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The involvement of α_{2A}-adrenoceptors in morphine analgesia, tolerance and withdrawal in mice

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Abstract

α_2-Adrenoceptor agonists potentiate opioid analgesia and alleviate opioid withdrawal. The effects of two α_2-adrenoceptor agonists, clonidine (2 mg/kg) and dexmedetomidine (20 and 100 μg/kg), and the α_1-adrenoceptor antagonist prazosin (0.5 mg/kg) were tested on morphine analgesia, tolerance, and withdrawal in wild-type and α_{2A}-adrenoceptor knock-out (KO) mice. Analgesia and tolerance were assessed with the tail-flick test. Withdrawal was precipitated with naloxone. Prazosin potentiated morphine analgesia equally in both genotypes. Clonidine and dexmedetomidine had no analgesic effects in α_{2A}-adrenoceptor KO mice, but morphine analgesia and tolerance were similar in both genotypes. α_{2A}-Adrenoceptor KO mice exhibited 70% fewer naloxone-precipitated jumps than wild-type mice; weight loss was similar in both genotypes. The α_2-adrenoceptor agonists reduced opioid withdrawal signs only in wild-type mice. We conclude that α_{2A}-adrenoceptors are not directly involved in morphine analgesia and tolerance, and not critical for potentiation of morphine analgesia by prazosin, but that α_{2A}-adrenoceptors modulate the expression of opioid withdrawal signs in mice.

Keywords: Morphine withdrawal; Morphine tolerance; Analgesia; Prazosin; α_2-Adrenoceptor agonist; α_{2A}-Adrenoceptor

1. Introduction

Rapid development of tolerance and dependence, and many undesired pharmacological effects limit the clinical usefulness of morphine and other potent opioids in the treatment of pain. Opioid peptide receptors are members of the G-protein coupled receptor (GPCR) family, and three genes encode three distinct opioid peptide receptor subtypes: μ-, δ-, and κ-opioid peptide receptors. The analgesic efficacy and most of the typical opioidergic undesired effects of morphine are mainly mediated by μ-opioid peptide receptors. Studies on gene-targeted mice lacking individual opioid peptide receptor subtypes have shown that analgesia, analgesic tolerance to opioids, dependence and withdrawal symptoms, and many other classical opioid effects of morphine, such as hyperlocomotion and constipation, are absent in μ-opioid peptide receptor-deficient mice (Kieffer and Gaveriaux-Ruff, 2002).

The development of opioid tolerance and dependence involve complex molecular and cellular adaptation mechanisms. There are several possible explanations for the development of opioid tolerance and dependence, including opioid peptide receptor desensitization (uncoupling from G-proteins, internalization and down-regulation of receptors), up-regulation of the cAMP pathway (supersensitization of adenylyl cyclases, receptor coupling with stimulatory G-proteins), and protein kinase-dependent neuroadaptative changes (phosphorylation) in signal transduction cascades (second messenger-dependent protein kinases, G protein-coupled receptor kinases and mitogen-activated protein kinases) (Liu and Anand, 2001).

The brain noradrenergic system plays an important role in opioid actions. α_2-Adrenoceptor agonists potentiate morphine analgesia, and there is analgesic synergism between endogenous α_2-noradrenergic and opioidergic mechanisms in the spinal cord of mice (Fairbanks and

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Wilcox, 1999; Ossipov et al., 1990a) and rats (Ossipov et al., 1990b). Substance P-induced behavioural nociceptive responses are inhibited by intrathecally administered opioid agonists and α_2-adrenoceptor agonists in mice (Roerig et al., 1992). In addition, the α_2-adrenoceptor antagonist, yohimbine, attenuates the analgesic effects of opioid agonists in the rat (Iglesias et al., 1992). Increased central and peripheral noradrenergic neuronal activity have been reported in morphine withdrawal (Devoto et al., 2002; Delfs et al., 2000; Fuentealba et al., 2000), and the sympatholytic and sedative α_2-adrenoceptor agonist, clonidine, is used clinically to treat morphine withdrawal symptoms (Gowing et al., 2002; Maldonado, 1997).

Three α_2-adrenoceptor subtypes have been identified in humans and in mice, i.e., α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. All three are coupled to $G_{i/o}$-type G-proteins, and their activation induces similar second messenger responses as those mediated by opioid peptide receptor activation, i.e., inhibition of adenylyl cyclases, activation of hyperpolarizing K^+ channels and inhibition of Ca^{2+} channels. In the central nervous system (CNS), presynaptically localized α_2-adrenoceptors inhibit the release of noradrenaline and several other neurotransmitters. The α_{2A}-adrenoceptor is the principal α_2-adrenoceptor subtype found in the CNS. The analgesic and sedative properties of α_2-adrenoceptor agonists are mainly attributed to α_{2A}-adrenoceptor activation, as evidenced by recent studies on mice lacking functional α_{2A}-adrenoceptors (Lakhlani et al., 1997; Hunter et al., 1997; Lähdesmäki et al., 2002; Stone et al., 1997).

Clonidine is a subtype-nonselective α_2-adrenoceptor agonist, and it can also activate α_1-adrenoceptors (Millan et al., 2000) and I_1-type imidazoline receptors (Bousquet, 1997). It has analgesic activity, and it decreases blood pressure and inhibits gastrointestinal motility and secretion. Dexmedetomidine is another subtype-nonselective α_2-adrenoceptor agonist, but with negligible effects at α_1-adrenoceptors (Millan et al., 2000) and imidazoline receptors in the relevant dose range (Parini et al., 1996). It has potent sympatholytic, analgesic, sedative and anesthetic effects in mice (Hunter et al., 1997; Lähdesmäki et al., 2003a) and in humans (Scheinin et al., 1992), and it is in clinical use as a sedative agent in the intensive care setting (Bhana et al., 2000). Dexmedetomidine decreases the turnover of the monoamine neurotransmitters noradrenaline, dopamine and serotonin (5-HT) in brains of rats (MacDonald et al., 1988) and mice (Lähdesmäki et al., 2003a), but this effect was absent in mice lacking α_{2A}-adrenoceptors (Lähdesmäki et al., 2003a). Also, other typical α_2-adrenoceptor mediated effects of dexmedetomidine, such as inhibition of locomotor activity and hypothermia, were very markedly attenuated, but not totally abolished, in α_{2A}-adrenoceptor deficient mice (Hunter et al., 1997; Lähdesmäki et al., 2003a).

