Anesthesiology 2006; 105:574–80

Enhancement of Morphine Analgesic Effect with Induction of μ-Opioid Receptor Endocytosis in Rats

Tatsuya Hashimoto, M.D., Yoji Saito, M.D., Ph.D.,† Kazuo Yamada, Ph.D.,‡ Nobumasa Hara, Ph.D.,§ Yumiko Kirihara, D.V.M.,‖ Mikako Tsuchiya, M.D., Ph.D.#*

Background: **Morphine can desensitize μ-opioid receptor (MOR), but it does not cause internalization of the receptor after binding. Acute desensitization of MOR impairs the efficiency of signaling, whereas the receptor internalization restores the cell responsiveness to the agonists. Thereby, the property of morphine may limit the analgesic effects of this opiate drug. It has been shown that [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO), a potent MOR agonist inducing the internalization, facilitates morphine to internalize MOR, suggesting that MOR agonists with low relative activity *versus* endocytosis (RAVE) values such as DAMGO can potentiate analgesic effects of morphine through stimulating MOR internalization. The authors examined whether the acute analgesic effect of morphine can be potentiated by low relative activity *versus* endocytosis agonists DAMGO and fentanyl.**

Methods: **Rats injected intrathecally with opioids were subjected to a hot plate test for antinociceptive effect. Immunostained spinal dorsal horn was analyzed by confocal microscopy.**

Results: **Fentanyl induced MOR internalization to a lesser extent than DAMGO at equianalgesic doses. Coadministration of fentanyl promoted morphine-induced MOR internalization. The analgesic effect of morphine was greatly potentiated together with decrease in the relative activity *versus* endocytosis value when MOR internalization was induced by coadministration of a subanalgesic dose of DAMGO or fentanyl. In contrast, the combination of DAMGO and fentanyl increased neither the analgesic effect nor the internalization of MOR.**

Conclusions: **The results suggest that the coadministration of morphine with MOR-internalizing agonist is clinically applicable to develop successful pain-management regimens to achieve satisfactory analgesia using less morphine.**

MORPHINE is a highly effective analgesic that has been used for centuries. It remains the accepted standard for clinical pain treatment. Nevertheless, its therapeutic use is often limited by severe side effects such as respiratory depression, tolerance, and dependence.[1] Because those adverse effects occur through the use of large doses of the drug, enormous effort has been invested to find a means of enhancing its analgesic efficacy and reducing undesirable side effects of morphine.[2]

Analgesic effects of morphine are mediated mainly by the μ-opioid receptor (MOR). The receptor, widely distributed throughout the central nervous system, including the spinal dorsal horn,[3] is a member of the G protein–coupled receptor family.[4] Exposure of G protein–coupled receptors to their agonists often results in rapid attenuation of receptor responsiveness.[5,6] The process, acute desensitization, impairs signaling efficiency. It is usually followed by internalization of the receptor to resensitize or rapidly recover the cell responsiveness to agonists.[7,8] However, morphine, a partial agonist of MOR, differs profoundly from full opioid agonists such as [D-Ala,^{2}MePhe,^{4}Gly-ol^5]enkephalin (DAMGO): The opiate binds to MOR without causing rapid internalization of the receptor in MOR-transfected cells.[9–11] That remarkable phenomenon has also been observed in organotypic cultures[12] or whole animal studies.[13,14]

Responsiveness to morphine can be enhanced by induction of MOR endocytosis if the unusual property limits acute analgesic effects of this opiate drug by desensitizing MOR while neither internalizing nor resensitizing the receptor. It has recently been demonstrated that morphine-induced MOR endocytosis can be evoked by coadministration of low concentrations of DAMGO in rat spinal dorsal horn as well as a cell culture model.[14] However, following studies have not reproduced the striking effect of DAMGO.[15–17] Therefore, whether the coadministration with DAMGO enhances morphine-induced internalization remains controversial.

This study examined whether DAMGO and a clinically available agonist, fentanyl, were able to facilitate morphine-induced MOR endocytosis and analgesic effect in rats. We first characterized fentanyl-induced internalization of MOR in the spinal dorsal horn *in vivo*. Then, we tested effect of fentanyl on morphine-induced MOR internalization. Finally, we investigated whether the acute analgesic effect of morphine could be potentiated when MOR internalization was induced by coadministration of DAMGO or fentanyl.

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

Materials and Methods

Materials

Fentanyl was purchased from Sankyo Co. Ltd. (Tokyo, Japan). Morphine hydrochloride was from Takeda Pharmaceutical Co. Ltd. (Osaka, Japan). DAMGO and other reagents were obtained from Sigma Corp. (St. Louis, MO).

* Staff Anesthesiologist, † Professor and Chairman, Department of Anesthesiology, ‡ Staff Biochemist, § Assistant Professor, # Professor, Department of Biochemistry, Shimane University Faculty of Medicine. ‖ Research Associate, Department of Experimental Animals, Center for Integrated Research in Science, Shimane University Faculty of Medicine.

