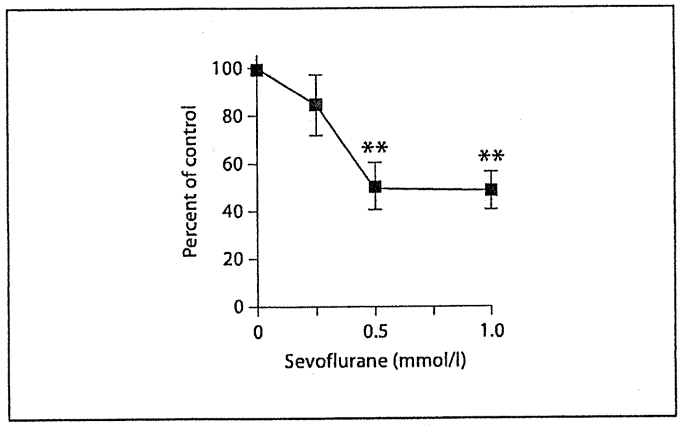


Fig. 2. Concentration-response curve for the inhibitory effects of sevoflurane on DAMGO (1 µmol/l)-induced Cl^- currents in oocytes expressing µOR-G_{qi5}. ** $p < 0.01$ vs. control. Values are expressed as means ± SEM.

of 20 nmol/l [20], produced enhancement of the initial Cl^- currents activated by DAMGO (1 µmol/l) (fig. 4a). After a 1-hour incubation of GF109203X, the response of DAMGO (1 µmol/l) increased to 2 times the initial currents (205 ± 27.6% of control), and this enhancement continued for 2 h (fig. 4b) (n = 6). Sevoflurane (1 mmol/l) inhibited DAMGO (1 µmol/l)-induced Ca^{2+}-activated Cl^- currents to 61.2 ± 15.8% of the control value (n = 6). However, the inhibitory effects of sevoflurane (1 mmol/l) on DAMGO (1 µmol/l)-induced currents were abolished after 2 h of pretreatment with GF109203X (115.8 ± 26.8% of control) (fig. 5).

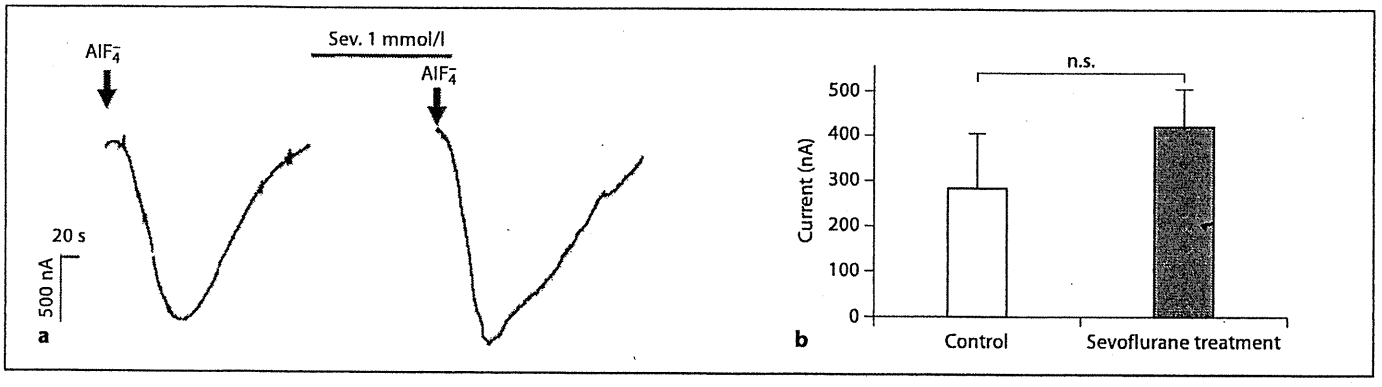


Fig. 3. Effects of sevoflurane (Sev.) on AlF_4^--induced currents in *Xenopus* oocytes. **a** Tracings were obtained from a single oocyte showing the effect of sevoflurane on AlF_4^--induced currents in oocytes expressing $\mu OR\text{-}G_{qi5}$. **b** Oocytes were injected with 30 nl test solution (20 mmol/l NaF and 60 μmol/l $AlCl_3$) in the presence (Sev. treatment) (n = 6) or absence (control) (n = 6) of 1 mmol/l sevoflurane. Data are expressed as means ± SEM of peak currents (nA).

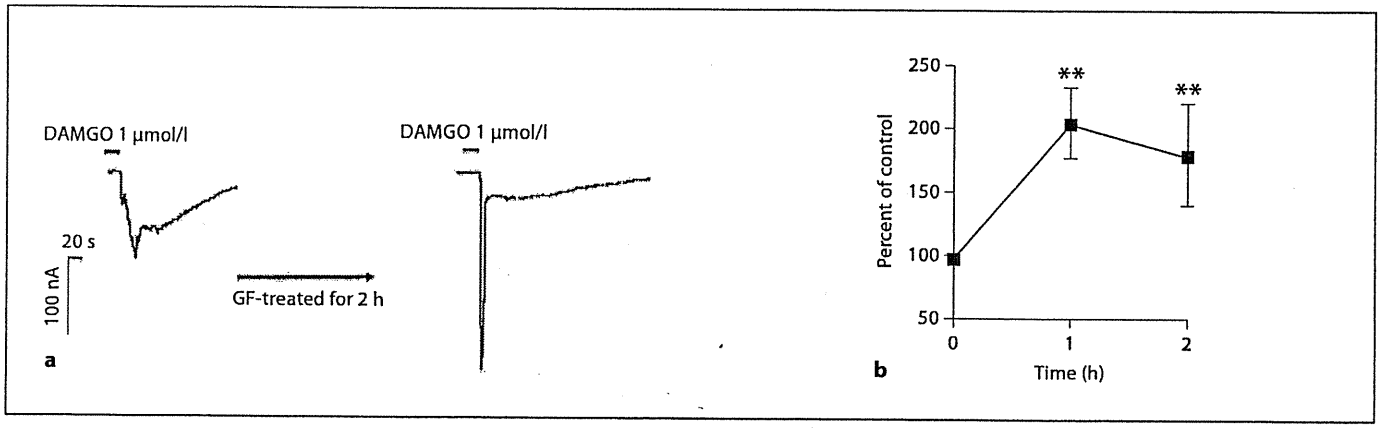
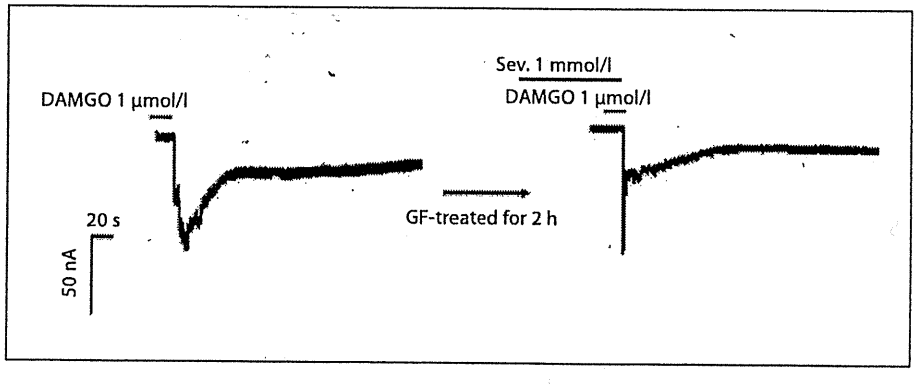


Fig. 4. Effects of bisindolylmaleimide I (GF109203X) on DAMGO-induced Cl^- current in oocytes expressing $\mu OR\text{-}G_{qi5}$ receptor. **a** Tracings were obtained from a single oocyte showing the DAMGO (1 μmol/l)-induced currents in oocytes expressing $\mu OR\text{-}G_{qi5}$ receptors before and after treatment with GF109203X (GF). Oocytes were incubated with 200 nmol/l GF for 2 h and were then stimulated by DAMGO. **b** Time course of effects of GF on DAMGO-induced Cl^- current in oocytes expressing $\mu OR\text{-}G_{qi5}$ receptor. Oocytes were incubated with GF (200 nmol/l) for 120 min. DAMGO (1 μmol/l) was applied at 60 and 120 min during treatment of GF. Data represent means ± SEM of 6 oocytes. ** $p < 0.01$ vs. time at starting incubation with 200 nmol/l GF (0 h).

