Activation of delta-opioid receptor contributes to the antinociceptive effect of oxycodone in mice

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Abstract
Oxycodone has been used clinically for over 90 years. While it is known that it exhibits low affinity for the multiple opioid receptors, whether its pharmacological activities are due to oxycodone activation of the opioid receptor type or due to its active metabolite (oxymorphone) that exhibits high affinity for the mu-opioid receptors remains unresolved. Ross and Smith (1997) reported the antinociceptive effects of oxycodone (171 nmol, i.c.v.) are induced by putative kappa-opioid receptors in SD rat while others have reported oxycodone activities are due to activation of mu- and/or delta-opioid receptors. In this study, using male mu-opioid receptor knock-out (MOR-KO) mice, we examined whether delta-opioid receptor was involved in oxycodone antinociception. Systemic subcutaneous (s.c.) administration of oxycodone (above 40 mg/kg) could induce a small but significant antinociceptive effect in MOR-KO mice by the tail flick test. Delta-opioid receptor antagonist (naltrindole, 10 mg/kg or 20 mg/kg, i.p.) could block this effect. When oxycodone was injected directly into the brain of MOR-KO mice by intracerebroventricular (i.c.v.) route, oxycodone at doses of 50 nmol or higher could induce similar level of antinociceptive responses to those observed in wild type mice at the same doses by i.c.v. Delta-opioid receptor antagonists (naltrindole at 10 nmol or ICI 154,129 at 20 μg) completely blocked the supraspinal antinociceptive effect of oxycodone in MOR-KO mice. Such oxycodone antinociceptive responses were probably not due to its active metabolites oxymorphine because (a) the relative low level of oxymorphone was found in the brain after systemically or centrally oxycodone injection using LC/MS/MS analysis; (b) oxymorphine at a dose that mimics the level detected in the mice brain did not show any significant antinociceptive effect; (c) oxycodone exhibits equal potency as oxymorphine albeit being a partial agonist in regulating [Ca²⁺]i transients in a clonal cell line expressing high level of mu-opioid receptor. These data suggest that oxycodone by itself can activate both the mu- and delta-opioid receptors and that delta-opioid receptors may contribute to the central antinociceptive effect of oxycodone in mice.

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1. Introduction

Oxycodone (14-hydroxy-7,8-dihydrocodeinone), a pure semi-synthetic opioid agonist derived from a naturally occurring alkaloid, thebaine, has been in clinical use since 1917 for the management of moderate and severe acute or chronic pain. With a methoxy group at the 3- position of the aromatic ring A, a position hypothesized by Beckett and Casy that must contain a hydroxy moiety for the receptor binding and analgesic potency of opioid drugs [1], it is surprising that oxycodone is more potent than morphine in vivo considering that mu-opioid receptor binding affinity of oxycodone is much less than that of morphine in the rat brain homogenate [2,3]. In humans, oral oxymorphone is 10-fold more potent than oral morphine based on effective dose [4]. Oxycodone has approximately 1.5 times more potent analgesic than morphine when administered intravenously or orally for the relief of post-operative and cancer pain [5,6]. In rats, oxycodone has an analgesic...
potency 2–4 times that of morphine after systemic administration [subcutaneous (s.c.) or intraperitoneal (i.p.)] [7]. However, oxycodone has been shown to induce significantly less antinociception compared with morphine when given intrathecally (i.t.) or intracerebroventricularly (i.c.v.) in rats [8–10]. Oxycodone is metabolized by cytochrome P450 in the liver into noroxycodone and oxymorphone, and the oxymorphone’s affinity for mu-opioid receptor is higher than oxycodone [11,12]. Activation of mu-opioid receptor by oxymorphone could contribute to the observed oxycodone adverse effects associated with mu-opioid receptor activation such as respiratory depression, miosis, euphoria, constipation, tolerance, dependence and addiction [13]. However, only 10% of oxycodone is O-demethylated to oxymorphone [12]. Noroxycodone and oxymorphone undergo further O-demethylation and N-demethylation, respectively to form norexymorphone [14]. Noroxycodone is the major metabolite of oxycodone in rats [15] and in humans [16]. Moreover, in pharmacokinetic studies, plasma concentrations of oxymorphone are either very low or absent following the systemic or oral administration of oxycodone in human [7,12,16,17]. The absence of detectable level of metabolites suggests oxycodone by itself or metabolites other than oxymorphone could activate one or multiple opioid receptor types.

Ross and Smith [18] reported that oxycodone is a putative kappa-opioid agonist based on studies in which i.c.v. pre-treatment of rats with the kappa-opioid selective antagonist, nor-binaltorphimine (nor-BNI) abolished i.c.v. oxycodone but not morphine antinociception. In contrast, Nozaki et al. [19] reported that systemic antinociceptive effect of oxycodone is mainly mediated by mu-opioid receptor in nondiabetic mice, whereas in diabetic mice, these effects were mediated by mu- and kappa-opioid receptors. However, the involvement of receptors other than mu-opioid receptor in oxycodone antinociceptive activity has been controversial. Recent animal studies have failed to show the reduction of the antinociceptive effect of oxycodone with selective kappa- and delta-opioid receptor antagonists [3,10]. Nielsen and colleagues [2] also suggested that the observed oxycodone pharmacology may represent the binding and activation of an opioid receptor dimer, such as the delta/kappa. Whether delta-opioid receptor is involved in oxycodone antinociception remains unresolved. However, because oxycodone produces effects typical of mu-opioid agonists, the central role of mu-opioid receptor in oxycodone activity is unequivocal.

