ABSTRACT

Opioids are used in humans in the management of chronic osteoarticular pains, but the development of tolerance to the analgesic effects after continuous administration is still not well understood. Our aim was to characterize morphine tolerance in a murine model of arthritis that mimics the sequence of events occurring in humans. Inflammation was induced by the intraplantar injection of complete Freund's adjuvant (CFA) and tolerance by the implantation of a 75-mg morphine pellet. We assessed the antihyperalgesic (plantar and Randall-Selitto tests), antiallodynic (Von Frey test), and antiexudative (Evans blue) effects of morphine, the μ-opioid receptor (MOR) mRNA levels in dorsal root ganglia (DRG), and MOR protein levels in DRG and plantar tissue. Inflammation induced plasma extravasation, and it significantly increased the antihyperalgesic effects of morphine (p < 0.05). Morphine pellet implantation decreased morphine potency in all tests. ED_{50} values decreased 4.4 and 7.3 times in the absence and presence of inflammation in the plantar test and 2.7 and 5.3 times in the Randall-Selitto test, whereas plasma extravasation decreased 4.2 times. MOR mRNA levels in the DRG were not affected 7 days after inflammation, whereas chronic morphine administration induced a discrete increase (p < 0.05). MOR protein in the DRG or the paw was unchanged. The results show that inflammation enhances the development of tolerance to the antihyperalgesic and antiexudative effects of morphine. At the molecular level, our results suggest that these effects are not mediated by changes in MOR expression but by other changes in receptor activation/internalization.

Tolerance to the Antinociceptive and Antiexudative Effects of Morphine in a Murine Model of Peripheral Inflammation

Víctor Fernández-Dueñas, Olga Pol, Paula García-Nogales, Laura Hernández, Eulàlia Planas, and Margarita M. Puig

Anesthesiology Research Unit, Department of Anesthesiology, Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona, Spain (V.F.-D., P.G.-N., L.H., M.M.P.); Molecular Neuropharmacology Laboratory, Hospital de la Sta Creu i Sant Pau and Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain (O.P.); and Pharmacology Unit, Department of Pathology and Experimental Therapeutics, Universitat de Barcelona, Barcelona, Spain (E.P.)

Received December 20, 2006; accepted April 25, 2007

Opioids are used in humans in the management of inflammatory pain in humans, but the development of tolerance to their analgesic effects is still controversial. The present study used a mouse model that reproduces the events that occur when patients receive chronic opioids for the symptomatic management of osteoarticular pain, to determine possible changes that could explain the decrease in morphine potency after chronic exposure.

In humans, tolerance to the analgesic effect of morphine has been described in patients with cancer pain (McQuay, 1999). In chronic noncancer pain, lower doses of opioids are administered over extended periods, and dose-escalation is seldom observed (Jensen et al., 2006). However, a recent report evaluating experimentally induced pain in patients with chronic low back pain shows the development of tolerance to oral morphine (Chu et al., 2006). The presence of tolerance could be a limiting factor for the clinical use of opioids in the management of chronic pain.

Opioid tolerance to antinociception has been demonstrated in animal models, both in control conditions (Raehal and Bohn, 2005) and during inflammation (Liang et al., 2006); however, tolerance to other peripheral events such as the antiexudative effect, have not been reported. During opioid tolerance, the reports on MOR mRNA levels and protein expression are contradictory. In the central nervous system, an increase (Fábián et al., 2002), a decrease (Meuser et al., 2003), and no change (Castelli et al., 1997) have been described, whereas in the peripheral nervous system, down-regulation of MOR was described after chronic morphine administration in the rat (Meuser et al., 2003).

Several studies have shown that peripheral inflammation
enhances the antinociceptive effects of opioids (Labuz et al., 2006), although controversy exists regarding the mechanisms involved. An increase in the expression of MOR mRNA in the DRG shortly after complete Freund’s adjuvant (CFA) injection (Puehler et al., 2004) as well as changes in the transport of these receptors to the periphery (Mousa et al., 2001) has been described. However, the time course of MOR mRNA and protein expression after CFA inflammation in the presence of morphine tolerance has not been fully explored.

In the present study, an inflammatory injury in the paw of mice was induced by the intraplantar injection of CFA, and after the inflammation becomes stabilized (chronic), the animals are exposed to constant plasma levels of morphine for a period of 3 days, by the implantation of a subcutaneous morphine pellet. To measure the development of tolerance, we assessed the requirements of acute morphine to induce antinociception/antiestravasation. The aim of our study was to evaluate the development of tolerance to the antinociceptive (antihyperalgesic and antiallodynic) and antiexudative effects of morphine in a murine model of chronic articular inflammation. We also investigated MOR mRNA and protein levels as a possible explanation for the behavioral changes in the potency of morphine. These experiments could be useful to establish the basis for chronic opioid administration in patients with osteoarticular pain.