Three different subtypes of α_1-adrenoceptors have been described, i.e., α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors. All three have been found in the brain and in the spinal cord, and all activate phospholipase C through the $G_{q/11}$ family of G-proteins, release stored Ca^{2+}, and activate protein kinase C (Zhong and Minneman, 1999). Activation of α_{1A}-adrenoceptors potentiates spinal nociceptive reflexes (Hedo and Lopez-Garcia, 2001), and central α_1-adrenoceptor stimulation functionally antagonizes the hypnotic response to dexmedetomidine (Guo et al., 1991). Prazosin, a potent subtype non-selective α_1-adrenoceptor antagonist, has significant antagonistic potency also at α_{2B}- and α_{2C}-adrenoceptors. In a previous study, we showed in normal mice that prazosin potentiates morphine analgesia, decreases morphine tolerance and also reverses some morphine withdrawal signs (Özdoğan et al., 2003).

Genetic mouse models have been widely used to study receptor functions. There is ample evidence that the brain noradrenergic system is involved in many of the actions of morphine (Airio and Ahtee, 1999; Bentley et al., 1983; Browning et al., 1982; Dambisya et al., 1991; Robson et al., 1983; Sierralta et al., 1996), but the involvement of the six α-adrenoceptor subtypes and the mechanisms of these interactions are still unclear. In this study, we have investigated the involvement of the α_{2A}-adrenoceptor subtype in opioid actions. Morphine analgesia and the development of morphine tolerance and dependence were compared in mice lacking the gene encoding the α_{2A}-adrenoceptor (α_{2A}-adrenoceptor knock-out (KO); Altman et al., 1999) and in C57Bl/6J control mice. In addition, the analgesic efficacy of clonidine, dexmedetomidine and prazosin and their effects on morphine analgesia, tolerance and withdrawal were evaluated.

2. Materials and methods

2.1. Animals

Male C57Bl/6J control mice and mice with targeted disruption of the α_{2A}-adrenergic receptor gene (α_{2A}-adrenoceptor KO) weighing 20–35 g were used. The generation of an α_{2A}-adrenoceptor KO mouse line has been described previously (Altman et al., 1999). The α_{2A}-adrenoceptor KO mice were backcrossed to C57Bl/6J mice for a minimum of five generations to produce a congenic line. Animals were allowed free access to food (RM 3 standard pellets, SDS, UK) and tap water, and were kept under artificial light for 12 h each day in a room with controlled temperature (21 °C) and humidity (50±10%). All experiments were approved by the local committee for animal welfare and were in accordance with the European Communities Council Directive of 24 November 1986 (86/906/EEC). The same groups of mice were subjected to the entire test protocol. The group *n* was 9 or 10.

2.2. Drugs

Morphine HCl and clonidine HCl (Sigma, St. Louis, MO), prazosin HCl (Tocris, Ellisville, MO), dexmedetomi-

dine HCl (Orion Pharma, Turku, Finland), and naloxone HCl (RBI, Natick, MA) were used. Prazosin was dissolved in polyethylene glycol and other drugs were dissolved in physiological saline. Appropriate vehicle controls were used. All drugs were administered in a volume of 10 ml/kg s.c. or i.p. as applicable.

2.3. Antinociceptive assay, acute analgesic effects of the test drugs, and their modulatory effects on morphine analgesia

The tail-flick method of D'Amour and Smith (1941) was used with a cut-off time of 10 s, using a commercial tail-flick analgesiameter (Ugo Basile, Comercio, Italy). The basal tail-flick latencies were first measured, followed by a second tail-flick measurement 30 min after i.p. administration of the test drugs (prazosin 0.5 mg/kg, clonidine 2 mg/kg, or dexmedetomidine 20 or 100 μg/kg) or saline. Then, a 5 mg/kg test dose of morphine was administered to all mice, and 30 min later, tail-flick latencies were measured for the third time. Tail-flick latencies were converted to %Maximal Possible Effect (MPE): %MPE=(measured tail flick score−average tail-flick score of saline-treated control group)/(cut-off time−average tail-flick score of saline-treated control group)×100%.

2.4. Induction of morphine tolerance and withdrawal

A 3-day cumulative dosing regimen was used for the induction of morphine tolerance and dependence. The treatment schedule consisted of twice daily s.c. doses of morphine given at 30 mg/kg (a.m.) and 45 mg/kg (p.m.) on day 1; 60 and 90 mg/kg on day 2; and 120 mg/kg twice on day 3. Animals were assessed for both tolerance and dependence on the 4th day, as described by Way et al. (1969). Tolerance was assessed based on loss of the antinociceptive effects of a test dose (5 mg/kg, s.c.) of morphine, using the tail-flick test. Morphine withdrawal was assessed as the occurrence of withdrawal signs following naloxone (1 mg/kg, s.c.) administration. In saline-treated mice, saline was administered twice daily for 3 days according to the same injection schedule.

