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Abolished thermal and mechanical antinociception but retained visceral chemical antinociception induced by butorphanol in μ -opioid receptor knockout mice

Soichiro Ide^{a,b}, Masabumi Minami^c, Kumatoshi Ishihara^b, George R. Uhl^d, Masamichi Satoh^e, Ichiro Sora^{a,d,f}, and Kazutaka Ikeda^{a,*}

^aDivision of Psychobiology, Tokyo Institute of Psychiatry, Tokyo 156-8585, Japan

^bLaboratory of Neuropharmacology, Faculty of Pharmaceutical Sciences, Hiroshima International University, Kure 737-0112, Japan

^cDepartment of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

^dMolecular Neurobiology, National Institute on Drug Abuse, Baltimore, Maryland 21224, USA

^eDepartment of Pharmacy, Yasuda Women's University, Hiroshima 731-0153, Japan

^fDivision of Psychobiology, Department of Neuroscience, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

Abstract

Butorphanol is hypothesized to induce analgesia via opioid pathways, although the precise mechanisms for its effects remain unknown. In this study, we investigated the role of the μ -opioid receptor (MOP) in thermal, mechanical, and visceral chemical antinociception induced by butorphanol using MOP knockout (KO) mice. Butorphanol-induced thermal antinociception, assessed by the hot-plate and tail-flick tests, was significantly reduced in heterozygous and abolished in homozygous MOP-KO mice compared with wildtype mice. The results obtained from our butorphanol-induced mechanical antinociception experiments, assessed by the Randall-Selitto test, were similar to the results obtained from the thermal antinociception experiments in these mice. Interestingly, however, butorphanol retained its ability to induce significant visceral chemical antinociception, assessed by the writhing test, in homozygous MOP-KO mice. The butorphanol-induced visceral chemical antinociception that was retained in homozygous MOP-KO mice was completely blocked by pretreatment with nor-binaltorphimine, a κ -opioid receptor (KOP) antagonist. *In vitro* binding and cyclic adenosine monophosphate assays also showed that butorphanol possessed higher affinity for KOPs and MOPs than for δ -opioid receptors. These results molecular pharmacologically confirmed previous studies implicating MOPs, and partially KOPs, in mediating butorphanol-induced analgesia.

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*Corresponding author: Kazutaka Ikeda, Ph.D., Division of Psychobiology, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan, Tel: +81-3-3304-5701, Fax: +81-3-3329-8035, ikedak@prit.go.jp.

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Keywords

Opioid receptor; Knockout mice; Butorphanol; Antinociception

1. Introduction

Butorphanol (17-cyclobutylmethyl-3,14-dihydroxymorphinan) tartrate, known by the trade name Stadol, is a widely used synthetic opioid analgesic used for Step 2 pain management in the World Health Organization's pain ladder. Butorphanol binds μ -, κ -, and δ -opioid receptors (MOP, DOP, and KOP) in rat brain membranes with high affinity (Chang et al., 1981) and acts as a partial agonist. Several reports have indicated that the effects of butorphanol are mediated via its agonistic action at the MOP. Butorphanol exhibited cross-tolerance with morphine in rats (Picker et al., 1990). Butorphanol also has been reported to act as a MOP partial agonist in the antinociceptive effects assessed in the tail-flick test in mice (Garner et al., 1997), in the warm-water tail withdrawal assay in rhesus monkeys (Butelman et al., 1995), and in responses to shock titration in squirrel monkeys (Dykstra, 1990). In addition, the involvement of the KOP in the antinociceptive effects of butorphanol in responses to shock titration in squirrel monkeys and in tail-flick and writhing tests in rats has been reported (Dykstra, 1990; Feng et al., 1994). Although these previous studies suggest that butorphanol acts both as a MOP partial agonist and a KOP partial agonist, several contradictory results remain. Nor-binaltorphimine (nor-BNI), a selective KOP antagonist, precipitated withdrawal in butorphanol-dependent rats. Pretreatment with nor-BNI inhibited the development of butorphanol dependence, and δ -funaltrexamine, a MOP-selective antagonist, was less effective than nor-BNI at precipitating withdrawal in butorphanol-dependent rats (Jaw et al., 1993a, b). These reports suggest that KOPs have a more significant role than MOPs in mediating physical dependence on butorphanol. In contrast, the degree of dependence caused by chronic butorphanol administration into the lateral cerebral ventricle is decreased by δ -funaltrexamine treatment in rats (Oh et al., 1992). Furthermore, the most selective ligands for a specific subtype of opioid receptors (i.e., δ -funaltrexamine for MOP, naltrindole for DOP, and nor-BNI for KOP) possess certain affinities for other subtypes (Newman et al., 2002). Thus, the precise molecular mechanisms underlying the antinociceptive effects of butorphanol have not been clearly delineated by traditional pharmacological studies that only used selective ligands.

