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The antinociceptive effect of morphine is reversed by okadaic acid in morphine-naive but not in morphine-tolerant mice

María Ocaña, José M. Entrena, José M. Baeyens, Esperanza Del Pozo *

Department of Pharmacology and Neuroscience Institute, School of Medicine, University of Granada, Avenida de Madrid 12, E-18012 Granada, Spain

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Abstract

The activation of specific subtypes of serine/threonine protein phosphatases (PPs) plays a role in the antinociceptive effect of acute morphine, but it is not known whether these enzymes are involved in morphine-induced antinociception in morphine-tolerant animals. We evaluated the effects of both okadaic acid (a selective inhibitor of some serine/threonine PPs) and its inactive analogue L-norokadaone on the antinociception induced by morphine in morphine-naive and -tolerant female mice in the tail-flick test. Okadaic acid (0.01 and 1 pg/mouse, i.c.v.), but not L-norokadaone (1 pg/mouse, i.c.v.), antagonized in a dose-dependent way the antinociception induced by morphine (1–16 mg/kg, s.c.) in morphine-naive animals. However, both okadaic acid (0.01 and 1 pg/mouse, i.c.v.) and L-norokadaone (1 pg/mouse, i.c.v.) were unable to modify the antinociceptive effect of morphine in morphine-tolerant mice. These results suggest that in morphine-induced thermal analgesia, the role of serine/threonine PPs highly sensitive to okadaic acid is different in morphine-tolerant and morphine-naive female mice.
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Keywords: Antinociception; Morphine tolerance; Okadaic acid; L-norokadaone; Serine/threonine protein phosphatases

1. Introduction

The reversible phosphorylation and dephosphorylation of proteins, catalyzed by protein kinases and protein phosphatases, respectively, play important roles in the control of synaptic and intracellular cell events (Bauman and Scott, 2002). The most abundant protein phosphatases in mammalian systems are the serine/threonine protein phosphatases (PPs) (Price and Mumby, 1999). Okadaic acid (OA), a cell-permeable agent that inhibits several types of PPs, is especially potent against PP2A and its structural homologue PP4 (IC_{50} less that 1 nM) (Honkanen and Golden, 2002). This product has been used as a research tool to investigate the role of these PPs in several physiological processes.

We previously showed that the central administration of selective inhibitors of PPs (OA and cantharidin at very low doses) antagonized the antinociceptive effects induced by the acute injection of the μ-opioid receptor agonist morphine in mice (Moncada et al., 2003), and these findings were corroborated by other authors in experiments with OA and antisense oligodeoxynucleotides directed against PP2A and PP5 (Maeda et al., 2005). These effects of OA appear to be specific, because OA did not modify the antinociceptive effects of U50,488-H, a prototypic agonist of κ-opioid receptors (Moncada et al., 2005). Together, these findings suggest that PPs highly sensitive to OA are involved in the acute antinociception induced by μ-opioid receptor stimulation with morphine.

Phosphorylation events participate in the expression of tolerance to the antinociceptive effects of morphine (Smith et al., 2003), but the role of phosphatases has been little studied. The aim of this study was to determine whether the observed inhibitory effect of OA on acute morphine-induced antinociception is modified during the development of tolerance. We then compared the effect of the intracerebroventricular (i.c.v.) injection of OA on morphine antinociception in animals pretreated repeatedly with morphine (morphine-tolerant group) and in animals pretreated with morphine-vehicle (morphine-naive group). To rule out possible false-positive

* Corresponding author. Tel.: +34 958 243539; fax: +34 958 243537.
E-mail address: edpozo@ugr.es (E. Del Pozo).

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results, we also tested the effects of L-norokadaone (L-nor), an analogue of OA devoid of activity against PPs (Honkanen et al., 1994), in both groups of animals.

2. Methods

2.1. Animals

Female CD-1 mice (Charles River, Barcelona, Spain) weighing 25–30 g were used for all experiments. The animals were housed with food and water available ad libitum in a temperature-controlled room with a 12-h light/12-h dark cycle (lights on at 7:00 a.m). The experiments were performed during the light phase (9:00–15:00). Experimental protocols were approved by the Ethics Committee at the University of Granada and performed in strict accordance with EU regulations for the use of experimental animals (86/609/ECC).

