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Buprenorphine Antinociception is Abolished, but Naloxone-Sensitive Reward is Retained, in μ-Opioid Receptor Knockout Mice

Soichiro Ide[1,2], Masabumi Minami[2], Masamichi Satoh[2], George R Uhl[3], Ichiro Sora[1,3,4] and Kazutaka Ikeda*[,1]

[1]Department of Molecular Psychiatry, Tokyo Institute of Psychiatry, Tokyo, Japan; [2]Department of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan; [3]Molecular Neurobiology, National Institute on Drug Abuse, Baltimore, MD, USA; [4]Department of Neuroscience, Division of Psychobiology, Tohoku University Graduate School of Medicine, Sendai, Japan

Buprenorphine is a relatively nonselective opioid receptor partial agonist that is used in the management of both pain and addiction. To improve understanding of the opioid receptor subtypes important for buprenorphine effects, we now report the results of our investigation on the roles of μ-, δ-, and κ-opioid receptors in antinociceptive responses and place preferences induced by buprenorphine. Buprenorphine antinociception, assessed by hot-plate and tail-flick tests, was significantly reduced in heterozygous μ-opioid receptor knockout (MOR-KO) mice and abolished in homozygous MOR-KO mice. In contrast, buprenorphine retained its ability to establish a conditioned place preference (CPP) in homozygous MOR-KO, although the magnitude of place preference was reduced as the number of copies of wild-type μ-opioid receptor genes was reduced. The remaining CPP of buprenorphine was abolished by pretreatment with the nonselective opioid antagonist naloxone, but only partially blocked by pretreatment with either the δ-selective opioid antagonist naltrindole or the κ-selective opioid antagonist norbinaltorphimine. These data, and biochemical confirmation of buprenorphine actions as a partial δ-, μ-, and κ-agonist, support the ideas that μ-opioid receptors mediate most of analgesic properties of buprenorphine, but that μ- and δ- and/or κ-opioid receptors are each involved in the rewarding effects of this drug.
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INTRODUCTION

Buprenorphine is a relatively long-acting nonselective partial agonist of opioid receptors that has been widely used as an analgesic and an antiaddiction therapeutic. Previous reports suggest that systemically administered buprenorphine can produce μ-opioid receptor-mediated antinociceptive actions and also antagonize morphine antinociception (Cowan *et al*, 1977; Kamei *et al*, 1995; 1997). Intrathecal (i.t.) buprenorphine administration produces antinociception that can be antagonized by κ-opioid antagonists, and it also blocks the antinociceptive effects of κ-opioid agonists in the acetic acid writhing test (Kamei *et al*, 1995; Leander, 1988; Tejwani and Rattan, 2002). Although Neilan *et al* (1999) reported buprenorphine to be a partial δ-opioid receptor agonist, Pick *et al* (1997) did not find such an effect. Each opioid receptor subtype has thus been implicated in buprenorphine antinociception, but with several inconsistencies.

Buprenorphine is also used as a therapeutic agent for patients with opioid dependence (Cheskin *et al*, 1994; Lintzeris *et al*, 2002), even though its own abuse liability is manifest by findings including its self-administration by laboratory animals (Mello *et al*, 1988; Winger and Woods, 2001). The precise molecular mechanisms underlying the therapeutic and rewarding effects of buprenorphine have not been clearly delineated, although investigators have estimated its antinociceptive and rewarding effects by using selective agonists and antagonists. Recent success in developing knockout mice with μ-opioid receptor gene deletions have allowed definition of the loss of the analgesic and rewarding effects of morphine that occurs in mice in the absence of μ-opioid receptors (Kieffer, 1999; Loh *et al*, 1998; Sora *et al*, 1997b, 2001). DPDPE, an agonist active at δ-opioid receptors with some affinity for μ-opioid receptors, has a much weaker analgesic effect in homozygous μ-opioid receptor knockout (MOR-KO) mice (Matthes *et al*, 1998; Sora *et al*, 1997a). These observations are especially interesting since the distribution of δ- and κ-opioid receptors is nearly normal in MOR-KO mice (Loh *et al*, 1998; Matthes *et al*, 1996; Sora *et al*, 1997b).

*Correspondence: Dr K Ikeda, Department of Molecular Psychiatry, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan, Tel: +81-3-3304-5701, Fax: +81-3-3329-8035, E-mail: ikedak@prit.go.jp
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We now report herein the results of further investigations into the molecular mechanisms that underlie antinociceptive and rewarding effects of buprenorphine, which we conducted by using various pharmacological agents, MOR-KO mice, and cDNAs for μ-, δ-, or κ-opioid receptors. We found abolition of buprenorphine-elicited thermal analgesia in homozygous MOR-KO mice, but retention of some naloxone-sensitive buprenorphine rewarding effects in these animals. These observations are supplemented by *in vitro* data that document partial buprenorphine agonism at δ- as well as μ- and κ-opioid receptors. Our results indicate that μ-opioid receptors play mandatory roles in buprenorphine antinociception and that δ-, κ-, and μ-opioid receptors are involved in buprenorphine reward.