Recent success in developing mice lacking the MOP gene has made possible the discovery of molecular mechanisms underlying opioid effects (Loh et al., 1998; Matthes et al., 1996; Sora et al., 1997b, 2001). Both the analgesic effects of morphine in the tail-flick and hot-plate tests and the rewarding effects of morphine in self-administration tests are abolished in MOP knockout (KO) mice (Loh et al., 1998; Sora et al., 1997b, 2001). Buprenorphine, a nonselective opioid receptor partial agonist, has no analgesic effect in the tail-flick and hot-plate tests but a significant rewarding effect in the conditioned place preference tests in homozygous MOP-KO mice (Ide et al., 2004). These observations are especially interesting because the distributions of DOP and KOP are not apparently altered in MOP-KO mice (Loh et al., 1998; Matthes et al., 1996; Sora et al., 1997b). Using MOP-KO mice in tail-flick and hot-plate tests, the antinociceptive effects of tramadol, an analgesic possessing both opioid and non-opioid activity, were shown to be mediated mainly by MOPs and adrenergic α_2 receptors (Ide et al., 2006). Although several compensatory changes might occur in KO animals, they have potential utility in investigating *in vivo* roles of specific proteins. Thus, the use of MOP-KO mice has provided novel theories on the molecular mechanisms underlying the effects of opioid ligands. The present study investigated the molecular mechanisms underlying the antinociceptive effects of butorphanol using MOP-KO mice and human MOP, DOP, and KOP cDNA.

2. Methods

2.1. Animals

The present study used wildtype, heterozygous, and homozygous MOP-KO mouse littermates from heterozygous/heterozygous MOP-KO crosses on a C57BL/6J genetic background (backcrossed at least 10 generations) as previously described (Sora et al., 2001). The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee, and all animal care and treatment were in accordance with our institutional animal experimentation guidelines. Naive adult (>10 weeks old) male and female mice were group housed in an animal facility maintained at $22 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ relative humidity under a 12 h/12 h light/dark cycle with lights on at 8:00 am and off at 8:00 pm. Food and water were available *ad libitum*.

2.2. Drugs

Butorphanol tartrate and nor-BNI dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). For *in vitro* assays, [D-Ala²,N-MePhe⁴,Gly-ol⁵]enkephalin (DAMGO), a MOP-selective agonist, and [D-Pen²,D-Pen⁵]enkephalin (DPDPE), a DOP agonist, were purchased from Peninsula Laboratories Ltd. (Merseyside, UK). (+)-(5,7,8)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide (U69593), a KOP-selective agonist, was a gift from Upjohn (Kalamazoo, MI). [tyrosyl-3,5-³H(N)]DAMGO (50.5 Ci/mmol), [phenyl-3,4-³H]U69593 (47.5 Ci/mmol), and [tyrosyl-2,6-³H(N)]DPDPE (33.0 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA).

2.3. Antinociceptive tests

Thermal antinociception was evaluated using the hot-plate and tail-flick tests. Hot-plate testing was performed according to the method of Woolfe and MacDonald (1944) with slight modifications. A commercially available apparatus consisting of an acrylic resin cage (20 × 25 × 25 cm: width × length × height) and a temperature-controlled aluminum plate (Model MK-350A, Muromachi Kikai Co., Tokyo, Japan) were used for this test. Mice were placed on a $52 \pm 0.2^\circ\text{C}$ hot-plate, and latencies to hind-paw-lick or jump were recorded. We selected a relatively lower temperature (52°C) to examine the mild thermal antinociceptive effects of opioid partial agonists (Ide et al., 2004). The cut-off time was 60 s. Tail-flick testing was carried out according to the method of D'Amour and Smith (1941) with slight modifications using a commercially available apparatus consisting of an irradiator for heat stimulation and a photosensor for detection of tail-flick behavior (Model MK-330A, Muromachi Kikai Co., Tokyo, Japan). Mice were loosely wrapped in a felt towel. Their tails were heated, and tail-flick latencies were automatically recorded. The cut-off time was 15 s. Mechanical antinociception was evaluated using the hind-paw pressure test according to the method of Randall and Selitto (1957) with slight modifications using a commercially available apparatus (Pressure Analgesy-Meter, Model MK-201D, Muromachi Kikai Co., Tokyo, Japan). Mice were loosely wrapped in a felt towel. Their hind paws were gradually pressed, and hind-paw drawing or struggle latencies were automatically recorded. The cut-off pressure was 250 mmHg. The drug injection volume was 10 ml/kg.