2.5. Effects of the test drugs on expression of morphine tolerance and withdrawal

On the 4th day, 12 h after the second 120 mg/kg dose of morphine or saline injection, a test drug or saline was given i.p. 30 min before the morphine test dose (5 mg/kg) for assessment of tolerance. Two different doses of dexmedetomidine (20 and 100 μg/kg), prazosin (0.5 mg/kg) and clonidine (2 mg/kg) were tested. On the 4th day, after the assessment of tolerance, a final dose of morphine (120 mg/kg) or saline was given. Ten h later, test drugs or saline were given. Thirty min after the drug administration, naloxone (1 mg/kg, s.c.) was given to precipitate morphine withdrawal, and the mice were observed in groups of five for 15 min in a transparent cylinder (16 cm height, 12 cm diameter) for the occurrence of withdrawal jumping. They were then kept in well-ventilated cages without access to food and water for another 2 h 45 min. The weight loss rate was calculated for each animal.

2.6. Analysis of morphine, morphine-3-glucuronide, catecholamines and catecholamine metabolites in brain hemispheres of wild-type and α_{2A}-adrenoceptor KO mice

Following the last measurements, 210 min after test drug administration, the mice were decapitated under CO_2 anesthesia. Brains were taken out immediately and divided into left and right hemispheres, and stored at about −70 °C.

For analysis of morphine and morphine-3-glucuronide concentrations, brain halves were thawed, weighed, and homogenized with 200 μl of internal standard solution (codeine) and 900 μl of buffer solution (20 mM ammonium formate, 20 mM NaCl, pH 4.2). The homogenate was carefully transposed into a fresh tube. The first tube was rinsed two times with 500 μl of water and the water was added to the homogenate. The mixture was stirred, centrifuged and subjected to solid-phase extraction using Sep-Pak® Vac 1cc (100 mg) tC18 columns (Waters, Milford, MA). The analytes were eluted with 1 ml of methanol and 1 ml of acetonitrile. The eluate was evaporated to dryness under a stream of nitrogen at 50 °C. The dry sample was dissolved in 100 μl of a solution containing 5% methanol/95% 7 mM ammonium formate, pH 4.2 (v/v).

Mass spectrometric analysis of morphine and morphine-3-glucuronide was performed on a PE Sciex API 365 Triple Quadrupole LC/MS/MS System equipped with two PE Series 200 Micro pumps and a PE Series 200 Autosampler. The reversed phase-high performance liquid chromatography (RP-HPLC) column used was Symmetry C_8 2.1×100 mm (3.5 μm) (Waters). The mobile phase consisted of methanol (A) and 20 mM ammonium formate, pH 4.2 (B). The following gradient was used: from 0 to 0.5 min, 1% A, and from 0.5 to 13 min, from 1% A to 22% A. The flow rate was 200 μl min^{-1}. MS/MS detection was performed by positive ion spray ionization in single quadrupole mode. The needle potential was set to 2000 V, the declustering potential to 36 V, the focusing potential to 117 V, the entrance potential to 5.0 V and the collision energy to 30 V for morphine and codeine and 39 V for morphine-3-glucuronide. The molecular ion/fragment ion pairs measured (m/z) were 286.1/201.1 for morphine, 300.1/215.1 for codeine and 462.1/286.1 for morphine-3-glucuronide. The calibration standards and control samples contained morphine-6-glucuronide (morphine-6-glucuronide dihydrate, Sigma) instead of morphine-3-glucuronide. The results were calculated assuming that the glucuronides behave similarly during sample preparation and analysis. The retention times (t_R) were about 2.0 min for morphine-3-glucuronide, 4.9 min for morphine-6-glucuronide, 5.7 min for morphine and 11.8 min for codeine.

For determination of dihydroxyphenylglycol (DHPG), noradrenaline, dihydroxyphenylacetic acid (DOPAC) and dopamine, the brain samples were homogenized in 500 μl of 0.1 M perchloric acid containing 0.05% Na_2EDTA and 0.1% $Na_2S_2O_5$ and diluted with 0.1 M perchloric acid in a proportion of 1:9. The mixture was centrifuged, and 65 μl of the clear supernatant was used for the analysis, as described in an earlier publication (Lähdesmäki et al., 2003a).

2.7. Statistical analysis

Analgesia was measured as tail-flick latencies and converted to %MPE. Naloxone-precipitated morphine withdrawal was evaluated based on the number of jumps and percentage loss of body weight. The effects of each drug were tested using three-way analysis of variance (ANOVA) followed by Scheffé post-hoc tests using SPSS 8.0 for Windows programs (SPSS, Chicago, IL). Different drugs were compared using independent samples *t*-tests. All data are presented as means±S.E.M. The level of significance was set at $P<0.05$.

3. Results

3.1. Effects of the test drugs on tail-flick latency and morphine analgesia in wild-type and α_{2A}-adrenoceptor KO mice

A test dose of morphine (5 mg/kg, s.c.), given to saline-treated mice, induced similar analgesic responses in wild-type control and α_{2A}-adrenoceptor KO mice (57±5% and 60±6% of MPE, respectively) (Fig. 1). In contrast, single doses of dexmedetomidine (20 and 100 μg/kg) and clonidine (2 mg/kg), given to saline-treated mice, induced analgesic responses (30±12%, 64±13% and 51±12% of MPE, respectively) in wild-type mice but had no effects on tail-flick latency in saline-treated α_{2A}-adrenoceptor KO mice (Fig. 2).

Prazosin given alone had no effects on tail-flick latency, neither in saline-treated nor in morphine-treated mice (Fig. 2). A single dose of prazosin (0.5 mg/kg) administered 30 min before a test dose (5 mg/kg) of morphine increased the analgesic efficacy of the morphine test dose in saline-treated mice, as evidenced by increased reaction latencies in the tail-flick test ($P<0.01$). This effect was similar in wild-type mice and in α_{2A}-adrenoceptor KO mice (Fig. 1). When dexmedetomidine or clonidine were given to saline-treated mice preceding the 5 mg/kg test dose of morphine, the combined treatment showed greater analgesic efficacy in control mice than either treatment alone (88±6%, 99±1%, and 94±5% of MPE after dexmedetomidine 20, 100 μg/kg and clonidine 2 mg/kg; $P<0.01$), but no statistically significant analgesic additivity was seen in α_{2A}-adrenoceptor KO mice (50±5%, 60±7%, and 73±5% of MPE) (Fig. 1).