Therefore, the aim of the present study is to examine the contribution of the central delta-opioid receptor in the oxycodone’s antinociceptive activity and to also investigate whether oxymorphone, the metabolite of oxycodone, plays an important role in this effect of oxycodone in mice. We evaluated the antinociceptive effects of systemic or supraspinal administered oxycodone in MOR-KO mice [20] in order to determine the possible central molecular target(s) of oxycodone in the absence of mu-opioid receptor. Selective delta-opioid receptor antagonists were used to investigate the possible role of delta-opioid receptor for the antinociceptive effect of oxycodone. Except determination of the brain levels of oxycodone and its active metabolite oxymorphone following systemic or supraspinal administration of oxycodone, we also injected oxymorphone directly in its pure form into the brain, in order to evaluate the role of oxymorphone in the antinociceptive response of oxycodone.

2. Materials and methods

2.1. Ethical statement

All experimental procedures were approved by the Institutional Animal Care and Use Committee at an AAALAC international-accredited animal facility: the Animal Center of National Defense Medical Center, Taiwan, R.O.C. (Approval Number: IACUC-09-070).

2.2. Animals

In order to remove the factor of mu-opioid receptor on the effect of oxycodone and investigate the role of delta-opioid receptor on the effect of oxycodone, we used both MOR-KO mice and selective delta-opioid receptor antagonists in our study.

MOR-KO mice [20] were kindly provided by Dr. John Pintar at Robert Wood Johnson Medical School, Piscataway, New Jersey, USA. These mice derived from heterozygote/heterozygote mating were viable and fertile. Knockout mice used in the present study were littermates derived from mating of homozygous MOR-1 knockout mice. Wild-type mice were of the parental C57BL/6j strain. Adult male wild-type and MOR-KO mice, weighing 20–30 g (8–12 weeks) were used in this study. All mice were kept in an animal room with a 12 h light/dark cycle, at a temperature of 25 ± 2 °C and humidity of 55% at an AAALAC international-accredited animal facility: the Animal Center of National Defense Medical Center, Taiwan, R.O.C. Standard diet and water were provided ad libitum during the experiment. The care of animals were carried out in accordance with institutional and international standards (Principles of Laboratory Animal Care, NIH) and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Defense Medical Center, Taiwan, ROC. All studies involving animals are reported in accordance with the ARRIVE guidelines [21,22]. In order to minimize the animals used, the sample sizes usually were 6–10 in our study. For dose response study of the systemic administered saline or drug, we only used 6 animals per group. On the other hand, for those experiments to see the effects of antagonist on oxycodone or i.c.v. injection of drug(s), we have used 10 animals per group in order to minimize the variation or loss of animals due to the i.c.v. injection. In some later studies, we only used 4 animals per group due to the limited number of male MOR-KO mice we could get. Male mice after weaning were randomly housed in 4 per polypropylene cage in clean convention facility. One week before experiment, mice were randomly assigned into different experimental groups.

2.3. Drug administrations for antinociception study

2.3.1. Systemic administration

S.c. injections of saline or oxycodone (1–30 mg/kg for wild type mice; 30–80 mg/kg for MOR-KO mice) were administered. Systemic antagonists (naloxone or naltrindole) were administered i.p. 30 min before test drug administration.

2.3.2. Intracerebroventricular (i.c.v.) injections

Different doses of oxycodone (10–100 nmol) were acutely administered into the right lateral ventricle of mice by i.c.v. injection using the method of Laursen et al. [23]. The procedure included cutting the scalp of the mouse, locating bregma and injecting (saline or drug) 2 mm lateral to bregma at a depth of 2 mm by using a 50-μl Hamilton microliter syringe (Hamilton Co., Reno, NV) in a volume of 5-μl with a 30-gauge needle of appropriate length. The syringe was held manually at an approximate 45° angle to the skull, with the bevel of needle facing up and pointing toward the tail of the animal. Each mouse received one injection only.

2.4. Determination of the antinociceptive effect of drugs

Drug-induced antinociception was evaluated by the tail-flick test [24]. The light intensity of tail-flick apparatus (Model: Ugo Basile, Italy) was set to allow the basal tail-flick latencies of most mice were within the range of 2.5–3.5 s. A cut-off time of 10 s was
used to avoid tissue damage. The tail-flick latency was recorded at 5, 15, 30, 45, 60, 90, 120 and 180 min after drug administration. The area-under curve of the time-response curve (AUC) was regarded as an index of the antinociceptive effect of the drug(s). The AUC value was obtained by calculating the area under the time-response curve of the tail flick test (test latency—basal latency) from 0 to 180 min after the administration of the drugs as shown in Fig. 1.

2.5. Rotarod test

In order to know whether the high dose of oxycodone in MOR-KO mice (40 mg/kg, s.c.) would affect the motor coordination of animals, rotarod tests were done in MOR-KO mice. In this test, mice were habituated in their home cages and acclimated to the testing room for at least 15 min. They were placed on a bar rotating at a constant speed for 4 rpm for 60 s and subsequently at an acceleration speed from 4 to 40 rpm in 300 s. The latency to fall from a rotating rod is scored automatically with infrared sensors in a RT-01 Rotarod Treadmill (SINGA, Taiwan). The test was repeated three times with 10-min intervals for each animal.

2.6. Determination of the amount of oxycodone and oxymorphone in the brain

2.6.1. Sample preparation

Mice were sacrificed at the peak time after administration of oxycodone (i.e., 10 min after oxycodone at 3 mg/kg, s.c. in wild type mice or 50 nmole, i.v. in wild type and MOR-KO mice; 30 min after oxycodone at 40 mg/kg, s.c. in MOR-KO mice) and the whole brains were rapidly removed. Each brain (0.43–0.46 g) was homogenized with 5-fold volume (w/v) of 0.1 M perchloric acid and then centrifuged at 3000 rpm for 10 min. The supernatant was obtained and the levels of oxycodone and oxymorphone were determined by LC/MS/MS analysis.

