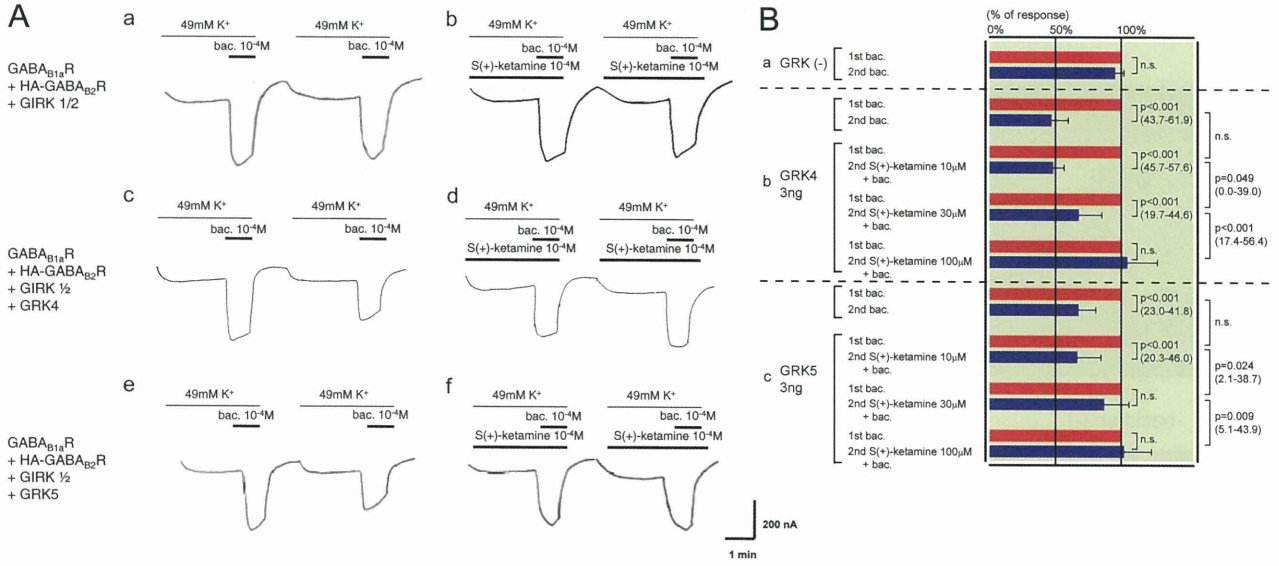

Fig. 1. Effects of *S*(+)-ketamine on the desensitization of γ-aminobutyric acid type B receptor ($GABA_BR$)–mediated G protein–activated inwardly rectifying K^+ channel (GIRK) currents in *Xenopus* oocytes. (*A*) Typical tracing of GIRK currents induced by the first and second application of baclofen (bac) (100 μM) for 1 min in a time lag of 4 min in oocytes coexpressing $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), hemagglutinin (HA)–$GABA_{B2}$ subunit (GB_2R), and GIRK1/2 without (*a*) or with (*b*) *S*(+)-ketamine (100 μM) before (2 min) and during (1 min) application of a second preapplication of bac. Typical tracing of GIRK currents induced by the first and second application of bac (100 μM) for 1 min in a time lag of 4 min in oocytes coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and G protein–coupled receptor kinase (GRK) 4 or 5 without (*c* and *e*) or with (*d* and *f*) *S*(+)-ketamine (100 μM) before (2 min) and during (1 min) application of a second preapplication of bac 49 mM k^+: 49 mM K^+ (high potassium) solution. (*B*) Summary of the effects of *S*(+)-ketamine on $GABA_BR$ desensitization. Each bar represents the mean ± SD of the peak GIRK currents induced by second application, expressed as percentage to each current induced by first application of bac in oocytes. (*a*) A group coexpressing $GB_{1a}R$, HA-GB_2R, and GIRK1/2, n = 8, (*b*) groups coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and GRK 4 (n = 10 for each group), (*c*) groups coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and GRK5 (n = 10 for each group). Statistical results are represented as *P* values (95% confidence interval for the differences in the two conditions). ns = not significant.

Statistical Analysis

Data are expressed as mean ± SD. For comparisons of the peak GIRK currents induced by second application of baclofen with those by first application of baclofen in *Xenopus* oocytes coexpressing $GB_{1a}R$, HA-GB_2R, and GIRK1/2 with or without GRK 4 or 5, two-tailed paired *t* tests were performed and the 95% confidence intervals (CIs) are depicted. The effects of *S*(+)-ketamine on the percentages of GIRK currents induced by second application of baclofen to each current induced by first application of baclofen were compared using one-way ANOVA, followed by the Tukey test. For comparison of FRET efficiency in BHK cells coexpressing $GB_{1a}R$, GB_2R-Venus, and GRKs-Cerulean, with or without *S*(+)-ketamine application before and during baclofen stimulation, two-tailed unpaired *t* tests were performed. Statistical significance was accepted at $P < 0.05$. All analyses were performed using computer software (IBM SPSS Statistics 18; IBM Corp, Armonk, NY).

Results

S(+)-Ketamine Inhibits the Desensitization of $GABA_B$ Receptor-Mediated Signaling by GRK 4 or 5 in *Xenopus* Oocytes

It was previously reported that baclofen elicited a GIRK conductance in *Xenopus* oocytes coexpressing heterodimeric $GABA_BR$ ($GB_{1a}R$ and HA-tagged GB_2R [HA-GB_2R]) with GIRKs 1 and 2 (GIRK1/2).[7] In addition, $GABA_BR$ desensitization was observed after repeated application of baclofen at 100 μM, which was a submaximum concentration to elicit inward K^+ current through GIRK1/2 to oocytes, coexpressing GRK 4 or 5 but not 2, 3, or 6.[7]

As previously demonstrated,[7] no desensitization was observed after repeated application of baclofen at 100 μM (for 1 min, each application) to oocytes coexpressing the $GB_{1a}R$ and HA-GB_2R with GIRK1/2 (fig. 1, A and B). When either GRK 4 (3 ng) or 5 (3 ng) cRNA was coinjected with heterodimeric $GABA_BR$ and GIRK1/2 cRNA, the amplitude of first baclofen-induced K^+ currents was almost the same as that in oocytes coexpressing $GABA_BR$ and GIRK1/2 without GRKs, whereas that of the second K^+ currents induced by baclofen was attenuated to 47.2 ± 12.7% (n = 8) in oocytes coexpressing GRK4 and to 67.6 ± 13.1% (n = 8) in oocytes coexpressing GRK5. This indicates that GRK 4 or 5 induced $GABA_BR$ desensitization (fig. 1, A and B). *S*(+)-Ketamine (100–300 μM) by itself had no effects on both the 49-mM K^+- and baclofen-induced K^+ currents in oocytes expressing $GABA_BR$ and GIRK1/2 without GRKs (fig. 1A and data not shown).

When *S*(+)-ketamine at a concentration of 10, 30, or 100 μM was applied before (2 min) and during the second application of baclofen (1 min) to oocytes coexpressing heterodimeric $GABA_BR$ and GIRK1/2 with GRK 4 or 5, the attenuation of the second baclofen-induced K^+ currents was