Materials and Methods

Animals and Control of Variation

Male Swiss CD1 mice weighing 25 to 30 g were used in all experiments. The study protocol was approved by the local Committee of Animal Use and Care of our Institution, in accordance with the International Association for the Study of Pain guidelines on ethical standards for investigation in animals. To control variation, we used the same strain, gender, and weight of mice in all the groups. Diet, cycle of light/dark (12/12-h), temperature (22°C), and humidity (60%) were also similar. Animals had free access to food and water, and they were used after a minimum of 4 days of acclimatization to the housing conditions. All experiments were performed under the same laboratory conditions between 9:00 AM and 5:00 PM, and the same housing conditions. All experiments were performed under the same strain, gender, and weight of mice in all the groups.

To carry out the experiments, we placed a pool of mice in a cage, and from these animals were arbitrarily assigned to different groups and subgroups. A specific method of randomization (e.g., code or table) was not used in the study. For each set of experiments, controls (animals with no inflammation, injected with saline, or implanted with a placebo pellet) were carefully planned before behavioral testing. In all instances, control and treated animals were assessed the same day in similar experimental conditions. Blinding was not feasible for the two main variables of the study (inflammation and morphine administration), since the treatments could be clearly identified by the experienced investigator (i.e., visibly inflamed paw and peculiar behavior after morphine). Blinding of the experiments using opioid antagonists was not performed.

Paw Inflammation

Mice received a single intraplantar injection of 30 μl of CFA in the right hind paw, according to the method described by Larson et al. (1986). These animals developed a local inflammatory reaction that remained confined to the injected paw. The presence of inflammation was assessed by paw weight (electronic scale ADJ150L; Mettler-Toledo AG, Greifensee, Switzerland) and diameter (Fine Science Tools, Heidelberg, Germany), plasma extravasation (Evans blue), and nociceptive behavior (see below). The initial experiments were performed 4 h and 4, 7, and 14 days after CFA, and at the same time points in control mice (noninjected). Saline was not injected in control animals, because we showed that it induces a slight but significant inflammatory reaction (Planas et al., 1995). However, since the results obtained in control mice and in the contralateral paw were similar, we used the latter as a true control in all subsequent testing.

Due to the fact that mechanical allodynia significantly decreased 14 days after CFA injection, all experiments were performed 7 days after CFA.

Plasma extravasation was determined by the modified method of Udaka et al. (1970). Mice were briefly anesthetized with halothane, and then they were injected with 50 mg/kg Evans blue in 0.1 ml of saline in the retroorbital plexus. After 15 min, animals were killed by cervical dislocation, and both hind paws were removed, weighed, and placed in 1 ml of formamide at 60°C for 24 h. The concentration of Evans blue present in the supernatant was determined by spectrophotometry (Smart Spec 3000; Bio-Rad, Hercules, CA) at 620 nm. Results are expressed as AU per gram of wet tissue. To determine inflammation-related plasma extravasation, the concentration of Evans blue in the contralateral paw was subtracted from the value obtained in the inflamed paw (the contralateral paw served as control, since no significant extravasation of Evans blue occurs in the absence of inflammation). The inhibitory effects of s.c. morphine on plasma extravasation was determined according to the following equation: % inhibition of extravasation = [(baseline – morphine)/(baseline)] × 100.

Behavioral Testing

Mechanical nociceptive thresholds (Randall-Selitto test) were evaluated using an Analoges-Meter (Ugo Basile, Comerio, Italy), as described by Stein et al. (1988). Mice were gently held, and incremental pressure (maximum of 250 g) was applied to the dorsal surface of the hind paw. The pressure required to elicit paw withdrawal (paw-pressure threshold) was determined.

Thermal nociceptive thresholds were assessed by the plantar test (Hargreaves et al., 1988), which involves the application of a light beam directed at the center of the plantar surface of the hind paw. The time for purposeful withdrawal of the paw from the light beam was registered. A 15-s cut-off time was established to prevent tissue damage. In both nociceptive tests, the mean of three consecutive measurements separated by a period of 5 min was used.

Morphine-induced antinociception is expressed as the percent maximal possible effect, calculated according to the following equation: percent maximal possible effect (%MPE) = [(morphine – baseline)/(cut-off – baseline)] × 100, where the test latencies before (baseline) and after morphine administration are compared.

Mechanical or punctate allodynia was determined using Von Frey filaments (Sommers-Weinstein Von Frey Aesthesiometer for Touch Assessment; Stoelting Co., Wood Dale, IL), according to the method of Chaplan et al. (1994). Filaments of increasing strength (1.57–39.20 mN) were applied 10 times alternatively to each hind paw for approximately 1 s, and paw withdrawal was assessed. For each hind paw, percentage of responses was calculated and graphically represented against the log of filament strength; using this approach, we obtained the area under the curve (AUC), which was used to quantify allodynia. The inhibitory effects of s.c. morphine were calculated according to the equation % inhibition = [(AUCbaseline – AUCmorphine)/AUCbaseline] × 100.

Morphine tolerance was induced by the s.c. implantation of a 75-mg morphine pellet (Pol and Puig, 1997; Bohn et al., 2002), whereas control animals received a placebo pellet. Under halothane anesthesia, a small skin pocket was dissected in the back of the animal, where a single pellet was inserted, and the skin was closed with surgical sutures. In all instances, experiments were performed 3 days after pellet implantation. During inflammation, pellets were...
Implanted 4 days after CFA, and experiments were performed on day 7 (3 days after pellet implantation).