2.6.2. LC/MS/MS analysis of oxycodone and oxymorphone

The chromatographic system consisted of an Agilent 1200 series LC system and an Agilent ZORBAX Eclipse XDB-C8 column (5 µm, 3.0 × 150 mm) interfaced to an MDS Sciex API4000 tandem mass spectrometer, equipped with an ESI in the positive scanning mode at 600 °C. Data acquisition was via multiple reactions monitoring (MRM). The MS/MS ion transitions monitored were m/z 316.2/298.3 and 302.2/284.2 for oxycodone and oxymorphone, respectively. The collision energy were 27, 28 V for oxycodone and oxymorphone, respectively. An isotropic HPLC method was employed for separation. Mobile phase A consisted of 10 mM ammonium acetate aqueous solution containing 0.1% formic acid and mobile phase B consisted of acetonitrile. The mobile phase C system was 70%/30% (A/B). The flow rate was set at 1.5 ml/min. The autosampler was programmed to inject 8 μl sample aliquots every 3 min. The retention time of oxycodone and oxymorphone was 0.483 and 0.426 min, respectively.

2.7. In vitro cell-based assay of mu- or delta-opioid receptor activation

The activation of mu-opioid receptor is determined by the ligands’ effect on intracellular Ca2+ transient as described by Chu et al. [25]. Briefly, the CHO-K1/OPRM1/Gα15 cells co-expressing the human MOR (in pEntry) and Gα15 (M00304, GenScript) or HEK293/OPRD1/G16 (transfection of HEK293 cell line hDOR (in pcDNA3) with G16 lentivirus) expressing the human DOR and G16 were cultured in CORNING® black with clear flat bottom 96-well assay plate with 200 μl of HAM’s/F12 medium containing 10% fetal calf serum, pen/strep, 1 x Glutamax (Gibco), zeocin (200 μg/ml) and hygromycin (100 μg/ml) [CHO-K1/OPRM1/Gα15] or Advanced DMEM medium (InvitrogenTM) containing 5% fetal calf serum, pen/strep, 1xGlutamax (Gibco) and G418 (200 μg/ml) under 5% CO2 atmosphere until confluence. On the day of assay, 100 μl of medium was removed from each well, and 50 μl of FLIPR® calcium assay reagent (Molecular Devices Corp) dissolved in 1x reagent buffer (1x HANKS buffer with 20 mM HEPES, pH 7.4 with 5 mM probenecid) was added to each well. The plate was incubated at 37 °C for an hour to allow loading of the Ca2+ dye. Opioid agonists were dissolved and serial diluted in the assay buffer [HBSS (KCl, 5 mM, KH2PO4, 0.3 mM, NaCl, 138 mM, NaHCO3, 4 mM, Na2HPO4, 0.3 mM, D-glucose, 5.6 mM) with the addition of 20 mM HEPES, 2.5 mM probenecid and 13 mM CaCl2]. After the 1 h of dye loading, the plate was placed into a FLEX station (Molecular Devices Corp), and the [Ca2+]i fluorescence was monitored every 1.52 s with excitement wavelength at 485 nm and with emission wavelength at 525 nm for 30 s in order to establish the baseline. Then 50 μl of different concentrations of agonists were injected robotically and the transient increases in the [Ca2+]i were monitored. Normally, the [Ca2+]i transient peaked at 15 s, and returned baseline within 30 s after injection. In order to determine the area under the curve (AUC), the [Ca2+]i transient was monitored for additional 90 s at a 1.52 s intervals. The fluorescence intensity from 3 to 4 wells of cells injected with a single concentration of agonist were averaged and amount of Ca2+ release was determined by integrating the AUC of the [Ca2+]i fluorescence averages. The EC50 values of the agonists were then determined from analyzing 3–5 individual dose-response curves using the GraphPad Prism4 software.

2.8. Chemicals

Morphine hydrochloride was purchased from the National Bureau of Controlled Drugs, Food and Drug Administration, Taipei, Taiwan. Oxycodone hydrochloride was obtained as a gift from QRxPharma (USA). Oxymorphone, DAMGO and etorphine were obtained from National Institute on Drug Abuse (USA). Naloxone hydrochloride, naltrindole hydrochloride, and probenecid were purchased from Sigma Chemicals (St. Louis, MO, USA). ICI 154,129 was purchased from Tocris, MO, USA. All drugs were dissolved in sterile saline.

2.9. Data analysis and statistical assessment

Data were expressed as the mean ± SEM. Analysis of variance was used to access the statistical significance for repeated measures of the data, and the differences between the individual mean values in different groups were analyzed by One way ANOVA and the Newman-Keuls test. The differences were considered to be significant at p < 0.05.
3. Results

3.1. Systemic oxycodone induced antinociception in wild type and MOR-KO mice

As shown in Fig. 2A, systemic administration of oxycodone in wild type mice produced a dose-dependent antinociceptive response at doses of 3, 5, 10 and 30 mg/kg (area under the curve (AUC) = 472.4 ± 56.8 min × s, 651.2 ± 50.8 min × s, 937.3 ± 72.1 min × s, 1037.1 ± 61.9 min × s respectively; p < 0.001 vs. saline group, AUC = 88.9 ± 14.3 min × s). However, not at doses of 1, 1.5 and 2 mg/kg of oxycodone. In MOR-KO mice, oxycodone also produced minimal but significant antinociceptive effects at doses higher than 30 mg/kg (40, 60 and 80 mg/kg, s.c.; AUC = 114.7 ± 18.2 min × s, 117.6 ± 19.0 min × s, 119.4 ± 20.0 min × s; p < 0.01 vs. saline group, AUC = 38.9 ± 12.9 min × s). The oxycodone antinociceptive effects were not dose-dependent and were about 10% of those observed in the wild type mice (Fig. 2B). Oxycodone at doses we used in this study induced hyperactivity in wild type mice but did not affect the measurement of tail-flick test. The high dose of oxycodone (40 mg/kg, s.c.) we used in MOR-KO (MOR −/−) mice did not affect the activity or motor coordination in mice as determined by the rotarod test and the data was shown in Table 1.