Fig. 5. Effects of bisindolylmaleimide I (GF109203X) on the inhibitory effects of sevoflurane (Sev.) on DAMGO (1 μmol/l)-induced currents. Tracings were obtained from a single oocyte showing the effect of sevoflurane on 1 μmol/l of DAMGO-induced currents in oocytes expressing $\mu OR\text{-}G_{qi5}$ receptor before and after treatment with GF109203X (GF). Oocytes were incubated with 200 nmol/l GF for 2 h, and were then stimulated by DAMGO (1 μmol/l) in the presence of sevoflurane (1 mmol/l).



Discussion

We showed that sevoflurane had inhibitory effects on DAMGO-induced Cl^- currents in oocytes expressing μOR-G_{qi5}. In clinical situations, the free plasma concentration of sevoflurane was approximately 0.5 mmol/l [22, 23]. Sevoflurane suppressed DAMGO-induced Cl^- currents in oocytes expressing μOR-G_{qi5} at concentrations more than 0.5 mmol/l. Consistent with these reports, our present results suggest that anesthetic concentrations of sevoflurane would have inhibitory effects on μOR.

Our study raises the question of how sevoflurane inhibits μOR function. In our results, sevoflurane had little effect on AlF_4^--induced currents, suggesting that sevoflurane may not interfere with the signaling pathways downstream of activation of G proteins, such as phospholipase C activation, intracellular Ca^{2+} release, and Ca^{2+}-activated Cl^- channels. From these results, the action site of sevoflurane would be OR.

There is considerable evidence that PKC plays an important role in the regulation of OR function. A number of studies have reported that PKC is involved in morphine-induced tolerance in vivo [24–27]. In our present results, the PKC inhibitor GF109203X enhanced DAMGO-induced currents. These results suggested that PKC would inhibit the OR function. Moreover, the PKC inhibitor GF109203X abolished the inhibitory effects of sevoflurane on μOR function, suggesting that sevoflurane would inhibit μOR function by PKC-mediated pathways. In our study, unfortunately, we could not study how sevoflurane activates the PKC because of difficulties in measuring the activities of PKC in *Xenopus* oocyte preparation. However, there are several lines of evidence which reveal that sevoflurane activated PKC [28, 29]. To confirm this hypothesis, it could be required to investigate the region of μOR responsible for PKC action by using mutated μOR whose serine/threonine sites were point mutated.

In conclusion, we demonstrated that sevoflurane has significant inhibitory effects on the function of μOR at clinically relevant concentrations, and the inhibition might be mediated via PKC pathways. Although several investigations have reported the effects of opioids on sevoflurane anesthesia, the nature of the interaction between opioids and sevoflurane remains unclear. Our present results showed the inhibitory effects on μOR. To clarify the interaction between sevoflurane and opioid in the clinical situation, further study would be necessary.

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Short Communication

Possible Involvement of β-Endorphin in a Loss of the Coordinated Balance of μ-Opioid Receptors Trafficking Processes by Fentanyl

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KEY WORDS internalization/recycling pathway; opioids; receptor trafficking; fentanyl

BACKGROUND

It has been considered that opioid tolerance is, in part, the end result of a coordinated balance between processes that govern the desensitization, internalization, and resensitization of μ-opioid receptors (MOR) (Claing et al., 2002; Gainetdinov et al., 2004). However, a several line of evidence suggests that the trafficking properties of MORs driven by MOR agonists may depend on intrinsic characters of each agonist, and are still complicated. Previous biochemical studies on cultured enteric neurons have indicated that fentanyl induces either the functional desensitization or internalization of MORs (Minnis et al., 2003). In contrast, under the same condition, morphine does not promote the detectable internalization of MORs in cultured cells after prolonged or acute treatment in healthy animals, although it has been well-established that morphine causes the development of tolerance to its pharmacological actions (Minnis et al., 2003). However, recent studies have demonstrated that morphine activates MORs with promoting internalization of MORs via β-arrestin-2-dependent mechanisms in striatal neurons (Haberstock-Debic et al., 2005).

In the previous study, we demonstrated that repeated treatment with fentanyl, but not morphine, causes a rapid desensitization to its ability to block the hyperalgesia associated with the attenuation of MOR resensitization in mice with inflammatory pain (Imai et al., 2006). Based on this study, we hypothesized that released β-endorphin within the spinal cord under a chronic pain-like state may be implicated in the rapid development of tolerance to fentanyl, but not morphine and oxycodone. Namely, these findings raise the possibility that β-endorphin could attenuate the resensitization of MOR after the treatment with fentanyl, resulting in the high degree of tolerance to fentanyl-induced antihyperalgesic effects under long-lasting pain state. To further address this issue, this cell culture study was performed to investigate the effects of fentanyl on MOR internalization and resensitization in the presence or absence of β-endorphin.

MATERIALS AND METHODS

Baby hamster kidney (BHK) cells (Riken Cell Bank, Tsukuba, Japan) were grown in Dulbecco's

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modified eagle medium (DMEM: Invitrogen™) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Transient transfection was then performed with Effectene transfection reagent (Qiagen, Tokyo, Japan) in 0.2 μg of each cDNA according to the protocol provided by the manufacturer. Cells were used in confocal microscopy 16–24 h after transfection. cDNA for rat MOR was kindly provided by Dr. Dascal (Tel Aviv University). Venus, a brighter variant of yellow fluorescent protein (Nagai et al., 2002) was obtained from Dr. T. Nagai (Riken, Wako, Japan). Primers (5′-GGG GTA CCC CAT GGA CAG CAG CAC-3′) and (5′-GCG GCC GCG GGG CAA TGG AGC AGT-3′) were engineered to ligate the N-terminus of MOR by using standard molecular approaches with the polymerase chain reaction (PCR). Venus-fused MOR was created by ligating the MOR cDNA sequences into the *NotI* site of the corresponding Venus site. cDNA for transfection in BHK cells was subcloned into pcDNA3.1 (Invitrogen™ Life Technologies, CA). cDNA for rat β-arrestin 2 was generously provided by Dr. Y. Nagayama (Nagasaki University, Japan). For the analysis of the agonist-induced internalization of MORs, BHK cells that had been transfected with Venus-fused MORs and β-arrestin-2 were incubated in the absence or presence of 100 nM β-endorphin for 30 min at 37°C, and then treated with 10 μM morphine, 100 nM fentanyl or 10 μM oxycodone. To investigate the resensitization of MORs, the cells were incubated with 100 nM fentanyl or 10 μM oxycodone in the presence or absence of β-endorphin, and then apposed for 30 min, 90 min, 3 h, or 6 h at 37°C. The cells were subsequently fixed and examined by confocal microscopy as previously reported (Corbani et al., 2004). Venus was excited by a 488-nm laser was used to detect Venus fluorescence with a 505- to 530-nm band-pass filter, and images were obtained by placing the dish on the stage of an inverted Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany). Data were stored on the hard disc with and analyzed with the Zeiss LSM software Zen 2009. For the quantitative analysis of agonist-induced internalization of MORs, BHK cells were fixed with 4% paraformaldehyde in PBS and stored at 4°C. The numbers of cells expressing Venus-fused MORs were counted. For counting cells whether Venus fluorescence was at the plasma membrane or in cytosol (internalization), we basically followed by Corbani et al. (2004). Localization of Venus-fused MORs in BHK cells was categorized as "mainly expressed at the plasma membrane," "not detected in plasma membrane but detected in cytosol," or "not detected" (whose localization was not belong to the former category), separated with a software Zen 2009 equipped with Zeiss LSM510 META confocal microscope, with reference to the control, not stimulated BHK cells. A total of 100 cells (counted mean 200–250 cells in sum of "the plasma membrane," "in the cytosol," plus "not detected") in six independent each dish. % Internalization was described as cytosol × 100/[plasma membrane + cytosol (total 100 cells)]. The drugs used in this study were fentanyl citrate (Hisamitsu Pharmaceutical, Tokyo, Japan), morphine hydrochloride