3.2. Effects of the test drugs on morphine tolerance

Tolerance to the analgesic effect of the test dose of morphine was evident in both types of mice pretreated with morphine for 3 days as indicated by the effect of morphine ($P<0.001$) in three-way ANOVA. Tolerance was similar in both genotypes (6±2% of MPE in wild-type and 6±3% of MPE in α_{2A}-adrenoceptor KO mice, vs. 57±5% and 60±6% of MPE in saline-treated wild-type and α_{2A}-adrenoceptor KO mice; $P<0.001$; *t*-tests, the effect of morphine test dose between saline-treated and morphine-treated mice of each genotype) (Fig. 1).

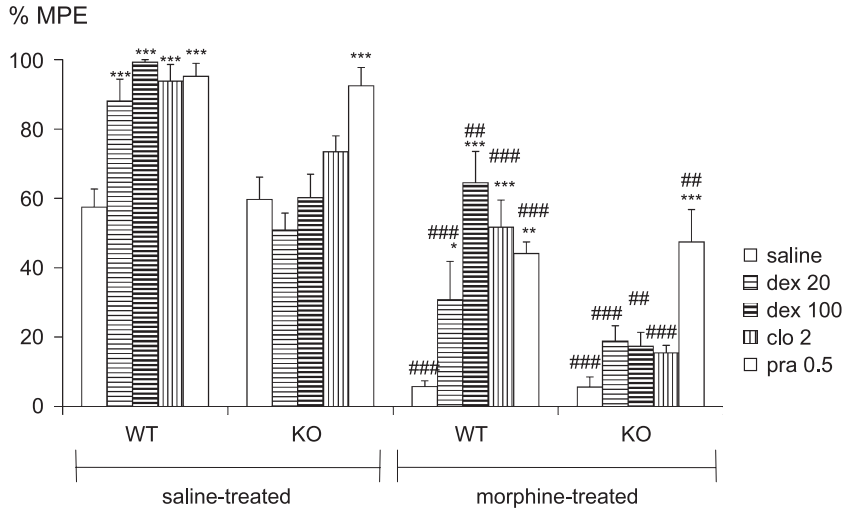

Fig. 1. Tail-flick responses to a test dose (5 mg/kg) of morphine after administration of saline or single doses of dexmedetomidine (20 and 100 μg/kg), clonidine (2 mg/kg) or prazosin (0.5 mg/kg). The experiments were carried out in two groups of mice, pretreated either with repeated injections of saline (saline-treated) or with ascending doses of morphine (morphine-treated). Tail-flick latencies were converted to percentage of Maximal Possible Effect (%MPE). Means±S.E.M.; * denotes significant differences compared to control (saline) groups (Scheffé tests) and # indicates significant differences compared to saline-treated groups (*,# $P<0.05$, **, ## $P<0.01$, ***,### $P<0.001$).

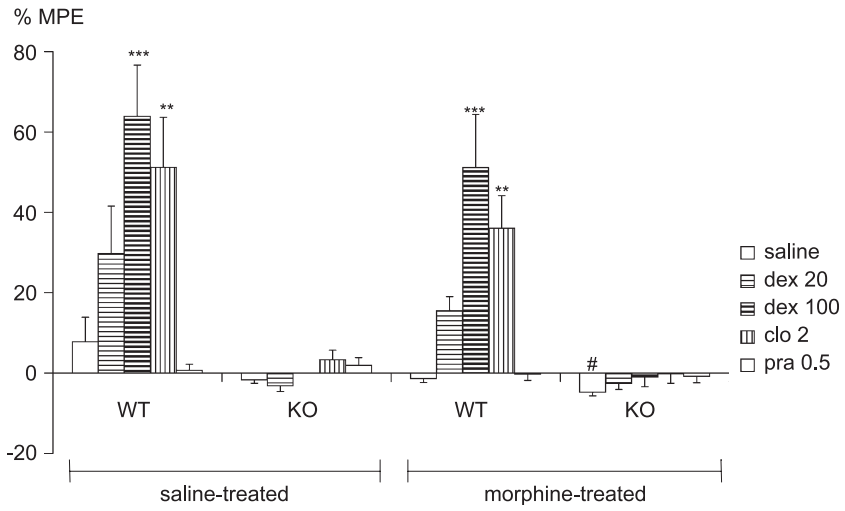

Fig. 2. Tail-flick responses after acute administration of saline or single doses of dexmedetomidine, clonidine or prazosin in saline- and morphine-treated, wild-type and α_{2A}-KO mice. Statistical significance as indicated in Fig. 1.

Dexmedetomidine- and clonidine-induced analgesia tended to be somewhat attenuated in morphine-tolerant wild-type mice (16±3%, 51±12%, and 36±1% of MPE after dexmedetomidine 20, 100 μg/kg and clonidine 2 mg/kg) (Fig. 2).

Morphine tolerance was opposed by prazosin in both types of mice (P<0.01). Prazosin 0.5 mg/kg given 30 min before the test dose of morphine restored the analgesic efficacy of the test dose of morphine in morphine-treated mice to 44±3% of MPE in wild-type and to 47±9% of MPE in α_{2A}-adrenoceptor KO mice, but not to the level observed in saline-treated mice receiving both prazosin and 5 mg/kg morphine (95±4% of MPE in wild-type and 92±5% of MPE in α_{2A}-adrenoceptor KO mice) (Fig. 1). Reflecting the significant genotype x drug interaction (P<0.001) in ANOVA, dexmedetomidine and clonidine did not significantly potentiate the analgesic effect of the 5 mg/kg test dose of morphine in morphine-treated α_{2A}-adrenoceptor KO mice (19±4%, 17±5% and 15±2% of MPE), while the analgesic effects of dexmedetomidine and clonidine were retained in morphine-treated wild-type mice (Fig. 1).