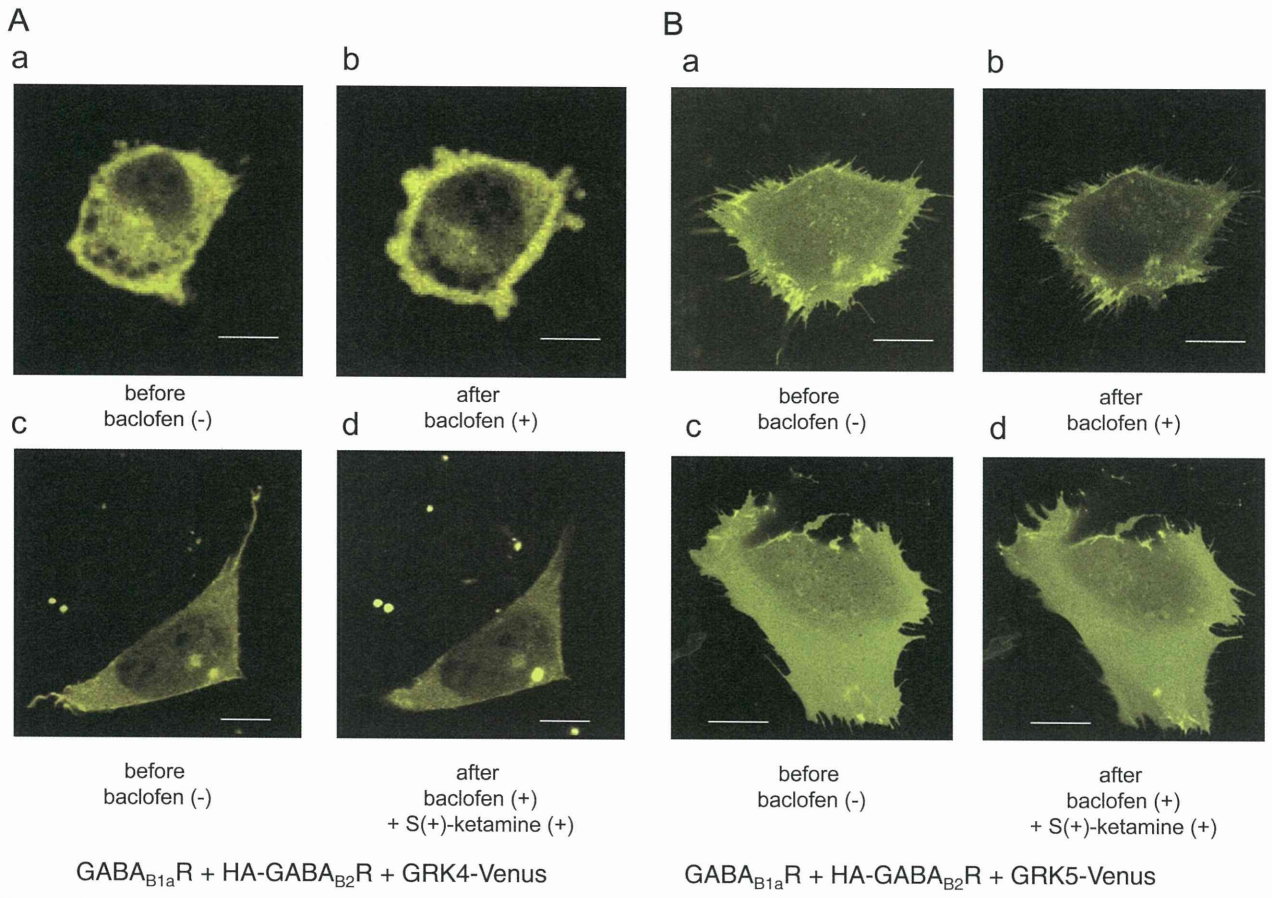

Fig. 2. Confocal imaging showing the effects of *S*(+)-ketamine on the translocation of G protein–coupled receptor kinase (GRK) 4–Venus or GRK5-Venus to the plasma membranes in baby hamster kidney (BHK) cells coexpressing the γ-aminobutyric acid $(GABA)_{B1a}$ receptor subunit ($GB_{1a}R$), hemagglutinin (HA)–$GABA_{B2}$ subunit (GB_2R), and GRKs-Venus. Each bar represents 10 μm. (*A*) Visualization of GRK4-Venus in the cells before (*a* and *c*) and after stimulation of baclofen (100 μM) for 5 min with (*d*) or without (*b*) previous application of *S*(+)-ketamine (100 μM) for 5 min in BHK cells coexpressing $GB_{1a}R$, HA-GB_2R, and GRK4-Venus. (*B*) Visualization of GRK5-Venus in BHK cells before (*a* and *c*) and after stimulation of baclofen for 5 min with (*d*) or without (*b*) previous application of *S*(+)-ketamine for 5 min in BHK cells coexpressing $GB_{1a}R$, HA-GB_2R, and GRK5-Venus.

significantly restored in a concentration-dependent manner (fig. 1, A and B). The amplitude of K^+ currents induced by the second application of baclofen with 10-, 30-, or 100-μM *S*(+)-ketamine was 48.3 ± 8.4%, 67.9 ± 17.4%, and 104.8 ± 22.7% in oocytes coexpressing GRK4 (n = 10 each) and 66.8 ± 17.9%, 87.2 ± 18.7%, and 102.4 ± 20.6% in oocytes coexpressing GRK5 (n = 10 each) of those induced by the first application of baclofen, respectively (fig. 1, A and B). When typical GIRK currents were not obtained by first application of baclofen, such data were excluded. Overall, approximately 67–83% of recording data in each group of oocytes were obtained for statistical analyses.

Translocation of Venus-Fused GRK 4 or 5 to the Plasma Membranes after Activation of $GABA_BR$ Is Inhibited in the Presence of S(+)-Ketamine

To determine the effects of *S*(+)-ketamine on the translocation of GRK 4 or 5 in response to baclofen in BHK cells, we cotransfected GRK4-Venus or GRK5-Venus cDNA with $GB_{1a}R$ and HA-GB_2R cDNAs and determined the intracellular distribution and translocation properties of GRK4-Venus or GRK5-Venus. We then applied baclofen with or without *S*(+)-ketamine application to living BHK cells. As shown in figure 2, A and B, GRK4-Venus or GRK5-Venus was diffusely distributed in the cytosol without agonist stimulation in BHK cells but was translocated to the plasma membranes gradually in 5 min after application of baclofen (100 μM). When *S*(+)-ketamine (100 μM) was applied to such cells 2.5 min before and during application of baclofen, the translocation of GRK4-Venus or GRK5-Venus to the plasma membranes was almost inhibited (fig. 2, A and B). Treatment of *S*(+)-ketamine (100 and 300 μM) alone for 10 min did not affect translocation properties of both GRK4-Venus and GRK5-Venus in BHK cells coexpressing heterodimeric $GABA_BR$ with GRK4-Venus or GRK5-Venus (data not shown).

FRET and Acceptor Photobleaching Analysis of BHK Cells Coexpressing GRK 4 or 5 with Heterodimeric $GABA_BR$

Previously, we showed that functional $GABA_BR$ formed heterodimers with $GB_{1a}R$ and GB_2R by analysis with FRET and