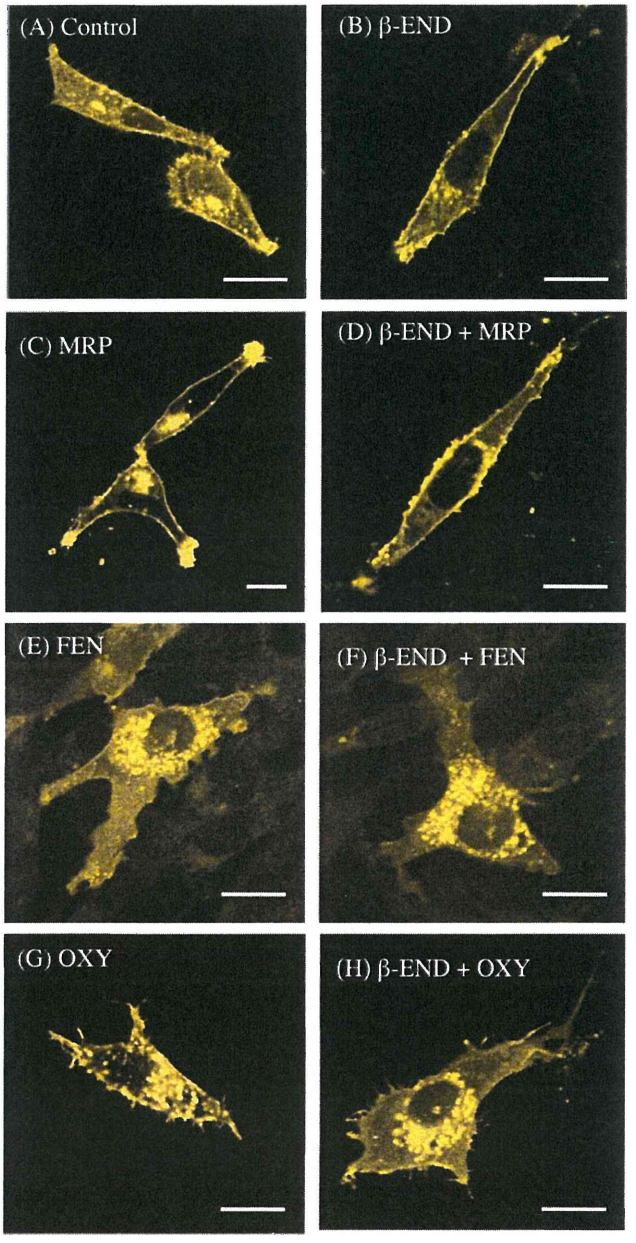


Fig. 1. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. The cells were incubated in the absence (**A**, **C**, **E**, and **G**) or presence (**B**, **D**, **F**, and **H**) of 100 nM β-endorphin (β-END) for 30 min at 37°C and then treated with 10 μM morphine (MRP; C, D), 100 nM fentanyl (FEN; E, F), or 10 μM oxycodone (OXY; G, H). The cells were subsequently fixed and examined by confocal microscopy. Yellow fluorescence from Venus indicates the localization of MORs in BHK cells. Scale bars, 10 μm.

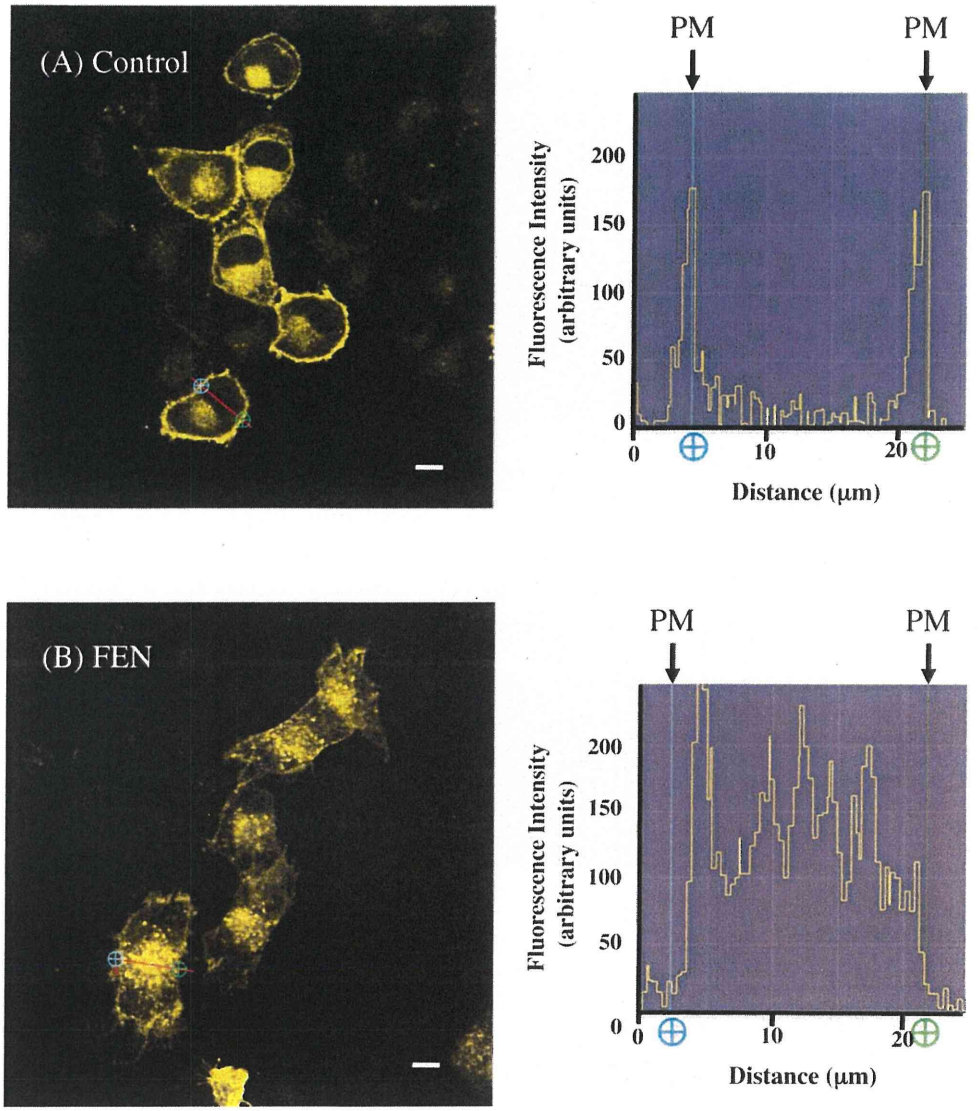
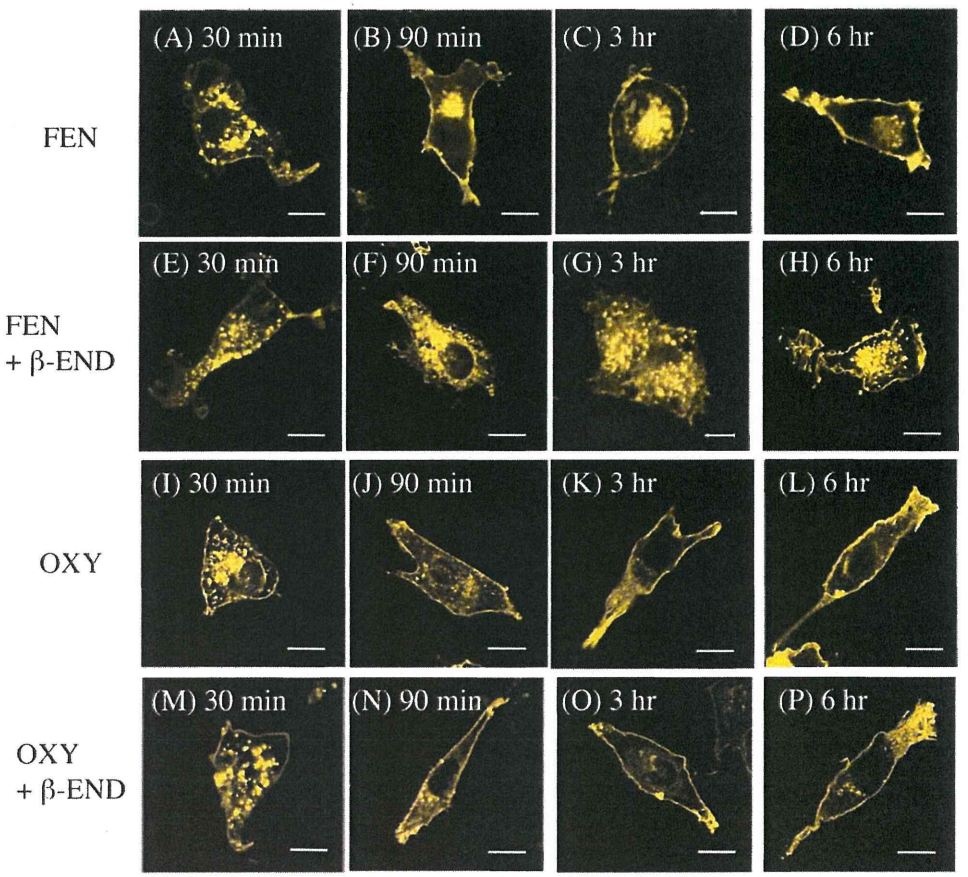


Fig. 2. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. Typical cells where most of MOR-Venus intensity was at the plasma membranes, [**A**, control cells (Control)] or in the cytosolic fraction [**B**, 100 nM fentanyl-stimurated for 30 min (FEN)]. PM; plasma membranes in BHK cells. Scale bars, 10 μm.