Received from the Department of Anesthesiology, Shimane University Faculty of Medicine, Shimane, Japan. Submitted for publication October 20, 2005. Accepted for publication March 7, 2006. Supported by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (No. 17791029), Tokyo, Japan. Presented in part at 11th World Congress on Pain, Sydney, Australia, August 21–26, 2005.

Address correspondence to Dr. Hashimoto: Department of Anesthesiology, Shimane University Faculty of Medicine, 89-1 Enya, Izumo, Shimane 693-8501, Japan. hashit@med.shimane-u.ac.jp.

Animal Preparation and Surgical Procedure

Our animal care and use committee approved the protocol (permission No. 03-041; Shimane University, Izumo, Japan). Male Sprague-Dawley rats (SLC Inc., Hamamatsu, Japan) weighing 250–280 g were maintained on a 12-h light–dark schedule and were housed individually with free access to food and water.

All animals were handled in the test situation for at least three times before intrathecal catheterization and testing to reduce the influences of handling on nociceptive responses. The surgical procedure was performed as described previously.[18,19] Briefly, during sodium pentobarbital anesthesia, the rats were implanted with intrathecal catheters in the caudal direction at the level of L3–L4. The effectiveness of each catheter was confirmed by injection of 15 μl lidocaine, 2%, the day before the experiment. Only rats developing paralysis of both hind paws because of the local anesthetic were used in this study. After catheterization, at least 3 days were allowed for recovery before the study. Rats that had motor deficits as a result of catheter placement, infection, or other health problems were excluded.

Nociceptive Test

All drugs were dissolved in 10 μl sterile saline and injected, followed by 10 μl saline to flush the catheter. The hot plate (HP) test was performed to measure the response to heat stimuli by monitoring latency until hind paw licking at the indicated time after injection. The floor of the apparatus (model MK-350B; Muromachi Kikai Co., Ltd., Tokyo, Japan) was heated to 52.0°C. A cutoff latency of 60 s was used to prevent tissue damage. The tail flick (TF) test was performed to measure the response to heat stimuli by monitoring latency to withdrawal from a heat source focused on the tail approximately 5 cm from the tip. The apparatus (model DS20; Ugo Basile, Comerio-Varese, Italy) was calibrated to give an average baseline latency of approximately 4 s. A cutoff latency was 10 s.

The latencies were converted to the percentage of the maximal possible effect, calculated as (postdrug value − baseline value)/(cutoff value − baseline value) × 100.

Tissue Preparation

Immediately after the behavioral test, the rats were anesthetized deeply with sodium pentobarbital and then perfused intracardially with 100 ml saline, followed by 300 ml cold 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. The L4–L5 segment of the rat spinal cord was dissected, removed, and then postfixed for 4 h in the same fixative at 4°C. Finally, it was cryoprotected overnight in 30% sucrose buffer solution. Transverse sections (40 μm) of the spinal cord were cut on a freezing microtome.

Immunohistochemistry and Quantification of MOR Internalization

Spinal sections were blocked in 5% normal goat serum in 0.1 M phosphate buffered saline with 0.5% Triton X-100 for 90 min and then incubated in a rabbit anti-MOR antibody (Diasorin, Stillwater, MN) and mouse anti-NeuN antibody (Chemicon International Co. Ltd., Temecula, CA) both at a 1:5,000 dilution in 0.1 M phosphate-buffered saline with 0.2% Triton X-100 overnight at room temperature. The mouse anti-NeuN antibody was used to identify the neurons in the section.[20] Sections were washed extensively in 0.02 M phosphate-buffered saline with 0.1% Tween and incubated in goat anti-rabbit IgG-Alexa-488 (Molecular Probes Inc., Eugene, OR) and goat anti-mouse IgG-Alexa-594 (Molecular Probes Inc., Eugene, OR) both at a 1:1,000 dilution for 2 h at room temperature. The sections were then washed and mounted on slides. MOR distribution in the spinal dorsal horn was examined with a confocal laser scanning microscope (Fluoview FV300; Olympus Optical Co. Ltd., Tokyo, Japan) using a 60× PlanApo 1.40 oil immersion objective. MOR internalization in lamina II was quantified by calculating the percentage of immunoreactive neurons that showed internalization in relation to the total number of immunoreactive neurons examined. Neuronal soma with more than 10 endosomes were considered to have internalized MOR (*i.e.*, the categorization was all or none). The person examining the neurons was blinded to the treatment. At least 100 MOR neurons were examined per rat.[21,22] Images were processed and labeled using Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA).

Relative Activity versus *Endocytosis Value*

To determine values of relative activity *versus* endocytosis (RAVE), defined as the relative activity (efficacy) *versus* the ability of a ligand to induce endocytosis,[11] the ratio of the activity expressed as percentage of the maximal possible effect to the endocytosis as percentage of neurons with internalized MOR measured using immunohistochemistry was calculated. The peptide DAMGO was defined as having activity and endocytosis of 1.