Three days after pellet implantation, dependence on morphine was assessed after the s.c. administration of 10 mg/kg naloxone in animals receiving a morphine or placebo pellet. After naloxone, animals were observed (30 min) for increased spontaneous activity, tremors, jumping, or rotating movements, which were considered signs of withdrawal (Pol and Puig, 1997).

Morphine plasma concentrations were determined daily for a period of 7 days after pellet implantation in animals with and without inflammation. Under halothane anesthesia, blood samples (50–60 μl/day) were obtained from an incision in the tail, and morphine plasma concentrations were determined by gas chromatography/mass spectrometry (Solans et al., 1995).

**MOR mRNA Determination by Real-Time PCR**

Tissue from the DRG between L₄ and L₆ was removed from mice after sacrifice, and it was frozen in liquid nitrogen. All tissues were homogenized in ice-cold buffer (Ultra-Turf, T8; Ika Werke, Staufen, Germany), and the total RNA was isolated with TRIzol (Invitrogen, Renfrewshire, UK).

**Reverse Transcription.** In all experiments, 4 to 5 μg of total RNA was transcribed into cDNA using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen).

**TaqMan Probe Real-Time PCR.** The expression of the MOR was determined by relative real-time PCR using premade TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for this gene (Mm00440568_m1). A probe against PGK-1 (Mm00435617_m1) was used as endogenous control. PCR reactions were set up in 384-well plates containing the corresponding cDNA, 2× Universal Master Mix (Applied Biosystems), the forward and reverse primers, and the TaqMan probe. The assays were conducted with the Applied Biosystems ABI Prism 7900HT Sequence Detection System. Each sample was tested in triplicate. Relative expression of the target gene was calculated using the comparative threshold cycle method. Data were plotted as the fold change in mRNA levels compared with the control (untreated).

**MOR Protein Analysis by Western Blot**

After sacrifice, DRG and the soft tissue of the paw were removed and frozen in liquid nitrogen. Tissues were homogenized in an SDS sample buffer containing protease inhibitors. For the Western analysis, protein samples were separated on a SDS-polyacrylamide gel electrophoresis gel and transferred to polyvinylidene difluoride filters. The filters were incubated with a polyclonal anti-MOR antibody (Chemicon International, Temecula, CA) followed by a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK), developed in enhanced chemiluminescence solution (PerkinElmer Life and Analytical Sciences) and exposed onto hyperfilm (GE Healthcare).

**Statistical Evaluation**

Data are expressed as a group mean ± S.E.M. ED₅₀ values were determined by linear regression analysis of dose-response relations based on at least six to eight animals per dose. In the present study, the ED₅₀ is defined as the dose that produces 50% of the maximal effect (Eₘₐₓ) obtained from the double reciprocal plot. Statistical analysis for significant differences between two groups was obtained by Student’s t test, and significant differences between multiple groups were determined by one-way ANOVA followed by a post hoc Student-Newman-Keuls test. A p < 0.05 was considered statistically significant.

**Experiments**

**Behavioral Experiments.** The aims of the behavioral experiments were to 1) assess the effects of systemic morphine after peripheral inflammation, 2) assess the reversibility of the effects by centrally and peripherally acting opioid antagonists, and 3) establish whether the presence of inflammation would modify the extent of morphine tolerance. Approximately 350 mice were required to perform these experiments.

The antinoceptive (plantar, Randall-Selitto, and Von Frey tests) and anticeutative (Evans blue) effects of morphine (1–100 mg/kg) were evaluated (n = 6–8 animals/dose) as follows: 1) dose-response relationships to s.c. morphine were established in the presence and absence of CFA-induced inflammation; 2) dose-response relationships to s.c. morphine were obtained in mice 3 days after pellet implantation (morphine or placebo), in the presence and absence of inflammation; and 3) the antagonism of the effects of morphine was evaluated after the s.c. administration (immediately before morphine) of 0.1 mg/kg naloxone (a central and peripherally acting antagonist) or 0.3 mg/kg naloxone-methiodide (a peripheral antagonist). For each behavioral test and experimental condition (control, CFA), we evaluated the inhibitory effect of the ED₅₀ values of morphine derived from the respective dose-response curves. Given that morphine ED₅₀ values are different in the presence or absence of inflammation, non-CFA-injected animals (instead of the contralateral paw) were used as control. In view of the fact that the percentage of inhibition obtained experimentally in the presence of antagonists was at times very low, for the sake of simplicity, the inhibitory effects of morphine (ED₅₀ values) were normalized and considered to induce a 100% effect.

**Molecular Experiments.** MOR mRNA was determined in the DRG. For each sample, DRG tissue from three to four animals was pooled for RNA isolation. For the receptor protein extraction, DRG tissue was used and exposed onto hyperfilm (GE Healthcare).

**TABLE 1**

Local inflammatory reaction induced by the intraplantar injection of CFA

Results are shown as mean values of six to eight animals ± S.E.M.