(Daiichi-Sankyo, Tokyo, Japan), oxycodone hydrochloride (a kind gift from Shionogi Pharmaceutical, Osaka, Japan), and β-endorphin (Sigma-Aldrich, St Louis, MO), which were dissolved in assay buffer.

RESULTS AND DISCUSSION

In this study, we assessed whether β-endorphin could affect the trafficking properties of MORs using immunocytochemical methods in BHK cells with confocal microscope. Confocal imaging of the BHK cells expressing Venus-fused MOR with β-arrestin-2 revealed that the yellow fluorescence was largely confined to the plasma membrane (Figs. 1A and 2A). In both the presence and absence of 100 nM β-endorphin, at which concentration there did not cause any internalization of MORs (Figs. 1B and 1C), cells expressing MORs treated with 10 μM morphine (Figs. 1C and 1D) showed little internalization of MORs, while the cells treated with 100 nM fentanyl (Figs. 1E, 1F, and 2B) and 10 μM oxycodone (Figs. 1G and 1H) showed robust internalization of the receptor. These findings were consistent with previous reports that fentanyl and etorphine caused partial internalization, while morphine failed to induce detectable MOR endocytosis (Koch et al., 2005). We next investigated the resensitization properties of MORs after the washing-out of agonists. In the absence of β-endorphin, internalized MOR returned to the plasma membrane from 90 min after the washing-out of fentanyl (Figs. 3B–3D). However, in the presence of β-endorphin, the internalized MOR induced by fentanyl



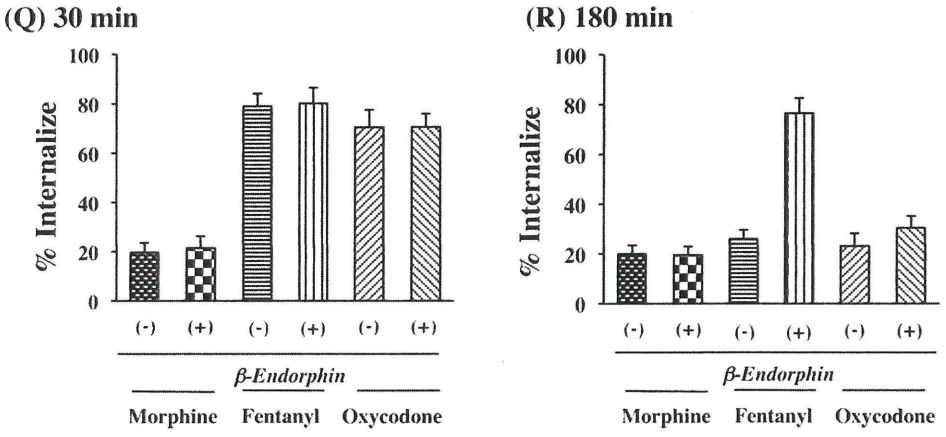


Fig. 3. Confocal imaging of resensitization of MORs in BHK cells expressing Venus-fused MORs. Cells were incubated with 100 nM fentanyl (**A–H**) or 10 μM oxycodone (**I–P**) in the absence (A-D and I-L) or presence (E-H and M-P) of β-endorphin, and then apposed for 30 min, 90 min, 3 h, or 6 h at 37 C. The cells were then fixed and counted by confocal microscopy. Yellow fluorescence from Venus indicates the cellular localization of MOR in BHK cells. Scale bars, 10 μm. Quantitative analysis of the % of the internalized cells expressing Venus-fused MORs treated with the drugs for 30 min (**Q**) or 180 min (**R**), respectively. The agonist concentrations represent the dose required to induce the maximal effect on receptor endocytosis for each drug. Each value represents the mean ± SEM of six separate experiments.

remained in the cytosolic fraction at 3–6 h after the washing-out of β-endorphin and fentanyl (Figs. 3F–3H). However, in both the presence and absence of β-endorphin, the internalized MOR induced by oxycodone returned to the plasma membrane after the washing-out of agonist in a time-dependent manner (Figs. 3I–3P). We performed quantitative analysis of the agonist-induced internalization of MORs after the washing-out of each agonist shown in Materials and Methods. At 30 min after the washing-out of agonists,

cells treated with fentanyl or oxycodone showed robust internalization of MORs (fentanyl: 79.0 ± 5.14%, β-endorphin fentanyl: 80.2 ± 3.7%, oxycodone: 70.5 ± 7.09%, β-endorphin oxycodone: 70.7 ± 5.35%), which was not seen in morphine-treated cells (morphine: 19.67 ± 3.93%, β-endorphin morphine: 21.5 ± 4.76%; Fig. 3Q). However, while there was no difference in the degree of oxycodone-induced MOR internalization between the presence and absence of β-endorphin 3 h after washing-out (oxycodone: 23.17 ± 5.12%, β-endorphin oxycodone: 30.5 ± 4.72%), in fentanyl-treated cells, β-endorphin caused the prolonged internalization of MORs and fluorescence was stayed in the cytosolic fraction (fentanyl: 27.67 ± 5.47%, β-endorphin fentanyl: 76.5 ± 6.02%; Fig. 3R).

It has been widely accepted that receptor desensitization, internalization and trafficking appear to play a key role in the development of opioid tolerance (Claing et al., 2002; Gainetdinov et al., 2004). The initial process in these events is the phosphorylation of intracellular domains of MOR. Phosphorylated MORs are mostly internalized via clathrin-coated pits into early endosomes and subsequently dephosphorylated by intracellular protein phosphatases. The dephosphorylated MORs might either be recycled to the plasma membrane or transported to lysosomes for degradation. A growing body (Smalheiser and Lugli) of evidence suggests that among diverse serine/threonine (Thr) residues of the intracellular domain of MOR, the phosphorylation of Ser 375 in the mouse MOR is essential for the internalization of MORs (Schulz et al., 2004). In a previous study, we found that repeated treatment with fentanyl, but not morphine, resulted in an increase in the levels of phosphorylated-MOR (Ser 375) associated with the enhanced inactivation of protein phosphatase 2A and a reduction in Rab4-dependent MOR resensitization in the spinal cord of mice that showed inflammatory pain (Imai et al., 2006). However, several lines of evidence indicate that, in response to pain stimulus, endogenous β-endorphin is released within some brain regions (Zubieta et al., 2001). We previously reported that β-endorphin released in the ventral tegmental area is a key factor in regulating the dysfunction of MOR to negatively modulate opioid reward under a neuropathic pain-like state (Niikura et al., 2008, 2010). Taken together, although further studies are still needed, these findings support the idea that inhibition of the resensitization system of MOR following chronic treatment with fentanyl in the presence of β-endorphin may be associated with antihyperalgesic tolerance to fentanyl under a chronic pain-like state.

In conclusion, we demonstrated here that unlikely morphine, either fentanyl or oxycodone induced a robust MOR internalization and, in turn, its resensitization. In the presence of β-endorphin, the internalized MOR induced by fentanyl, but not oxycodone, remained within the cytosolic fraction even after washing out. These findings strongly support that idea that fentanyl has different pharmacological profile form that of morphine or oxycodone.

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Addiction Biology

PRECLINICAL STUDY

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Possible involvement of prolonging spinal μ-opioid receptor desensitization in the development of antihyperalgesic tolerance to μ-opioids under a neuropathic pain-like state

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ABSTRACT

In the present study, we investigated the possible development of tolerance to the antihyperalgesic effect of μ-opioid receptor (MOR) agonists under a neuropathic pain-like state. Repeated treatment with fentanyl, but not morphine or oxycodone, produced a rapid development of tolerance to its antihyperalgesic effect in mice with sciatic nerve ligation. Like the behavioral study, G-protein activation induced by fentanyl was significantly reduced in membranes obtained from the spinal cord of nerve-ligated mice with in vivo repeated injection of fentanyl. In β-endorphin-knockout mice with nerve ligation, developed tolerance to the antihyperalgesic effect of fentanyl was abolished, and reduced G-protein activation by fentanyl after nerve ligation with fentanyl was reversed to the normal level. The present findings indicate that released β-endorphin within the spinal cord may be implicated in the rapid development of tolerance to fentanyl under a neuropathic pain-like state.