Statistical Analysis

Data are presented as mean ± SEM unless otherwise indicated. Statistical analyses were one-way analysis of variance followed by Scheffé post test. *P* values less than 0.05 were considered to be statistically significant.

Results

Intrathecal Fentanyl Produced MOR Internalization in Spinal Dorsal Horn Neurons

Previous studies demonstrated that the synthetic opioid peptide DAMGO produced MOR internalization *in*

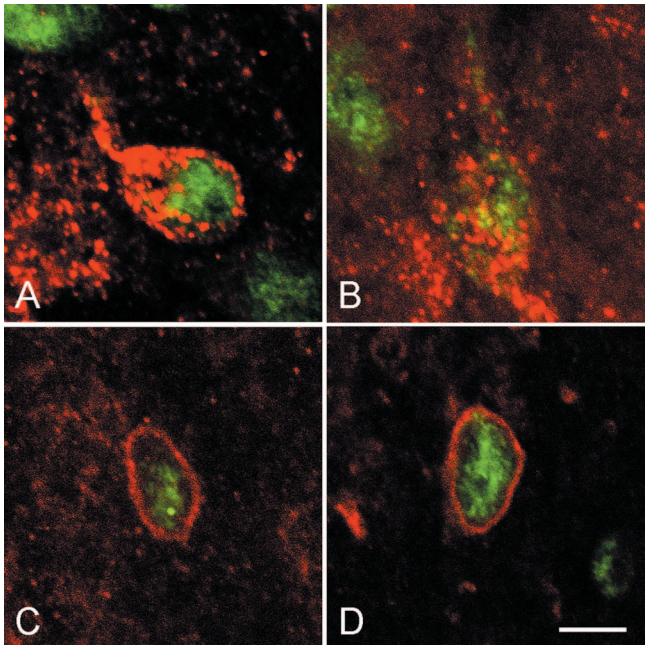

Fig. 1. Confocal microscopic images of μ-opioid receptor (MOR) internalization in lamina II of the spinal dorsal horn. Rats were perfused with a fixative 15 min after the intrathecal injection of 10 μg fentanyl (*A*), 100 ng [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO) (*B*), 20 μg morphine (*C*), or saline (*D*), to observe MOR internalization in the spinal dorsal horn neurons. Neuronal soma and MOR were stained with anti-NeuN antibody (*green*) and anti-MOR antibody (*red*), respectively. Confocal images (60×, zoom of 5) were two or three optical sections through the center of the neuron at intervals of 0.5 μm. *Scale bar* = 10 μm.

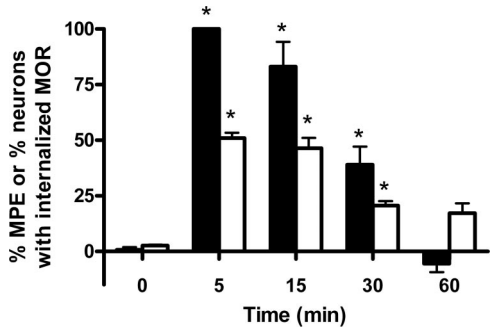

Fig. 2. Time courses of μ-opioid receptor (MOR) internalization and analgesia produced by fentanyl. Rats were injected intrathecally with 5 μg fentanyl, subjected to the hot plate test for antinociceptive effect (percent maximum possible effect [% MPE]; *closed bars*) at the indicated time, and then quickly fixed to determine the percentage of MOR immunoreactive neurons with internalization (*open bars*) (n = 5–8). **P* < 0.05 compared with 0 min.

vivo[13,14] as well as *in vitro*.[10,21] A clinically used MOR agonist fentanyl has been shown to internalize the receptor *in vitro* [23] but not yet *in vivo*. Therefore, we first assessed whether fentanyl can induce MOR internalization in rat spinal dorsal horn neurons after the intrathecal administration of this agonist. Rats were killed 15 min after intrathecal administration of opioid agonists. In 10 μg fentanyl–treated rats, MOR immunoreactivity was depleted from the plasma membrane and observed in numerous endosomes within the dorsal horn neurons (fig. 1A). The pattern was comparable to that induced by 100 ng DAMGO (fig. 1B). In 20 μg morphine–treated rats, however, MOR immunoreactivity was primarily concentrated at the plasma membrane of the dorsal horn neurons, indistinguishable from that of saline-treated rats (figs. 1C and D).

Next, rats were injected intrathecally with 5 μg fentanyl; tested for antinociceptive effect 5, 15, 30, and 60 min after administration; and then quickly fixed to quantify MOR internalization. We found that the ratio of MOR internalized neurons in the total MOR-positive neurons at 5 and 15 min was approximately 50%, the maximum value among those determined. It subsequently declined to approximately 20% at 60 min. Correlated with the internalization, the antinociceptive effect of fentanyl on the HP test was 100% and 83% at 5 and 15 min, respectively. It returned to the control level at 60 min (fig. 2).