<table>
<thead>
<tr>
<th>Test</th>
<th>Time of Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Diameter paw (cm)</td>
<td>3.32 ± 0.02a</td>
</tr>
<tr>
<td>Evans blue (AU/g)</td>
<td>1.15 ± 0.14a</td>
</tr>
<tr>
<td>Plantar (s)</td>
<td>7.32 ± 0.22a</td>
</tr>
<tr>
<td>Randall-Selitto (g)</td>
<td>152 ± 4.2a</td>
</tr>
<tr>
<td>Von Frey (AUC)</td>
<td>3389 ± 58a</td>
</tr>
</tbody>
</table>

* For each test, different letters indicate significant differences between times of evaluation (basal, 4 h, and 4, 7, and 14 days), whereas the same letter indicates that the values were analogous (p < 0.05; one-way ANOVA followed by Student-Newman-Keuls test).
Naloxone-HCl was purchased from Sigma-Aldrich, and naloxone methiodide was from Sigma/RBI (Natick, MA). Drugs were dissolved in saline solution (0.9% NaCl) and administered s.c. at the nape of the neck, in a final volume of 10 ml/kg, 30 min before testing. TaqMan probes and reagents were purchased from Applied Biosystems.

Results

Local Inflammatory Reaction Induced by the Intraplantar Injection of CFA

The intraplantar injection of CFA induced a significant increase in paw diameter and plasma extravasation as well as a decrease in nociceptive thresholds in the plantar, Randall-Selitto, and Von Frey tests. The effects were statistically significant compared with baseline values at all time points except at 14 days for the Von Frey test (Table 1). Thus, CFA administration induced a local inflammatory response associated with hyperalgesia and punctuate allodynia that remained stable 4 and 7 days after injection. When the same parameters were evaluated in animals without inflammation (noninjected with CFA), no significant changes were observed in any of the parameters compared with the contralateral paw (Larson et al., 1986; data not shown). Therefore, subsequent experiments were performed 7 days after CFA, using the contralateral paw as control, which allowed a reduction in the number of animals used.

Effects Induced by the Subcutaneous Implantation of a 75-mg Morphine Pellet

Baseline Behavioral Values. In mice without inflammation, morphine pellet implantation significantly increased the nociceptive threshold for thermal hyperalgesia (plantar test), but no changes were observed in the other nociceptive tests. During inflammation, the morphine pellet returned the thresholds to baseline values, except in the Randall-Selitto test. Plasma extravasation increased during inflammation, but it was unaltered by the morphine pellet (Table 2).

Morphine plasma levels were determined daily for 7 days after the implantation of the morphine pellet in animals with and without CFA-induced inflammation. In both groups, peak plasma concentrations were obtained at day 2 (7.3 ± 1.4 and 7.5 ± 0.3 μg/ml in the absence and presence of inflammation, respectively), remained unaltered at day 3 (8.2 ± 1.3 and 7.8 ± 1.5 μg/ml) gradually decreasing thereafter. No significant differences (p > 0.05; Student’s t test) were observed between groups at any time point. Thus, the morphine pellet induced high plasma levels during the first 3 days after implantation, in animals with and without peripheral inflammation.

Withdrawal. Three days after the implantation of a placebo or morphine pellet, two groups of animals (n = 5 each group) received a s.c. injection of 10 mg/kg naloxone. All mice implanted with the morphine pellet, and none with the placebo pellet, showed increased spontaneous activity, tremors, jumping, and rotating movements. Withdrawal was not further quantified in the present investigation.

Body weight and temperature were assessed in animals implanted with placebo or morphine pellets, in the presence of CFA-induced inflammation. Both parameters significantly decreased after exposure to morphine for a period of 3 days (Table 3).

Antinociceptive Effects of Acute Subcutaneous Morphine during CFA-Induced Inflammation and Morphine Pellet Implantation

Dose-Response Curves to the Antinociceptive Effects of Morphine during CFA Inflammation. Dose-response curves to morphine were obtained in the plantar, Randall-Selitto, and Von Frey tests, 7 days after the injection of CFA (Fig. 1). In each test, the dose-response curves in the presence and absence of inflammation were parallel, and slopes were not significantly different. The results show that the antihyperalgesic (thermal and mechanical), but not the antiallodynic, effects of morphine were greater (approximately two times) in the inflamed compared with the noninflamed contralateral paw (control). Table 4 shows the ED_{50} values of morphine obtained from the dose-response curves (see Materials and Methods) and the E_{max} values, in the different experimental conditions. In Table 5, we show fold changes in the potency of morphine in the different tests and experimental conditions.