In the time-course analyses, the tail-flick, hot-plate, and hind-paw pressure tests were conducted before and 5, 10, 20, 40, 60, 120, and 180 min after subcutaneous (s.c.) injection of butorphanol (3.0 mg/kg). In the dose-response analyses, butorphanol was administered at doses of 0.3, 0.7, 2.0, and 7.0 mg/kg (s.c.), for cumulative doses of 0.3, 1.0, 3.0, and 10 mg/kg, respectively. Tail-flick, hot-plate, and hind-paw pressure tests were conducted 20 min after each drug injection. Drug injections occurred immediately after the previous test.

The hot-plate, tail-flick, and hind-paw pressure responses of each mouse in the drug-induced antinociception tests were converted to percentage of maximal possible effect (%MPE) according to the following formula:

$$\%MPE = (\text{postdrug latency} - \text{predrug latency}) / (\text{cut-off time or pressure} - \text{predrug latency}) \times 100\%$$

Visceral chemical antinociception was evaluated using the writhing test (Collier et al., 1968). Acetic acid (0.6% v/v, 10 ml/kg) was injected intraperitoneally (i.p.), and the mouse was placed in a large plastic cage. The intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 15 min after acetic acid injection. The writhing response consists of a contraction of the abdominal muscles. Nociception was expressed as writhing scores during the 15 min period. Butorphanol (3.0 mg/kg, s.c.) or saline was administered 10 min before acetic acid injection. nor-BNI (5.0 mg/kg, s.c.) was administered 24 h before butorphanol injection.

2.4. Stable expression of human opioid receptors in Chinese hamster ovary cells

Chinese hamster ovary (CHO) cell lines stably expressing human μ -, δ -, and κ -opioid receptors (MOP/CHO, DOP/CHO, and KOP/CHO, respectively) were established as previously described (Ide et al., 2004). K_d values of [3 H]DAMGO to MOP, [3 H]DPDPE to DOP, and [3 H]U69593 to KOP were 1.7 ± 0.3 nM ($n = 4$), 2.2 ± 0.2 nM ($n = 4$), and 2.5 ± 0.2 nM ($n = 3$), respectively. B_{max} estimates of receptor densities in these cell lines were 2300 ± 160 , 3000 ± 270 , and 5000 ± 450 fmol/mg protein, respectively.

2.5. Radioligand binding assay

Binding assays were performed as previously described (Katsumata et al., 1995) with slight modifications. Expressing cells were harvested after 65 h in culture, homogenized in 50 mM Tris buffer (pH 7.4) containing 10 mM MgCl₂ and 1 mM EDTA, pelleted by centrifugation for 20 min at $30\,000 \times g$, and resuspended in the same buffer. For saturation binding assays, cell membrane suspensions were incubated for 60 min at 25°C with various concentrations of [3 H]DAMGO for human MOP, [3 H]DPDPE for human DOP, or [3 H]U69593 for human KOP. Nonspecific binding was determined in the presence of 10 mM unlabeled ligands. For competitive binding assays, the cell membrane suspensions were incubated for 60 min at 25°C with 2 nM [3 H]DAMGO for human MOP, 2 nM [3 H]DPDPE for human DOP, or 3 nM [3 H]U69593 for human KOP in the presence of various concentrations of ligands. After incubation for 60 min, membrane suspensions were rapidly filtrated, and the radioactivity on each filter was then measured by liquid scintillation counting. K_d values of the radiolabeled ligands were obtained by Scatchard analysis of the data from the saturation binding assay. For the competitive binding assay, non-linear regression analysis using a one-competition model (GraphPad Prism, GraphPad, San Diego, CA) was conducted to estimate the inhibitory concentration at 50% (IC_{50}). K_i values were calculated from IC_{50} values obtained from the competitive binding assay in accordance with the equation $K_i = IC_{50} / (1 + [\text{radiolabeled ligand}] / K_d)$, where IC_{50} is the concentration of unlabeled ligand producing 50% inhibition of the specific binding of radiolabeled ligand. The binding assay results are presented as mean $\pm$ S.E.M. of 11 to 15 independent experiments.