Relation of MOR Internalization versus *Analgesic Effects*

We first examined the dose effects of three opioid agonists on the MOR internalization of dorsal horn neurons as well as analgesic response of rats to establish the relation between MOR internalization and analgesia. DAMGO increased not only the latency to licking of the hind paw on the HP test but also the ratio of the MOR-internalized neurons in a dose-dependent manner. The ratio of internalization was 30.3% and the antinociceptive effect was 34.3% when rats were treated with 20 ng DAMGO. In rats treated with 50 ng DAMGO, MOR internalization and the analgesic effect were 79.8% and 77.1%, respectively. The dose–response relation of DAMGO for MOR internalization in the spinal dorsal horn neurons matched with that for the antinociceptive effect (fig. 3A). Fentanyl also increased the HP response latency and the number of MOR internalized neurons in a dose-dependent manner. The antinociceptive effect of fentanyl on the HP test almost reached a maximum value at the dose of 5 μg, but the internalized neuron ratio was only 51.0% at that dosage (fig. 3B). Morphine caused a dose-dependent increase in the HP response latency but elicited no MOR internalization. Its effect on the internalization was indistinguishable from those of saline control at all dosages (fig. 3C). In addition to the HP test, we used TF test to evaluate analgesic effects under the same conditions, but the effects at all doses that we tested reached the cutoff latency in the TF test except 5 ng DAMGO (data not shown).

The order of potency to induce endocytosis was DAMGO > fentanyl >> morphine when these three drugs were compared at the equianalgesic dose (fig. 3D). When the relation between endocytosis and analgesic effect were expressed as RAVE value, the order of the

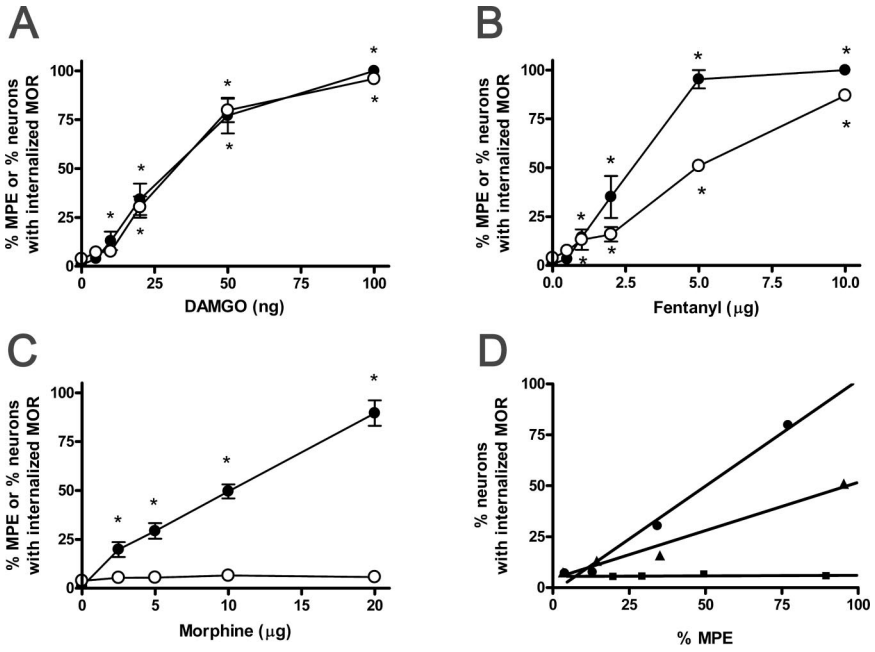

Fig. 3. Effects of opioid doses on μ-opioid receptor (MOR) internalization and analgesia. Rats were injected intrathecally with indicated doses of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO; *A*), fentanyl (*B*), or morphine (*C*). Then, they were tested for antinociception on the hot plate test 5 min after injection (percent maximum possible effect [% MPE]; *closed circles*) and fixed quickly to determine the percentage of MOR immunoreactive neurons with internalization (*open circles*) (n = 5–8). * $P < 0.05$ compared with saline. In *D*, data in *A–C* were replotted to explore the relationship between MOR internalization and analgesic effects of DAMGO (●), fentanyl (▲), and morphine (■).

values obtained from figure 3D was DAMGO (1) < fentanyl (1.8) << morphine (8.5) (table 1).