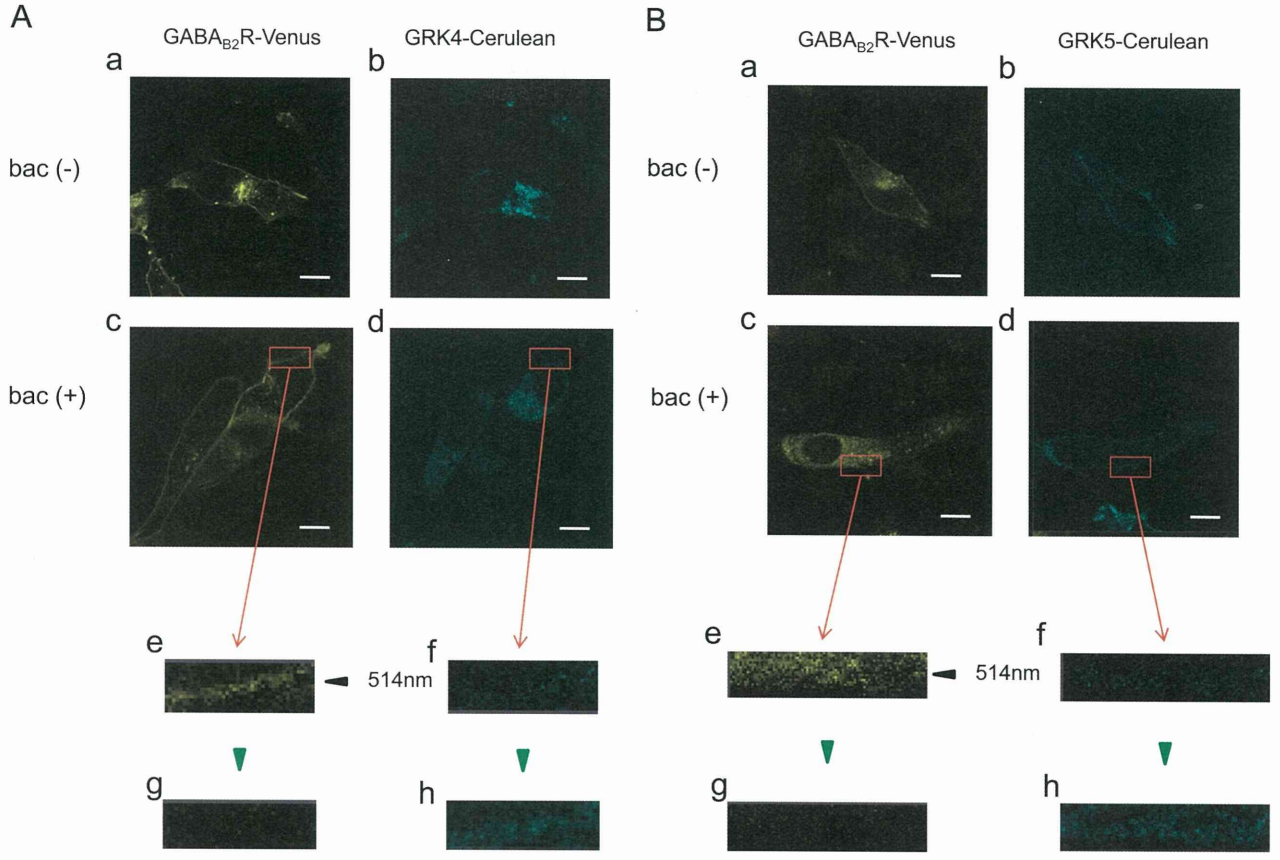

Fig. 3. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the protein complex formation of the γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing the $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), GB_2R-Venus, and GRKs-Cerulean. Each bar represents 10 μm. (*A*) Visualization of GB_2R-Venus and GRK4-Cerulean in nonstimulated (*a* and *b*) and baclofen (bac)-stimulated (100 μM, 5 min) BHK cells (*c* and *d*). Fluorescence changes by acceptor photobleaching (1-min application of 514-nm wavelength) in bac-stimulated BHK cells (*e–h*). (*B*) Visualization of GB_2R-Venus and GRK5-Cerulean in nonstimulated (*a* and *b*) and bac-stimulated (100 μM, 5 min) BHK cells (*c* and *d*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*e–h*).

acceptor photobleaching in BHK cells coexpressing $GB_{1a}R$-Venus and GB_2R-Cerulean.[7,20] We also showed that GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with the GB_2R subunit after $GABA_BR$ activation in the cells coexpressing Venus-fused $GB_{1a}R$ or GB_2R and Cerulean-fused GRKs.[7] We examined the effects of *S*(+)-ketamine on the formation of protein complexes of GRK 4 or 5 with GB_2R in BHK cells coexpressing $GB_{1a}R$, GB_2R-Venus, and GRK4-Cerulean (fig. 3A) or GRK5-Cerulean (fig. 3B). The fluorescence from GB_2R-Venus was mostly localized on the plasma membranes, whereas that from GRK4-Cerulean or GRK5-Cerulean was localized in the cytosol and to some extent on the plasma membranes (fig. 3A, a and b, and 3B, a and b). When cells were stimulated with baclofen (100 μM) for 5 min, the fluorescence of GRK4-Ceulean or GRK5-Cerulean and GB_2R-Venus was detected on and around the plasma membranes (fig. 3A, c and d, and 3B, c and d). Photobleaching analysis demonstrated that Venus fluorescence was reduced by application of a 514-nm wavelength at 100% intensity of the argon laser power to the indicated area (fig. 3A, e–h, and 3B, e–h). This application did not affect the fluorescent intensity of Venus and Cerulean in the unbleached area (data not shown). Acceptor photobleaching showed increased Cerulean fluorescence (donor) with decreased Venus fluorescence (acceptor) (fig. 3A, e–h, and 3B, e–h).

To determine the effects of *S*(+)-ketamine on the protein complex formation of GRK4-Cerulean or GRK5-Cerulean with GB_2-Venus plus $GB_{1a}R$, we applied *S*(+)-ketamine (100 μM) to the cells 5 min before application of baclofen (100 μM) and then simultaneously treated the cells for 5 min with baclofen and *S*(+)-ketamine. The fluorescence from GRK4-Cerulean or GRK5-Cerulean was detected diffusely in the cytosol and on the plasma membranes, whereas the fluorescence from GB_2R-Venus was mostly detected on the plasma membranes. Acceptor photobleaching demonstrated the reduction of the fluorescence from GB_2R-Venus; however, the fluorescence from GRK4-Cerulean or GRK5-Cerulean hardly changed (fig. 4, A and B; and fig. 5), which indicates that GRK4-Cerulean or GRK5-Cerulean and

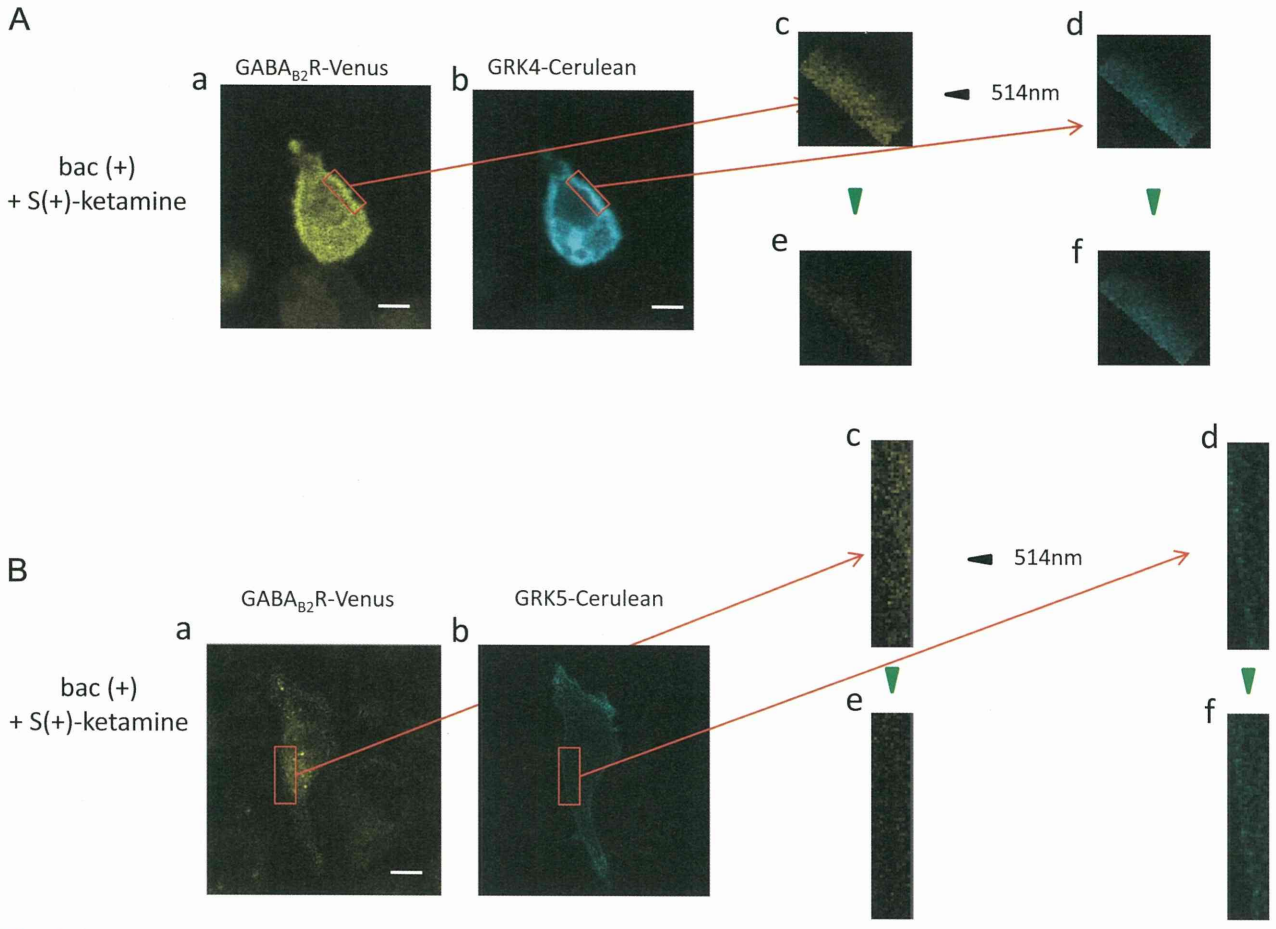

Fig. 4. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the effects of *S*(+)-ketamine on the interaction of γ-aminobutyric acid (GABA)$_{B2}$ subunit (GB$_2$R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing GABA$_{B1a}$ receptor subunit (GB$_{1a}$R), GB$_2$R-Venus, and GRKs-Cerulean. Each bar represents 10 μm. (*A*) Visualization of GB$_2$R-Venus and GRK4-Cerulean in a BHK cell treated by *S*(+)-ketamine (100 μM) before (5 min) and during (5 min) baclofen (bac) stimulation (*a* and *b*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*c–f*). (*B*) Visualization of GB$_2$R-Venus and GRK5-Cerulean in a BHK cell pretreated with *S*(+)-ketamine (100 μM) before (5 min) and during (5 min) bac stimulation (*a* and *b*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*c–f*).

GB$_2$R-Venus do not form baclofen-induced protein complexes in the presence of *S*(+)-ketamine.