2.6. cAMP assay

3',5'-Cyclic adenosine monophosphate (cAMP) assays were performed as previously described (Katsumata et al., 1995) with slight modifications. Briefly, 10^5 cells were placed into each well of a 24-well plate, grown for 24 h, washed, and incubated with 0.45 ml HEPES-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°C. Next, the cells were stimulated for 10 min by the addition of 50 μ l HEPES-buffered saline

containing 100 mM forskolin and 1 mM 3-isobutyl-1-methylxanthine in the presence or absence of various concentrations of opioid ligands and then disrupted by adding 0.5 ml ice-cold 10% trichloroacetic acid to each well. Concentrations of cAMP were measured by radioimmunoassay (Amersham, Buckinghamshire, UK). cAMP accumulation is presented as a fraction of the control value obtained without the addition of opiates. Inhibition curves were generated by a computer-generated non-linear least-squares fit using GraphPad Prism (GraphPad, San Diego, CA). IC_{50} values were calculated as the concentration of ligand producing 50% of maximal inhibition of cAMP accumulation. IC_{50} values and the maximal inhibitory effects (I_{max}) in the cAMP assays are presented as mean $\pm$ S.E.M. of 3 to 5 independent experiments, each performed in triplicate.

2.7. Statistical analyses

The time-courses of butorphanol effects were statistically analyzed by one-way, repeated-measures analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test. The dose-response functions of the antinociceptive effects of butorphanol in wildtype, heterozygous, and homozygous MOP-KO mice were statistically evaluated by two-way, mixed-design ANOVA and one-way, repeated-measures ANOVA followed by the Tukey-Kramer *post hoc* test. The visceral chemical antinociceptive effects of butorphanol were analyzed by one-way factorial ANOVA followed by the Tukey-Kramer *post hoc* test. Differences with $p < 0.05$ were considered statistically significant. In the analysis of differences among genotypes, no significant differences were observed between male and female mice in the antinociceptive effects of butorphanol (although the antinociceptive effects of butorphanol were slightly greater in male mice than in female mice), thus male and female data were combined.

3. Results

3.1. Antinociceptive effects

First, the time-courses of the thermal antinociceptive effects of butorphanol were analyzed in wildtype mice (Figs. 1a, b). The thermal antinociceptive effects of butorphanol (3 mg/kg, s.c.) were expressed early (<5 min), reached peak effects approximately 20 min after injection, and were long-lasting (>3 h) in both the hot-plate (Fig. 1a) and tail-flick (Fig. 1b) tests. Butorphanol showed a significant increase in %MPE in both the hot-plate and tail-flick tests in wildtype mice [one-way, repeated-measures ANOVA: $F_{(7,56)} = 2.27$, $p = 0.042$; $F_{(7,56)} = 9.63$, $p < 0.0001$; respectively]. *Post hoc* analysis revealed that the thermal antinociceptive effects of butorphanol were significant at all examined time-points in the tail-flick test and 20–180 min after butorphanol injection in the hot-plate test. Second, the thermal antinociceptive dose-response relationships of butorphanol were analyzed in wildtype, heterozygous, and homozygous MOP-KO mice (Figs. 1c, d). Butorphanol dose-dependently showed thermal antinociceptive effects in both wildtype and heterozygous MOP-KO mice, but not in homozygous MOP-KO mice. Two-way, mixed-design ANOVA revealed that the antinociceptive effects of butorphanol (%MPE) were significantly different among these genotypes in both the hot-plate test [significant difference between genotypes, $F_{(2,29)} = 8.99$, $p < 0.001$; no significant genotype $\times$ dose interaction, $F_{(8,116)} = 1.75$, $p = 0.095$; Fig. 1c] and tail-flick test [significant difference between genotypes, $F_{(2,29)} = 28.71$, $p < 0.0001$; significant genotype $\times$ dose interaction, $F_{(8,116)} = 9.08$, $p < 0.0001$; Fig. 1d]. The antinociceptive effects of butorphanol (%MPE) in heterozygous and homozygous MOP-KO mice were significantly lower than in wildtype mice, both in the hot-plate and tail-flick tests ($p < 0.05$, Tukey-Kramer *post hoc* test). One-way, repeated-measures ANOVA revealed that butorphanol induced significant increases in %MPE in both the hot-plate [$F_{(4,50)} = 3.40$, $p < 0.05$] and tail-flick tests [$F_{(4,50)} = 17.69$, $p < 0.0001$] in wildtype mice. By contrast, butorphanol induced significant increases in %MPE only in the tail-flick test in

heterozygous MOP-KO mice [$F_{(4,40)} = 2.77$, $p < 0.05$] and failed to significantly change %MPE in either the hot-plate or tail-flick test in homozygous MOP-KO mice at cumulative doses up to 10 mg/kg (Figs. 1c, d).