3.3. Effects of the test drugs on naloxone-precipitated morphine withdrawal signs in wild-type and α_{2A}-adrenoceptor KO mice

Naloxone-precipitated jumping was different in morphine-treated wild-type and α_{2A}-adrenoceptor KO mice, and it was differently affected by the drug treatments (P<0.0001, drug x morphine treatment x genotype interaction). The number of naloxone-precipitated vertical jumps was less in morphine-treated α_{2A}-adrenoceptor KO mice than in morphine-treated wild-type mice (19±3 and 62±6 jumps in 15 min; P=0.001) (Fig. 3). Single doses of clonidine (2 mg/kg) or prazosin (0.5 mg/kg) did not influence the number of naloxone-induced vertical jumps in either of the genotypes (Fig. 3). Single doses of

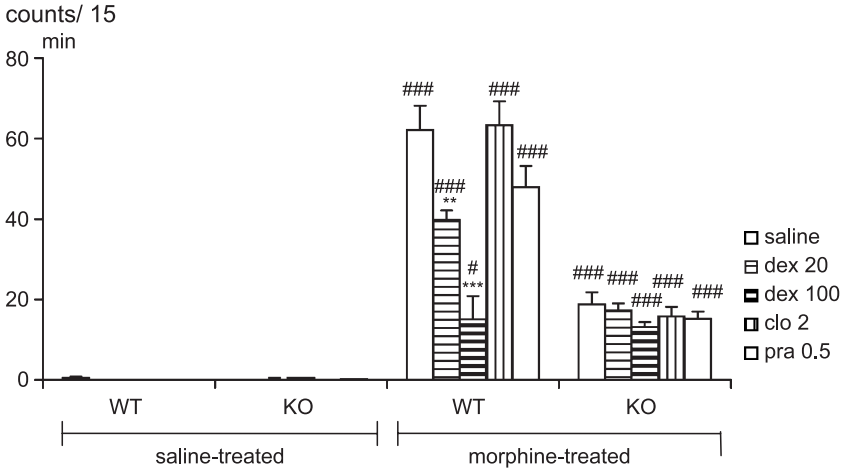

Fig. 3. Effects of acute administration of saline or single doses of dexmedetomidine, clonidine or prazosin on the number of vertical jumps after naloxone (1 mg/kg) administration to saline- and morphine-treated, wild-type and α_{2A}-adrenoceptor KO mice. Statistical significance as indicated in Fig. 1.

dexmedetomidine (20 and 100 μg/kg) decreased the number of naloxone-induced vertical jumps in wild-type mice (40±2 and 15±6 jumps in 15 min compared to 62±6 in the saline-injected group; $P<0.01$), but did not influence the number of naloxone-induced vertical jumps in α_{2A}-adrenoceptor KO mice (17±2 and 13±1 jumps in 15 min compared to 19±3 in the control group) (Fig. 3).

Weight loss was similarly increased after naloxone in morphine-treated α_{2A}-adrenoceptor KO mice and wild-type mice (7.8±0.4% and 6.4±0.9% in 3 h) (Fig. 4). The effects of the drug treatments were partly dependent on the genotype ($P<0.004$, genotype×drug treatment interaction). Naloxone-induced weight loss was effectively prevented by acute clonidine administration in wild-type mice (2.0±0.3% compared to 6.4±0.9% in the control group; $P<0.01$), but clonidine had no significant effect on weight loss in α_{2A}-adrenoceptor KO mice (6.7±0.5% compared to 7.8±0.4% in the control group). Naloxone-induced weight loss was dose-dependently prevented by acute dexmedetomidine administration in morphine-treated wild-type mice (5.4±0.9% and 3.4±0.6% compared to 6.4±0.9% in the control group; $P<0.01$ for 100 μg/kg dexmedetomidine), but again, there was no significant effect in α_{2A}-adrenoceptor KO mice (7.7±0.4% and 8.2±0.6% compared to 7.8±0.4% in the control group) (Fig. 4). Prazosin attenuated naloxone-induced weight loss similarly in morphine-dependent wild-type and α_{2A}-adrenoceptor KO mice ($P<0.05$) (Fig. 4).

3.4. The brain levels of morphine, noradrenaline, dopamine and their metabolites in wild-type and α_{2A}-adrenoceptor KO mice

The basal levels of DHPG were statistically significantly elevated in brains of α_{2A}-adrenoceptor KO mice compared to the wild-type controls (0.24±0.02 vs. 0.18±0.02 nmol/g; $P=0.01$), but noradrenaline, DOPAC and dopamine levels were similar in both genotypes. Subchronic morphine treatment had no clear effects on the brain levels of DHPG, noradrenaline and DOPAC. The concentrations of dopamine were increased by chronic morphine treatment ($P<0.001$; the effect of morphine in one-way ANOVA). The increase in dopamine levels tended to be greater in WT mice (from 8.6±0.4 nmol/g to 10.0±0.4 nmol/g) than in α_{2A}-adrenoceptor KO mice (from 8.3±0.3 to 8.9±0.4 nmol/g), but no significant genotype×morphine interaction was observed. Dexmedetomidine and clonidine slightly increased the brain noradrenaline (19% and 20% increases; $P<0.003$) and dopamine (17% and 16% increases; $P<0.008$) levels in saline-treated wild-type mice but not in α_{2A}-adrenoceptor KO mice. DHPG and DOPAC levels were not statistically significantly influenced by the α_2-adrenoceptor agonist drugs. Also, in morphine-treated wild-type mice, clonidine slightly increased the brain noradrenaline levels (16% increase; $P=0.008$). None of the drug treatments had significant effects on the brain DHPG, noradrenaline, DOPAC and dopamine levels in morphine-treated α_{2A}-adrenoceptor KO mice (results not shown).