Enhancement of Morphine-induced Internalization and Analgesia by Coadministration with DAMGO or Fentanyl

We next examined whether internalization-inducible MOR agonist DAMGO or fentanyl was able to potentiate morphine-induced MOR internalization in the spinal dorsal horn neurons. We used a submaximal antinociceptive dose of morphine (2.5 μg) in the HP test for this coadministration study. DAMGO alone (5 ng) produced neither detectable MOR internalization nor significant antinociception in the HP test (fig. 4). However, when the low dose of DAMGO was administered concurrently with 2.5 μg morphine, the peptide agonist potentiated the morphine-induced MOR endocytosis (fig. 4). The combination of morphine and the small amounts of DAMGO increased MOR internalization in the dorsal horn to 62.8% (fig. 4). Moreover, under the same conditions, the antinociceptive effect of the combination of morphine and DAMGO in the HP test was greatly enhanced to 94.0%, approximately five times as much as morphine alone (18.7%) (fig. 4). Without DAMGO, eight times as much morphine was required to show similar analgesic effect (figs. 3C and 4).

Fentanyl, at a dose of 0.5 μg, produced neither the detectable MOR internalization in the dorsal horn nor significant antinociception in the HP test (fig. 4). The opioid facilitated the morphine-induced internalization as well when this low dose of fentanyl was administered concurrently with 2.5 μg morphine (fig. 4). The combination of morphine and the low dose of fentanyl achieved MOR internalization of 33.3% (fig. 4). The analgesic effect of this combination in the HP test was also increased to 70.0%, approximately 3.7 times as much as morphine alone (fig. 4). Without fentanyl, six times as much morphine was required to show a similar analgesic effect (figs. 3C and 4).

Table 1. RAVE Values of Drugs and Drug Combinations

Agonist	RAVE Value
DAMGO	1.0*
Fentanyl	1.8*
Morphine	8.5*
Morphine + DAMGO	1.5†
Morphine + fentanyl	2.1†

* The value was 1/slope of each line in figure 3D. † The value was determined by analgesic effect versus endocytosis in figure 4.

DAMGO = [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin; RAVE = relative activity *versus* endocytosis.

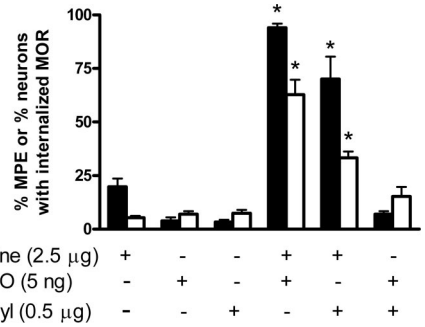

Fig. 4. Effects of [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO) or fentanyl on morphine-induced analgesia and μ-opioid receptor (MOR) internalization. Rats were subjected to the hot plate test 5 min after intrathecal injection of indicated agonists (percent maximum possible effect [%MPE]; *closed bars*). They were fixed quickly to determine the percentage of MOR immunoreactive neurons with internalization (*open bars*) (n = 5–8). * $P < 0.05$ compared with 2.5 μg morphine.

In the TF test, again, the analgesic effect of the combination of morphine with DAMGO or fentanyl as well as morphine only reached the cutoff latency. Therefore, by this test, the analgesic augmentation of morphine with DAMGO or fentanyl could not be detected. RAVE values of morphine in combination with DAMGO and fentanyl were 1.5 and 2.1, respectively (table 1). However, coadministration of 5 ng DAMGO and 0.5 μg fentanyl increased neither antinociceptive effects in the HP test nor MOR endocytosis compared with each agonist alone (fig. 4).

Discussion

Enhancement of Morphine-induced Analgesia Associated with MOR Internalization

In this study, we hypothesized that morphine-induced analgesic effect can be enhanced by induction of MOR internalization and tested the hypothesis using coadministration of morphine with small doses of endocytosis-inducing agonists to facilitate morphine-mediated MOR internalization.

We demonstrated that endocytosis-inducing agonists DAMGO and fentanyl enhanced morphine-induced MOR internalization of dorsal horn neurons in rats. We reproduced the remarkable, but previously somewhat controversial, effect of DAMGO to facilitate morphine-induced endocytosis reported by He *et al.* [14] and further showed that fentanyl, a clinically used opioid with internalization-inducing potency, has a similar effect on MOR internalization *in vivo*. More importantly, concomitant with the enhancement of MOR endocytosis, we observed that the acute analgesic effect of morphine evaluated by the HP test was greatly potentiated by coadministration of these agonists. As described in the Results, the increase in analgesia was not detected in TF test, the method used in the previous report.[14] Probably because of the longer cutoff latency, *i.e.*, broader range in analgesic extent of the HP test, we were able to find the analgesic potentiation, although contribution of the difference in mechanism between the two tests could not be excluded. It is suggested that the effect is synergistic rather than additive because DAMGO or fentanyl alone at the concentration used for the coadministration with morphine caused no internalization of MOR or analgesic effect on the HP test. Methadone, another clinically available MOR-internalizing agonist,[11,24] has also been shown to synergistically enhance the analgesic effect of morphine.[25] However, the combination of two endocytosis-inducing agonists, such as DAMGO and fentanyl (our data) or methadone and fentanyl,[25] showed no such synergistic increase as that shown by combination with morphine. These observations support our proposal that facilitation of MOR internalization by endocytotic agonists can restore morphine responsiveness.