Next, the time-course of the mechanical antinociceptive effects of butorphanol was analyzed in wildtype mice. Similar to the thermal antinociceptive effects, the mechanical antinociceptive effects of butorphanol (3 mg/kg, s.c.) were expressed early (<5 min), reached peak effects approximately 20 min after injection, and were long-lasting (>3 h) in the hind-paw pressure test (Fig. 2a). Butorphanol showed a significant increase in %MPE in the hind-paw pressure test in wildtype mice [one-way, repeated-measures ANOVA: $F_{(7,56)} = 11.35$, $p < 0.0001$]. *Post hoc* analysis revealed that the mechanical antinociceptive effects of butorphanol were significant at all examined time-points (5–180 min after butorphanol injection). The mechanical antinociceptive effects of butorphanol also were analyzed in wildtype, heterozygous, and homozygous MOP-KO mice (Fig. 2b). Butorphanol showed dose-dependent mechanical antinociceptive effects in both wildtype and heterozygous MOP-KO mice, but not in homozygous MOP-KO mice. Two-way, mixed-design ANOVA revealed that the antinociceptive effects of butorphanol (%MPE) were significantly different between genotypes in the hind-paw pressure test [significant difference between genotypes, $F_{(2,30)} = 66.05$, $p < 0.0001$; significant genotype $\times$ dose interaction, $F_{(8,120)} = 14.67$, $p < 0.0001$]. The antinociceptive effects of butorphanol (%MPE) in heterozygous and homozygous MOP-KO mice were significantly lower than in wildtype mice in the hind-paw pressure test ($p < 0.05$, Tukey-Kramer *post hoc* test). One-way, repeated-measures ANOVA revealed that butorphanol induced significant increases in %MPE in the hind-paw pressure test in both wildtype mice [$F_{(4,45)} = 64.88$, $p < 0.0001$] and heterozygous MOP-KO mice [$F_{(4,55)} = 15.82$, $p < 0.0001$]. By contrast, butorphanol failed to significantly change %MPE in the hind-paw pressure test in homozygous MOP-KO mice at cumulative doses up to 10 mg/kg (Fig. 2b).

The visceral chemical antinociceptive effects of butorphanol (3 mg/kg, s.c.) were analyzed in wildtype, heterozygous, and homozygous MOP-KO mice. The 3 mg/kg butorphanol dose was chosen because it induced almost maximal antinociceptive effects in wildtype mice in the hot-plate, tail-flick, and hind-paw pressure tests (Figs. 1, 2). Interestingly, butorphanol induced chemical antinociceptive effects not only in wildtype and heterozygous MOP-KO mice, but also in homozygous MOP-KO mice. One-way factorial ANOVA revealed that butorphanol induced significant decreases in writhing (Fig. 3) in wildtype mice [$F_{(1,14)} = 265.0$, $p < 0.0001$], in heterozygous MOP-KO mice [$F_{(1,16)} = 210.0$, $p < 0.0001$], and in homozygous MOP-KO mice [$F_{(1,17)} = 9.50$, $p < 0.001$], although significant differences were observed between these genotypes [$F_{(2,24)} = 16.43$, $p < 0.0001$]. The remaining visceral chemical antinociceptive effects of butorphanol in homozygous MOP-KO mice were diminished by pretreatment with nor-BNI (5 mg/kg, s.c.). One-way factorial ANOVA revealed no significant difference between writhing counts in MOP-KO mice treated with nor-BNI and butorphanol and in MOP-KO mice treated with nor-BNI and saline. Furthermore, one-way factorial ANOVA revealed a significant difference between writhing counts in MOP-KO mice treated with butorphanol alone and writhing counts in MOP-KO mice treated with nor-BNI and butorphanol [$F_{(1,23)} = 6.57$, $p < 0.05$].

3.2. Binding characteristics

Butorphanol competition experiments using membranes prepared from MOP/CHO, DOP/CHO, and KOP/CHO cells revealed apparent binding affinities for each opioid receptor subtype (Fig. 4a, Table 1). Butorphanol bound with higher affinity than morphine to membranes prepared from MOP/CHO, DOP/CHO, and KOP/CHO cells. The morphine results were obtained from previous data (Ide et al., 2004) that were analyzed according to

the present methods. The affinities of butorphanol for MOP and KOP were equivalent and higher than for DOP.