Coimmunoprecipitation and Western Blot Analysis of GRK 4 or 5 Using BHK Cells Coexpressing FLAG-GRKs, HA-GB$_2$R, and GB$_{1a}$R

Previously, it was shown that FLAG-GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with HA-GB$_2$R after baclofen stimulation (100 μM, 5 min) in BHK cells determined with coimmunoprecipitation and Western blot analysis.[7] We investigated whether *S*(+)-ketamine has an effect on the protein complex formation of GRK 4 or 5 with GB$_2$R induced by baclofen. Western blot analysis was performed with proteins extracted from BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, GB$_{1a}$R, and HA-GB$_2$R after immunoprecipitation with anti-HA. In the precipitate using anti-HA from the BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB$_2$R, and GB$_{1a}$R, the band intensity of the immune complex determined with anti-HA was similar in nonstimulated and baclofen-stimulated (100 μM, 5 min) BHK cells (fig. 6A). On the other hand, the immune complex determined with anti-FLAG was stronger in baclofen-stimulated cells than that in nonstimulated cells (fig. 6B).

To determine the effect of *S*(+)-ketamine on the protein complex formation of FLAG-GRK4 or FLAG-GRK5 with GB$_2$R, we treated *S*(+)-ketamine (100 μM) to the cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB$_2$R, and GB$_{1a}$R 5 min before and during the stimulation of baclofen (5 min, 100 μM). In the precipitate using anti-HA from the cells coexpressing either FLAG-GRK4 or FLAG-GRK5 with HA-GB$_2$R and GB$_{1a}$R, the intensity of the immune complex with anti-HA was similar among nonstimulated and baclofen-stimulated cells with or without *S*(+)-ketamine treatment (fig. 6A). On the other hand, the intensity of the immune complex determined with anti-FLAG was less in baclofen-stimulated cells with *S*(+)-ketamine treatment than in baclofen-stimulated cells without *S*(+)-ketamine treatment; and the intensity in baclofen-stimulated cells with

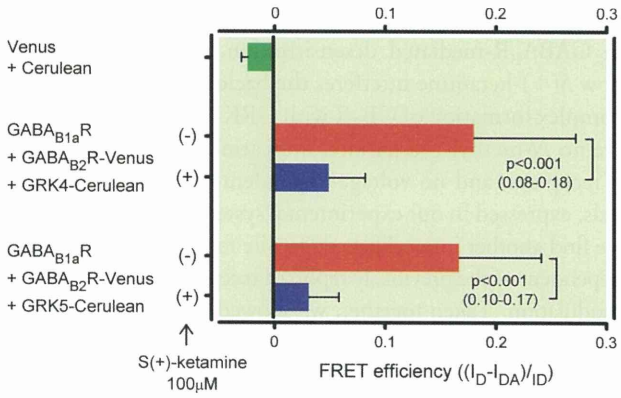

Fig. 5. Comparison of fluorescence resonance energy transfer (FRET) efficiency in baby hamster kidney (BHK) cells expressing γ-aminobutyric acid $(GABA)_{B1a}$ receptor subunit ($GB_{1a}R$), $GABA_{B2}$ subunit (GB_2R)–Venus, and G protein–coupled receptor (GRK) 4–Cerulean or GRK5-Cerulean, with or without previous stimulation of *S*(+)-ketamine (n = 8 for each group). The FRET efficiency was calculated from emission spectra. Each bar represents the mean ± SD. Statistical results are represented as *P* values (95% confidence interval for the differences in the two conditions). I_D = peak of donor emission in presence of sensitized acceptor; I_{DA} = peak of donor emission in presence of acceptor.

S(+)-ketamine was almost similar to that in nonstimulated cells (fig. 6B). In the total lysate, the intensity of the immune complex determined with anti-FLAG was similar among nonstimulated and baclofen-stimulated cells with or without *S*(+)-ketamine treatment (fig. 6C). *S*(+)-Ketamine treatment alone (100 μM) did not affect the intensity of the immune complex determined with anti-HA (HA-$GABA_{B2}R$) and that determined with anti-FLAG (FLAG-GRK4 and FLAG-GRK5) (data not shown).

Discussion

Previously, it was demonstrated that the desensitization of $GABA_BR$-mediated responses was associated with the formation of protein complexes of the GB_2R subunit with GRK 4 or 5 on the plasma membranes, which may cause signal disconnection from the receptors to downstream transducers, such as G proteins.[7] In the current study, the same desensitization was observed by the second application of baclofen in *Xenopus* oocytes coexpressing heterodimeric $GABA_BR$ and GIRKs in the presence of GRK 4 or 5. We demonstrated that pretreatment of *S*(+)-ketamine significantly suppressed such desensitization. Furthermore, our results showed that the translocation of GRK4-Venus or GRK5-Venus to the plasma membranes after stimulation of baclofen was inhibited by pretreatment of *S*(+)-ketamine in BHK cells. In addition, FRET analysis showed that *S*(+)-ketamine inhibited the protein complex formation of GB_2R-Venus with GRK4-Cerulean or GRK5-Cerulean in the cells. Such an inhibitory effect of protein complex formation by *S*(+)-ketamine was also confirmed by coimmunoprecipitation and Western blot analysis in cells coexpressing HA-GB_2R, $GB_{1a}R$, and FLAG-

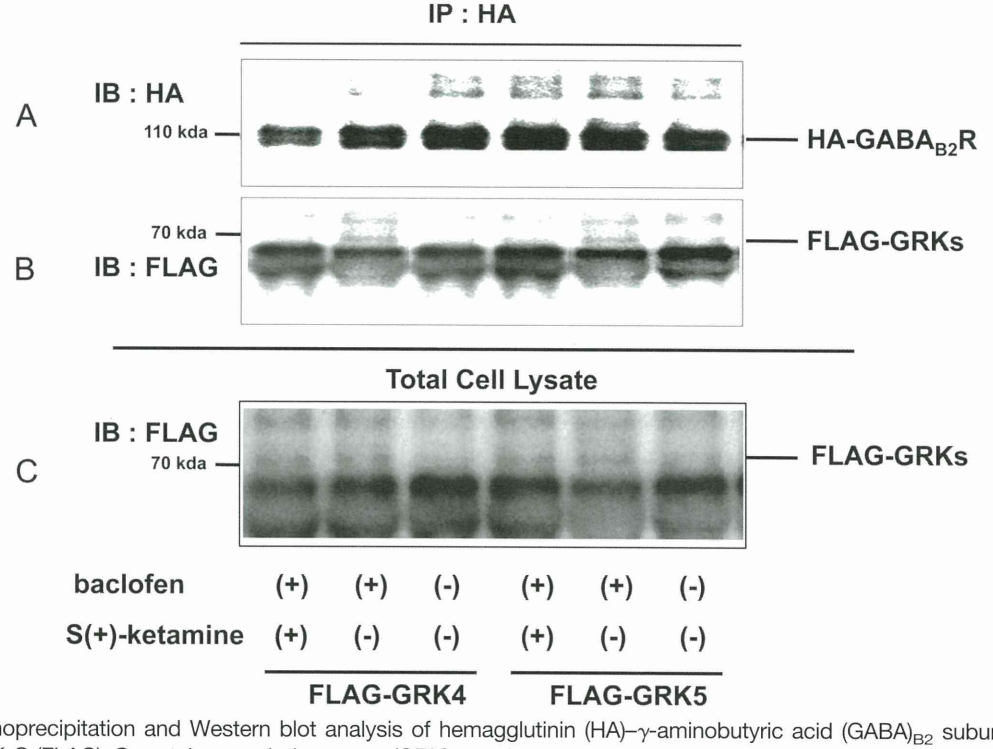

Fig. 6. Immunoprecipitation and Western blot analysis of hemagglutinin (HA)–γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) and N-DYKDDDDK-C (FLAG)–G protein–coupled receptor (GRK) proteins extracted from nonstimulated cells, baclofen-stimulated cells (100 μM, 5 min), or baclofen-stimulated cells (100 μM, 5 min) with previous stimulation of *S*(+)-ketamine (100 μM, 5 min), coexpressing $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), HA-GB_2R, and FLAG-GRKs. Western blot of anti–HA immunoprecipitates from FLAG-GRK4– or FLAG-GRK5–expressing cells determined with anti-HA (*A*) and anti-FLAG (*B*) and with anti-FLAG in the total lysate (*C*).

GRK4 or FLAG-GRK5. Collectively, these results suggest that *S*(+)-ketamine could suppress the GRK 4– or 5–induced $GABA_BR$ desensitization, at least in part, by interfering with the protein complex formation of GRK 4 or 5 with the GB_2R subunit.