The brain morphine and morphine-3-glucuronide levels were similar in morphine-treated α_{2A}-adrenoceptor KO and wild-type mice (64±8 and 56±9 ng/g for morphine; and 15±2 and 14±1 ng/g for morphine-3-glucuronide). In both genotypes, the concentrations of morphine in brain were somewhat lowered by the test drug treatments ($P<0.001$; effect of drug, three-way ANOVA).

4. Discussion

4.1. Analgesia

α_2-Adrenoceptor agonists, such as dexmedetomidine and clonidine, have potent analgesic and sedative effects in

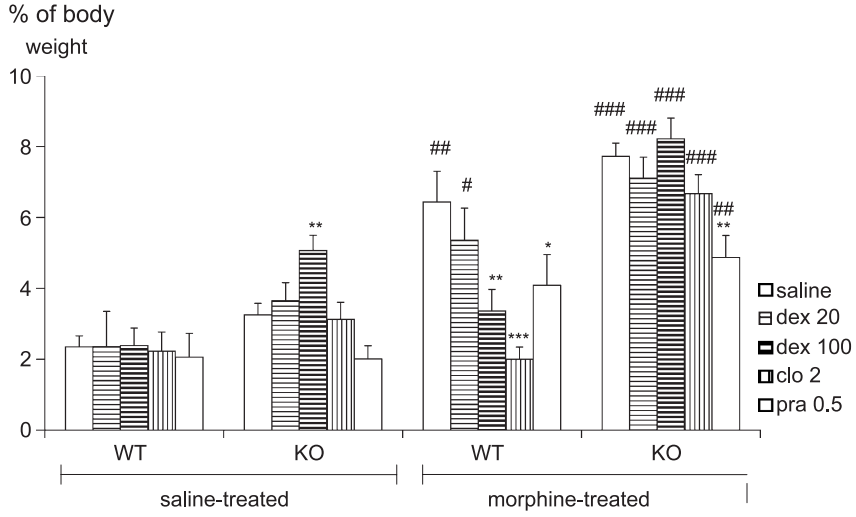

Fig. 4. Effects of acute administration of saline or single doses of dexmedetomidine, clonidine or prazosin on naloxone-induced weight loss (percent of body weight in 3 h) in saline- and morphine-treated, wild-type and α_{2A}-adrenoceptor KO mice. Statistical significance as indicated in Fig. 1.

rodents. These agonists do not discriminate between the three α_2-adrenoceptor subtypes, but studies on KO mice have revealed that their sedative-anesthesic and analgesic effects are mainly mediated by the α_{2A}-adrenoceptor subtype (Lähdesmäki et al., 2002, 2003a; Malmberg et al., 2001). Also, our results confirm that the acute analgesic effects of clonidine and dexmedetomidine are lost in α_{2A}-adrenoceptor KO mice.

Activation of supraspinal opioid peptide receptors in the central gray matter, the nucleus raphe magnus and the locus coeruleus results in increased activity of descending inhibitory pathways that inhibit the processing of nociceptive information in the dorsal horn of the spinal cord (Reisine and Pasternak, 1996). Both noradrenaline and 5-HT mediate this descending inhibition, and it can be antagonized by intrathecal (Sawynok and Reid, 1992) and systemically administered α_2-adrenoceptor antagonists (Liu and Zhao, 1992; Schreiber et al., 1998, 1999). The analgesia produced by agonists of μ-opioid peptide receptors and α_2-adrenoceptors involves both postsynaptic inhibition via G protein-coupled inwardly rectifying potassium channels (GIRKs) and presynaptic inhibition of neurotransmitter release through regulation of voltage-gated Ca^{2+} channels (Mitrovic et al., 2003). The analgesic response to α_2-adrenoceptor agonists is mediated at both supraspinal and spinal sites in mice (Lakhlani et al., 1997; Li and Eisenach, 2001; Miranda et al., 2001).

The dorsal horn of the spinal cord represents an important site for the antinociceptive synergism between opioid and α_2-adrenergic mechanisms (Ossipov et al., 1989, 1990b). As demonstrated with α_2-adrenoceptor and μ-opioid peptide receptor antagonists, neither receptor mechanism is absolutely required for the antinociception mediated by the other. The analgesic activity of the μ-opioid peptide receptor agonist, morphine, was not altered in α_{2A}-adrenoceptor deficient mice (Lähdesmäki et al., 2003b). No similar direct evidence is yet available from μ-opioid peptide receptor KO mice. The interactions of opioid and α_2-adrenergic analgesic mechanisms may be very complex, and there are several partly conflicting reports on the importance and modulation of these interactions. The α_2-adrenoceptor antagonist yohimbine has been shown to antagonize morphine analgesia in rats (Iglesias et al., 1992), but the analgesia produced by the α_2-adrenoceptor agonist, clonidine, was not sensitive to the μ-opioid peptide receptor antagonist, naloxone (Hylden et al., 1991; Ossipov et al., 1989). On the other hand, it has been reported that yohimbine potentiated morphine analgesia in humans (Gear and Levine, 1995), and that dexmedetomidine-induced inhibition of C-fiber evoked responses was reversed by naloxone in rats (Sullivan et al., 1992). Mice with genetic inactivation of the noradrenaline transporter had increased sensitivity for morphine in the tail-flick assay, indicating that increased synaptic noradrenaline concentrations evoked potentiation of the opioid effects. This synergism was mediated by α_2-adrenoceptors and it was blocked by administration of yohimbine (Bohn et al., 2000b).