3.3. cAMP assay

Butorphanol effects on forskolin-stimulated cAMP accumulation in MOP/CHO, DOP/CHO, and KOP/CHO cells also were tested. Butorphanol concentration-dependently suppressed forskolin-stimulated cAMP accumulation in all three cell types (Fig. 4b). I_{max} values for butorphanol were apparently lower than those of morphine for all cell lines (Table 1). IC_{50} values of butorphanol were lower than those of morphine for MOP/CHO, DOP/CHO, and KOP/CHO cells (Table 1). The morphine results were obtained from previous data (Ide et al., 2004) that were analyzed according to the present methods.

4. Discussion

The thermal antinociceptive effects of butorphanol were significantly reduced in heterozygous and homozygous MOP-KO mice compared with wildtype mice in both the hot-plate and tail-flick tests. The thermal antinociceptive effects of butorphanol in both tests increased in a MOP gene dose-dependent fashion. The copy numbers of the MOP gene are zero in homozygous MOP-KO mice, one in heterozygous MOP-KO mice, and two in wildtype mice. In addition, the mechanical antinociceptive effects of butorphanol in these mice, analyzed by the Randall-Selitto test, were similar to the thermal antinociceptive effects. These results suggest that the MOP is the main opioid receptor involved in butorphanol-induced thermal and mechanical antinociception. This is consistent with previous reports. The selective MOP antagonist α -funaltrexamine was reported to antagonize the thermal antinociceptive effects of butorphanol in a tail-flick test, but pretreatment with nor-BNI did not reliably antagonize the butorphanol effects, and naltrindole, a DOP antagonist, failed to antagonize the effects of butorphanol (Garner et al., 1997).

The antinociceptive effects of morphine, an MOP agonist with low affinity for DOP and KOP, are reduced in several strains of heterozygous MOP-KO mice and completely abolished in homozygous MOP-KO mice (Loh et al., 1998; Sora et al., 1997b, 2001). Also, the antinociceptive effects of DPDPE, a DOP-preferring ligand with modest affinity for the MOP, are reduced in MOP-KO mice (Matthes et al., 1998; Sora et al., 1997a). CXBK mice, which express MOPs at approximately half of the level of C57BL/6 and BALB/c progenitor strains, show reduced analgesic effects in response not only to morphine but also to U50,488H, a μ -selective agonist (Ikeda et al., 1999). Furthermore, the thermal antinociception induced by buprenorphine, a nonselective partial agonist for opioid receptors, is abolished in MOP-KO mice, although the rewarding effects of the drug remain (Ide et al., 2004). In contrast, the antinociceptive effects of morphine are not altered in mice lacking DOPs (Zhu et al., 1999) or in mice lacking KOPs (Simonin et al., 1998). The present results, together with these previous reports, suggest that MOPs play a critical role in the thermal and mechanical analgesia induced by opioid partial agonists. MOP tolerance and inactivation and/or individual differences in MOP number are thus important for most of the variation in the degree of thermal and mechanical analgesia induced by opioids.

The thermal antinociceptive effects of butorphanol were weak when compared with the effects in the tail-flick test. This is consistent with a previous report showing that butorphanol had weak analgesic activity in a mouse hot-plate test (Pircio et al., 1976). Antinociception in the hot-plate test is hypothesized to reflect analgesia at supraspinal level, whereas antinociception in the tail-flick test is hypothesized to reflect analgesia at spinal levels. Thus, the present results suggest that butorphanol may exert its antinociceptive effects spinally rather than supraspinally. Further studies of butorphanol using intracerebroventricular (i.c.v.) and intrathecal (i.t.) microinjection may provide novel

insight. Butorphanol exhibited thermal antinociception more effectively with a low-temperature (50°C) stimulus than with a high-temperature (55°C) stimulus in warm-water tail-immersion tests in rhesus monkeys (Butelman et al., 1995). Further, our previous report showed that the thermal antinociceptive effects of buprenorphine in a hot-plate test was less effective than in a tail-flick test (Ide et al., 2004). Although we selected a relatively low temperature (52°C) in the hot-plate test, butorphanol-induced thermal antinociception was still weak in the hot-plate test. Far lower thermal stimuli (e.g., 48–50°C) may be preferable for assessing butorphanol effects in hot-plate tests.