METHODS

Animals

Wild-type, heterozygous, and homozygous MOR-KO mouse littermates from crosses of heterozygous/heterozygous MOR-KO mice with a C57BL/6J genetic background, as described previously (Sora *et al*, 2001), served as subjects. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee, and all animals were cared for and treated humanely in accordance with our institutional animal experimentation guidelines. Naive adult (>10 weeks old) mice were housed in an animal facility maintained at $24 \pm 1^{\circ}$C and 50% relative humidity under a 12/12 h light/dark cycle with lights on at 0800 and off at 2000. Food and water were available *ad libitum*.

Drugs

For *in vivo* assays, all drugs were dissolved in saline and injected into animals in volumes of 10 ml/kg. Buprenorphine hydrochloride, naloxone hydrochloride, naltrindole hydrochloride, and norbinaltorphimine dihydrochloride (norBNI dihydrochloride) were purchased from SIGMA Chemical Co. (St Louis, MO). Morphine hydrochloride was purchased from Sankyo Co. (Tokyo, Japan).

For *in vitro* assays, [D-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin (DAMGO), a μ-opioid receptor-selective agonist, and [D-Pen^2, D-Pen^5]enkephalin (DPDPE), a δ-opioid receptor agonist, were purchased from Peninsula Laboratories Ltd. (Merseyside, UK). (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspirol[4,5]dec-8-yl]benzeneacetamide (U69593), a κ-opioid receptor-selective agonist, was a gift from Upjohn (Kalamazoo, MI). [tyrosyl-3,5-^{3}H(*N*)]DAMGO (50.5 Ci/mmol), [phenyl-3,4-^{3}H]U69593 (47.5 Ci/mmol), and [tyrosyl-2,6-^{3}H(N)]DPDPE (33.0 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA).

Antinociceptive Tests

Hot-plate testing was performed according to the method of Woolfe and MacDonald (1944) with slight modifications. A commercially available apparatus consisting of acrylic resin cage ($20 \times 25 \times 25$ cm: width × length × height) and a thermo-controlled aluminum plate (Model MK-350A, Muromachi Kikai Co., Tokyo, Japan) were used for this test. Mice were placed on a $52 \pm 0.2^{\circ}$C hot plate, and latencies to paw licking were recorded with a cutoff time of 60 s. Tail-flick testing was carried out according to the method of D'Amour and Smith (1941) with slight modifications, by using a commercially available apparatus consisting of an irradiator for heat stimulation and a photosensor for the detection of the 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 with a cutoff time of 15 s. Tail-flick and then hot-plate testing were conducted 20 min after each subcutaneous (s.c.) drug injection. Buprenorphine was administered in doses of 0.1, 0.2, 0.7, and 2.0 mg/kg, for cumulative doses of 0.1, 0.3, 1.0, and 3.0 mg/kg, respectively. Morphine was injected s.c. at a dose of 10 mg/kg. The hot-plate and tail-flick responses of each mouse in the drug-induced antinociception were converted to the percent of maximal possible effect (%MPE) according to the following formula:

$$\%\text{MPE} = \frac{(\text{post drug latency} - \text{pre drug latency})}{(\text{cut-off time} - \text{pre drug latency})} \times 100\%$$

Conditioned Place Preference (CPP) Test

CPP test was carried out according to the method of Hoffman and Beninger (1989) with some modifications. For this test, we used a two-compartment plexiglass chamber, one compartment ($17.5 \times 15 \times 17.5$ cm: width × length × height) was black with a smooth floor and the other was of the same dimensions, but white with a textured floor. For pre- and postconditioning test phases, a T-style division with double 6×6 cm openings allowed access to both compartments. During the conditioning phases, the openings were eliminated to restrict mice to a single compartment. Locomotion and time spent in each compartment was recorded by using an animal activity monitoring apparatus equipped with an infrared detector (Neuroscience Inc., Osaka, Japan). The compartment chamber was placed in a sound- and light-attenuated box under conditions of dim illumination (about 40 lx). Conditioned place preferences were assessed by a protocol consisting of three phases (preconditioning, conditioning, and test phases). On days 1 and 2, the mice were allowed to freely explore the two compartments through the openings for 900 s and acclimatized to the apparatus. On day 3 (preconditioning phase), the same trial was performed and the time spent in each compartment was measured for 900 s. There was no significant difference between time spent in the black compartment with a smooth floor (464 ± 12 s, $n = 92$) and time spent in the white compartment with a textured floor (436 ± 12 s, $n = 92$), indicating that there was no preference before conditioning in the apparatus itself. We selected a counterbalanced protocol in order to nullify each mouse's initial preference, as discussed previously (Tzschentke, 1998). Biased mice that spent more than 80% of the time (ie 720 s) on one side on day 3 or more than 600 s on one side on day 2 and more than 600 s on the other side on day 3 were not used for further experiments. Conditioning was conducted once daily for 4 consecutive days (days 4–7). Mice were injected with either buprenorphine (1.0 mg/kg s.c.) or saline and immediately confined to the black or