3.2. The role of delta-opioid receptors in the systemic antinociceptive effects of oxycodone

To determine whether the mu- or delta-opioid receptors are involved in oxycodone-mediated systemic antinociception, we used mu- or delta-opioid receptor antagonists (naloxone or naltrindole) to block the antinociceptive responses in wild type and MOR-KO mice (Fig. 3). Systemic administration of oxycodone (5 mg/kg, s.c.) induced a significant antinociceptive effect in wild type mice (AUC = 651.2 ± 50.8 min × s; p < 0.001 vs. saline group). This effect of oxycodone was partially blocked by 1 mg/kg of naloxone (AUC = 492.6 ± 64.4 min × s; p < 0.05 vs. oxycodone group) and was completely blocked by 10 mg/kg of naloxone (AUC = 470.0 ± 27.8 min × s; p < 0.05 vs. oxycodone group) or 20 mg/kg of naltrindole at 10 or 20 mg/kg (i.p.) (AUC = 691.1 ± 14.3 min × s or 513.3 ± 5.1 min × s; p < 0.01 vs. oxycodone group; no significant difference from saline group) as shown in Fig. 3A-1. The representative time response curves were shown in Fig. 3A-2. However, in MOR-KO mice, the effect of oxycodone (40 mg/kg, s.c.) (AUC = 114.7 ± 18.2 min × s) was blocked by naloxone only at a very high dose at 100 mg/kg (i.p.) (AUC = 74.7 ± 16.7 min × s; p < 0.05 vs. oxycodone group) or by naltrindole at 10 or 20 mg/kg (i.p.) (AUC = 691.1 ± 14.3 min × s or 513.3 ± 5.1 min × s; p < 0.01 vs. oxycodone group; no significant difference from saline group) as shown in Fig. 3B-1. The representative time response curves were shown in Fig. 3B-2. These results indicated that the majority of the systemic oxycodone’s antinociceptive effect in wild type mice (5 mg/kg) was mediated by mu-opioid receptors and partially mediated by delta-opioid receptors. On the other hand, the
antinociceptive effect of oxycodone at high dose (40 mg/kg, s.c.) in MOR-KO mice was mediated by the delta-opioid receptors.

3.3. The role of delta-opioid receptors in the supraspinal antinociceptive effect of oxycodone

The supraspinal administration of oxycodone (i.c.v.) also produced a dose-dependent antinociception in wild type mice and reached a maximal effect at the dose of 50 nmol of oxycodone (AUC = 377.2 ± 26.7 min x s); p < 0.001 vs. saline group (AUC = 68.3 ± 11.4 min x s) as shown in Fig. 4A. Interestingly, 50 nmol of oxycodone also induced similar level of antinociceptive response in the MOR-KO mice (AUC = 368.9 ± 57.2 min x s); p < 0.001, vs. saline group (AUC = 75.6 ± 11.7 min x s) as those observed in the wild type mice. However there was no dose-dependent effect of oxycodone shown in MOR-KO mice (Fig. 4B).

To investigate whether the delta-opioid receptor plays a role for oxycodone-mediated supraspinal antinociception, mu- or delta-opioid receptor antagonists were co-administered with oxycodone by i.c.v. route. Co-treatment of naloxone (10 nmol, i.c.v.) with oxycodone (50 nmol, i.c.v.) significantly blocked the supraspinal effect of oxycodone in wild type mice (AUC = 107.2 ± 47.8 min x s; p < 0.001 vs. oxycodone group (AUC = 377.2 ± 26.7 min x s)). On the other hand, as shown in Fig. 5A-1, delta-opioid receptor antagonist, naltrindole at a dose of 1 nmol (i.c.v.) partially reduced the oxycodone-induced antinociception in wild type mice (AUC = 268.0 ± 41.0 min x s; p < 0.05, vs. oxycodone group) and almost completely abolished the central effect of oxycodone at a dose of 10 nmol of naltrindole (AUC = 100.9 ± 17.7 min x s; p < 0.001 vs. oxycodone group). Another selective peptide antagonist of delta-opioid receptor, ICI 154,129, at a dose of 20 μg (i.c.v.) also completely blocked oxycodone-induced antinociception in wild type mice (AUC = 63.4 ± 13.1 min x s; p < 0.001 vs. oxycodone group, no difference with saline group). The representative time response curves were shown in Fig. 5A-2. In MOR-KO mice, naloxone did not block the central antinociceptive effect elicited by oxycodone even at a very high dose (100 nmol, i.c.v.). However, the delta-opioid receptor antagonist, naltrindole, dose dependently blocked the central effect of oxycodone (1 nmol of naltrindole (AUC = 219.1 ± 34.0 min x s; p < 0.05 vs. oxycodone group); 10 nmol of naltrindole (AUC = 105.9 ± 29.1 min x s; p < 0.001 vs. oxycodone group) in the MOR-KO mice as shown in Fig. 5B-1. The selective peptide antagonist of delta-opioid receptor, ICI 154,129 (20 μg, i.c.v.) also completely blocked the supraspinal antinociceptive effect of oxycodone (AUC = 55.0 ± 13.9 min x s; p < 0.001 vs. oxycodone group, no difference with saline group). The representative time response curves were shown in Fig. 5B-2. These results further support a role of delta-opioid receptors in the supraspinal oxycodone-induced antinociceptive effects in mice.