2.2. Drugs and drug administration

To induce antinociception, animals were treated with morphine hydrochloride (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health) dissolved in saline and injected subcutaneously (s.c.) in a volume of 5 ml/kg. Okadaic acid (Sigma Química, Madrid, Spain) and its inactive analogue L-norokadaone (ICN Hubber, Madrid, Spain) were dissolved in 1% Tween 80 and injected i.c.v. in a volume of 5 µl/mouse, according to a procedure described in detail previously (Ocaña et al., 1995). Control animals received the same volume of vehicle. After the experiments were done the position of the injection was evaluated in each brain, and the results from animals in which the tip of the needle did not reach the lateral ventricle were discarded. The accuracy of the injection technique was evaluated, and the percentage of correct injections was 99%.

2.3. Morphine tolerance protocol

To induce tolerance to morphine, animals were treated with morphine 20 mg/kg intraperitoneally (i.p.), in a volume of 5 ml/kg, twice daily (9:00 h and 19:00 h) for 3 consecutive days (Oliverio and Castellano, 1974). Control animals received the same schedule of injections with the vehicle of morphine (morphine-naive animals). In all cases the assays for antinociception were performed 15 h after the last injection of morphine or its vehicle.

2.4. Antinociception experiments

The tail-flick test, used to evaluate the antinociceptive effects of the drugs, was performed as described in detail previously (Robles et al., 1996; Moncada et al., 2003). Before the test, animals were allowed to adapt to the conditions of the laboratory room for at least 1 h. After that, animals were immobilized and kept so for 30 min before the test. Restrained animals were placed on the tail-flick apparatus (LI 7100, Letica, S.A., Barcelona, Spain), a noxious beam of light was focused on the tail about 4 cm from the tip, and tail-flick latency was recorded automatically. The intensity of the radiant heat source was adjusted to yield baseline latencies between 2 and 4 s; this intensity was never changed and any animal whose baseline latency was outside the pre-established limits was excluded from the experiments. In order to minimize injury in the animals, a cut-off time of 10 s was used.

Tail-flick latencies were recorded 10 min before and immediately before all injections, and the mean of these two measures was used as the baseline tail-flick latency for each mouse. Once baseline latencies were obtained, the animals received an s.c. injection of morphine or its vehicle, and immediately thereafter an i.c.v. injection of OA or L-nor, or

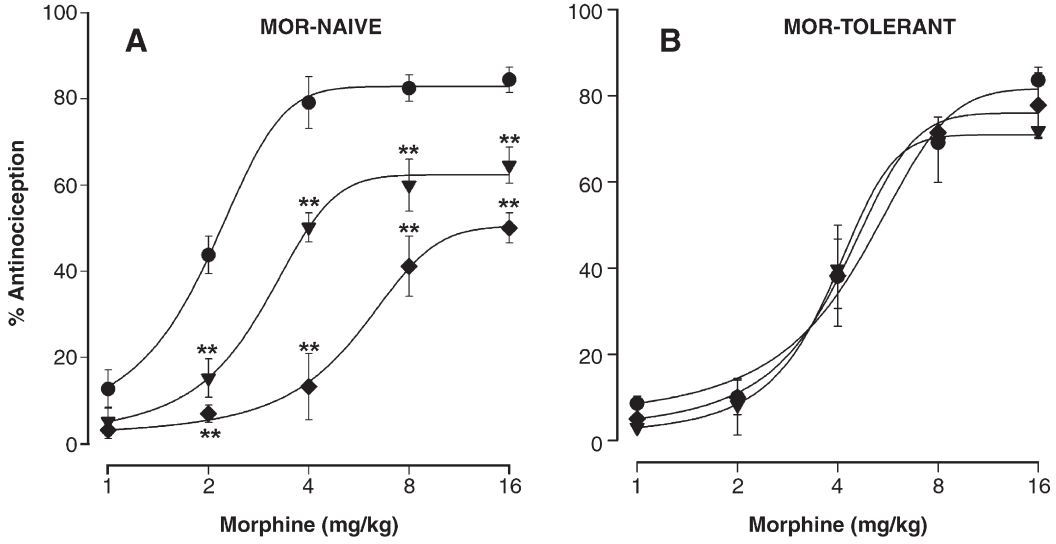

Fig. 1. Effects of the i.c.v. administration of okadaic acid (OA) or its vehicle on the antinociception induced by morphine (MOR) s.c. in the tail-flick test in morphine-naive [A] or morphine-tolerant [B] female mice. Effects of morphine plus: vehicle (●), OA 0.01 pg/mouse (▼) or OA 1 pg/mouse (◆). The percentage of antinociception was calculated from the area under the curve (AUC) of antinociception (see Methods). Each point and vertical line represents the mean±SEM (n=8–12). Statistically significant differences in comparison to morphine plus vehicle: $^{**}P<0.01$ (two-way ANOVA followed by Newman–Keuls test).