white compartment for 50 min on day 4. On day 5, the mice were injected with alternate saline or buprenorphine (1.0 mg/kg s.c.) and immediately confined to the opposite compartment for 50 min. On days 6 and 7, the same conditioning as on days 4 and 5 was repeated. Assignment of the conditioned compartment was performed randomly and counterbalanced across subjects. Naloxone (1.0 mg/kg s.c.), naltrindole (2.5 mg/kg s.c.), or norBNI (5.0 mg/kg s.c.) was injected 10 min before the injection of buprenorphine (1.0 mg/kg s.c.) or saline. During the test phase on day 8, the time spent in each compartment was measured for 900 s without drug injection. The CPP score was designated as the time spent in the drug-paired compartment on day 8 minus the time spent in the same compartment in the preconditioning phase on day 3. The scores were expressed as means ± the standard error of the mean (SEM).

Stable Expression of Human Opioid Receptors in Chinese Hamster Ovary (CHO) Cells

CHO cells were grown in F-12 medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C. The human opioid receptor cDNAs were cloned from poly(A) + RNA obtained from human cerebrum donated by Dr R Takahashi (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) by using an RT-PCR-based method, subcloned into pcDNA3 (Invitrogen, Carlsbad, CA), and confirmed by sequencing using an ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, CA). CHO cells were transfected with these plasmids by using lipofectin (GibcoBRL, Gaithersburg, MD) and selected by being cultured in the presence of 500 μg/ml G418. Stable expression was confirmed by conducting binding experiments using the appropriate selective tritiated ligands.

Radioligand Binding Assay

Binding assays were performed as 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 $MgCl_2$ and 1 mM EDTA, pelleted by centrifugation for 20 min at 30 000 *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 μ-opioid receptor, [^{3}H]DPDPE for human δ-opioid receptor, or [^{3}H]U69593 for human κ-opioid receptor. Nonspecific binding was determined in the presence of 10 μM 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 μ-opioid receptor, 2 nM [^{3}H]DADLE for human δ-opioid receptor, or 3 nM [^{3}H]U69593 for human κ-opioid receptor 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. K_i values were calculated from the 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 a 50% inhibition of the specific binding of radiolabeled ligand. The results of binding assays were presented as the mean ± SEM of 11–15 independent experiments.

cAMP Assay

cAMP assays were performed as 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 of HEPES-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°C. Next, they were stimulated for 10 min by the addition of 50 μl of HEPES-buffered saline containing 100 μM 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 of ice-cold 10% trichloroacetic acid to each well. Concentrations of adenosine 3′, 5′-cyclic monophosphate (cAMP) were measured by radioimmunoassay as described (Amersham, Buckinghamshire, UK). cAMP accumulation was presented as a fraction of the control value obtained without addition of opiates. IC_{50} values were calculated as the concentration of ligand producing 50% of the maximal inhibition of cAMP accumulation. The values of IC_{50} and the maximal inhibitory effects (I_{max}) in cAMP assays were presented as the mean ± SEM of three to five independent experiments, each performed in triplicate.

Statistical Analyses

We combined the data of male and female mice because there were no statistically significant differences between male and female mice in the antinociceptive and rewarding effects of buprenorphine (paired *t*-test). The antinociceptive effects of buprenorphine and morphine were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls *post hoc* test. Comparisons between genotypes at each dose were analyzed by the Tukey–Kramer test. Time spent in the drug-paired compartment during pre- and postconditioning phases of CPP test were analyzed by within-group paired *t*-tests. Factors of 'genotypes' and 'treatments' were compared by the one-way ANOVA followed by the Fisher's PLSD *post hoc* test. Differences with $p < 0.05$ were considered significant.