In order to determine whether central delta-opioid receptors are involved in oxycodone-mediated systemic antinociception, we administered delta-opioid receptor antagonists (naltrindole) by i.c.v. route to see whether it would block the systemic antinociceptive responses of oxycodone in MOR-KO mice. We found that the antinociceptive effect of systemic high dose oxycodone (40 mg/kg, s.c.) in MOR-KO mice could be completely blocked by i.c.v. injection of naltrindole (NTI, 10 nmol) as shown in Fig. 6.

3.4. The brain level of oxycodone and its metabolite oxymorphone after systemic or supraspinal administration of oxycodone in wild type and MOR-KO mice

It is possible that the observed antinociceptive effects of oxycodone were due to one of its metabolites, i.e. oxymorphone. Hence, brain samples were collected at the peak time (10 min) after s.c. or i.c.v. administration of saline or oxycodone (3 mg/kg, s.c. or 50 nmol, i.c.v.) in the wild type mice for the analysis of oxycodone and its metabolite oxymorphone in brain by LC/MS/MS. As shown in Table 2, the oxycodone levels in brain were 730.6 ± 72.4 pmol after systemic injection of oxycodone (3 mg/kg, s.c.) and 848.5 ± 82.5 pmol after supraspinal administration of oxycodone (50 nmol, i.c.v.) in wild type mice. Meanwhile, low levels of oxymorphone in brain were detected (13.8 ± 2.3 pmol after systemic and 12.5 ± 0.6 pmol after central administration of oxycodone) in wild type mice. The ratio of oxymorphone/oxycodone was calculated to be approximately 1.9% by systemic administration of oxycodone and 1.5% by central oxycodone administration in wild type mice. On the other hand, brain samples were collected 10 min after supraspinal administration of oxycodone (50 nmol, i.c.v.) or 30 min after systemic administration of oxycodone (40 mg/kg, s.c.) in MOR-KO mice for the analysis of oxycodone and its metabolite oxymorphone in brain. As shown in Table 3, the levels of oxycodone in brain were 1764.9 ± 99.9 pmol after systemic administration of oxycodone (40 mg/kg, s.c.) or 1439.8 ± 149.5 pmol after supraspinal administration of oxycodone (50 nmol, i.c.v.) in MOR-KO mice. Oxymorphone levels in the brain were 149.1 ± 7.9 pmol after systemic administration of oxycodone (about 8% of oxycodone) and 12.9 ± 1.3 pmol (about 0.9% of oxycodone) after supraspinal administration of oxycodone.

In order to further investigate whether central oxymorphone plays an important role in the antinociceptive effect of oxycodone, we injected a dose of oxymorphone (300 pmol) directly into the brain by i.c.v. route and found that it did not induce any antinociceptive effect (Fig. 7).
values was injected with non-selective opioid receptor antagonist (naloxone; 10 or 100 nmol, i.c.v.), delta-opioid receptor antagonist (naltrindole; 1 nmol or 10 nmol, i.c.v.) or a selective delta-opioid receptor antagonist (ICI 154,129; 20 μg, i.c.v.) and oxycodone (50 nmol, i.c.v.). The antinociceptive effects were measured by tail flick test. The area-under curve values (AUC) of wild type (MOR +/+ ) mice (A-1), MOR-KO (MOR −/− ) mice (B-1), and the representative time response curves of wild type (MOR +/+ ) mice (A-2), MOR-KO (MOR −/− ) mice (B-2). Data were presented as means ± S.E.M. (n = 7–10). One way ANOVA and Newman-Keuls test were used to analyze the data. **P < 0.001 when compared to saline; *p < 0.05; ***p < 0.001 when compared to oxycodone.

Fig. 5. Delta-opioid receptors play a role on the central oxycodone induced antinociception in both wild type and MOR-KO mice. The wild type and MOR-KO mice were injected with systemic oxycodone (40 mg/kg, s.c.) or oxymorphone (40 mg/kg, s.c.) + central delta-opioid receptor antagonist (naltrindole; 10 nmole, i.c.v.). The antinociceptive effects were determined by tail flick test. The area-under curve values (AUC) (A), and the time response curves (B) were demonstrated. Data were presented as means ± S.E.M. (n = 4–8). Student t-test was used to analyze the data in (A). ***P < 0.01 when compared to oxycodone group.

Table 2 The brain levels of oxycodone and oxymorphene at time of peak effect after acute systemic or central administration of saline or oxycodone in wild type mice.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Route of administration</th>
<th>Brain levels of drugs (pmol)</th>
<th>Ratio [%] Oxymorphene/oxycodone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oxycodone</td>
<td>Oxy morphone</td>
</tr>
<tr>
<td>saline</td>
<td>s.c.</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Oxycodeine (3 mg/kg)</td>
<td>s.c.</td>
<td>730.6 ± 72.4</td>
<td>13.8 ± 2.3</td>
</tr>
<tr>
<td>saline</td>
<td>i.c.v.</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>Oxycodeine (50 nmol)</td>
<td>i.c.v.</td>
<td>848.5 ± 82.5</td>
<td>12.5 ± 0.6</td>
</tr>
</tbody>
</table>

The brains were removed 10 min after systemic (s.c.) or central (i.c.v.) injection of oxycodone. The brain levels of oxycodone and oxymorphone were analyzed by LC/MS/MS. Data were presented as means ± S.E.M. (n = 5–6).