The increase in acute antinociception of morphine has also been observed in mice lacking β-arrestin 2, which is involved in receptor desensitization and development of opioid tolerance, suggesting an important role of β-arrestin 2 in morphine-induced desensitization.[26,27] β-Arrestin is required not only for desensitization but also for subsequent internalization of MOR.[28] Overexpression of this protein allows the morphine-activated receptors to internalize.[29] Therefore, the enhancement of antinociception caused by the deletion of β-arrestin 2 seems somewhat paradoxical. According to Bohn *et al.*[30] and Koch *et al.*,[17] morphine-bound MOR is a poor substrate for G protein–coupled receptor kinase 2 and thus less phosphorylated than the DAMGO-bound receptor, which results in recruitment of less β-arrestin, sufficient for desensitization but insufficient for endocytosis. Based on the molecular schema, both facilitating internalization by endocytosis-inducing agonists and the removal of β-arrestin would enhance the analgesic effect of morphine *via* the escape from β-arrestin–dependent desensitization. Addition of a low dose of DAMGO or fentanyl seems to allow the partial agonist morphine to behave as a full agonist, thereby improving the efficiency of MOR signaling through internalization of the receptor. Consequently, it potentiates the morphine-induced analgesia. Although further studies at the cellular and molecular levels are required to elucidate the precise mechanism, our results yield implications to explain the improved response to morphine in molecular terms and to develop clinical strategies for more effective pain killing with morphine.

Fentanyl-induced MOR Internalization in Rat Spinal Dorsal Horn Neurons

We showed for the first time that intrathecal fentanyl produced MOR internalization in rat spinal dorsal horn neurons together with analgesic effect in a dose-dependent manner. MOR internalization caused by fentanyl has been demonstrated in an artificial expression system[31,32] and in organotypic cultures of the ileum.[23] Our results confirm that fentanyl can also induce internalization *in vivo*.

Comparison of the analgesia–internalization relation among three MOR agonists—morphine, DAMGO, and fentanyl—indicates that their relations differ greatly. For example, at equianalgesic doses, fentanyl produced less MOR internalization than DAMGO. That is, fentanyl has higher analgesic potency than DAMGO at the dose that yields equivalent MOR internalization. Morphine exerts its analgesic effect without leading MOR endocytosis *in vivo*, as reported previously.[13,14] For that reason, MOR internalization cannot function as an indicator of the analgesic effects. It is also the case even with internalization-inducing agonist DAMGO or fentanyl.

A concept RAVE is defined as the relative activity *versus* the ability of a ligand to induce endocytosis *in vitro*.[11] Morphine has a particularly high RAVE value as a consequence of its inability to promote receptor endo-

cytosis compared with DAMGO, which induces receptor endocytosis. To date, activity has been measured as potassium current or adenylate cyclase inhibition and endocytosis as the percentage of receptors internalized measured using fluorescence flow cytometry or enzyme-linked immunosorbent assay *in vitro*.[11,17] The RAVE concept can be extended to *in vivo*, as shown here. When the activity was expressed as the percentage of the maximal possible effect and endocytosis was expressed as the percentage of neurons with internalized MOR measured using immunohistochemistry, the order of the RAVE values was DAMGO < fentanyl << morphine, consistent with the *in vitro* data.[17]

Based on the hypothesis that potentiation of morphine-induced endocytosis can enhance its antinociceptive effect, MOR agonists with higher efficacy to induce MOR internalization, or with lower RAVE values, would be favorable to improve morphine analgesia. Actually, our results showed that DAMGO, which has a lower RAVE value than fentanyl, enhanced the analgesic effect of morphine more strongly than fentanyl. It is necessary to examine whether other opioids with low RAVE values *in vitro* also increase the morphine-induced analgesia *in vivo*. In this article, we also showed the relative decrease in the RAVE value of morphine by combination with subanalgesic and subendocytotic doses of low-RAVE agonist, DAMGO or fentanyl. For better analgesic augmentation of morphine, *i.e.*, to decrease RAVE value of morphine, previously determined RAVE values of various MOR agonists[17] would aid in the selection of a clinically useful combination of opioid agonists.

Evaluation of MOR Internalization

We examined at least 100 MOR-immunoreactive neurons per rat and calculated the percentage of neurons with internalization to avoid a bias for the selection of neurons to be sampled.[22] Trafton *et al.*[13] and He *et al.*[14] quantified the amount of MOR internalization by counting endocytic vesicles in confocal images of sample neurons for each condition. We found that the increase in the percentage of neurons with internalization was matched with the increase in the number of endocytic vesicles in each MOR immunoreactive neuron (data not shown). Results obtained with our method, dose–response curve of DAMGO and facilitation of morphine-induced MOR internalization with DAMGO, were consistent with those of Trafton *et al.*[13] and He *et al.*,[14] respectively. Therefore, the endocytosis of MOR and induction of endocytosis in rat spinal dorsal horn neurons seem to be reproducible.