RESULTS

Antinociceptive Effects

Buprenorphine antinociceptive dose–response relationships were analyzed in wild-type, heterozygous, and homozygous MOR-KO mice. Buprenorphine induced significant increases in the %MPE in both hot-plate (Figure 1a) and tail-flick (Figure 1b) tests in wild-type mice (ANOVA: $p < 0.0001$; $F = 8.38$; $df = 4, 75$, $p < 0.0001$; $F = 34.18$; $df = 4, 75$, respectively) and heterozygous MOR-KO mice (ANOVA: $p < 0.0001$; $F = 6.96$; $df = 4, 95$, $p < 0.0001$; $F = 16.83$; $df = 4, 95$, respectively). In contrast, buprenorphine failed to significantly change the %MPE in either hot-plate or tail-flick

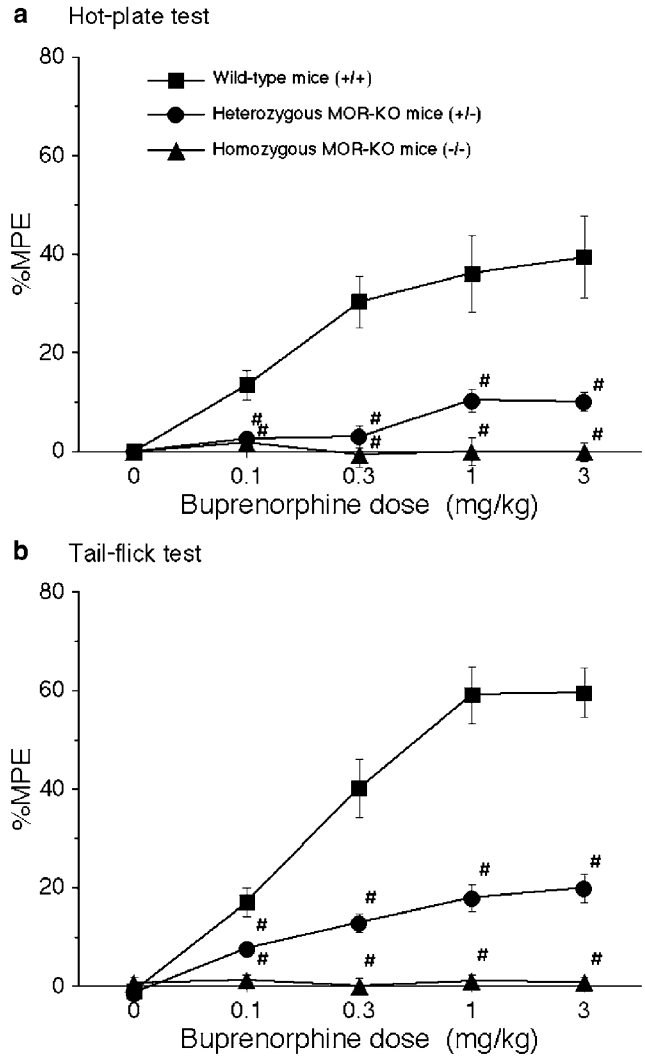

Figure 1 Antinociceptive effects of buprenorphine in wild-type, heterozygous, and homozygous MOR-KO mice. Buprenorphine-induced alterations of %MPE in the hot-plate (a) and tail-flick (b) tests in wild-type (+/+, square, $n=16$), heterozygous (+/−, circle, $n=20$), and homozygous (−/−, triangle, $n=15$) MOR-KO mice, under the cumulative dose–response paradigm. #A significant difference ($p<0.05$) from the corresponding values for wild-type mice. Data are presented as the mean ± SEM.

tests in homozygous MOR-KO mice at cumulative doses up to 3 mg/kg (Figure 1a and b). Antinociceptive effects of buprenorphine in wild-type mice were significantly ($p<0.05$) different from those of either heterozygous or homozygous MOR-KO mice in all doses in both hot-plate and tail-flick tests.

Morphine (10 mg/kg s.c.) caused a significant increase in the %MPE in both hot-plate (Figure 2a) and tail-flick (Figure 2b) tests in wild-type (ANOVA: $p<0.0001$; $F=74.79$; $df=1$, 24, $p<0.0001$; $F=7236.30$; $df=1$, 24, respectively) and heterozygous MOR-KO mice (ANOVA: $p<0.0005$; $F=19.25$; $df=1$, 26, $p<0.0001$; $F=31.18$; $df=1$, 26, respectively), whereas it had no significant effect on it in homozygous MOR-KO mice. In both hot-plate and tail-flick tests, the antinociceptive effects of morphine in wild-type mice were also significantly ($p<0.05$) different from those of heterozygous and homozygous mice at all doses.