Keywords Fentanyl, mouse, neuropathic pain, opioid tolerance, μ-opioid receptor, spinal cord.

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INTRODUCTION

Although drugs that act on μ-opioid receptor (MOR), such as morphine, fentanyl and oxycodone, have been used clinically as analgesics, these MOR agonists also have undesirable effects, such as tolerance, and physical and psychological dependence (Ventafridda and De Conno, 1981; Raynor *et al.* 1994). It has been considered that opioid tolerance is, in part, the end result of a coordinated balance between processes that govern the desensitization, internalization and resensitization of MORs (Claing *et al.* 2002; Gainetdinov *et al.* 2004). The initial process in these events is the phosphorylation of intracellular domains of MORs. Phosphorylated MORs are mostly internalized via clathrin-coated pits into early endosomes and subsequently dephosphorylated by intracellular protein phosphatases. The dephosphorylated MORs may either be recycled to the plasma membrane or transported to lysosomes for degradation. Previous biochemical studies on cultured enteric neurons have indicated that fentanyl induces either the functional desensitization or internalization of MORs (Minnis *et al.* 2003). In contrast, under the same condition, morphine does not promote the detectable internalization of MORs in cultured cells after prolonged or acute treatment in healthy animals, although it has been well-established that morphine causes the development of tolerance to its pharmacological actions (Minnis *et al.* 2003). On the other hand, recent studies have demonstrated that morphine activates MORs with promoting internalization of MORs via β-arrestin-2-dependent mechanisms in striatal neurons (Haberstock-Debic *et al.* 2005).

Thus, the mechanisms that underlie the development of analgesic tolerance to MOR agonists are very much complicated. To further understand properties of analgesic tolerance to MOR agonists, it has been necessary to investigate possible changes in analgesic efficacy following repeated treatment with MOR agonists at optimum doses just for the relief of chronic pain associated with physiological changes in the endogenous MOR system.

In a previous study, we demonstrated that repeated treatment with fentanyl caused a rapid desensitization to its ability to block hyperalgesia under an inflammatory pain state, whereas morphine did not have a similar effect (Imai *et al.* 2006). In addition, repeated treatment with fentanyl, but not morphine, resulted in the attenuation of MOR resensitization, and a subsequent increase in the levels of phosphorylated-MOR in the spinal cord of mice with inflammatory pain. These findings raise the possibility that chronic treatment with fentanyl may cause a different modulation of either the desensitization, internalization or resensitization of MORs in the spinal cord under a pain-like state compared with chronic treatment with morphine.

One mechanism for the MOR desnsitization or attenuation of MOR resensitization by fentanyl in the spinal cord under chronic pain could be a sustained increase in release of the endogenous μ-opioid neuropeptide β-endorphin after sciatic nerve ligation. In fact, it has been reported that β-endorphin is released within some brain regions during pain state (Zangen *et al.* 1998; Zubieta *et al.* 2001). In their reports, they mentioned that the extracellular levels of β-endorphin in the arcuate nucleus increased by 88% under pain-like state. Based on these findings, we assumed that β-endorphin might be released within the spinal cord, as well as brain regions, under pain-like state, as compensatory mechanism for the inhibition of pain transmisson. As sustained exposure to β-endorphin could results in receptor phosphorylation and uncoupling of receptors from effector systems, and thus desensitization, neuropathic pain associated with release of β-endorphin may interfere MOR resensitization by fentanyl.

To further understand the mechanisms that underlie the development of tolerance to this opioid analgesic-induced antihyperalgesic effect under chronic pain, we evaluated the effect of repeated administration of morphine, fentanyl or oxycodone on neuropathic pain-like hyperalgesia and the possible development of tolerance following sciatic nerve ligation. As in the mouse model of inflammatory pain, we demonstrated that repeated treatment with fentanyl, but not morphine or oxycodone, caused a rapid desensitization to its antihyperalgesic effect in nerve-ligated mice. Furthermore, we found that β-endorphin could be a key modulator for the high degree of antinociceptive tolerance to fentanyl caused by sciatic nerve injury. Based on this phenomenon, the present study was performed to investigate the effects of fentanyl on antihyperalgesic effect in β-endorphin knockout (KO) mice.

MATERIALS AND METHODS

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of Hoshi University, as adopted by the Committee on Animal Research of Hoshi University. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments.

Animals

Male and female β-endorphin derived from *proopiomelanocortin* (POMC) gene-KO mice (8–13 weeks old, 22–30 g) (The Jackson Laboratory, Bar Harbor, ME, USA), which had a C57BL/6J and 129S2/SvPas mixed genetic background as described previously (Niikura *et al.* 2008), their wild-type (WT) male and female C57BL/6J mice (8–13 weeks old, 22–30 g) (The Jackson Laboratory, Bar Harbor, ME, USA) and male ICR mice (7–9 weeks old, 20–25 g) (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) were used in the present study. Animals were housed in a room maintained at 23 ± 1°C with a 12-hour light–dark cycle. Food and water were available *ad libitum*. Each animal was used only once.

Drugs

The drugs used in the present study were fentanyl citrate (Hisamitsu Pharmaceutical Co., Inc., Tokyo, Japan), morphine hydrochloride (Daiichi-Sankyo Co., Tokyo, Japan), oxycodone hydrochloride (a kind gift from Shionogi Pharmaceutical Co. Inc., Osaka, Japan) and β-endorphin (Sigma-Aldrich Co., St. Louis, MO, USA), which were dissolved in 0.9% physiological saline (Otsuka Pharmaceutical Co. Inc., Tokyo, Japan) for *in vivo* experiments or assay buffer for *in vitro* experiments.

Neuropathic pain model

Mice were anesthetized with 3% isoflurane. We produced a partial sciatic nerve injury by tying a tight ligature with a 8–0 silk suture around approximately one-third to one-half the diameter of the sciatic nerve on the right side (ipsilateral side) under a light microscope (SD30, Olympus, Tokyo, Japan), as described previously (Seltzer *et al.* 1990; Malmberg and Basbaum 1998). In sham-operated mice, the nerve was exposed without ligation.

Guanosine-5′-o-(3-thio) triphosphate ([^{35}S]GTPγS) binding assay

For membrane preparation, the mouse spinal cord was quickly removed after decapitation and rapidly transferred to a tube filled with ice-cold bufferh. The membrane homogenate (3–8 μg protein/assay) was prepared as described previously (Narita *et al.* 2001) and incubated at 25°C for 2 hours in 1 ml of assay buffer with various concentrations of each agonist, 30 μM guanosine-5′-diphosphate and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The reaction was terminated by filtration using Whatman GF/B glass filters (Brandel, Gaithersburg, MD, USA) that had been presoaked in 50 μM Tris-HCl, pH 7.4, and 5 μM $MgCl_2$ at 4°C for 2 hours. The filters were washed three times with 5 ml of ice-cold Tris-HCl buffer, pH 7.4, and then transferred to scintillation-counting vials. Next, 4 ml of clear-sol 2 (Nacalai Tesque, Inc., Kyoto, Japan) was added to the vials and equilibrated for 12 hours. The radioactivity in the samples was determined with a liquid scintillation analyzer. Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS.

Measurement of thermal hyperalgesia and tactile stimulus

To assess the sensitivity to thermal stimulation, each of the hind paws of mice was tested individually using a thermal stimulus apparatus (UGO-BASILE, Biological Research Apparatus, Varese, Italy). The intensity of the thermal stimulus was adjusted to achieve an average baseline paw-withdrawal latency of approximately 9 to 12 seconds in naive mice. Only quick hind-paw movements (with or without licking of the hind paws) away from the stimulus were considered to be a withdrawal response. Paw movements associated with locomotion or weight-shifting were not counted as a response. The paws were measured alternating between the left and right with an interval of more than 3 minutes between measurements. The latency of paw withdrawal after the thermal stimulus was determined as the average of three measurements per paw.

Statistical analysis

The data from the [^{35}S]GTPγS binding assay are expressed as the mean ± standard error of the mean (SEM) of % Stimulation. The data regarding hyperalgesic responses are shown as the mean ± SEM of the paw-withdrawal latency. Receptor binding curves were fitted using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA, USA). The statistical significance of differences between groups was assessed by two-way analysis of variance followed by the Bonferroni/Dunn multiple comparison test or Student's *t*-test.