The selective $GABA_BR$ agonist baclofen is widely used as a spasmolytic drug. ITB therapy, proposed by Penn and Kroin[26] in 1984, is a method for the treatment of spasticity and rigidity of spinal and cerebral origin, approved by the Food and Drug Administration in 1992.[1] Recently, it was reported that ITB therapy is also effective in the management of various forms of chronic pain, with or without spasticity.[1–5] There is no doubt that ITB therapy will play a greater part in the management of chronic pain[1]; however, long-term management of ITB therapy has been reported to occasionally result in the development of tolerance to baclofen in both clinical[6] and animal[27] studies. Several reports have shown that intrathecal administration of morphine in place of baclofen for some period (the so-called baclofen holiday)[28] or a shift in treatment to continuous intrathecal morphine administration[29] was effective for pain management in patients who had developed tolerance against ITB therapy. However, the preventive measures for the development of baclofen tolerance have not been established yet.

Baclofen tolerance is the condition in that gradually increased doses of baclofen are required to keep the therapeutic effects stable. Many processes underlie baclofen tolerance *in vivo*, including adaptations in neural circuitry (*e.g.*, descending excitatory pathways) and changes in neurotransmitter signaling pathways surrounding the $GABA_BR$ neuron. In addition, cellular responses mediated by $GABA_BR$ are attributed to the development of baclofen tolerance. In the rat model, ITB down-regulated the number of $GABA_BR$ binding sites in the spinal cord.[30] Desensitization of $GABA_BR$-mediated signaling is one of the mechanisms of development of baclofen tolerance. The desensitization of $GABA_BR$ was induced after protein complex formation of GB_2R with GRK 4 or 5.[7,8] Ketamine is an agent that has widely been used as an analgesic for postoperative pain,[18] chronic noncancer pain,[31] and cancer pain.[32] Although it has been commonly acknowledged that ketamine shows an analgesic effect by blocking the *N*-methyl-D-aspartate receptors in the central nervous system, many other prospective targets are reported (*e.g.*, muscarinic acetylcholine receptors,[33] opioid receptors,[34] substance P receptors,[35] and voltage-dependent Na^+ and K^+ channels).[36] In animal studies, intrathecal[13] or subcutaneous[14] administration of ketamine attenuated the development of tolerance to morphine. The precise mechanisms of such phenomena were not understood; however, tolerance of opioids to μ-opioid receptors could be attributed by receptor desensitization, in which GRKs 2 and 3 were involved.[15–17] One possibility is that ketamine would inhibit μ-opioid receptor–mediated desensitization by modulation of GRK 2 or 3. Likewise, we expected, and suggested, that *S*(+)-ketamine would attenuate the development of tolerance to baclofen to the sites where GRK 4 or 5 is involved in $GABA_BR$-mediated desensitization.[7,8] It is not known how *S*(+)-ketamine interferes the baclofen-induced protein complex formation of GB_2R with GRK 4 or 5. Because there are no *N*-methyl-D-aspartate, muscarinic, opioid, substance P receptors, and no voltage-dependent Na^+ and K^+ channels, expressed in our experimental system, we could say that we find another intracellular target site for ketamine that is independent of the previously reported receptors and ion channel modulation. Taken together, we showed, for the first time to our knowledge, that desensitization of $GABA_BR$-mediated signaling was significantly attenuated by pretreatment of *S*(+)-ketamine, suggesting that S(+)-ketamine suppresses baclofen-induced $GABA_BR$ desensitization, possibly followed by greater antinociceptive effects when used in ITB therapy for long-term pain management.

Clinically, our results propose the possibility that combination intrathecal administration of *S*(+)-ketamine with ITB therapy provides high-quality pain relief without tolerance of ITB to patients experiencing chronic pain. Intrathecal ketamine has been administered in an animal model and to humans, but the safety of preservative-free ketamine through the intrathecal route remains controversial.[37–40] Although some reports have shown no neurotoxic damage after intrathecal administration of preservative-free ketamine using pig[37] and rabbit[38] models, recent animal studies have shown the severe neurotoxicity of intrathecal administration of ketamine with canine[39] and rabbit.[40] Pathologic findings also demonstrated subpial spinal cord vacuolar myelopathy after intrathecal ketamine in a terminally ill cancer patient who received continuous-infusion intrathecal ketamine for 3 weeks.[41] Furthermore, the continuous intrathecal administration of *S*(+)-ketamine, in combination with morphine, bupivacaine, and clonidine, resulted in adequate pain relief in a patient experiencing intractable neuropathic cancer pain; however, postmortem observation of the spinal cord and nerve roots revealed severe histologic abnormalities, including central chromatolysis, nerve cell shrinkage, neuronophagia, microglial up-regulation, and gliosis.[42] A recent report[43] indicates that the neurotoxicity of *S*(+)-ketamine is produced by blockade of *N*-methyl-D-aspartate receptors on the inhibitory neurons, resulting in an exicitotoxic injury through hyperactivation of muscarinic M_3 receptors and non–*N*-methyl-D-aspartate glutamate receptors in the cerebral cortex. Yaksh *et al.*[39] recently reported the detailed toxicology profile of an *N*-methyl-D-aspartate antagonist, including ketamine, delivered through long-term (28-day) intrathecal infusion in the canine model and suggested needs for reevaluation of the use of these agents in long-term spinal delivery. Clinical and pathologic results from an animal or clinical study with intrathecal administration of a combination of baclofen and ketamine have not been reported. Thus, carefully designed studies with an animal model and a clinical trial should be required to know how ketamine (*i.e.*, timing of administration, concentration, duration of adminis-

tration, and ratio of doses of ketamine and baclofen) is safely administered without pathophysiologic findings and how it might suppress the development of baclofen-induced tolerance clinically.

In conclusion, we demonstrated that *S*(+)-ketamine suppressed the baclofen-induced desensitization of $GABA_BR$-mediated signaling, at least in part, through inhibition of protein complex formation of the GB_2R subunit and GRK 4 or 5. If the safety of intrathecal administration of *S*(+)-ketamine is established, it could be a candidate for preventing the development of tolerance against ITB therapy in long-term spasticity and pain management.

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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