Table 1
ED_{50} (mg/kg) of different treatments in morphine-naive and morphine-tolerant female mice

Treatments [a]	Morphine-naive	Morphine-tolerant
Morphine+vehicle (control)	1.948±0.046	4.556±0.687 [b]
Morphine+OA 0.01 pg/mouse	2.887±0.187 [c]	3.789±0.074 [NS]
Morphine+OA 1 pg/mouse	5.566±0.316 [c]	4.031±0.214 [NS]
Morphine+L-nor 1 pg/mouse	1.724±0.086 [NS]	4.637±0.526 [NS]

[NS]The differences were not statistically significant in comparison to the respective control groups.
[a] Morphine or its vehicle were injected subcutaneously, whereas okadaic acid (OA), L-norokadaone (L-nor) or their vehicle were administered intracerebroventricularly.
[b] Statistically significant differences in comparison to the control morphine-naive group ($P<0.01$; non-paired Student's t test).
[c] Statistically significant differences in comparison to the control morphine-naive group ($P<0.01$; one-way ANOVA followed by Newman–Keuls test).

their vehicle. Completion of the last injection was considered time 0. Tail-flick latencies were recorded again 10, 20, 30, 45, 60, 90 and 120 min after the end of the last injection.

The degree of antinociception was calculated from the area under the curve (AUC) of tail-flick latency against time, which allowed us to obtain a global value of the antinociception induced during the 2-h experimental period according to the formula: % antinociception = $[(AUC_d - AUC_v) / (AUC_{max} - AUC_v)] \times 100$, where AUC_d and AUC_v are the areas under the curve for drug-treated and vehicle-treated animals respectively, and AUC_{max} is the area under the curve of maximum possible antinociception (10 s in each determination).

2.5. Data analysis

The area under the curve (AUC) of tail-flick latency against time was calculated with the GraphPad Prism version 3.00 program (GraphPad Sofware Inc., CA, USA). The ED_{50} values (dose of drug that produced half of the maximal antinociception) were calculated from the dose-response curves using nonlinear regression analysis of the equation for a sigmoid plot with the Sigma Plot 2000 version 6.00 program (SPSS Inc., IL, USA).

Differences between morphine ED_{50} values in the morphine-naive and -tolerant groups of animals were analyzed with Student's t test for non-paired data. The values of AUC and ED_{50} in the control group were compared against those in the treated groups with one-way or two-way analysis of variance (ANOVA) followed by the Newman–Keuls test. In all cases the differences between means were considered significant when the value of P was below 0.05.

3. Results

3.1. Effects of okadaic acid (OA) and L-norokadaone (L-nor) on morphine-induced antinociception in morphine-naive animals

As expected, the administration of morphine (1–16 mg/kg, s.c.) to morphine-naive animals induced a dose-dependent antinociceptive effect in the tail-flick test (Fig. 1A). The maximum effect (82.864±1.012% antinociception, measured as maximum possible AUC) was recorded with 16 mg/kg of morphine, and the ED_{50} was 1.948±0.046 mg/kg.

Intracerebroventricular injections of OA or L-nor, both at the highest dose administered (1 pg/mouse), did not modify the tail-flick latency values when either drug was given alone (data not shown). The association of OA (0.01 or 1 pg/mouse, i.c.v.) with morphine (1–16 mg/kg, s.c.) significantly decreased ($P<0.01$) the antinociceptive effect of the latter drug in morphine-naive animals, displacing the dose-response curve of morphine to the right (Fig. 1A), and significantly increased the ED_{50} of morphine to 2.887±0.187 mg/kg (morphine plus OA 0.01 pg/