In the present study, the thermal and mechanical antinociceptive effects of butorphanol were assessed under the cumulative dose-response paradigm similar to previous reports (Ide et al., 2004; Sora et al., 1997b). This paradigm has the advantage of reducing the number of animals necessary for experiments. Because it is difficult to obtain a large number of genetically modified animals, the paradigm is especially useful for studies using these animals. However, the results obtained from the cumulative dose-response paradigm may not be comparable to those from paradigms using different animals for each dose. The cumulative dose-response paradigm can be used when the effect of the drug is expressed prior to the next injection and is retained until the end of the test. The paradigm may not be appropriate for analyzing drugs that are characterized by rapid tolerance or sensitization. In the present study, the effects of butorphanol were expressed early (<5 min) and were long-lasting (>3 h). Thus, the cumulative dose-response paradigm seems to have been appropriate for assessing the antinociceptive effects of butorphanol in the present study. In addition, the antinociceptive effects of butorphanol at 3 mg/kg in the cumulative dose-response paradigm was weak compared with the time-course analysis in both the hot-plate (about 10%MPE weak) and tail-flick (about 20%MPE weak) tests, although these differences were not statistically significant in the present study. The decreased effects of butorphanol in the cumulative dose-response paradigm may be attributable to both acute tolerance and blood level reduction by metabolism of butorphanol injected earlier. Cumulative dose-response paradigms may have relative weaknesses compared with other paradigms, but the present results indicated robust differences in the effects of butorphanol between genotypes.

In contrast to thermal and mechanical antinociception, butorphanol showed significant visceral chemical antinociceptive effects in homozygous MOP-KO mice, although the visceral chemical antinociceptive effects of butorphanol increased in a MOP gene dose-dependent fashion. The residual visceral chemical antinociception induced by butorphanol was abolished by pretreatment with nor-BNI, a selective KOP antagonist. These results indicate that both MOP and KOP play dominant roles in butorphanol-induced visceral chemical antinociception. This is also consistent with previous reports. The effectiveness of a KOP agonist in reducing visceral pain has been reported (Riviere, 2004). The enhanced response of KOP-KO mice in the acetic acid writhing test also has been demonstrated (Simonin et al., 1998). The nociceptive responses in the acetic acid writhing test were not altered in homozygous MOP-KO mice in the present study. Although the precise molecular mechanisms have not been revealed, KOPs rather than MOPs may regulate intrinsic visceral nociceptive sensations induced by endogenous opioids, and MOPs may regulate visceral nociception only with exogenously administered opioid drugs. The present results, together with previous reports, suggest that both MOPs and KOPs play important roles in visceral chemical analgesia mediated by opioid partial agonists. Furthermore, the present results suggest that varied visceral chemical pain can be controlled by nonselective opioids, such as brexazocine, pentazocine, and buprenorphine, acting through both MOPs and KOPs.

The results of our *in vitro* experiments using human MOP, DOP, and KOP cDNA suggest that butorphanol induces antinociceptive effects mainly via MOPs, and partially KOPs, in humans. Butorphanol bound to human MOPs with moderate affinity, approximately 4.9-fold

greater than that displayed by morphine. Butorphanol binding affinity for MOPs was as high as that for KOPs and 5.1-fold higher than that for DOPs in human clones. In the cAMP assays, butorphanol also showed lower IC_{50} values for both MOPs and KOPs than for DOPs, although moderate I_{max} values were demonstrated for three opioid receptor subtypes. These results suggest that mainly MOPs, and partially KOPs but not DOPs, may be involved in the antinociceptive effects of butorphanol in humans as well as in rodents.

In conclusion, the present study demonstrated the abolition of thermal and mechanical antinociceptive effects of butorphanol in MOP-KO mice, suggesting that thermal and mechanical antinociception induced by butorphanol is completely mediated by partial agonistic effects of butorphanol on MOPs. We also demonstrated retention of butorphanol-induced visceral chemical antinociception in MOP-KO mice, abolition of butorphanol-induced visceral chemical antinociception by pretreatment with nor-BNI, and *in vitro* data showing that butorphanol more strongly acted on KOPs and MOPs than on DOPs, suggesting that butorphanol-induced visceral chemical antinociception is mediated by partial agonistic effects on both MOPs and KOPs. For clinical usage, butorphanol may be effective for controlling visceral pain, particularly in patients tolerant to MOP-selective opioid drugs or patients whose MOPs are hypoactive by genetic and/or other factors. Future studies elucidating the precise molecular mechanisms underlying the antinociceptive effects of butorphanol will contribute to the better usage of opioid drugs for pain management.

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Fig. 1.