Dose-Response Curves to the Antinociceptive Effects of Morphine in Mice Implanted with a Morphine Pellet. In these animals, dose-response curves to morphine

---

**TABLE 2**

Baseline behavioral values in mice implanted with a placebo or a morphine pellet, with and without CFA inflammation

Results are shown as mean values ± S.E.M. of six to eight animals per experimental condition. Experiments were performed 7 days after CFA and 3 days after pellet implantation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>No Inflammation</th>
<th>CFA Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Morphine</td>
</tr>
<tr>
<td>Plantar test (s)</td>
<td>7.28 ± 0.14a</td>
<td>8.55 ± 0.19b</td>
</tr>
<tr>
<td>Randall-Selitto (g)</td>
<td>157.5 ± 5.7a</td>
<td>162.5 ± 4.9a</td>
</tr>
<tr>
<td>Von Frey (AUC)</td>
<td>3449 ± 25a</td>
<td>3265 ± 70a</td>
</tr>
<tr>
<td>Evans blue (AU/g)</td>
<td>1.09 ± 0.7a</td>
<td>1.21 ± 0.06a</td>
</tr>
</tbody>
</table>

* For each test condition, different letters indicate significant differences between groups, whereas the same letter indicates that the values were analogous (p < 0.05; one-way ANOVA followed by Student’s-Newman-Keuls test).

---

**TABLE 3**

Body weight and core temperature in mice with CFA-induced inflammation implanted with a placebo or morphine pellet

Results are expressed as mean values ± S.E.M. of six to eight mice. Both parameters were evaluated in the same animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pellet</th>
<th>Day 0: CFA Injection</th>
<th>Day 7: Behavioral Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (g)</td>
<td>Placebo</td>
<td>27.6 ± 0.4a</td>
<td>29.1 ± 0.4a</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>Placebo</td>
<td>37.2 ± 0.2a</td>
<td>37.4 ± 0.1a</td>
</tr>
</tbody>
</table>

* For each parameter and time of evaluation, different letters indicate significant differences between animals implanted with placebo or morphine pellets, whereas the same letter indicates that the values were analogous (p < 0.05; one-way ANOVA followed by Student’s-Newman-Keuls test).
in the plantar and Randall-Selitto tests were shifted to the right in a parallel manner (Fig. 1); morphine ED$_{50}$ values were significantly increased, both in the absence and the presence of CFA inflammation (Table 4). The results show that the presence of inflammation enhances the extent of morphine tolerance. In the plantar test, morphine pellet implantation decreased the potency of morphine 4.4 and 7.3 times (without and with inflammation, respectively), whereas in the same experimental conditions the potency of morphine decreased 2.7 and 5.3 times in the Randall-Selitto test (Table 5). In the Von Frey test, we could not obtain a dose-response curve for morphine in animals implanted with an active pellet.

**Antiexudative Effects of Acute Subcutaneous Morphine during CFA-Induced Inflammation and Morphine Pellet Implantation**

**Dose-Response Curves to the Antiexudative Effect of Morphine during CFA Inflammation.** Morphine induced a dose-dependent inhibition of plasma extravasation in the inflamed paw. Increasing doses of morphine produced a bi-phasic response (ascending and descending slopes), with an $E_{\text{max}}$ of 47.6 ± 4.3%, obtained with a 10-mg/kg dose (Fig. 2). The ED$_{50}$ of morphine derived from the ascending aspect of the curve was 2.5 ± 0.1 mg/kg, a value that is similar to the ED$_{50}$ of morphine for the inhibition of thermal hyperalgesia (Table 4).

**Dose-Response Curves to the Antiexudative Effect of Morphine in Mice Implanted with a Morphine Pellet.** Pellet implantation induced a rightward shift of the dose-response curve to the antiexudative effects of morphine. The shape and $E_{\text{max}}$ of the curve were similar to those obtained in naive animals, and the calculated ED$_{50}$ value of the ascending aspect of the curve (10.6 ± 0.3 mg/kg) was 4.2 times higher than in naive animals (Fig. 2; Tables 4 and 5). The results demonstrate that continuous exposure to morphine in the presence of CFA inflammation induces tolerance to its antiexudative effects.

**Antagonism of the Antinociceptive and Antiexudative Effects of Morphine during CFA-Induced Inflammation**

**Effects of Naloxone and Naloxone-Methiodide Administered Individually in the Different Nociceptive Tests.** The administration of naloxone or naloxone-methiodide individually to control mice had no effect on nociceptive thresholds in any of the tests. During inflammation, naloxone and naloxone-methiodide, each individually, significantly decreased nociceptive thresholds in the plantar test by 7 and 9%, respectively, compared with baseline values ($p < 0.05$; Student's $t$ test); this effect was not observed in the Randall-Selitto or Von Frey tests. Thus, the antagonists induced a slight but detectable thermal hyperalgesia in the presence of inflammation.

**Antagonism of the Antinociceptive Effects of Morphine by 0.1 mg/kg Naloxone and 0.3 mg/kg Naloxone-Methiodide.** In these experiments, we tested the ED$_{50}$ values of morphine obtained in the different tests in the presence and absence of inflammation (Table 4). In animals without inflammation, naloxone completely antagonized the effects of morphine in the plantar and Von Frey tests; however, in the Randall-Selitto test, the effect of morphine was reduced to 28.2% (Table 6). Naloxone-methiodide did not
were analogous (H11021p of morphine in all tests. Naloxone-methiodide significantly treated animals, naloxone completely antagonized the effects mainly mediated by peripheral opioid receptors. In CFA-the effects of morphine on mechanical hyperalgesia are 31.2% in the Randall-Selitto test. This finding suggests that significantly alter the effects of morphine in the plantar and Randall-Selitto tests, but it was unable to antagonize the antiallodynic effects of morphine (Table 6).