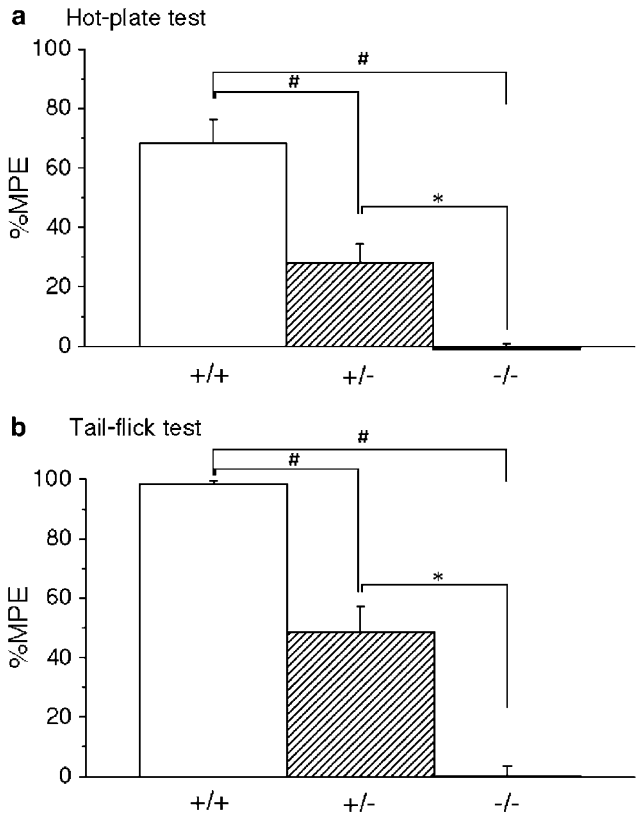

Figure 2 Antinociceptive effects of morphine in wild-type, heterozygous, and homozygous MOR-KO mice. Morphine (10 mg/kg s.c.)-induced alterations of %MPE in the hot-plate (a) and tail-flick (b) tests in wild-type (+/+, white column, $n=13$), heterozygous (+/−, hatched column, $n=14$), and homozygous (−/−, black column, $n=11$) MOR-KO mice. #A significant difference ($p<0.05$) from the values for wild-type mice. *A significant difference ($p<0.05$) from the values for homozygous MOR-KO mice. Data are presented as the mean ± SEM.

Rewarding Effects

Preferences for the places paired with 1 mg/kg buprenorphine s.c. were analyzed in wild-type, heterozygous, and homozygous MOR-KO mice. Buprenorphine induced significant increases in time spent on the previously drug-paired side in wild-type mice, as anticipated (CPP score = 154 ± 18, paired *t*-test, $p<0.0001$). This was also true for both heterozygous (CPP score = 96 ± 24, paired *t*-test, $p<0.005$) and homozygous (CPP score = 73 ± 18, paired *t*-test, $p<0.001$) MOR-KO mice (Figure 3). One-way ANOVA revealed significant differences between these genotype groups ($p<0.05$; $F=4.33$; $df=2$, 53). *Post hoc* comparison revealed that the buprenorphine-induced increase in CPP score for the wild-type mice was significantly higher than that for either heterozygous or homozygous MOR-KO mice ($p<0.05$). However, there was no significant difference in the place preference induced by buprenorphine between heterozygous and homozygous MOR-KO mice.

Next, we tested the influences of opioid antagonists. Mice were injected s.c. with 1.0 mg/kg of nonselective opioid antagonist naloxone, 2.5 mg/kg of δ-opioid receptor-selective antagonist naltrindole or 5.0 mg/kg of κ-opioid receptor-selective antagonist norBNI, and some of them

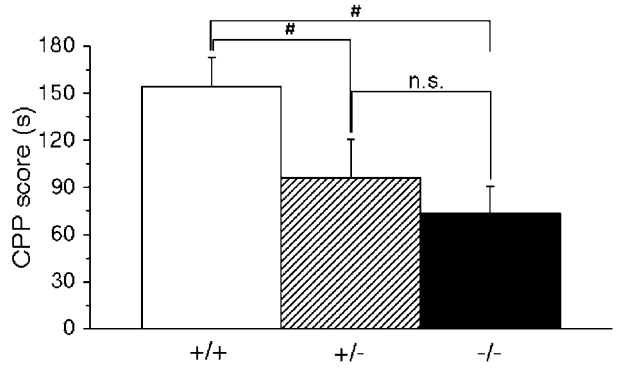

Figure 3 Rewarding effects of buprenorphine in wild-type, heterozygous, and homozygous MOR-KO mice. The CPP scores of wild-type (+/+, white column, $n=18$), heterozygous (+/−, hatched column, $n=18$) and homozygous (−/−, black column, $n=20$) MOR-KO mice. #A significant difference ($p<0.05$) from the values for wild-type mice. NS, not significant. Data are presented as the mean ± SEM.