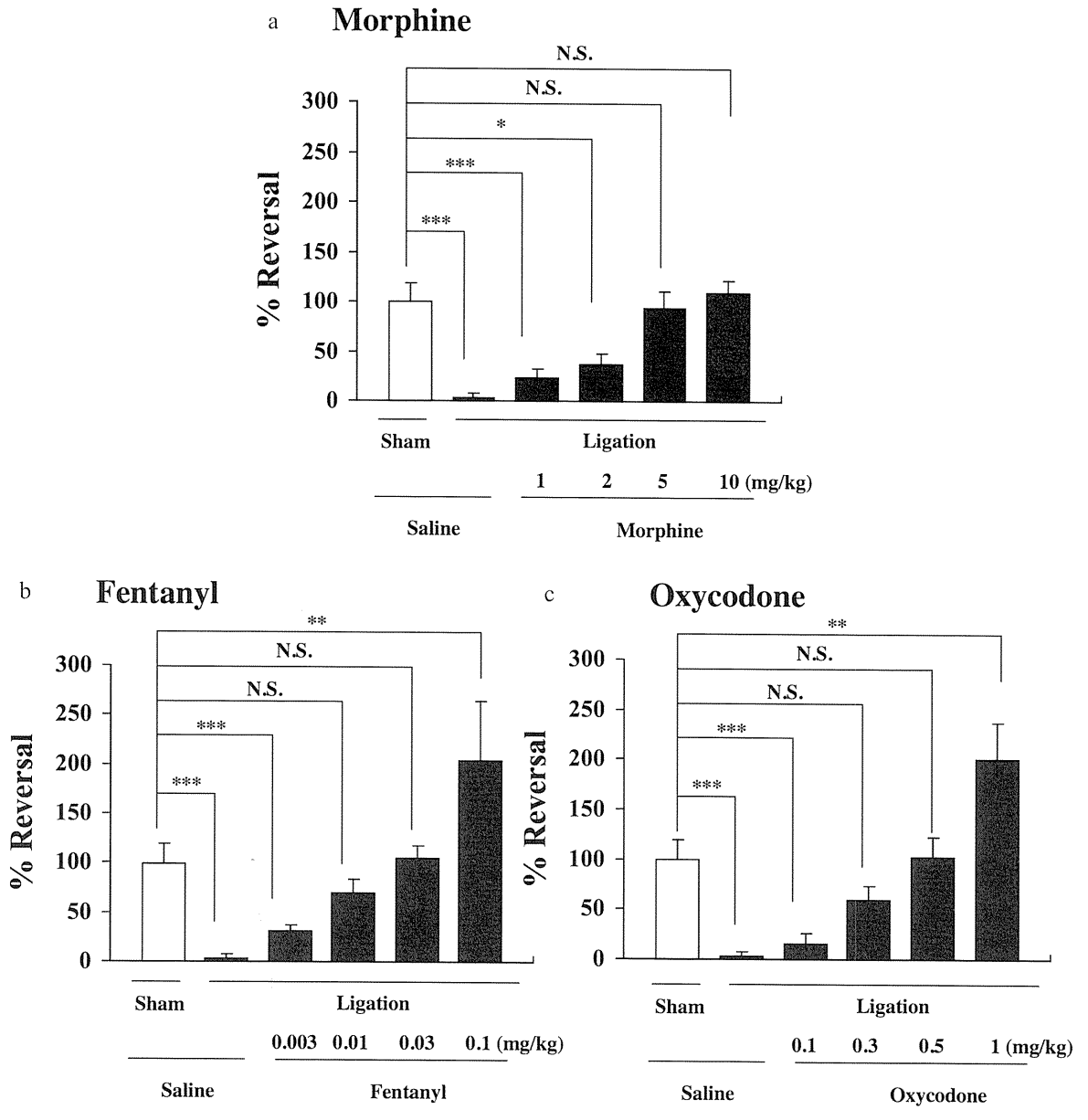


Figure 1 Effect of s.c. injection of morphine, fentanyl or oxycodone on the latency of paw withdrawal in response to a thermal stimulus on the ipsilateral side in sham-operated or sciatic nerve-ligated ICR mice. The thermal threshold was measured just before and 30, 15 or 15 minutes after s.c. injection 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

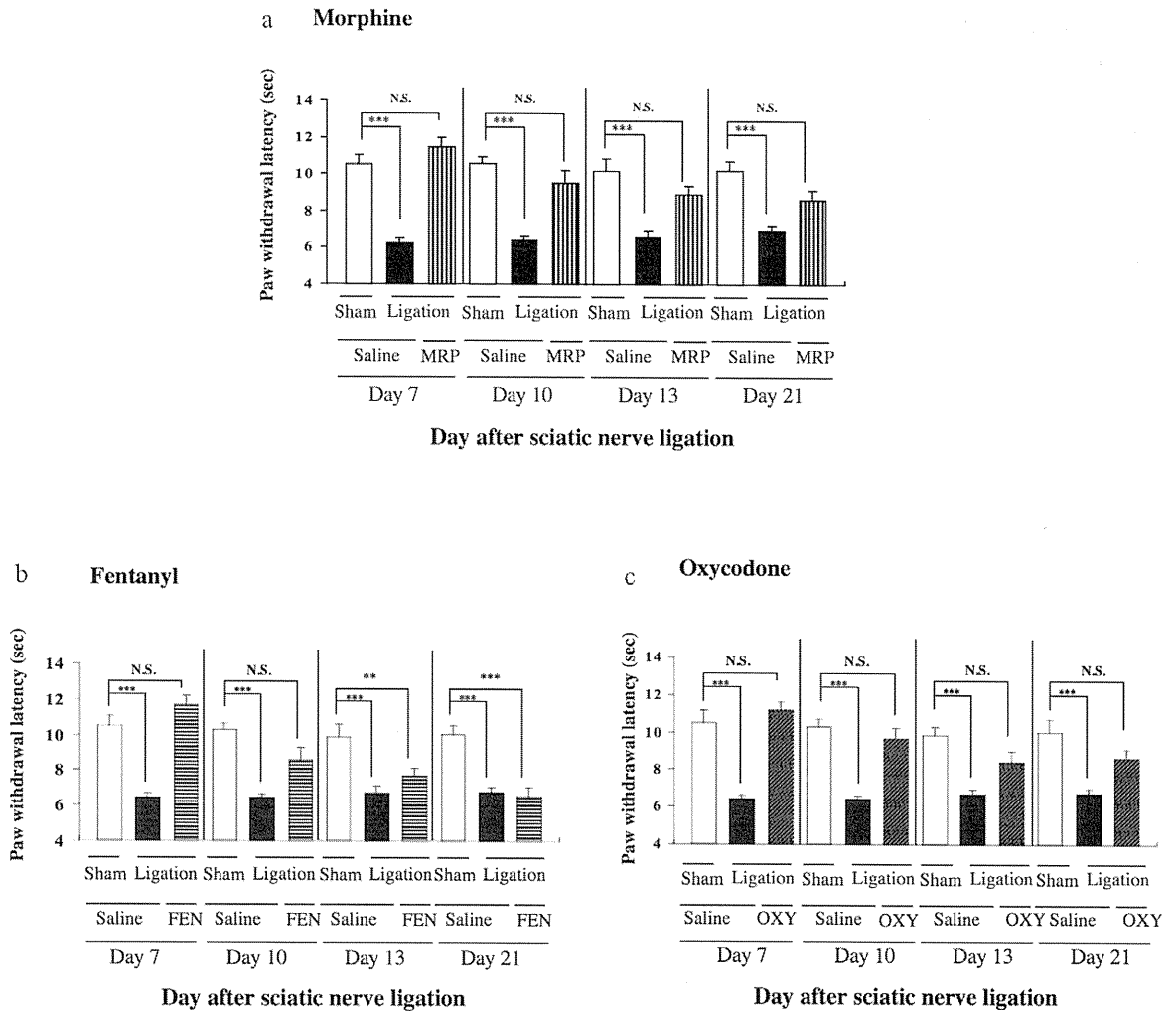


Figure 2 Effect of repeated s.c. injection of morphine (MRP) (a), fentanyl (FEN) (b) or oxycodone (OXY) (c) on the latency of paw withdrawal in response to a thermal stimulus on the ipsilateral side in sciatic nerve-ligated ICR mice. Repeated s.c. injection of saline, morphine (5 mg/kg), fentanyl (0.03 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