Antagonism of the Antiexudative Effects of Mor-

phine by Opioid Antagonists. The antiexudative effects of the ED$_{50}$ values of morphine were similarly decreased by naloxone and naloxone-methiodide (19.4 and 28.8% effect), suggesting that peripheral MOR mediates this effect.

MOR mRNA Expression in the DRG

MOR mRNA expression in the DRG was evaluated in the same experimental conditions as the nociceptive behavior: in untreated animals (control without inflammation, CTL), 7 days after CFA inflammation (CFA), in animals exposed to chronic morphine (MP) and in animals with inflammation plus chronic morphine (CFA + MP). In addition, MOR mRNA was determined daily for 7 days after the intraplantar injection of CFA. The results show that MOR mRNA levels remained unaltered during the first 7 days after CFA (data not shown). However, chronic exposure to morphine in the absence of inflammation induced a discrete (1.4-fold) but significant up-regulation of MOR mRNA that persisted (Fig. 3) in the presence of inflammation (CFA + MP).

MOR Protein Expression in DRG and Plantar Tissue

MOR protein expression levels were determined by Western blot in the DRG and the plantar tissue under the same experimental conditions described above. No significant differences were found in MOR protein levels among experimental conditions in the two tissues studied (Fig. 4).

Discussion

The aim of our study was to determine whether continuous exposure to morphine in the presence of chronic inflammation would affect the development of tolerance. Our results show that tolerance to the effects of morphine is enhanced in the presence of inflammation and that a nonfunctional MOR up-regulation (a discrete increase in mRNA but no change in MOR protein expression) occurs in the presence of morphine tolerance.

The behavioral experiments described in our study may have a bias related to inappropriate randomization and blinding (de Aguilar-Nascimento, 2005). However, our results (reporting the effects of CFA inflammation, acute/chronic morphine administration, and opioid antagonists) are supported by previous work performed by others and our group in similar experimental conditions; thus, they can be considered reproducible; conversely, most of the studies pub-

<table>
<thead>
<tr>
<th>Test</th>
<th>Parameter</th>
<th>CTL</th>
<th>CFA</th>
<th>MP</th>
<th>CFA + MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randall-Selitto</td>
<td>ED$_{50}$</td>
<td>12.8 ± 0.2a</td>
<td>6.3 ± 0.3b</td>
<td>34.5 ± 0.6c</td>
<td>33.1 ± 0.8c</td>
</tr>
<tr>
<td></td>
<td>$E_{max}$</td>
<td>51.7 ± 5.1a</td>
<td>66.3 ± 2.8b</td>
<td>33.1 ± 5.1c</td>
<td>35.3 ± 4.0c</td>
</tr>
<tr>
<td>Plantar</td>
<td>ED$_{50}$</td>
<td>4.9 ± 0.2a</td>
<td>2.7 ± 0.1b</td>
<td>21.5 ± 0.4c</td>
<td>19.8 ± 0.6c</td>
</tr>
<tr>
<td></td>
<td>$E_{max}$</td>
<td>22.1 ± 3.1a</td>
<td>40.2 ± 1.7b</td>
<td>29.7 ± 4.4a</td>
<td>29.8 ± 2.3a</td>
</tr>
<tr>
<td>Von Frey</td>
<td>ED$_{50}$</td>
<td>4.9 ± 0.1a</td>
<td>5.1 ± 0.2a</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>$E_{max}$</td>
<td>23.8 ± 2.7a</td>
<td>22.8 ± 1.8a</td>
<td>5.5 ± 1.9b</td>
<td>6.7 ± 1.7b</td>
</tr>
<tr>
<td>Extravasation</td>
<td>ED$_{50}$</td>
<td>2.5 ± 0.1a</td>
<td>47.6 ± 4.3a</td>
<td>10.6 ± 0.3b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$E_{max}$</td>
<td>47.6 ± 4.3a</td>
<td>49.1 ± 3.0a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* For each test and parameter, different letters indicate statistically significant differences between treatment groups, whereas the same letter indicates that the values were analogous (p < 0.05; Student’s t test or one-way ANOVA followed by Student’s-Newman-Keuls test).

Fig. 2. Effects of s.c. morphine on plasma extravasation during CFA-induced inflammation in mice implanted with a placebo or morphine pellet. Each point represents the mean value of six to eight animals, and the vertical bars indicate the S.E.M. For the ascending part of the curves, the asterisk (*) indicates significant differences between the effects induced by the same dose of morphine in animals implanted with a placebo or a morphine pellet (p < 0.05; Student’s t test).

significantly alter the effects of morphine in the plantar and Von Frey tests, but it decreased the effect of morphine to 31.2% in the Randall-Selitto test. This finding suggests that the effects of morphine on mechanical hyperalgesia are mainly mediated by peripheral opioid receptors. In CFA-treated animals, naloxone completely antagonized the effects of morphine in all tests. Naloxone-methiodide significantly decreased the effects of morphine to 38.8 and 35.7% in the plantar and Randall-Selitto tests, but it was unable to antagonize the antiallodynic effects of morphine (Table 6).