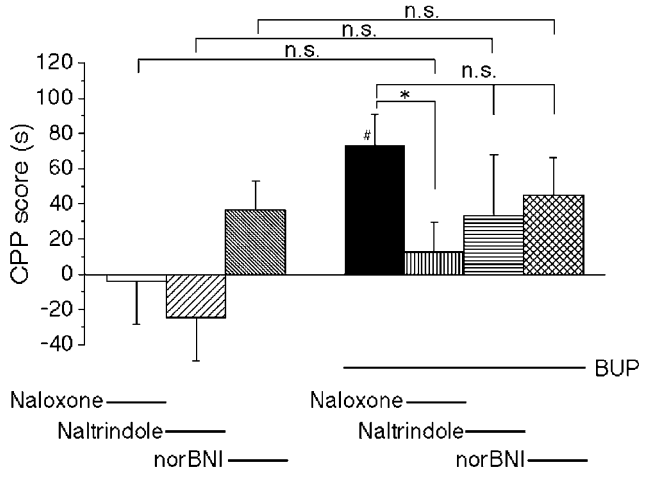

Figure 4 Inhibitory effects of naloxone, naltrindole, and norBNI on buprenorphine-induced rewarding effects in homozygous MOR-KO mice. Shown are the CPP scores of mice conditioned with naloxone ($n=18$), naltrindole ($n=8$), norBNI ($n=8$), or buprenorphine ($n=20$) alone and those of mice pretreated with naloxone ($n=18$), naltrindole ($n=10$), or norBNI ($n=10$) and conditioned with buprenorphine (BUP). #A significant difference ($p<0.05$) in the time spent in the drug-paired compartment between preconditioning and test phases in MOR-KO mice. *A significant difference ($p<0.05$) between the bracketed values. NS, not significant. Data are presented as the mean ± SEM.

were then administered buprenorphine in the CPP conditioning phase (Figure 4). When given alone, naloxone (1.0 mg/kg s.c.) did not alter place preference in homozygous MOR-KO mice (CPP score $=-4\pm29$), as reported previously (Skoubis *et al*, 2001). Neither naltrindole nor norBNI significantly altered place preference when administered alone, although they produced trends toward conditioned place aversion (CPA; naltrindole CPP score $=-25\pm25$) and place preference (norBNI CPP score $=36\pm18$).

Pretreatment with naloxone (1.0 mg/kg s.c.) 10 min before buprenorphine injections in the place preference conditioning phases did not change the increase in time spent on the buprenorphine-paired side in homozygous MOR-KO mice (CPP score $=13\pm17$). One-way ANOVAs demonstrated significant differences among homozygous MOR-KO mouse groups that were treated with naloxone alone, buprenorphine alone, and both buprenorphine and naloxone ($p<0.05$; $F=3.72$; $df=2, 53$). *Post hoc* comparison also revealed that naloxone pretreatment diminished buprenorphine-induced CPP in homozygous MOR-KO mice ($p<0.05$). In contrast, pretreatment with naltrindole (2.5 mg/kg s.c.) or norBNI (5.0 mg/kg s.c.) prior to buprenorphine injection did not significantly change the time spent in the buprenorphine-paired compartment after conditioning (the CPP score $=33\pm35$ and 45 ± 22, respectively). Thus, although pretreatment with naltrindole or norBNI each conferred tendencies toward lower buprenorphine place preference, one-way ANOVAs for the variously treated homozygous MOR-KO groups demonstrated no significant difference between treatment with naltrindole alone and that with it plus buprenorphine or between norBNI alone and that with it plus buprenorphine.

Binding Characteristics

In order to confirm the receptor specificity of buprenorphine, we established cell lines that stably expressed human μ-, δ-, and κ-opioid receptors (MOR/CHO, DOR/CHO, and KOR/CHO, respectively). Radiolabeled subtype-selective ligands, [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]U69593, respectively, displayed saturable, high-affinity binding to membranes from these cells. K_d values of [^{3}H]DAMGO to the μ-opioid receptor, [^{3}H]DPDPE to the δ-opioid receptor, and [^{3}H]U69593 to the κ-opioid receptor 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.

Buprenorphine competition experiments using membranes prepared from MOR/CHO, DOR/CHO, and KOR/CHO cells revealed apparent binding affinities for each opioid receptor subtype (Figure 5a, Table 1). Buprenorphine bound to membranes prepared from μ-opioid receptor-expressing cells with affinity almost as high as those of morphine. In contrast, the affinities of buprenorphine for δ- and κ-opioid receptors were moderate and higher than those of morphine.

cAMP Assay

Buprenorphine effects on forskolin-stimulated cAMP accumulation in MOR/CHO, DOR/CHO, and KOR/CHO cells were also tested. Buprenorphine suppressed forskolin-stimulated cAMP accumulation in a concentration-dependent manner in all three types of cells (Figure 5b). I_{max} values for buprenorphine were lower than those of morphine for MOR/CHO and KOR/CHO cells and were slightly lower for DOR/CHO cells (Table 1). IC_{50} values of buprenorphine were apparently lower than those of morphine for all cell lines, especially for DOR/CHO cells.