RESULTS

Effect of single or repeated subcutaneous (s.c.) injections of morphine, fentanyl or oxycodone on the neuropathic pain-like state induced by nerve injury in mice

In the present study, mice with partial sciatic nerve ligation exhibited marked neuropathic pain-like behavior only for the ipsilateral side at 7 days after nerve ligation (***$P < 0.001$ versus sham-saline group, Fig. 1). The persistent painful state caused by sciatic nerve ligation lasted for more than 21 days after surgery in mice (Fig. 2). A single s.c. injection of either morphine (1–10 mg/kg), fentanyl (0.003–0.01 mg/kg) or oxycodone (0.1–1 mg/kg) at 7 days after sciatic nerve ligation recovered the decreased thermal threshold observed on the ipsilateral side in sciatic nerve-ligated mice in a dose-dependent manner, and maximal antihyperalgesic responses were seen at 30, 15 or 15 minutes after the injection of morphine, fentanyl or oxycodone, respectively (*$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$ versus sham-saline group, Fig. 1). At a dose of 5.0 mg/kg, 0.03 mg/kg or 0.5 mg/kg, s.c. administration of morphine, fentanyl or oxycodone almost completely reversed the decrease in the thermal threshold without excessive effects in sciatic nerve-ligated mice. Therefore, we proposed that the optimal doses for the morphine-, fentanyl- or oxycodone-induced antihyperalgesic effect in nerve-ligated mice were 5.0, 0.03 or 0.5 mg/kg, respectively. As shown in Fig. 2a and c, the thermal hyperalgesia observed on the ipsilateral side after nerve ligation was clearly reversed by each repeated s.c. injection of morphine (5 mg/kg) or oxycodone (0.5 mg/kg) once a day for 14 consecutive days from 7 days after nerve ligation. In contrast, the antihyperalgesic effect following repeated treatment with fentanyl (0.03 mg/kg) was gradually tolerated (**$P < 0.01$ or ***$P < 0.001$ versus sham-saline group; Fig. 2b).

Changes in G-protein activation induced by repeated subcutaneous (s.c.) injection of morphine, fentanyl or oxycodone in the spinal cord of mice with nerve ligation

We investigated the ability of morphine, fentanyl or oxycodone to activate G-proteins through the stimulation of MOR in membranes of the ipsilateral side of the spinal cord obtained from mice treated with saline, morphine, fentanyl or oxycodone once a day for 14 consecutive days from 7 days after sham operation or nerve ligation (Fig. 3). The activation of G-proteins induced by morphine (0.001–10 μM), fentanyl (0.001–100 μM) or

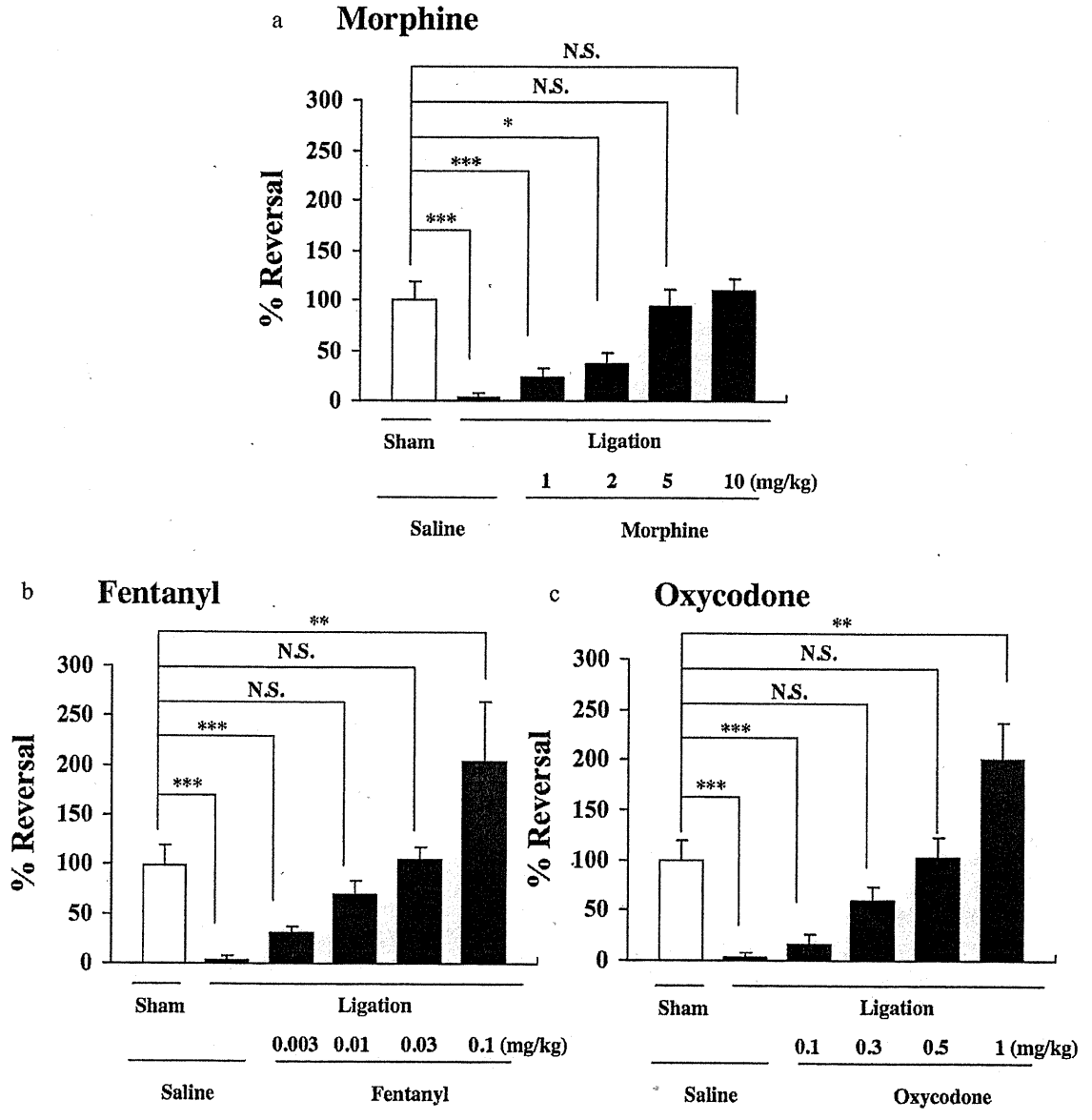

Figure 1 Effect of s.c. injection of morphine, fentanyl or oxycodone on the latency of paw withdrawal in response to a thermal stimulus on the ipsilateral side in sham-operated or sciatic nerve-ligated ICR mice. The thermal threshold was measured just before and 30, 15 or 15 minutes after s.c. injection of morphine, fentanyl or oxycodone, respectively. Groups of mice were treated s.c. with morphine (1–10 mg/kg) (a), oxycodone (0.003–0.1 mg/kg) (b) or fentanyl (0.003–0.01 mg/kg) (c) 7 days after the operation. Each column represents the mean ± standard error of the mean of 8–10 mice. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus sham-saline group. N.S. = not significant

oxycodone (0.001–10 μM) on the ipsilateral side of the spinal cord was examined by monitoring the binding of [^{35}S]GTPγS to membranes. Morphine, fentanyl and oxycodone each produced a concentration-dependent increase in the binding of [^{35}S]GTPγS to spinal cord membranes obtained from sham-operated mice (Fig. 3). In sciatic nerve-ligated mice following repeated injection of saline, the levels of [^{35}S]GTPγS binding stimulated by fentanyl, morphine or oxycodone were similar to that found in sham-operated mice (Fig. 3a-c). The binding of [^{35}S]GTPγS stimulated by fentanyl was significantly decreased in nerve-ligated mice by the repeated s.c. injection of an optimal dose of fentanyl compared with the findings in sham-operated mice [$F(2,81) = 141.7$; $P < 0.001$ versus sham-saline group, Fig. 3c]. In contrast, there was no difference in G-protein activation in the spinal cord between sham-operated and nerve-ligated mice with the repeated s.c. injection of an optimal dose of morphine or oxycodone (Fig. 3a or c). Furthermore, the maximal G-protein stimulation by fentanyl was significantly decreased in nerve-ligated mice with the repeated s.c. injection of an optimal dose of fentanyl