TABLE 4

Changes in the potency of morphine in the different tests and experimental conditions

The table shows the ratios of the ED$_{50}$ values of morphine derived from the dose-response curves under the different experimental conditions (Fig. 1; Table 4). A value of 1 (no change) indicates the value of reference that has been used to calculate the ratios. The arrows (↑, ↓) indicate the trend of the fold changes.

<table>
<thead>
<tr>
<th>Test</th>
<th>Condition</th>
<th>CTL</th>
<th>CFA</th>
<th>MP</th>
<th>CFA + MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randall-Selitto</td>
<td>CTL</td>
<td>2.03 (↑)</td>
<td>2.69 (↑)</td>
<td>2.58 (↑)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>1</td>
<td>5.48 (↑)</td>
<td>5.25 (↑)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>1</td>
<td>1</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>Plantar</td>
<td>CTL</td>
<td>1.81 (↑)</td>
<td>4.38 (↑)</td>
<td>4.04 (↑)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>1</td>
<td>7.96 (↑)</td>
<td>7.33 (↑)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>1</td>
<td>1</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>Von Frey</td>
<td>CTL</td>
<td>1</td>
<td>1.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extravasation</td>
<td>CFA</td>
<td>1</td>
<td>4.24 (↑)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In these experiments, we tested the ED$_{50}$ values of morphine obtained in the different experimental conditions (Table 4). Non-CFA-injected animals were used as control. Since the percentage of inhibition obtained experimentally in the presence of antagonists is at times very low, for the sake of clarity, we have normalized the effects of morphine (ED$_{50}$ values) and considered to induce a 100% effect. For each test and experimental condition (CTL and CFA), the results are shown as the mean values of six to eight animals ± S.E.M.

### Table 6

<table>
<thead>
<tr>
<th>Test</th>
<th>Plantar</th>
<th>Randall-Sciitte</th>
<th>Von Frey</th>
<th>Extravasation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>CFA</td>
<td>CTL</td>
<td>CFA</td>
</tr>
<tr>
<td>ED$_{50}$ morphine, mg/kg</td>
<td>4.9</td>
<td>2.7</td>
<td>12.8</td>
<td>6.3</td>
</tr>
<tr>
<td>% Effect morphine + saline</td>
<td>100 ± 2.9a</td>
<td>100 ± 5.7a</td>
<td>100 ± 2.7a</td>
<td>100 ± 3.0a</td>
</tr>
<tr>
<td>% Effect morphine + naloxone</td>
<td>0.0 ± 2.8b</td>
<td>3.4 ± 1.9b</td>
<td>28.2 ± 3.6b</td>
<td>9.0 ± 2.1b</td>
</tr>
<tr>
<td>% Effect morphine + naloxone methiodide</td>
<td>70.5 ± 3.1a</td>
<td>38.8 ± 2.5e</td>
<td>31.2 ± 3.8b</td>
<td>35.7 ± 2.0c</td>
</tr>
</tbody>
</table>

*a* Different letters indicate statistically significant differences between the effects of morphine (+ saline) and after antagonist administration ($p < 0.05$; one-way ANOVA followed by Student-Newman-Keuls test).

Doses of morphine that induced antinociception also produced inhibition of plasma extravasation with a biphasic response that has been reported previously by our group and others (Stein et al., 2001a; Romero et al., 2005). The dual response could be related to high- and low-affinity states of the MOR or to different functional subtypes or splice variants of the receptors (Pasternak, 2001).

To assess the contribution of central and peripheral MOR, we tested the ED$_{50}$ values of morphine alone and after the administration of naloxone or naloxone-methiodide. By comparing the effects, we could show significant differences in the reversal induced by the antagonists, although pA$_{2}$ values were not obtained to avoid unnecessary use of animals. The antagonists alone did not affect mechanical thresholds, but they showed a slight but significant pronociceptive effect in the plantar test, suggesting the release of endogenous opioids. Our experiments were performed 7 days after CFA at...
which time opioid release could be of a lesser magnitude than in earlier phases of inflammation, when the pronociceptive effects of the antagonists have been demonstrated (Planas et al., 2000; Stein et al., 2001b).

In the absence of inflammation, naloxone-methiodide antagonized the effects of morphine in the Randall-Selitto test but not in the plantar or Von Frey test, suggesting that mechanical hyperalgesia has an important peripheral component. In CFA-treated mice, naloxone completely blocked the antinociceptive and antiangiogenetic effects of morphine; however, naloxone-methiodide significantly decreased the antihyperalgesic effects of morphine, but it was unable to antagonize its antiallodynic effects. Naloxone-methiodide totally reversed the inhibition of plasma extravasation, suggesting that peripheral MOR mainly mediates the effect.

It could be hypothesized that the increased antihyperalgesic potency of morphine during CFA inflammation is partially mediated by the sensitization/up-regulation of MOR located in the peripheral terminals of Aδ- and C-fibers and that MOR located in the central nervous system mediates the antiallodynic effects. Alldynia may be mediated by different fibers (Aδ) with diverse sensitivity to drugs (Ossipov et al., 1999); since these fibers do not overexpress MOR during inflammation, a central, but not peripheral, mechanism could be postulated (Ständer et al., 2002; Li and Zhao, 2003).