3.5. Oxycodone can activate both mu and delta-opioid receptors

With the majority of the administered oxycodone remained not being metabolized, whether oxycodone itself could activate the mu- and delta-opioid receptors was examined. We used the cell-based [Ca2+]i transient assay to monitor opioid receptor activation. Since opioid receptors coupled to G(i)/G(o) proteins and not Gq, activation of opioid receptor resulting in the increase in [Ca2+]i requires the presence of Gα15/G16, the G proteins that ubiquitously coupled to multiple GPCRs, to activate the [Ca2+]i transient...
Table 3
The brain levels of oxycodone and oxymorphone at time of peak effect after acute systemic of saline or oxycodone in MOR-KO mice.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Route of administration</th>
<th>Brain levels of drugs (pmol)</th>
<th>Ratio (%) Oxymorphone/oxycodone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>s.c.</td>
<td>Oxycodone: 0.0 ± 0.0</td>
<td>Oxymorphone: 0.1 ± 0.1</td>
</tr>
<tr>
<td>Oxycodone (40 mg/kg)</td>
<td>s.c.</td>
<td>1764.9 ± 99.9</td>
<td>149.1 ± 7.9</td>
</tr>
<tr>
<td>Oxymorphone (0.3 mmol)</td>
<td>i.c.v.</td>
<td>1439.8 ± 149.5</td>
<td>12.9 ± 1.3</td>
</tr>
</tbody>
</table>

The brains were removed 30 min after systemic injection of oxycodone (s.c.) or 10 min after central injection of oxymorphone (i.c.v.). The brain levels of oxycodone and oxymorphone were analyzed by LC/MS/MS. Data were presented as means ± S.E.M. (n = 4–5).

Fig. 7. Central oxymorphone, the metabolite of oxycodone, did not induce antinociception in MOR-KO mice. The MOR-KO mice were injected with saline, oxycodone (50 nmol) or oxymorphone (0.3 nmol) by i.c.v. route. The antinociceptive effects were determined by tail flick test. Data were presented as means ± S.E.M. (n = 5–8).

Fig. 8. Dose-responses of opioid receptor agonists in the regulation of [Ca2+] transient in CHO-K1/ORPM1/Ga15 cells. The ability of various opioid ligands to regulate the [Ca2+] transient in the clonal cell line is measured as described in Methods. The inset (upper right figure) represents the time-dependent measurements of the averaged Ca2+ dye relative fluorescence unit (RFU) from 4 wells after the addition of either saline (●), 10 μM oxycodone (Δ) or 10 nM DAMGO (□). The area under the curve (AUC) of the time-dependent RFU were determined for 8 different concentrations of agonist, and the dose-dependent curve was analyzed with GraphPad Prism program. The dose-responses represent the summary from 3 to 5 independent experiments.

Fig. 9. Dose-responses of opioid receptor agonists in the regulation of [Ca2+] transient in HEK293/OPRD1/G16 cells. The ability of various opioid ligands to regulate the [Ca2+] transient in the clonal cell line is measured as described in Methods. The inset (upper right figure) represents the time-dependent measurements of the averaged Ca2+ dye relative fluorescence unit (RFU) from 4 wells after the addition of either saline (●), 10 nM DPDPE (○) or 10 μM oxycodone (Δ). The area under the curves (AUC) of the time-dependent RFU were determined for 8 different concentrations of agonists (DPDPE, oxycodone, oxymorphone), and the dose-dependent curves were analyzed with GraphPad Prism program. The dose-responses represent the summary from 3 independent experiments.

The increase in [Ca2+] transient was 6.0 ± 1.9 nM and 4.1 ± 0.6 nM respectively, oxycodone and oxymorphone exhibited similar potency as etorphine (6.8 ± 1.3 nM) and exhibited greater potency than DAMGO (21 ± 0.7 nM) and morphine (79 ± 3.0 nM). Similar to morphine, oxymorphone exhibited partial agonistic properties in this CHO cell line (Fig. 8). However, in another clonal cell line, the HEK293 cells that express the same human MOR and G16, but with a lower density of receptors, etorphine remained the agonist having the highest potency (EC50 = 0.47 ± 0.26 nM) and oxycodone remained a partial agonist. However, the relative potency of oxymorphone (EC50 = 10.7 ± 0.16 μM) was 20-fold lower than that of oxycodone (EC50 = 0.48 ± 0.13 μM). All the ligand-induced [Ca2+] transient increase in both cell lines can be blocked by the addition of the non-selective μ-opioid antagonist naloxone (100 nM).

In a parallel HEK293 cells expressing the human delta-opioid receptor and G16, addition of oxycodone and other opioid ligands elicited a transient increase in [Ca2+] transient (Fig. 9) that was dose-dependent. Both oxymorphone and oxycodone exhibited partial agonistic activity in eliciting the [Ca2+] transient (Fig. 9). In this HEK293/OPRD1/G16 cells, DPDPE elicited [Ca2+] transient increase with a potency of 43 ± 23 nM, while oxymorphone and oxycodone EC50 values were determined to be 680 ± 160 nM and 27 ± 23 μM respectively. The oxymorphone was 16-fold less potent than DPDPE, while oxycodone was 40-fold less potent than oxymorphone in eliciting the [Ca2+] transient increase. Similarly, all

Note: The text above contains a mixture of numbers and scientific terms related to the study of opioids. The figures mentioned in the text correspond to graphical representations that are not included in the text but are referenced throughout the document. The figures illustrate various responses and measurements related to the brain levels of oxycodone and oxymorphone after administration, as well as the dose-responses of opioid receptor agonists in CHO and HEK293 cells.
the ligand-induced response can be blocked by the addition of naloxone (10 μM).