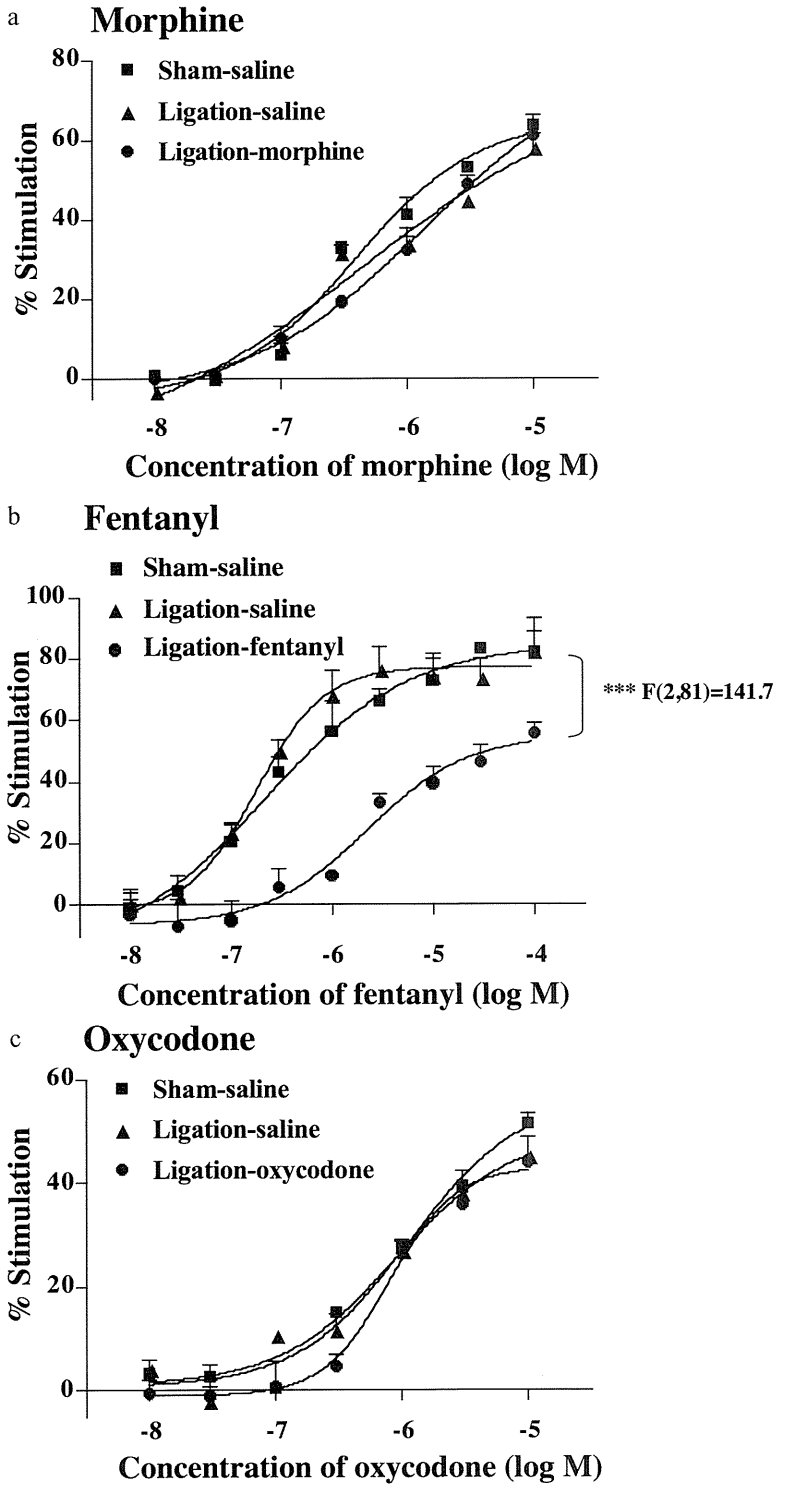


Figure 3 Effect of repeated injection of morphine (a), fentanyl (b) or oxycodone (c) on the morphine-, fentanyl- or oxycodone-induced increase in [^{35}S] GTPγS binding to membranes of the ipsilateral side of the spinal cord obtained from sham-operated and sciatic nerve-ligated ICR mice. Repeated s.c. 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

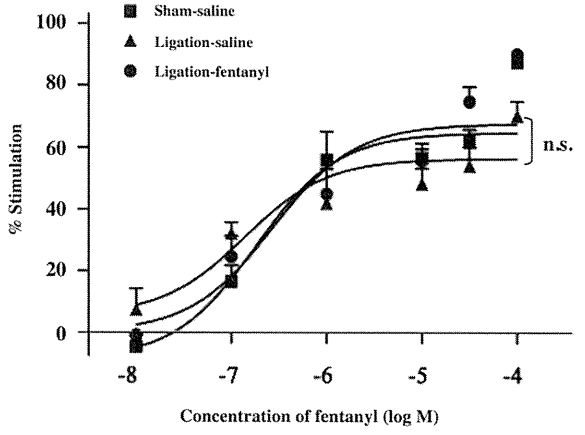


Figure 4 Effect of repeated injection of fentanyl on the fentanyl-induced increase in [^{35}S] GTPγS binding to membranes of the ipsilateral side of the spinal cord obtained from sham-operated and sciatic nerve-ligated β-endorphin knockout (KO) mice. Repeated s.c. injection of fentanyl was started 7 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

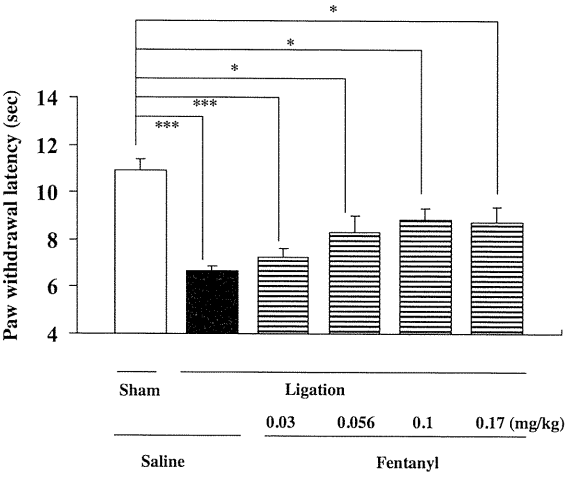
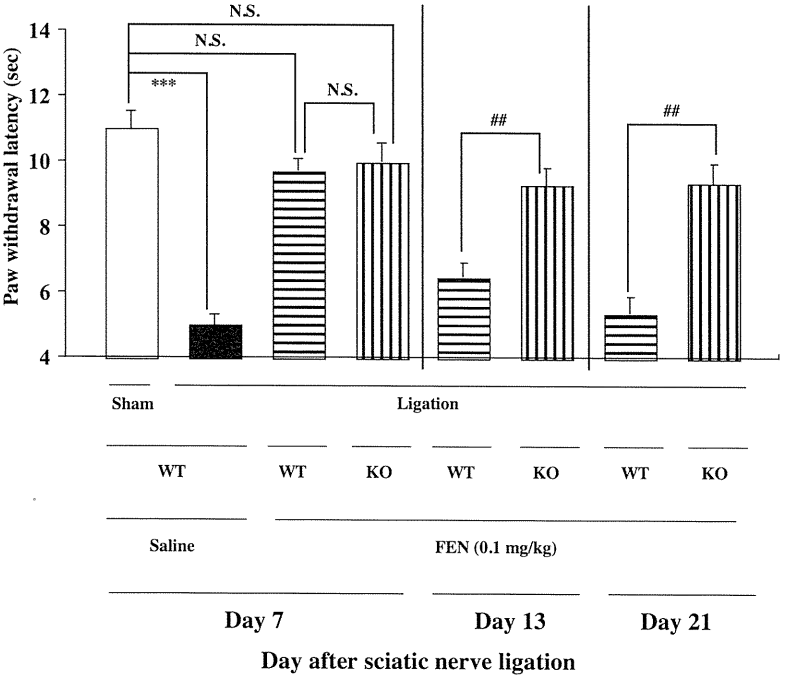


Figure 5 Effect of s.c. injection of fentanyl (0.03–0.17 mg/kg) on the latency of paw withdrawal in response to a thermal stimulus on the ipsilateral side in sciatic nerve-ligated ICR mice with the administration of fentanyl (0.03 mg/kg) for 14 consecutive days. The thermal threshold was measured 15 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



nerve-ligated mice was higher than that under inflammatory pain, whereas the optimal doses for fentanyl and oxycodone under a neuropathic pain-like state and an inflammatory pain-like state were similar. Under these conditions, the antihyperalgesic effect induced by fentanyl in mice with sciatic nerve ligation rapidly disappeared during the consecutive administration of fentanyl (0.03 mg/kg), whereas the potencies of morphine (3 mg/kg) and oxycodone (0.5 mg/kg) with regard to their antihyperalgesic effects were preserved in nerve-ligated mice even after repeated s.c. treatment with morphine or oxycodone. Furthermore, even relatively higher doses of fentanyl (0.056–0.17 mg/kg) failed to reverse the hyperalgesia in sciatic nerve-ligated mice under the consecutive administration of fentanyl (0.03 mg/kg). Consistent with these results, the dose-response curve for G-protein activation induced by fentanyl was significantly shifted to the right and its maximal response was dramatically decreased in membranes of the spinal cord of nerve-ligated mice following the repeated injection of fentanyl (ligation-fentanyl group) compared with those in the sham-fentanyl and ligation-saline group. In contrast, these phenomena were not observed in nerve-ligated mice with the repeated administration of morphine or oxycodone. These findings provide evidence that the consecutive injection of fentanyl, unlike morphine and oxycodone, may extensively induce the development of tolerance to its antihyperalgesic effect under a persistent pain state. This event could be associated with the repeated administration of fentanyl-induced functional desensitization of MORs under a neuropathic pain-like state.

Several lines of evidence indicated that, in response to a 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). Therefore, we next examined using β-endorphin KO mice whether a lack of β-endorphin expression could affect fentanyl-induced tolerance to antinociception under a neuropathic pain-like state. These β-endorphin KO mice showed no changes in the expression of other peptide products (e.g. ACTH and MSH) from the *POMC* gene (Rubinstein *et al.* 1996). With β-endorphin KO mice, we began by investigating whether a deletion of the β-endorphin gene could influence the development of a neuropathic pain-like state induced by sciatic nerve ligation in mice. As a result, there were no differences in decreased thermal hyperalgesia or increased tactile allodynia between β-endorphin KO and WT mice. Under these conditions, the fentanyl-induced antihyperalgesic tolerance under sciatic nerve ligation was abolished in β-endorphin KO mice. In addition, the reduced activation of G-proteins by fentanyl observed in the spinal cord of nerve-ligated mice after the repeated s.c. injection of fentanyl was dramatically suppressed in the spinal cord of nerve-ligated β-endorphin KO mice treated with the optimum dose of fentanyl for 14 days. These results suggest that released endogenous β-endorphin, in response to long-lasting pain, may play a critical role in the fentanyl-induced antihyperalgesic tolerance under a neuropathic pain-like state.