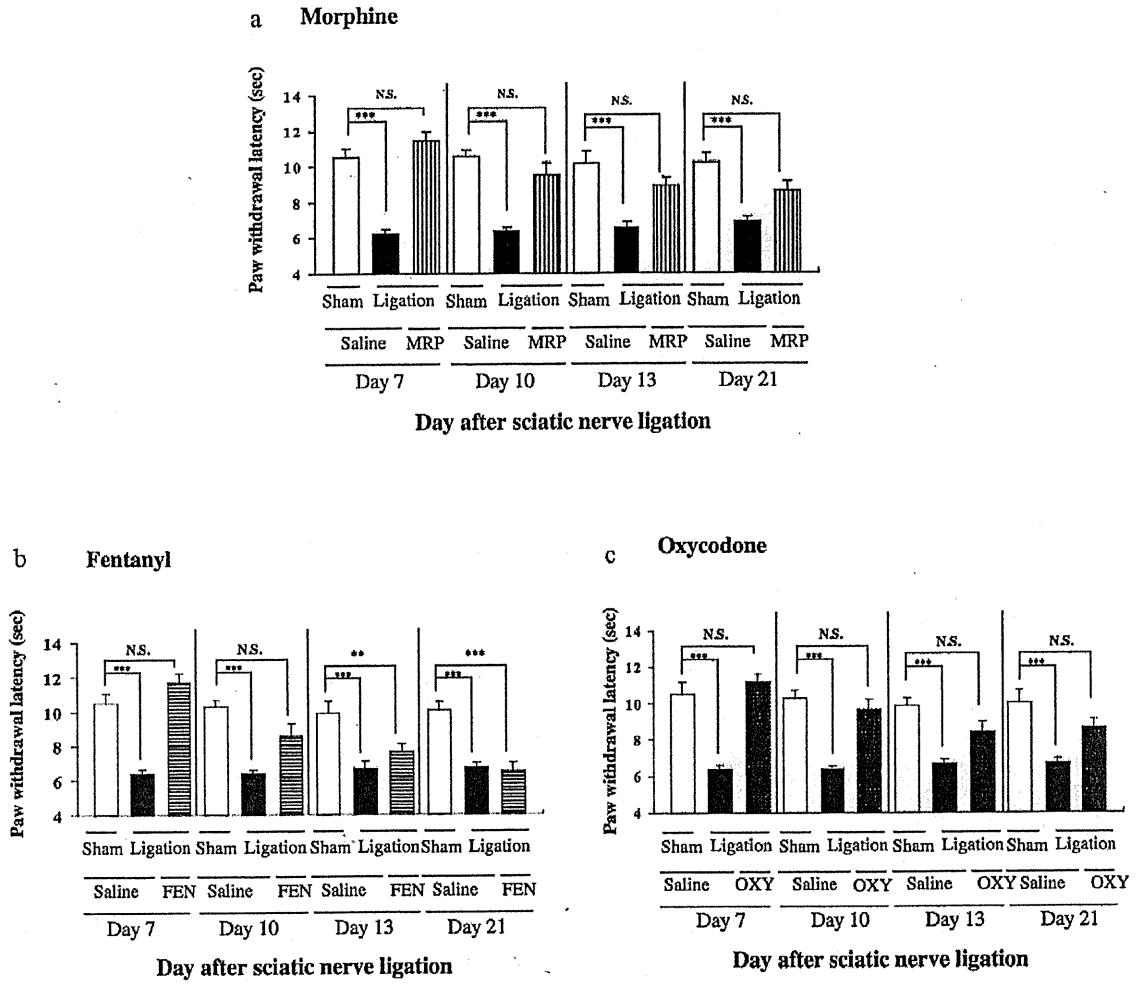

Figure 2 Effect of repeated s.c. injection of morphine (MRP) (a), fentanyl (FEN) (b) or oxycodone (OXY) (c) on the latency of paw withdrawal in response to a thermal stimulus on the ipsilateral side in sciatic nerve-ligated ICR mice. Repeated s.c. injection of saline, morphine (5 mg/kg), fentanyl (0.03 mg/kg) or oxycodone (0.5 mg/kg) was started 7 days after sciatic nerve ligation. ICR mice were repeatedly injected with saline, morphine, fentanyl or oxycodone once a day for 14 consecutive days. During the first 6 days after surgery, mice were not treated with saline, morphine, fentanyl or oxycodone. The thermal threshold was measured 7, 10, 13 and 20 days after ligation. Each column represents the mean ± standard error of the mean of 8–10 mice. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus Sham-saline group on day 1. N.S. = not significant

($^{***}P < 0.001$ versus sham-saline group, Fig. 3b). This reduction was not observed in the nerve-ligated β-endorphin KO mice treated with the optimum dose of fentanyl for 14 days (Fig. 4).

We further examined whether a single s.c. injection of fentanyl at relatively higher doses (0.03–0.17 mg/kg) could produce an antihyperalgesic effect in mice by using repeated treatment with an optimal dose of fentanyl under a neuropathic pain-like state (Fig. 5). Mice were repeatedly injected with saline or an optimal dose of fentanyl (0.03 mg/kg) for 14 consecutive days beginning at 7 days after nerve ligation. One day after the last injection of fentanyl, mice were challenged with fentanyl (0.03–0.17 mg/kg, Fig. 5). Fentanyl (0.056–0.17 mg/kg) failed to recover the decreased thermal threshold in nerve-ligated mice following the repeated injection of an optimal dose of fentanyl ($^{*}P < 0.05$ versus sham-saline group, Fig. 5).

Involvement of β-endorphin in the tolerance to fentanyl-induced antihyperalgesia under a pain-like state

We compared the potency of the antihyperalgesic effect induced by the repeated injection of fentanyl between nerve-ligated WT and β-endorphin KO mice (Fig. 6). In the present study, both WT and β-endorphin KO mice with partial sciatic nerve ligation exhibited a marked neuropathic pain-like behavior to almost the same degree ($^{***}P < 0.001$ versus sham-saline group Fig. 6). Under