Clinical Relevance

We found that the combination with fentanyl increased the analgesic effect approximately 2.5 times as much as morphine alone and thus reduced the amount of morphine to only one sixth to obtain an analgesic effect comparable to that of morphine alone. The current results, showing that fentanyl enhanced morphine-induced analgesia together with MOR internalization *in vivo*, strongly support that the combination of morphine with fentanyl is clinically applicable to enhance the acute analgesic effect of morphine.

In clinical practice, the combined administration of morphine with several different opioid agonists, such as fentanyl[33] or sufentanil,[34] has been attempted to improve analgesic onset and duration or to reduce adverse effects. Although these studies have generally obtained favorable results, there are few reports examining the potentiation of morphine analgesic effect by the addition of other opioid. The favorable effects of the combination may be explained, at least in part, by the spinal interactions of morphine and μ opioids, which can produce a substantial increase in analgesia. Our hypothesis in this article will be useful to search better combinations of opioid agonists with morphine.

The enhancement of the analgesic effect by the combination of morphine with other opioids could reduce the amount of morphine to obtain satisfactory antinociception and thus may prevent the development of tolerance to morphine.[14] Also, the combination-induced enhancement of analgesic effect may be one of the mechanisms of the restored opioid responses observed on opioid switching. On switching to an alternative opioid, or on opioid rotation, a dose of the second opioid, such as the endocytosis-inducing agonist methadone, can be much less than that expected by their relative potencies in naive patients.[35,36] The reduced amount of the second drug required for the original analgesia implies the enhancement of a morphine analgesic effect with the second drug *via* facilitation of MOR internalization.

This study demonstrated that coadministration of a low dose of MOR-internalizing agonist resulted in the potentiation of morphine-induced endocytosis and analgesia. Although further studies are needed to determine the minimum effective combination dose as well as the combinations and administration patterns of the drug *in vivo*, our observations might elucidate unique antinociceptive properties of morphine and help to develop clinically successful pain-management regimens such as combination or rotation with several different opioids to achieve satisfactory analgesia without excessive adverse effects.

The authors thank Juan Carlos G. Marvizon, Ph.D. (Assistant Professor, Center for Neurovisceral Sciences and Women's Health, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California), for helpful advice on quantification of MOR internalization. They also thank Yukihiko Yasui, D.D.S., Ph.D. (Professor, Department of Anatomy), and Shigefumi Yokota, Ph.D. (Staff Anatomist, Department of Anatomy), for helpful instruction in immunohistochemical procedures and Harumi Osago (Research Associate, Department of Biochemistry) and Tomoko Shibata (Research Associate, Department of Biosignaling and Radioisotope Experiment) for assistance with this study at Shimane University Faculty of Medicine, Izumo, Japan.