DISCUSSION

Antinociceptive effects of buprenorphine were significantly reduced in heterozygous MOR-KO mice and virtually absent from homozygous MOR-KO mice in both hot-plate

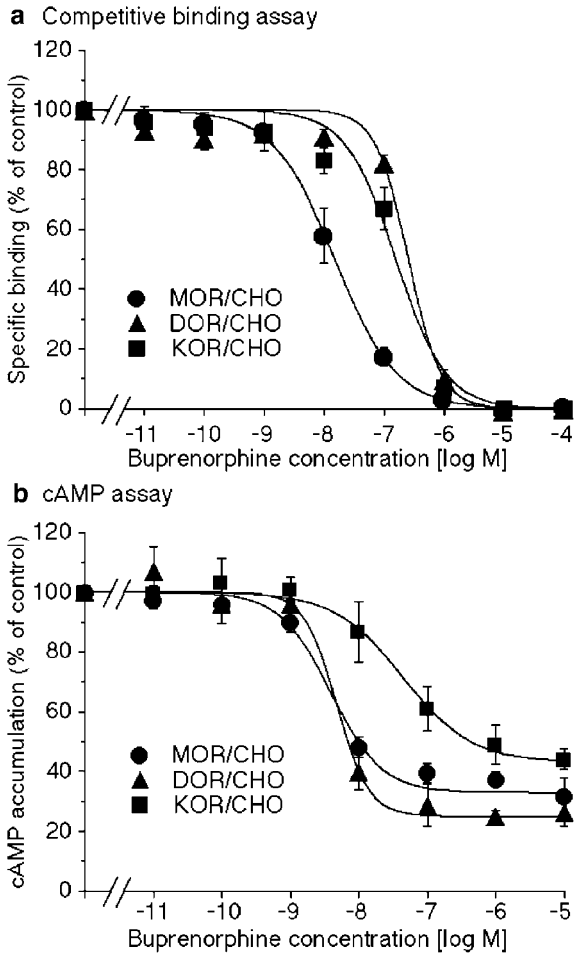

Figure 5 (a) Binding properties of buprenorphine for displacement of the specific binding of 2 nM [^{3}H]DAMGO, 2 nM [^{3}H]DADLE, and 3 nM [^{3}H]U69593 to the membranes of MOR/CHO (circle), DOR/CHO (triangle), and KOR/CHO (square) cells, respectively. The specific binding for MOR/CHO, DOR/CHO and KOR/CHO were 1800±310, 2800±340, and 4300±440 fmol/mg protein, respectively. Data are presented as the mean±SEM. $n=11$–15. (b) Agonistic effects of buprenorphine on forskolin-stimulated cAMP production in MOR/CHO (circle), DOR/CHO (triangle) cells, and KOR/CHO (square). Intracellular cAMP levels in the cells incubated with 10 μM forskolin alone served as the controls (100%). The control levels of cAMP in MOR/CHO, DOR/CHO, and KOR/CHO were 77±13, 78±6, and 75±7 pmol/well, respectively. Data are presented as the mean±SEM, $n=3$–5.

and tail-flick tests. These antinociceptive effects decreased in a μ-opioid receptor gene dose-dependent manner, even though buprenorphine activity at δ- and κ- as well as μ-opioid receptors was reconfirmed in the present study and was previously found in other *in vitro* experiments (Blake *et al*, 1997; Bot *et al*, 1998). Our data agree with those of Lutfy *et al* (2003), who also noted the absence of a thermal antinociceptive effect of buprenorphine in the tail-flick test conducted on a different strain of homozygous MOR-KO mice. Taken together, these results thus support a large role for μ-opioid receptors in both spinal and supraspinal thermal antinociceptive properties of buprenorphine. It thus seems likely that many of the nonselective opioids with moderate affinities for all subtypes of opioid receptors, such as bremazocine, pentazocine, and butorphanol, may also produce most of their analgesia through actions at the μ-opioid receptor.

Table 1 Binding Properties and Agonistic Effects of Buprenorphine and Morphine on Human Opioid Receptor Subtypes

	MOR/CHO	DOR/CHO	KOR/CHO
Competitive binging assay			
K_i value (nM)			
Buprenorphine	12.4±3.5	154±27	108±27
Morphine	21.0±3.7	524±83	247±13
cAMP assay			
IC_{50} (nM)			
Buprenorphine	3.7±0.5	5.5±1.3	20.6±6.4
Morphine	25.0±9.0	610±220	340±160
I_{max} (%)			
Buprenorphine	66.0±4.7	75.7±2.8	57.3±4.1
Morphine	88.0±3.1	83.7±2.7	84.3±3.3