Thermal antinociceptive effects of butorphanol in wildtype, heterozygous, and homozygous MOP-KO mice. (a, b) Butorphanol (3 mg/kg, s.c.)-induced alterations of %MPE in the hot-plate (a) and tail-flick (b) tests in wildtype mice [$n = 8$ (male, 4; female, 4)] under the time-course paradigm. * $p < 0.05$, significantly different from pre-injection (0 min). (c, d) Butorphanol-induced alterations of %MPE in the hot-plate (c) and tail-flick (d) tests in wildtype [+/+, closed square, $n = 11$ (male, 6; female, 5)], heterozygous [+/-, closed circle, $n = 12$ (male, 7; female, 5)], and homozygous [-/-, closed triangle, $n = 9$ (male, 6; female, 3)] MOP-KO mice under the cumulative dose-response paradigm. # $p < 0.05$, significantly different from wildtype mice. * $p < 0.05$, significantly different from heterozygous MOP-KO mice. Data are expressed as mean $\pm$ S.E.M.

Fig. 2.

Mechanical antinociceptive effects of butorphanol in wildtype, heterozygous, and homozygous MOP-KO mice. (a) Butorphanol (3 mg/kg, s.c.)-induced alterations of %MPE in the hind-paw pressure test in wildtype mice [$n = 8$ (male, 4; female, 4)] under the time-course paradigm. * $p < 0.05$, significantly different from pre-injection (0 min). (b) Butorphanol-induced alterations of %MPE in the hind-paw pressure test in wildtype [+ / +, closed square, $n = 10$ (male, 4; female, 6)], heterozygous [+ / -, closed circle, $n = 12$ (male, 7; female, 5)], and homozygous [- / -, closed triangle, $n = 11$ (male, 5; female, 6)] MOP-KO mice under the cumulative dose-response paradigm. # $p < 0.05$, significantly different from wildtype mice. * $p < 0.05$, significantly different from heterozygous MOP-KO mice. Data are expressed as mean $\pm$ S.E.M.

Fig. 3.

Visceral chemical antinociceptive effects of butorphanol in wildtype, heterozygous, and homozygous MOP-KO mice. Writhing counts induced by 0.6% acetic acid (i.p.) with saline pretreatment in wildtype [+/+, $n = 9$ (male, 5; female, 4)], heterozygous [+/-, $n = 10$ (male, 5; female, 5)], and homozygous [-/-, $n = 7$ (male, 4; female, 3)] mice, butorphanol pretreatment (3 mg/kg, s.c.) in wildtype [+/+, $n = 8$ (male, 5; female, 3)], heterozygous [+/-, $n = 8$ (male, 4; female, 4)], and homozygous [-/-, $n = 12$ (male, 5; female, 7)] MOP-KO mice, and nor-BNI (5 mg/kg, s.c.) and butorphanol (3 mg/kg, s.c.) [-/-, $n = 13$ (male, 7; female, 6)] or saline pretreatment [-/-, $n = 13$ (male, 4; female, 5)] in homozygous MOP-KO mice. # $p < 0.05$, significantly different from wildtype mice. * $p < 0.05$, significantly different from saline pretreatment. & $p < 0.05$, significantly different from nor-BNI pretreatment. n.s., not significant. Data are expressed as mean $\pm$ S.E.M.

Fig. 4.

(a) Binding properties of butorphanol for displacement of specific binding of 2 nM [^3H]DAMGO, 2 nM [^3H]DPDPE, and 3 nM [^3H]U69593 to the membranes of MOP/CHO (open circle, $n = 15$), DOP/CHO (open triangle, $n = 11$), and KOP/CHO (open square, $n = 12$), respectively. Data are expressed as mean $\pm$ S.E.M. (b) Agonistic effects of butorphanol on forskolin-stimulated cAMP production in MOP/CHO (open circle, $n = 5$), DOP/CHO (open triangle, $n = 3$), and KOP/CHO (open square, $n = 5$) cells. Intracellular cAMP levels in the cells incubated with 10 mM forskolin alone served as controls (100%). Data are expressed as mean $\pm$ S.E.M.

Table 1

Binding properties and agonistic effects of butorphanol and morphine on human opioid receptor subtypes.

	MOP/CHO	DOP/CHO	KOP/CHO
Competitive binding assay			
K _i value (nM)			
Butorphanol	6.8 ± 2.3	34.7 ± 10.2	7.8 ± 4.1
Morphine	33.2 ± 7.2	355 ± 61	224 ± 11
cAMP assay			
IC ₅₀ (nM)			
Butorphanol	5.8 ± 2.9	64.4 ± 43.9	4.4 ± 3.0
Morphine	25.5 ± 9.7	364 ± 200	252 ± 231
I _{max} (%)			
Butorphanol	59.3 ± 10.8	77.9 ± 4.2	68.8 ± 8.2
Morphine	87.5 ± 2.8	83.6 ± 2.8	83.0 ± 1.3

Morphine data were obtained from reanalysis of data in Ide et al. (2004).