During inflammation, pre-existing or newly synthesized MOR in the DRG of sensory neurons is axonally transported to the inflamed tissue and the spinal cord (Ballet et al., 2003). Increased peripheral MOR expression has been reported up to 3 days after inflammation (Mousa et al., 2001; Truong et al., 2003), and an increase in MOR mRNA has been described in the DRG 1 to 2 h after CFA injection (Puehler et al., 2004). However, these changes have not been studied during chronic inflammation; thus, it is unknown whether peripheral MOR up-regulation is related to de novo synthesis. Our results show that there are no changes in MOR mRNA levels 7 days after CFA injection, ruling out that the enhanced antinociceptive/antiangiogenetic effects of morphine are related to an increase in synthesis. Alternatively, peripheral inflammation could sensitize MOR and/or enhance the axonal transport of a pre-existing neuronal pool of MOR proteins that are later expressed at both terminals of sensory neurons (Hassan et al., 1993; Ji et al., 1995; Ballet et al., 2003). We did not observe significant changes in MOR protein levels in the DRG or the paw, although an increased axonal transport cannot be excluded due to insufficient sensitivity of the Western blot to detect small changes in MOR levels.

For the induction of tolerance, a 75-mg morphine pellet was implanted subcutaneously (Pol and Puig, 1997) that induced steady morphine plasma levels without the intermittent periods of abstinence or changes in nociceptive behavior observed when a daily injection protocol is used (Li and Clark, 2002). This method of morphine administration closely reproduces the clinical situation where patients are exposed to therapeutic plasma levels of opioids (e.g., slow-release formulations and transdermal patches) capable of controlling pain in a continuous manner. Morphine plasma levels were elevated during 3 days after pellet implantation, and they were unaltered by inflammation. During this period, we also observed decreased body weight and temperature, which are considered direct effects of chronic opioid exposure (Houshyar et al., 2001). The naloxone-induced severe withdrawal 3 days after pellet implantation was another sign of effective chronic exposure to morphine. However, some studies consider this period too short to obtain complete tolerance (Li and Clark, 2002), whereas others agree with the 3-day protocol (Bohn et al., 2002).

It is presently accepted that chronic exposure to opioids and tissue injury induce similar adaptive changes in the nervous system, resulting in central and peripheral nociceptive sensitization and abnormal pain (Mao and Mayer, 2001; Gardell et al., 2006). Thus, it would seem likely that concurrence of both factors would result in complete tolerance, as reported in the present investigation. Our protocol did not permit the assessment of the pro-nociceptive effects of morphine, since tolerance experiments were performed in the presence of high morphine plasma concentrations that in baseline conditions, induced a minor degree of antinociception (Table 2).

The present results show that inflammation enhanced morphine tolerance. In the plantar test, morphine pellet implantation decreased morphine potency 4.4 and 7.3 times in the absence and presence of inflammation, whereas in the Randall-Selitto test morphine potency decreased 2.7 and 5.3 times. Our results agree with those of Li et al. (1999) who reported that inflammation could facilitate tolerance development. Tolerance was also shown to the antiallodynic effect of morphine, although the decrease in the potency of morphine could not be estimated due to the limitations of the test. This finding together with the failure of naloxone-methiodide to reverse the antiallodynic effect of morphine suggests differences in the development of tolerance in the central and peripheral nervous systems. Controversy still exists regarding the development of tolerance to the peripheral effects of morphine, and some investigations have reported that peripheral opioid receptors do not become “tolerant” after continuous exposure to endogenous opioids released from immune cells (Stein et al., 2001b). Our results clearly indicate that during CFA inflammation, morphine tolerance develops both to the central and peripheral (antinociceptive and antiangiogenetic) effects of morphine.

During CFA inflammation, MOR mRNA and protein expression after chronic exposure to morphine has not been fully explored. Here, we show that chronic morphine exposure resulted in a modest increase in MOR mRNA levels that did not correlate with an increase in protein expression in the DRG or the injured tissue. Morphine-induced down-regulation of MOR has been implicated in opioid tolerance (Meuser et al., 2003), but our results do not support this hypothesis; the discrepancy could be related to the much higher sensitivity of the real-time PCR assay compared with the Western blot.

In conclusion, our study shows that morphine tolerance to its antinociceptive and antiangiogenetic effects is enhanced during peripheral inflammation. The functional MOR up-regulation in morphine-tolerant mice indicates that different regulatory mechanisms may be involved in the overexpression of MOR during chronic opioid administration. These results suggest that opioid tolerance may be a relevant factor affecting the adequate management of osteoarticular pain in humans.
Acknowledgments

We thank Dr. R. de la Torre for the determination of morphine plasma levels and C. Zamora for excellent technical help.

References


Address correspondence to: Dr. Margarita M. Puig, Department of Anesthesiology, UAB, Hospital del Mar, Paseo Marítimo 25, 08003 Barcelona, Spain. E-mail: mpuig@imas.imim.es