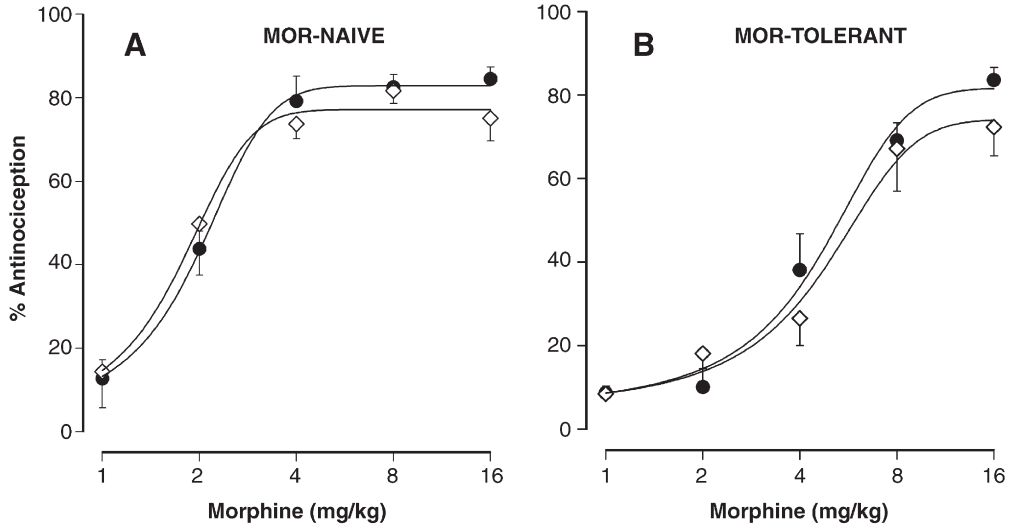

Fig. 2. Antinociceptive effects of the s.c. administration of several doses of morphine (MOR) plus L-norokadaone (L-nor) 1 pg/mouse i.c.v. (◇) or its vehicle i.c.v. (●) in the tail-flick test in morphine-naive [A] or morphine-tolerant [B] female mice. The percentage of antinociception was calculated from the area under the curve (AUC) of antinociception (see Methods). Each point and vertical line represent the mean±SEM (n=8–12). No statistically significant differences in comparison to morphine plus vehicle were observed.

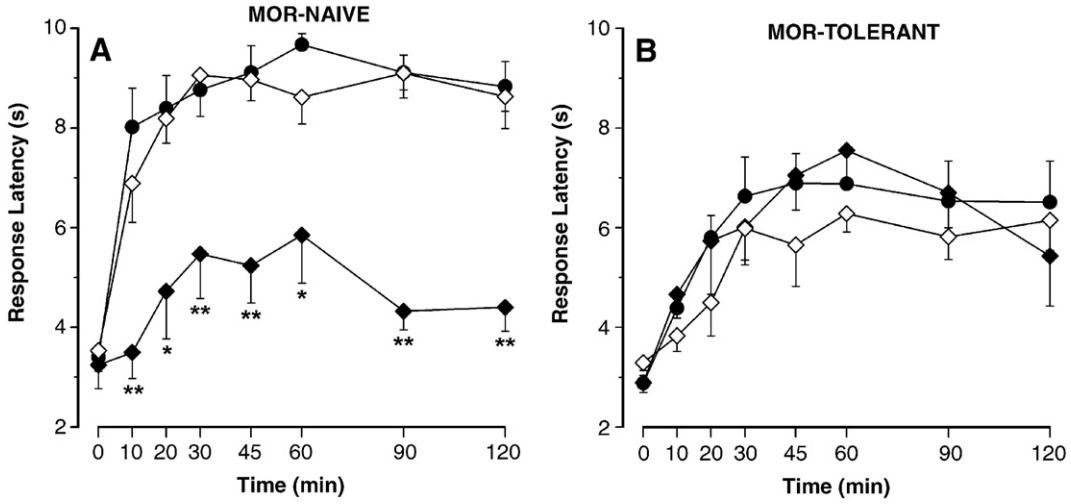

Fig. 3. Time-courses of the antinociceptive effects in the tail-flick test in morphine (MOR)-naive [A] or morphine-tolerant [B] female mice treated with morphine 4 mg/kg, s.c. plus vehicle i.c.v. (●), L-norokadaone 1 pg/mouse, i.c.v. (◇) or okadaic acid 1 pg/mouse, i.c.v. (◆). Each point and vertical line represents the mean±SEM (n=8–12). Statistically significant differences in comparison to morphine plus vehicle: *P<0.05; **P<0.01 (two-way ANOVA followed by Newman–Keuls test).

mouse) or to 5.566±0.316 mg/kg (morphine plus OA 1 pg/mouse) (Table 1). However, the association of morphine with L-nor (1 pg/mouse, i.c.v.) did not significantly modify the effects obtained with morphine+vehicle (Fig. 2A), and the ED_{50} of morphine plus L-nor (1.724±0.086 mg/kg) did not differ significantly (P>0.05) from that of morphine plus vehicle (Table 1).