It has been widely accepted that receptor desensitization appear to play a key role in the development of opioid tolerance (Bohn *et al.* 2000; Gainetdinov *et al.* 2004; Walwyn *et al.* 2004). Furthermore, it has been considered that opioid tolerance is, in part, the end result of internalized MORs (Whistler & von Zastrow, 1998, 1999; Claing *et al.* 2002; Kieffer & Evans 2002; Koch *et al.* 2005; Zollner *et al.* 2008). 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 of evidence suggests that among diverse serine (Ser)/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). Althoug further studies are still needed, the present study raise the possibility that released β-endorphin within the spinal cord may result in a loss of the coordinated balance between processes that govern the desensitization, internalization and resensitization of MORs. This phenomenon could be associated with the mechanism that underlies the rapid development of tolerance to fentanyl under a neuropathic pain-like state.

CONCLUSION

We have demonstrated that repeated treatment with fentanyl at an excessive dose causes a rapid antihyperalgesic tolerance in sciatic nerve-ligated mice, whereas morphine and oxycodone do not produce this phenomenon. This condition may reflect the clinical observation that tolerance to morphine analgesia is not a major concern when patients suffer from severe pain. In addition, the discrepancy between the present findings and classical basic understanding that chronic morphine treatment is

believed to lead to severe analgesic tolerance may result from the fact that most previous studies concerning molecular events in opioid tolerance have been performed using an excessive dose of MOR agonists in naive rodents. Furthermore, the present findings strongly indicate that β-endorphin within the spinal cord may be involved in the prolongation of the fentanyl-induced desensitization of MORs. This phenomenon may explain the high degree of tolerance to fentanyl-induced antihyperalgesia under a neuropathic pain-like state in rodents.

Authors Contribution

NM, HM, UY, DLA, ST designed the study. NM, IS wrote the manuscript. IS, NA, OA, AM, RM, SY, KN performed the experiments. IS, NA, OA, AM, RM, SY, KN performed the data analysis. All authors have critically reviewed content and approved final version submitted for publication.

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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

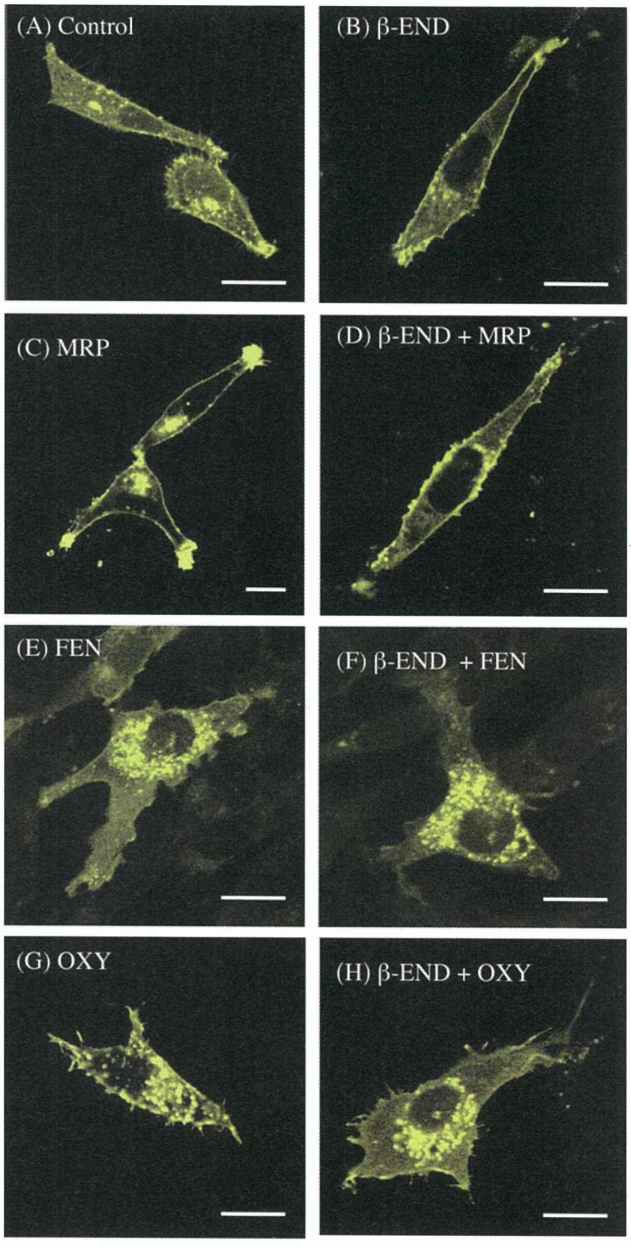

Fig. 1. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. The cells were incubated in the absence (**A**, **C**, **E**, and **G**) or presence (**B**, **D**, **F**, and **H**) of 100 nM β-endorphin (β-END) for 30 min at 37°C and then treated with 10 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.

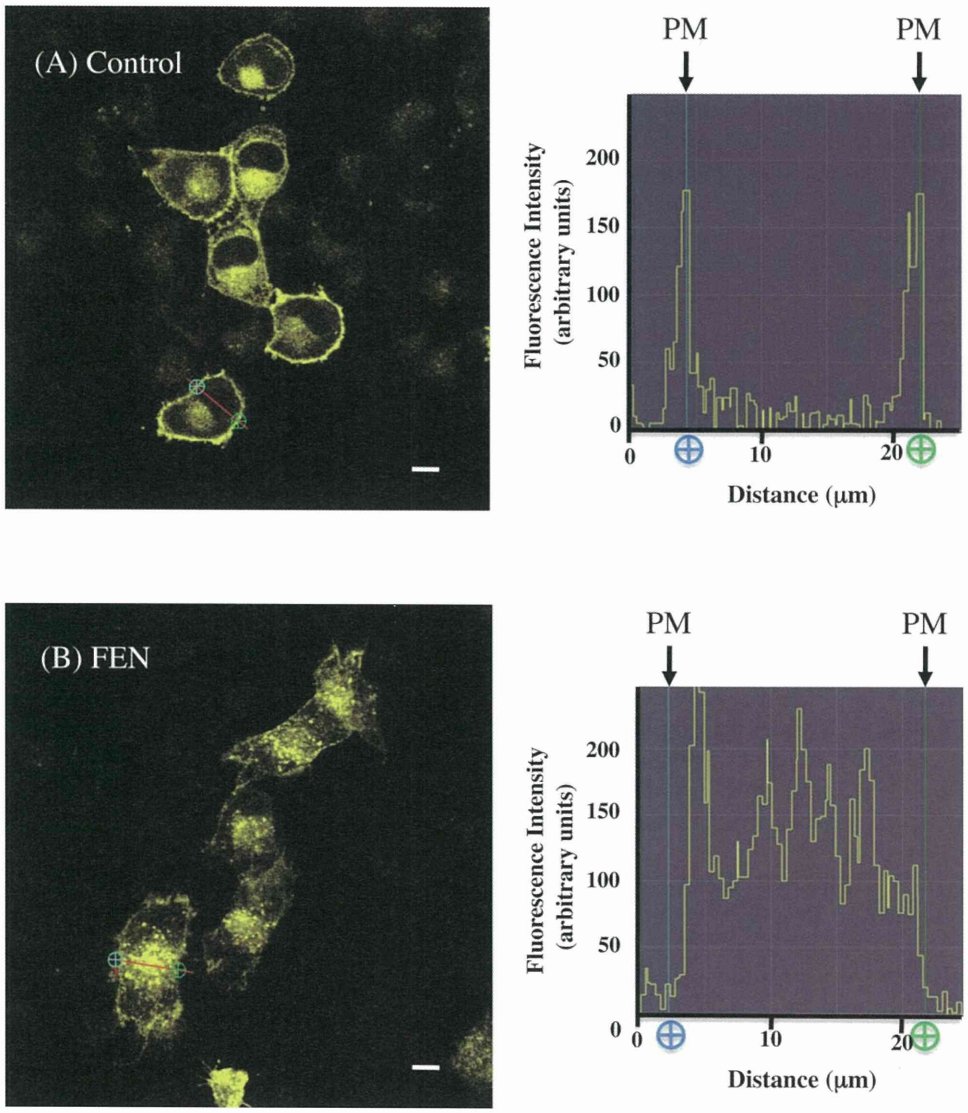

Fig. 2. Confocal imaging of agonist-induced internalization of MORs in BHK cells expressing Venus-fused MORs. Typical cells where most of MOR-Venus intensity was at the plasma membranes, [**A**, control cells (Control)] or in the cytosolic fraction [**B**, 100 nM fentanyl-stimurated for 30 min (FEN)].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