Previous experiments and our present observations all suggest that the antinociceptive effects of morphine, a μ-opioid receptor agonist with low affinities for δ- and κ-opioid receptors, are reduced in each of several strains of heterozygous MOR-KO mice and completely diminished in homozygous MOR-KO mice (Loh *et al*, 1998; Sora *et al*, 1997b; 2001). We and others have identified reduced antinociceptive effects of DPDPE, a δ-opioid receptor-preferring ligand with modest affinity for the μ-opioid receptor, in MOR-KO mice (Matthes *et al*, 1998; Sora *et al*, 1997a). CXBK mice, which express μ-opioid receptors at approximately half of the level of C57BL/6 and BALB/c mouse strains, also showed reduced analgesic effects of morphine and the κ-selective agonist U50488H (Ikeda *et al*, 1999; 2001). In contrast, the antinociceptive effects of morphine were not altered in either mice lacking δ-opioid receptors (Zhu *et al*, 1999) or in those lacking κ-opioid receptors (Simonin *et al*, 1998). The present results thus add to the previous suggestions that the μ-opioid receptor is an especially key site for the analgesic effects of many opioid ligands. μ-Opioid receptor tolerance and inactivation and/or individual differences in μ-opioid receptor numbers are thus likely of importance in most of the analgesia induced by opiates.

In contrast to the abolition of buprenorphine antinociception in homozygous MOR-KO mice, significant rewarding effects were still existent. These results provide a sharp contrast to the virtually complete loss of rewarding effects of morphine in place preference assays using either these or other strains of homozygous MOR-KO mice (Matthes *et al*, 1996; Sora *et al*, 2001). Our current observations that the rewarding effects of buprenorphine in homozygous MOR-KO mice were abolished by pretreatment with naloxone, a nonselective opioid antagonist, suggest δ- and/or κ-opioid receptor involvement. Both δ- and κ-involvement in buprenorphine reward are supported by trends toward efficacies of pretreatment with naltrindole, a δ-opioid receptor

selective antagonist, and norBNI, a κ-opioid receptor selective antagonist, to reduce buprenorphine CPP in homozygous MOR-KO mice.

Previous reports documented that treatment with κ-opioid receptor-selective agonists induced CPA (Funada *et al*, 1993; Sante *et al*, 2000) and that δ-opioid receptor-selective agonists caused CPP (Longoni *et al*, 1998) in wild-type animals. A κ-opioid receptor antagonist was also reported to induce CPP in wild-type rats (Iwamoto, 1985), suggesting that dynorphin, an endogenous κ-opioid ligand, might constitutively produce aversive feelings and/or reduce rewarding feelings. Thus, μ- and δ-opioid receptors appear well poised to play positive roles, and the κ-opioid receptor, a negative role, in reward systems. Conceivably, buprenorphine could produce reward through the activation of μ- and δ-opioid receptors and inhibition of κ-opioid receptors. This κ antagonistic property of buprenorphine was also documented by the weak inhibition by buprenorphine in the CHO cells expressing κ-opioid receptors and by the complete displacement of the κ-selective ligand by buprenorphine.

The results of our *in vitro* experiments using cDNAs for human μ-, δ-, and κ-opioid receptors also suggest that buprenorphine induces rewarding effects via δ- and κ-opioid receptors in humans. Buprenorphine binds to human δ-opioid receptors with a moderate affinity, approximately 3.4-fold greater than that displayed by morphine. The ratio of buprenorphine binding affinities for μ- and δ-opioid receptors (K_i value for δ/K_i value for μ) was 12.4 in human clones and 15.8 (calculated from our unpublished results) in rodent clones. In the cAMP assays, buprenorphine showed lower IC_{50} value for δ-opioid receptors than morphine. Furthermore, buprenorphine showed the highest I_{max} value for δ-opioid receptors among the subtypes. These results suggest that not only μ- but also κ- and especially δ-opioid receptors may be involved in the rewarding effect of buprenorphine in humans as well as in rodents.

It was earlier reported that buprenorphine can serve as a reinforcer not only in laboratory animals (Mello *et al*, 1988; Winger and Woods, 2001) but also in humans (Comer *et al*, 2002), although buprenorphine has been widely used in clinical management for the detoxification in opioid abusers (Cheskin *et al*, 1994; Gibson *et al*, 2003; Lintzeris *et al*, 2002). Since the rewarding effects of buprenorphine are likely to be mediated by δ- and κ-opioid receptors in addition to μ-opioid receptors, buprenorphine might conceivably provide a prototype for clinical effectiveness through decreased μ-opioid receptor availability (Greenwald *et al*, 2003; Zubieta *et al*, 2000). Such μ-opioid receptor-selective partial agonists might even provide good adjuncts during detoxification.

In conclusion, we demonstrated abolition of antinociceptive effects of buprenorphine but retention of at least much of the rewarding effect in MOR-KO mice. Abolition of buprenorphine reward by pretreatment with naloxone and the *in vitro* data showing that buprenorphine acted significantly on δ- as well as μ- and κ-opioid receptors each support the idea that the antinociceptive effects of buprenorphine are completely dependent on μ-opioid receptor, but that its rewarding effects are mediated by its properties of being a δ- as well as μ-opioid receptor agonist and a κ-opioid receptor antagonist.

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