When we compared the time courses of the effects of drugs in morphine-naive animals, we found that OA (1 pg/mouse, i.c.v.) significantly diminished the antinociception induced by morphine (4 mg/kg, s.c.) from 10 to 120 min after administration (Fig. 3A). In contrast, the i.c.v. administration of L-nor (1 pg/mouse, i.c.v.) did not modify the effects of morphine at any of the times when the results were recorded (Fig. 3A).

3.2. Effect of okadaic acid (OA) and L-norokadaone (L-nor) on morphine-induced antinociception in morphine-tolerant animals

In animals pretreated with repeated injections of morphine, the acute injection of morphine (1–16 mg/kg, s.c.) induced antinociception, although the effect was weaker than that produced in morphine-naive animals. The ED_{50} of morphine in morphine-tolerant animals was 4.556±0.687 mg/kg, a value significantly higher (P<0.01) than that obtained in morphine-naive mice (Table 1). The i.c.v. injection of OA and L-nor at 1 pg/mouse did not modify the pain threshold in this group of animals (data not shown).

In contrast to the findings in morphine-naive mice, the i.c.v. administration of OA in morphine-tolerant mice (0.01 and 1 pg/mouse) did not modify the antinociception induced by morphine (Fig. 1B). The ED_{50} values for morphine plus OA 0.01 or 1 pg/mouse were 3.789±0.074 or 4.031±0.214 mg/kg, respectively (Table 1), which were not significantly different (P>0.05) from the ED_{50} in the morphine-tolerant control group (morphine plus OA vehicle). The time course of the effects of OA 1 pg/mouse associated to morphine 4 mg/kg was similar to that of morphine 4 mg/kg+OA vehicle (Fig. 3B). As expected, the i.c.v. injection of 1 pg/mouse of L-nor did not modify morphine antinociception in morphine-tolerant mice (Figs. 2B, 3B, Table 1).

4. Discussion

In this study we found that very low doses of OA inhibited the thermal analgesia induced by morphine in morphine-naive but not in morphine-tolerant female mice, in the tail-flick test. We also observed that L-nor was inactive against the antinociceptive effect of morphine in both groups of animals. Since OA is a specific inhibitor of serine/threonine PPs whereas L-nor is an analogue of OA devoid of activity in these PPs (Honkanen et al., 1994; Honkanen and Golden, 2002), our results suggest that activation of specific subtypes of serine/threonine PPs plays a role in morphine-induced thermal analgesia in morphine-naive but not in morphine-tolerant female mice. In morphine-naive animals, OA decreased not only the potency of morphine, but also its maximum antinociceptive effect. This suggests a noncompetitive interaction, as might be expected bearing in mind that OA does not bind to opioid receptors (Moncada et al., 2003).

The maximal dose of OA used in our study was 1 pg/mouse, which corresponds to roughly 0.124 nM assuming that the volume of cerebrospinal fluid in mice is about 100 µL (Bernstein and Welch, 1998; Moncada et al., 2003). This low concentration of OA is within the range of IC_{50} values found to inhibit PP2A and PP4 activity (0.1–0.3 nM) (Honkanen and Golden, 2002). Intracerebroventricular treatment of mice with cantharidin, another preferential blocker of PP2A and PP4, and with antisense oligodeoxynucleotides against PP2A, also antagonized morphine antinociception in morphine-naive mice in the tail-flick test (Moncada et al., 2003), and in the tail-pinch test (Maeda et al., 2005), respectively.

The modulatory effect of i.c.v. injections of OA on systemic morphine-induced analgesia measured by us in the tail-flick test can be produced at the supraspinal or spinal level of the

nociceptive system. Other authors have demonstrated that a single dose of morphine increases OA-sensitive PP activity in specific areas of the mouse brain related with antinociception at both the supraspinal (PAG) and spinal level (spinal cord) (Maeda et al., 2005). Taken together, these results suggest that PP2A activation at different levels of the CNS is involved in analgesia by morphine in morphine-naïve mice, and that OA can modulate the antinociception of morphine in morphine-naive mice by inhibiting the increase in the activity of specific subtypes of PPs that occurs after the acute injection of morphine.