Earlier studies have not noted differences in baseline nociceptive mechanisms between α_{2A}-adrenoceptor deficient and wild-type control mice, nor have the acute analgesic effects of morphine been altered in α_{2A}-adrenoceptor KO animals in normal or inflamed conditions (Lähdesmäki et al., 2003b; Malmberg et al., 2001). Also, the current results confirm that the acute morphine-induced prolongation of tail-flick latency was similar between the genotypes. This agrees with earlier findings suggesting that acute morphine analgesia is not dependent on α_{2A}-adrenoceptor activation.

Potentiation of morphine-induced analgesia by prazosin was evident also in α_{2A}-adrenoceptor KO mice. Thus, the α_{2A}-adrenoceptor subtype is not involved in the prazosin-induced potentiation of morphine analgesia. Rather, the explanation for this recently reported (Özdoğan et al., 2003) phenomenon might depend directly on blockade of α_1-adrenoceptors by prazosin, or α_2-adrenoceptor subtypes other than the α_{2A}-adrenoceptor may be involved. For example, antagonism of non-α_{2A}-adrenoceptors by prazosin has been reported to reverse the inhibitory effect of clonidine in the stress-induced β-endorphin release in the rat (Zelena et al., 1999). A reasonable body of evidence, however, suggests that also α_1-adrenoceptors modulate nociceptive processing in the dorsal horn of the spinal cord (Millan, 2002). α_1-Adrenoceptors and α_2-adrenoceptors thus differentially influence nociceptive processing in the dosal horn of the spinal cord, and they have different roles in the expression of dorsal facilitation and dorsal inhibition. Pronociceptive properties of α_1-adrenoceptors are important for two reasons. First, in addition to a spinal role in mediating dorsal facilitation, α_1-adrenoceptors in other structures may likewise potentiate transmission of nociceptive information. Second, as a corollary, the degree of residual activity of centrally acting α_2-adrenoceptor agonists at α_1-adrenoceptors becomes a crucial issue (Millan, 2002).

4.2. Tolerance

Tolerance to the analgesic effect of the test dose of morphine was evident in subchronically morphine-treated mice. Clonidine and dexmedetomidine did not modify the expression of morphine tolerance in wild-type and α_{2A}-adrenoceptor KO mice. It has been reported that chronic administration of opioids or α_2-adrenoceptor agonists leads to decreased analgesic synergism between the drug classes. Cross-tolerance has been reported between morphine and the α_2-adrenoceptor agonists, clonidine (Kalso et al., 1993; Roerig, 1995), xylazine (Ware and Paul, 2000) and dexmedetomidine (Hayashi et al., 1996; Idänpään-Heikkilä et al., 1996). Cross-tolerance between morphine and dexmedetomidine has been reported both in the tail-flick test and in electrophysiological tests in the spinal cord of the rat (Kalso et al., 1993). Peripheral cross-tolerance was demonstrated between α_2-adrenoceptors and μ-opioid peptide receptors in the paw-withdrawal test in rats (Aley and

Levine, 1997), but the involvement of subtypes of α_2-adrenoceptors and other than the μ-type of opioid peptide receptors in such cross-tolerance is still not clear. Some cross-tolerance was evident in this study, but a longer treatment may be required for the assessment of the maximal possible extent of cross-tolerance. Prazosin appeared to attenuate the expression of morphine tolerance in wild-type mice, as we also reported earlier (Özdoğan et al., 2003), and also in α_{2A}-adrenoceptor KO mice. Although prazosin alone evoked no analgesia, it potentiated morphine analgesia in both saline-treated and morphine-treated wild-type and α_{2A}-adrenoceptor KO mice. This may have been caused by antagonism of α_1-adrenoceptors involved in dorsal facilitation, but other explanations remain possible.

The cellular mechanisms of opioid tolerance comprise changes at the receptor level as well as at down-stream sites (Taylor and Fleming, 2001). Adaptational changes directly affecting the receptors involve their phosphorylation by G protein-coupled receptor kinases (GRK) and subsequent binding of β-arrestin, resulting in uncoupling of the receptor from its associated G-proteins (receptor desensitization). Subsequent to receptor uncoupling, cell surface-located receptors may become internalized and either dephosphorylated and recycled back to the cell surface (resensitization) or targeted to lysosomes for degradation (downregulation) (Tsao and von Zastrow, 2001). Accordingly, uncoupling and internalization effectively contribute to desensitization of opioid peptide receptor signaling and, thus, to the phenomenon of opioid tolerance.

In addition, there are also other alterations of opioid peptide receptor signaling. Acute administration of opioid agonists decreases cellular cAMP levels, but prolonged treatment triggers upregulation of adenylyl cyclase activity (Nestler and Aghajanian, 1997). This may be one of the cellular adaptations that underlie neuronal hyperactivity during opioid tolerance and withdrawal (Nestler et al., 1996). Sustained stimulation of μ-opioid peptide receptors also leads to activation and subsequent desensitization of GIRK currents (Blanchet and Luscher, 2002).

The μ-opioid peptide receptor response to the antinociceptive drug morphine can be differently regulated at different levels of the pain perception system. It appears that β-arrestin-mediated mechanisms of desensitization are major contributors to the development of tolerance at both supraspinal and spinal sites. Elimination of β-arrestin-mediated mechanisms by gene targeting has revealed that a protein kinase C (PKC)-mediated mechanism also contributes to the development of morphine tolerance at the spinal level (Bohn et al., 2000a, 2002).

It has been shown that the analgesic and sedative effects of the α_2-adrenoceptor agonist, clonidine, are attenuated in chronic administration (Hayashi et al., 1996). This may involve GRK-mediated cellular adaptation mechanisms analogously to μ-opioid peptide receptor desensitization, or supersensitivity of α_1-adrenoceptors (Özdoğan et al., 2003; Paalzow, 1978) may be involved in clonidine-induced tolerance. It has also been shown that PKCγ-mediated phosphorylation contributes to the tolerance to the analgesic effects of both morphine and clonidine, based on experiments with PKCγ KO mice (Zeitz et al., 2001). However, tolerance was not totally abolished in these mice, indicating that other second messenger or GRK-mediated intracellular adaptation mechanisms also play important roles in clonidine tolerance as well as in morphine tolerance.