4. Discussion

The principal findings of our current studies are that the systemic or supraspinal administration of oxycodone produced marked thermal antinociception in both wild type and MOR-KO mice. Our results that systemic oxycodone (3–30 mg/kg) induced dose-dependent antinociceptive effects in wild type mice and minimal response in MOR-KO mice were consistent with other studies using systemic administration in a variety of analgesic tests previously reported [26,27]. Further, naloxone, a non-selective opioid receptor antagonist with higher affinity to mu-opioid receptors [28], produced greater antagonism than naltrindole, a selective delta-opioid receptor antagonist. These results suggested that the antinociceptive effects produced by systemic oxycodone were mainly mediated by mu-opioid receptors as evident also by the minimal antinociceptive response observed in MOR-KO mice. The other opioid receptor involved in oxycodone antinociceptive response is probably the delta-opioid receptor. This is concluded by the ability of low dose of naltrindole to partially block the systemic administered oxycodone antinociceptive responses in wild type mice, and that 10 mg/kg or 20 mg/kg of naltrindole could completely block while the relatively high dose of naloxone (100 mg/kg) was required to partially block the oxycodone antinociceptive effect in MOR-KO mice (40 mg/kg, s.c.). Furthermore, central administration of naltrindole (10 nmol, i.c.v.) completely blocked the antinociceptive effect of systemic oxycodone (40 mg/kg, s.c.) in MOR-KO mice as shown in Fig. 6. These results indicated that the antinociceptive effects of systemic oxycodone are mainly due to activation of the central delta-opioid receptor in the absence of mu-opioid receptor.

We further demonstrated that the central antinociceptive effect of oxycodone was different in wild type and MOR-KO mice. The supraspinal administration of oxycodone (i.c.v.) produced a dose-dependent antinociception in wild type mice and reached a maximal effect at the dose of 50 nmol of oxycodone. However there was no dose-dependent effect of oxycodone in MOR-KO mice. When the doses were lower than 50 nmol (i.e., 30 or 40 nmol), there were no antinociceptive effect at all. When the doses reached 50 nmol and higher, they induced maximal and similar antinociceptive effects as that in wild type mice (Fig. 4). The antinociceptive effects of central oxycodone (50 nmol, i.c.v) in wild type mice were antagonized by co-treatment with naloxone, naltrindole or ICI 154,129. It is probably that the antinociceptive effect of oxycodone results from the action at both mu- and delta-opioid receptors. Our data are consistent with the finding that naloxone or β-funaltrexamine completely abolished the supraspinal antinociceptive effect of oxycodone in rat and mice [5,29]. However, our results were inconsistent with the report by Ross and Smith [18], in which kappa opioid receptors but not delta-opioid receptors were inferred in the supraspinal oxycodone antinociceptive responses in rat. The reasons of difference between previous study by Ross and Smith and our study might be due to different experimental conditions, doses of drug and animal species. In our present study, the antinociceptive responses observed with 50 nmol of oxycodone injected i.c.v. in MOR-KO mice were almost completely antagonized by co-treatment with delta-opioid receptor antagonists, naltrindole or ICI 154,129, at doses that have been reported to antagonize the antinociceptive effects of delta-opioid receptor agonists (DPPDE and DSLET) but not morphine or the kappa-opioid receptor agonist U 50,488 in mice [30]. All these results indicated that the central delta-opioid receptors play a very important role for the antinociceptive effect of oxycodone.

Oxymorphone, the metabolite of oxycodone, produced by CYP2D6 (cytochrome p450 2D6), is an agonist with high affinity at mu-opioid receptor [12]. The brain level of oxycodone and its metabolite oxymorphone produced by systemic or supraspinal administration of oxycodone in mice has not been reported yet. In order to delineate whether oxycodone or its metabolite oxymorphone is accountable for the antinociceptive effect, we used LC/MS/MS for the quantification after systemic or supraspinal administration of oxycodone. Our data have shown that the oxycodone levels in brain after systemic administration of oxycodone in MOR-KO mice (1764.9 ± 99.9 pmol) was much higher than that in wild type mice (730.6 ± 72.4 pmol). It could be explained by the dose of oxycodone we used in MOR-KO mice was higher than that in wild type mice in order to produce antinociceptive effects (40 mg/kg in MOR-KO mice vs. 3 mg/kg in wild type mice). However with the same central doses (50 nmol, i.c.v.) which induced similar antinociceptive effects in MOR-KO and wild type mice, we still found a big difference in the brain levels of oxycodone (1439.8 ± 149.5 pmol in MOR-KO mice vs. 848.5 ± 82.5 pmol in wild type mice). These results imply that lack of mu-opioid receptors may change the pharmacokinetics of oxycodone by some unknown mechanisms. Furthermore, the ratios of oxymorphone as compared to oxycodone with systemic or supraspinal administration of oxycodone were similar in wild type mice. As summarized in Table 2, either systemically or centrally injection of oxycodone, relatively low amount of oxymorphone was found in the brain (1.5–1.9% of oxycodone). On the other hand, in MOR-KO mice, the ratio of oxymorphone as compared to oxycodone was much higher after systemic administration of oxycodone (8.4%) than that after central administration of oxycodone (0.8%). These results further indicate that mu-opioid receptors may play an important role on the pharmacokinetics of oxycodone. On the other hand, although the oxymorphone level was much higher after systemic administration than central administration of oxycodone in MOR-KO mice (Table 3, 149.1 ± 7.9 pmol vs. 12.9 ± 1.3 pmol), the antinociceptive effect of oxycodone after systemic administration was much less than central administration of oxycodone in MOR-KO mice (Figs. 2 and 4). Meanwhile, the brain oxycodone levels were similar with these two different administration routes (1764.9 ± 99.9 pmol vs. 1439.8 ± 149.5 pmol). We also injected oxymorphone directly in its pure form at a dose (300 pmol, i.c.v.) that doubles the level detected in the brain and found that there was no antinociceptive effect at all (Fig. 7). These data again indicated that oxymorphone does not play a role on the central effect of oxycodone. On the contrary, central oxymorphone might interfere the effect of oxycodone to the central delta-opioid receptors so that the antinociceptive effect of oxycodone after systemic administration in MOR-KO mice was less than that after central administration.