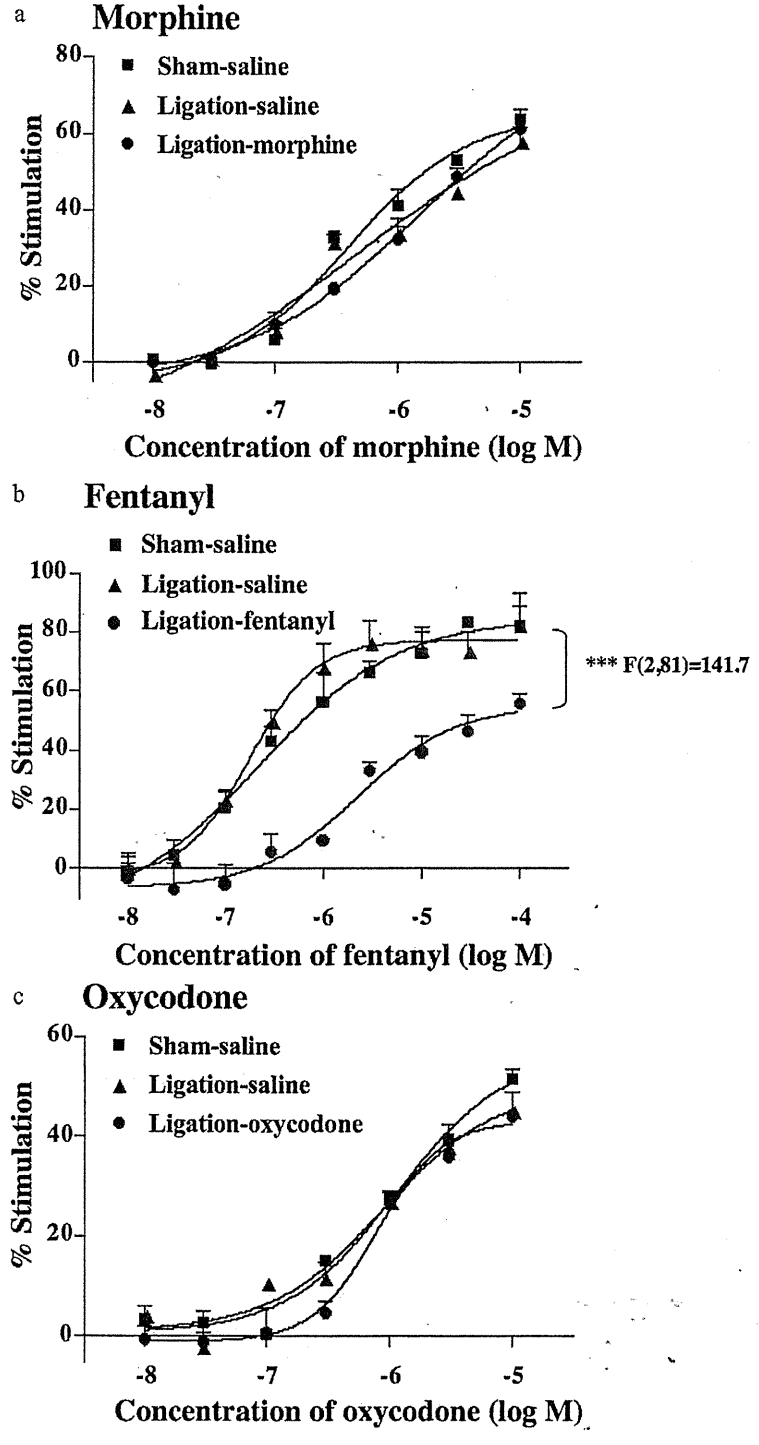

Figure 3 Effect of repeated injection of morphine (a), fentanyl (b) or oxycodone (c) on the morphine-, fentanyl- or oxycodone-induced increase in [^{35}S] GTPγS binding to membranes of the ipsilateral side of the spinal cord obtained from sham-operated and sciatic nerve-ligated ICR mice. Repeated s.c. injection of saline, morphine, fentanyl or oxycodone was started 7 days after sciatic nerve ligation. ICR mice were repeatedly injected with saline, morphine, fentanyl or oxycodone once a day for 14 consecutive days. During the first 6 days after surgery, mice were not treated with saline, morphine, fentanyl or oxycodone. Membranes were prepared at 21 days after nerve ligation. Each value represents the mean ± standard error of the mean of four samples

these conditions, the single s.c. injection of fentanyl (0.1 mg/kg) 7 days after nerve ligation almost completely reversed the decrease in the thermal threshold without excessive effects in sciatic nerve-ligated WT and β-endorphin KO mice, and maximal antihyperalgesic responses were seen at 15 minutes after fentanyl injection (Fig. 6). The antihyperalgesic effect following repeated treatment with fentanyl (0.1 mg/kg) was gradually tolerated from 14 days after sciatic nerve ligation in WT mice. In contrast, the potency of the antihyperalgesic effect of fentanyl was preserved in nerve-ligated β-endorphin KO mice under repeated s.c. treatment with fentanyl (##$P < 0.01$ versus knockout-ligation-fentanyl group; Fig. 6).

DISCUSSION

In the present study, a neuropathic pain-like state induced by partial sciatic nerve ligation was suppressed by the single s.c. injection of morphine, fentanyl or oxycodone in a dose-dependent manner. At doses of 5.0, 0.5 and 0.03 mg/kg, s.c. administration of morphine, oxycodone and fentanyl, respectively, completely reversed the decreased thermal threshold without excessive effects in nerve-ligated mice. Based on the present findings, we proposed that the optimal doses for the morphine-, oxycodone- and fentanyl-induced antihyperalgesic effects in sciatic nerve-ligated mice were 5 mg/kg, 0.5 mg/kg and 0.03 mg/kg, respectively. If we combine this result with our previous findings, the optimal dose for a morphine-induced antihyperalgesic effect in sciatic

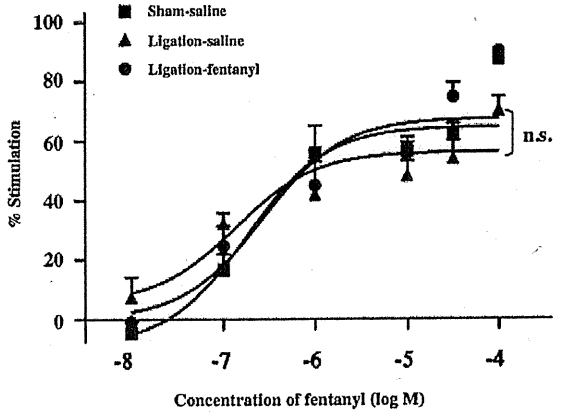

Figure 4 Effect of repeated injection of fentanyl on the fentanyl-induced increase in [^{35}S] GTPγS binding to membranes of the ipsilateral side of the spinal cord obtained from sham-operated and sciatic nerve-ligated β-endorphin knockout (KO) mice. Repeated s.c. injection of fentanyl was started 7 days after sciatic nerve ligation. Mice were repeatedly injected fentanyl once a day for 14 consecutive days. During the first 6 days after surgery, mice were not treated with fentanyl. Membranes were prepared at 21 days after nerve ligation. Each value represents the mean ± standard error of the mean of six samples. Each group was consisted of four males and two females. n.s. = not significant

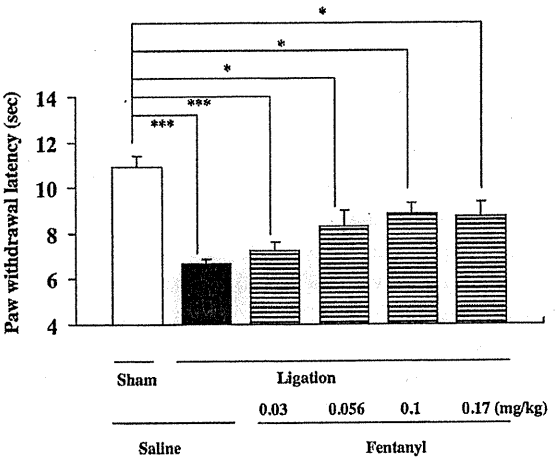

Figure 5 Effect of s.c. injection of fentanyl (0.03–0.17 mg/kg) on the latency of paw withdrawal in response to a thermal stimulus on the ipsilateral side in sciatic nerve-ligated ICR mice with the administration of fentanyl (0.03 mg/kg) for 14 consecutive days. The thermal threshold was measured 15 minutes after the s.c. injection of fentanyl. Each column represents the mean ± standard error of the mean of eight mice. *$P < 0.05$, and ***$P < 0.001$ versus Sham-saline group

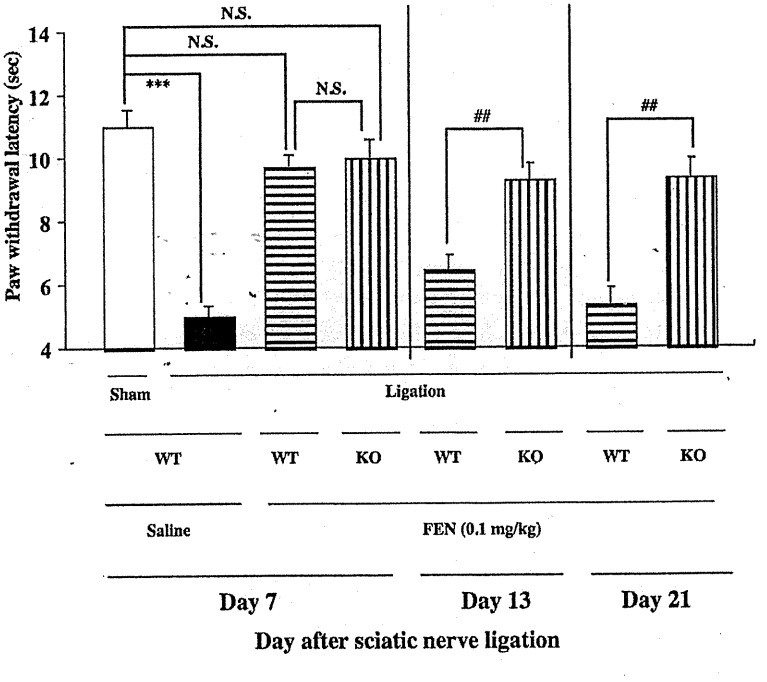

Figure 6 Effect of the repeated s.c. injection of fentanyl (0.1 mg/kg) on the latency of paw withdrawal in response to a thermal stimulus on the ipsilateral side in sciatic nerve-ligated, wild-type (WT) or β-endorphin knockout (KO) mice. Repeated s.c. injection of fentanyl was started 7 days after sciatic nerve ligation. Mice were repeatedly injected with fentanyl once a day for 14 consecutive days. During the first 6 days after surgery, mice were not treated with fentanyl. The thermal threshold was measured 7, 13 or 20 days after nerve ligation at 15 minutes after s.c. injection of fentanyl. Each column represents the mean ± standard error of the mean of five to six mice (consisting of two to three males and three females). ***$P < 0.001$ versus WT-sham-saline group. ##$P < 0.01$ versus β-endorphin KO-ligation-fentanyl group. N.S. = not significant