4.3. Withdrawal

The 3-day treatment regimen with increasing doses of morphine was sufficient to induce significant morphine dependence, as evidenced by clear withdrawal signs after the administration of naloxone. Our hypothesis was that opioid withdrawal symptoms would be accentuated in α_{2A}-adrenoceptor KO mice, since α_2-adrenoceptor agonists attenuate opioid withdrawal in mice (Antonelli et al., 1991; El-Kadi and Sharif, 1997) and in rats (Pinelli and Trivulzio, 1997). We assumed that a similar role would be revealed for endogenous noradrenaline acting through α_{2A}-adrenoceptors.

Contrary to our expectations, naloxone-precipitated vertical jumping was dramatically decreased in α_{2A}-adrenoceptor KO mice, although naloxone-induced weight loss was similar in morphine-treated α_{2A}-adrenoceptor KO mice compared to wild-type controls. Clonidine decreased the body weight loss in morphine-treated wild-type mice, but did not affect naloxone-precipitated vertical jumping. Dexmedetomidine dose-dependently decreased both naloxone-precipitated vertical jumping and body weight loss in morphine-treated wild-type mice. Clonidine and dexmedetomidine had no effects on morphine withdrawal signs in morphine-treated α_{2A}-KO mice. This was in general agreement with the hypothesis of the role of α_{2A}-adrenoceptors in α_2-adrenergic control of opioid withdrawal. The different effects of clonidine on weight loss and jumping may be explained by central α_1-adrenoceptor activation by the employed high dose of clonidine (Hascoet et al., 1991).

Activation of α_{2A}-adrenoceptors is known to cause inhibition of gastrointestinal motility and intestinal secretion (Scheibner et al., 2002). Gastrointestinal motility is increased in α_{2A}-adrenoceptor KO mice, and medetomidine failed to cause any reduction of the speed of gastrointestinal transit in α_{2A}-adrenoceptor KO mice (Scheibner et al., 2002). In line with this, the α_2-adrenoceptor agonists did not affect the weight loss rate in morphine-treated α_{2A}-adrenoceptor KO mice.

Hyperactivity of brain noradrenaline systems has long been implicated among the mechanisms of opioid withdrawal (Aston-Jones et al., 1999), and increased noradrenergic activity is associated with the behavioural symptoms of morphine withdrawal (Devoto et al., 2002; Fuentealba et al., 2000). Noradrenergic cells become hyperactive during opioid withdrawal, which results in enhanced noradrenaline release at target sites (Aston-Jones et al., 1999). We observed

that the basal levels of the brain noradrenaline metabolite, DHPG, were significantly increased in α_{2A}-adrenoceptor KO mice, as described earlier for another noradrenaline metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG) (Lähdesmäki et al., 2002, 2003a). As also reported earlier (Airio and Ahtee, 1999; Devoto et al., 2002), brain levels of noradrenaline and dopamine or their metabolites were not altered in morphine withdrawal in mice.

It has been reported that although the acute administration of the α_2-adrenoceptor antagonist, yohimbine, increased naloxone-induced withdrawal signs in morphine-dependent mice (Sharif and El-Kadi, 1997), the chronic application of yohimbine attenuated naloxone-induced opioid withdrawal in mice (El-Kadi and Sharif, 1997). Chronic administration of the α_2-adrenoceptor antagonist may have caused downregulation of α_1-adrenoceptors, as also prazosin, our α_1-adrenoceptor antagonist, was capable of attenuating opioid withdrawal. The effect of prazosin was not, however, very potent which would point to a lesser role of this mechanism. Also, other α_1-adrenoceptor antagonists have decreased some somatic signs of opioid withdrawal, and the selective α_1-agonist, ST587, has been reported to increase body weight loss during morphine abstinence (Maldonado, 1997). Downregulation of α_1-adrenoceptors may thus decrease the severity of opioid withdrawal signs. Noradrenaline acting on α_1-adrenoceptors depolarizes neurons in periaqueductal gray matter (Chieng and Christie, 1996), and the inhibitory effect of the α_1-adrenoceptor antagonist, prazosin, on morphine withdrawal may perhaps be explained by blockade of this mechanism. In any case, the current results indicate that the mechanisms by which prazosin attenuates morphine withdrawal are not dependent on α_{2A}-adrenoceptor activation. In line with this, the life-long absence of inhibitory α_{2A}-autoreceptors may have evoked functional desensitization of α_1-adrenoceptor-dependent mechanisms involved in opioid withdrawal.

4.4. Conclusions

The interactions between central opioidergic and noradrenergic mechanisms are complex. The localization of and the receptor subtypes involved in the interactions of these systems with regard to antinociception, analgesic tolerance, and opioid dependence and withdrawal are still unclear. The current results show that the α_{2A}-adrenoceptor is not necessary for morphine-induced analgesia, since morphine-induced analgesia was similar in wild-type and α_{2A}-adrenoceptor KO mice. The present results also confirm the previously reported role of the α_{2A}-adrenoceptor as the main subtype for α_2-adrenoceptor-induced analgesia and potentiation of morphine analgesia. Moreover, the α_{2A}-adrenoceptor is not critical for prazosin-induced potentiation of morphine analgesia or alleviation of morphine withdrawal. Lack of α_{2A}-adrenoceptors seems to attenuate morphine withdrawal-induced jumping but not weight loss. It is possible that life-long absence of autoinhibitory α_{2A}-adrenoceptors may have lead to desensitization of post-synaptic mechanisms mediating the withdrawal symptoms.

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