Previous studies with s.c. administration of oxycodone (2 mg/kg) in rats [31] or intravenous and intramuscular administration of oxycodone in human [17,32] also reported very low plasma level of oxymorphone. In human studies, the pharmacodynamic effects of oxycodone have been attributed to oxycodone alone [12,33]. Hence even with the mu-opioid receptor affinity of oxymorphone is >40-fold higher than that of oxycodone [12], probably, the observed oxycodone-induced antinociceptive responses in mice should be attributed to oxycodone, and not to oxymorphone.

If the observed antinociceptive response is elicited by oxycodone itself, the drug molecule must be able to activate the opioid receptors. With the presence of a methoxy group at the 3-position of ring A, comparing to that of oxymorphone, the affinity of oxycodone for the opioid receptor should be greatly reduced, as was predicted by the Beckett–Casy’s hypothesis [1]. However, as shown in Figs. 8 and 9, the ability of oxycodone to activate the mu- and
delta-opioid receptors could be clearly demonstrated in the regulation of [Ca\(^{2+}\)]\(_{i}\) transient. The Ca\(^{2+}\) transient assay provides an excellent measurement of response corresponding to receptor concentration. Although the cAMP measurement is the gold standard, the coupling efficiency is high that in instances when the receptor is over expressed, the partial agonistic property of a compound is not revealed. As for the 35SSTPgp assay, although it is widely used, the measured responses is dependent on the nucleotides concentration, and it is not a cell-based assay. We feel that a cell-based Ca\(^{2+}\) transient assay will provide a better representation of the drug action. In the CHO-K1/OPRM1/Ga15 cells, the potency of oxycodeone to increase the [Ca\(^{2+}\)]\(_{i}\) was similar to that of oxymorphone. The difference in oxycodeone and oxymorphone potency was apparent only in clonal cell lines expressing lower level of opioid receptors. At what level mu-opioid receptor is expressed at the nociceptive neurons has not been defined. If the receptor levels in the nociceptive neurons resemble that of CHO-K1/OPRM1/Ga15 and do not resemble that of HEK293-hMOR, then it is likely that with the relative low level of oxymorphone produced, oxycodeone by itself could activate both the mu- and delta-opioid receptors even with its low affinity for the receptor’s orthosteric sites. It is also interesting to note the large discrepancy between the receptor affinity and oxycodeone’s potency suggests that oxycodeone’s activity is not due to the drug interaction with the orthosteric sites of the mu-opioid receptor, but rather with the yet to be identified allosteric sites of the receptor.

The ability of delta-opioid receptor selective antagonists, naltrindole and ICI 154,129, to antagonize oxycodone antinociceptive responses when administered centrally in the wild type and MOR-KO mice established the involvement of delta-opioid receptor in oxycodone pharmacological activities. The involvement of delta- opioid receptor in the antinociceptive response has been clearly established by i.c.v. administration of DPDPPE [34] and in MOR-KO mice [35,36]. However, in current studies, the oxycodone potency to produce antinociceptive responses after i.c.v. injection was greatly different in wild type and MOR-KO mice (Fig. 4). Oxycodone did not elicit an antinociceptive response until 50 nM of drug was injected i.c.v. in MOR-KO mice, while 10 nM of oxycodone was able to produce robust antinociceptive response in wild type mice. Furthermore, in MOR-KO mice, oxycodone did not exhibit a dose-dependent responses, but the level of antinociceptive response was similar to that observed in wild type mice (Fig. 4). Such discrepancy in potency and lack of dose-response might be due to probable mu- and delta-opioid receptors’ interaction and influencing each other properties as reported previously [37]. Heterodimerization between MOR and DOR has been extensively reported [38]. Recent report from Gupta et al. [39] demonstrated the presence of heterodimers in pain pathway by using MOR-DOR heterodimer selective antibodies. Gomes et al. also suggested that mu-opioid receptor/delta-opioid receptor dimers possess functional and ligand binding synergy [40]. Previous studies also have shown that dimeric analogs of oxymorphine and enkephalin exhibit higher affinity and potency than their monomeric forms suggested that mu-opioid receptor function as dimers [41]. Thus, the absence of mu-opioid receptor within such probable dimers could reduce oxycodone eventual antinociceptive responses. Whether oxycodone acts on the mu/delta receptor complexes or the mu- or delta-receptor monomers need further investigation.

5. Conclusion

In summary, our study showed that the supraspinal antinociceptive effect of oxycodone in MOR-KO mice was almost completely antagonized by the antagonists of delta-opioid receptor. Not only the central administration, but also the systemic administration of oxycodone seems to activate central delta-opioid receptors to induce antinociception in MOR-KO mice. Based on these results, the antinociceptive effect of oxycodone in wild type mice might be mediated mainly by mu-opioid receptor when administered systemically, whereas supraspinal delta-opioid receptor might also play an important role in the antinociceptive effect of oxycodone.

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References