The involvement of PPs in the reactions at different levels of downstream traffic signaling after agonist stimulation of the opioid receptors has previously been demonstrated in vitro. Serine/threonine PPs modulate the activity of adenylyl-cyclase and outward potassium currents after μ-opioid receptor stimulation (Osborne and Williams, 1995; Wang et al., 1996). Moreover, dephosphorylation of the opioid receptors is catalyzed by G-protein-coupled receptor phosphatases (GRP) (Koch et al., 1998), and in mammalian (bovine) brain GRP activity is produced by an oligomeric form of PP2A sensitive to OA (Pitcher et al., 1995). Our results suggest that these biochemical and electrophysiological effects can have behavioral consequences for morphine-induced antinociception in morphine-naive mice.

Serine/threonine PPs that are highly sensitive to OA also are involved in the antinociceptive effects of other analgesic drugs such as the α_2-adrenoceptor agonist clonidine and the $GABA_B$ receptor agonist baclofen (Moncada et al., 2005). These PPs also play a role in the maintenance of sensitization to nociceptive stimuli as determined in experiments that measured mechanical and thermal hyperalgesia and mechanical allodynia following the intradermal injection of capsaicin (Zhang et al., 2003). It therefore seems that serine/threonine PPs that are highly sensitive to OA play a relevant role in different types of pain and in the antinociception induced by several types of drugs in morphine-naive animals.

In contrast to the effect in morphine-naive animals, we did not observe any inhibitory activity of OA upon morphine-induced antinociception in morphine-tolerant animals. The only antecedent for these findings is a study by Bernstein and Welch (1998), who showed that the i.c.v. injection of OA significantly increased the degree of antinociception induced by a single dose of morphine in morphine-tolerant mice. However, these authors did not use L-nor to test the specificity of the effect of OA, and injected a very high dose of OA five orders of magnitude higher than ours (0.2 μg/mouse instead of 1 pg/mouse in this study). The use of such high doses of OA can produce unspecific effects including toxicity. In fact, Bernstein and Welch (1998) observed seizures and barrel rolling with OA injected intrathecally at 0.2 μg/mouse, and we also observed seizures with OA i.c.v. at doses higher than 10 ng/mouse (unpublished results). Moreover, the high doses of OA used by these authors can produce complex effects, since it has been reported that the antagonism by OA of morphine-induced antinociception in morphine-naive animals shows a U-shaped dose–response relationship, the inhibitory effects of OA being counterbalanced at doses higher than 10 pg/mouse. Such high doses probably have additional effects aside from inhibiting highly sensitive PPs (Moncada et al., 2003; Maeda et al., 2005).

Our observation that OA, at doses that inhibit morphine antinociception in morphine-naive mice, did not antagonize morphine antinociception in morphine-tolerant mice suggests that during the development of morphine tolerance an adaptive change takes place. In fact, during the development of morphine tolerance several adaptive cellular processes have been described, mainly involving changes in neuronal ionic channels (Diaz et al., 1995; González et al., 2001), G proteins (Xu et al., 2005) and adenylyl-cyclase (Williams et al., 2001). In studies of the mouse brain that focused on enzymes that catalyze protein phosphorylation or dephosphorylation reactions, the acute administration of morphine induced significant upregulation of some G-protein receptor kinases (GRK) such as GRK2 and GRK5, whereas the repeated administration of this drug did not modify the expression of GRK5 genes, and downregulated transcript levels of GRK2 (Fan et al., 2002). Similar changes might occur in the protein phosphatases. In fact, it has been demonstrated that s.c. morphine increases the activity of OA-sensitive PPs in several areas in the CNS, but unfortunately no data have been published for morphine-tolerant animals, and the issue of the possible differences in the pharmacological activity of OA-sensitive PPs between naive and tolerant animals awaits further research. It has also been reported that chronic morphine treatment upregulates the expression of the kinase-enhanced PP1 inhibitor (KEPI), an inhibitory protein for serine/threonine PP1 (Liu et al., 2002). As we previously observed, the blockade of PP1 may counterbalance the effect of the blockade of PP2A in morphine-induced antinociception (Moncada et al., 2003), an effect which might account for the absence of effect of OA in morphine-tolerant mice.

In summary, we found that low doses of OA but did not L-nor antagonized morphine antinociception in morphine-naive female mice, whereas none of these drugs modified the antinociceptive effect of morphine in morphine-tolerant female mice. These results suggest a different role for highly-OA-sensitive PPs in thermal analgesia in morphine-tolerant and morphine-naive mice.

Acknowledgments

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