References

1. Simon EJ: Recent studies on opioid receptors: Heterogeneity and purification. Ann N Y Acad Sci 1986; 463:31–45
2. Cherny N, Ripamonti C, Pereira J, Davis C, Fallon M, McQuay H, Mercadante S, Pasternak G, Ventafridda V: Strategies to manage the adverse effects of oral morphine: An evidence-based report. J Clin Oncol 2001; 19:2542–54
3. Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano AH, Dado RJ, Loh HH, Law PY, Wessendorf MW, Elde R: Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. J Neurosci 1995; 15:3328–41
4. Kieffer BL: Recent advances in molecular recognition and signal transduction of active peptides: Receptors for opioid peptides. Cell Mol Neurobiol 1995; 15:615–35
5. Chakrabarti S, Law PY, Loh HH: Neuroblastoma Neuro2A cells stably expressing a cloned mu-opioid receptor: A specific cellular model to study acute and chronic effects of morphine. Brain Res Mol Brain Res 1995; 30:269–78
6. Freedman NJ, Lefkowitz RJ: Desensitization of G protein-coupled receptors. Recent Prog Horm Res 1996; 51:319–51
7. Yu SS, Lefkowitz RJ, Hausdorff WP: Beta-adrenergic receptor sequestration: A potential mechanism of receptor resensitization. J Biol Chem 1993; 268:337–41
8. Pippig S, Andexinger S, Lohse MJ: Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. Mol Pharmacol 1995; 47:666–76
9. Arden JR, Segredo V, Wang Z, Lameh J, Sadee W: Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged mu-opioid receptor expressed in HEK 293 cells. J Neurochem 1995; 65:1636–45
10. Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, von Zastrow M: Morphine activates opioid receptors without causing their rapid internalization. J Biol Chem 1996; 271:19021–4
11. Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M: Functional dissociation of mu opioid receptor signaling and endocytosis: Implications for the biology of opiate tolerance and addiction. Neuron 1999; 23:737–46
12. Sternini C, Spann M, Anton B, Keith DE Jr, Bunnett NW, von Zastrow M, Evans C, Brecha NC: Agonist-selective endocytosis of mu opioid receptor by neurons *in vivo*. Proc Natl Acad Sci U S A 1996; 93:9241–6
13. Trafton JA, Abbadie C, Marek K, Basbaum AI: Postsynaptic signaling *via* the μ-opioid receptor: Responses of dorsal horn neurons to exogenous opioids and noxious stimulation. J Neurosci 2000; 20:8578–84
14. He L, Fong J, von Zastrow M, Whistler JL: Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. Cell 2002; 108: 271–82
15. Bailey CP, Couch D, Johnson E, Griffiths K, Kelly E, Henderson G: Mu-opioid receptor desensitization in mature rat neurons: Lack of interaction between DAMGO and morphine. J Neurosci 2003; 23:10515–20
16. Schulz S, Mayer D, Pfeiffer M, Stumm R, Koch T, Hollt V: Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. Embo J 2004; 23:3282–9
17. Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G, Hollt V: Receptor endocytosis counteracts the development of opioid tolerance. Mol Pharmacol 2005; 67:280–7
18. Saito Y, Kaneko M, Kirihara Y, Kosaka Y, Collins JG: Intrathecal prostaglandin E1 produces a long-lasting allodynic state. Pain 1995; 63:303–11
19. Kirihara Y, Saito Y, Sakura S, Hashimoto K, Kishimoto T, Yasui Y: Comparative neurotoxicity of intrathecal and epidural lidocaine in rats. ANESTHESIOLOGY 2003; 99:961–8
20. Mullen RJ, Buck CR, Smith AM: NeuN, a neuronal specific nuclear protein in vertebrates. Development 1992; 116:201–11
21. Marvizon JC, Grady EF, Waszak-McGee J, Mayer EA: Internalization of mu-opioid receptors in rat spinal cord slices. Neuroreport 1999; 10:2329–34
22. Song B, Marvizon JC: Peptidases prevent mu-opioid receptor internalization in dorsal horn neurons by endogenously released opioids. J Neurosci 2003; 23:1847–58
23. Minnis JG, Patierno S, Kohlmeier SE, Brecha NC, Tonini M, Sternini C: Ligand-induced mu opioid receptor endocytosis and recycling in enteric neurons. Neuroscience 2003; 119:33–42
24. Haberstock-Debic H, Wein M, Barrot M, Colago EE, Rahman Z, Neve RL, Pickel VM, Nestler EJ, von Zastrow M, Svingos AL: Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons. J Neurosci 2003; 23:4324–32
25. Bolan EA, Tallarida RJ, Pasternak GW: Synergy between mu opioid ligands: evidence for functional interactions among mu opioid receptor subtypes. J Pharmacol Exp Ther 2002; 303:557–62
26. Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT: Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science 1999; 286:2495–8
27. Zuo Z: The role of opioid receptor internalization and beta-arrestins in the development of opioid tolerance. Anesth Analg 2005; 101:728–34
28. Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG: Desensitization of G protein-coupled receptors and neuronal functions. Annu Rev Neurosci 2004; 27:107–44
29. Whistler JL, von Zastrow M: Morphine-activated opioid receptors elude desensitization by beta-arrestin. Proc Natl Acad Sci U S A 1998; 95:9914–9
30. Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG, Barak LS: Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. Mol Pharmacol 2004; 66:106–12
31. Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ, von Zastrow M: μ-Opioid receptor internalization: Opiate drugs have differential effects on a conserved endocytic mechanism *in vitro* and in the mammalian brain. Mol Pharmacol 1998; 53:377–84
32. Zaki PA, Keith DE Jr, Brine GA, Carroll FI, Evans CJ: Ligand-induced changes in surface mu-opioid receptor number: Relationship to G protein activation? J Pharmacol Exp Ther 2000; 292:1127–34
33. Tanaka M, Watanabe S, Endo T, Okane M, Hamaya Y: Combination of epidural morphine and fentanyl for postoperative analgesia. Reg Anesth 1991; 16:214–7
34. Mason N, Gondret R, Junca A, Bonnet F: Intrathecal sufentanil and morphine for post-thoracotomy pain relief. Br J Anaesth 2001; 86:236–40
35. Lawlor PG, Turner KS, Hanson J, Bruera ED: Dose ratio between morphine and methadone in patients with cancer pain: A retrospective study. Cancer 1998; 82:1167–73
36. Ripamonti C, Groff L, Brunelli C, Polastri D, Stavrakis A, De Conno F: Switching from morphine to oral methadone in treating cancer pain: What is the equianalgesic dose ratio? J Clin Oncol 1998; 16:3216–21