TOLERANCE TO THE ANTINOCICEPTIVE AND ANTI-EXUDATIVE EFFECTS OF MORPHINE IN A MURINE MODEL OF PERIPHERAL INFLAMMATION

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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 intra-plantar injection of complete Freund’s adjuvant (CFA), and tolerance by the implantation of a 75-mg morphine pellet. We assessed the anti-hyperalgesic (plantar and Randall-Selitto tests), anti-allodynic (Von Frey) and anti-exudative (Evan’s 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 significantly increased the anti-hyperalgesic effects of morphine ($p<0.05$). Morphine pellet implantation decreased morphine potency in all tests. ED$_{50}$’s 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, while plasma extravasation decreased 4.2 times. MOR mRNA levels in the DRG were not affected seven days after inflammation, while 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 anti-hyperalgesic and anti-exudative 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.
Introduction

Opioids are used 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 evaluate the effects of opioid tolerance in pain control. We also investigated MOR expression, 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 non-cancer pain, lower doses of opioids are administered over extended periods of time, 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 anti-exudative 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 (Fabian et al., 2002), a decrease (Meuser et al., 2003) and no change (Castelli et al., 1997) have been described. While 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 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 three 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 / anti-extravasation. The aim of our study was to evaluate the development of tolerance to the antinociceptive (anti-hyperalgesic and anti-allodynic) and anti-exudative 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.
Methods

Animals and control of variation

Male Swiss CD1 mice weighing 25-30 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-h/12-h light/dark), temperature (22ºC) and humidity (60%) were also similar. Animals had free access to food and water and were used after a minimum of four days acclimatization to the housing conditions. All experiments were performed in the same laboratory conditions between 9 AM – 5 PM, and the same qualified investigator collected data. For the duration of the study, body weight, rectal temperature (CheckTemp, Hanna Instruments, USA) and animal well-being (posture, grooming, motor activity) were verified every other day.

To carry out the experiments, we placed a pool of mice in a cage and from there animals were arbitrarily assigned to different groups and sub-groups. A specific method of randomization (code, table, other) 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 intra-plantar injection of 30 µl of Complete Freund’s Adjuvant (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 (Mettler-Toledo AG electronic scale AJ150L, Greifensee Switzerland) and diameter (Fine Science Tools, Germany), plasma extravasation (Evan’s blue) and nociceptive behaviour (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 (non-injected). 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 anaesthetized with halothane, and injected with Evan’s blue (50 mg/kg in 0.1 ml saline) in the retro orbital plexus. After 15 minutes, animals were killed by cervical dislocation and both hindpaws removed, weighed, and placed in 1 ml formamide at 60°C, for 24 h. The concentration of Evan’s blue present in the supernatant was determined by spectrophotometry (Smart Spec 3000, Bio-Rad, USA) at 620 nm. Results are expressed as absorbance units per gram of wet tissue (AU/g). To determine inflammation-related plasma extravasation, the concentration of Evan’s 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 Evan’s 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 (AU/g) = [(baseline – MORPHINE) / (baseline)] x 100

**Behavioral testing**

*Mechanical nociceptive thresholds* (Randall-Selitto test) were evaluated using an Analgesy-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 percentage of maximal possible effect (% MPE), calculated according to the following equation:

\[
\text{% MPE} = \left(\frac{\text{MORPHINE} - \text{baseline}}{\text{cut-off} - \text{baseline}}\right) \times 100,
\]

where the test latencies before (baseline) and after morphine administration are compared.

*Mechanical or punctate allodynia* was determined using Von Frey filaments (Semmes-Weinstein Touch test Sensory Evaluation, Stoelting North Coast, USA), according to the method of Chaplan et al. (1994). Filaments of increasing strength (1.57 to 39.20 mN) were applied ten times alternatively to each hind paw for approximately 1 sec, and paw withdrawal assessed. For each hind paw, percent responses were 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:

\[
\text{% inhibition} = \left(\frac{\text{AUC}_{\text{BASELINE}} - \text{AUC}_{\text{MORPHINE}}}{\text{AUC}_{\text{BASELINE}}}\right) \times 100
\]
Morphine tolerance was induced by the s.c. implantation of a 75-mg morphine pellet (Pol and Puig, 1997; Bohn et al., 2002), while control animals received a placebo pellet. Under halothane anesthesia, a small skin pocket was dissected in the animal’s back, 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 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 naloxone (10 mg/kg) 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 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 frozen in liquid nitrogen. All tissues were homogenized in ice-cold buffer (Ultra-Turf, T8; Ika Werke, Staufen, Germany) and the total RNA isolated with TRIzol (Invitrogen, Renfrewshire, England).

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

TaqMan probe real-time polymerase chain reaction (PCR). The expression of the MOR was determined by relative real-time PCR using pre-made TaqMan® Gene Expression
Assays (Applied Biosystem, CA, USA) 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 Biosystem, CA, USA), the forward and reverse primers and the TaqMan probe. The assays were conducted with the Applied Biosystem 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 (Ct) method. Data were plotted as the fold change in mRNA levels compared to 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 a SDS sample buffer containing protease inhibitors. For the western analysis, protein samples were separated on a SDS-PAGE gel and transferred to PVDF filters. The filters were incubated with a polyclonal anti-MOR antibody (Chemicon Intl, Inc, USA) followed by an HRP-conjugated secondary antibody (Amersham Biosciences, Germany), developed in ECL solution (NEN) and exposed onto hyperfilm (Amersham Biosciences, Germany).

**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 a 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 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 assess i) the effects of systemic morphine after peripheral inflammation; ii) the reversibility of the effects by centrally and peripherally-acting opioid antagonists; and iii) to establish if the presence of inflammation would modify the extent of morphine tolerance. Approximately 350 mice were required to perform these experiments.

The antinociceptive (plantar, Randall-Selitto, and Von Frey tests) and anti-exudative (Evan’s blue) effects of morphine (1-100 mg/kg) were evaluated (6-8 animals per dose) as follows:

- Dose-response relationships to s.c. morphine were established in the presence and absence of CFA-induced inflammation.
- 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.
- 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 ED$_{50}$’s of morphine derived from the respective dose-response curves. Given that morphine ED$_{50}$’s are different in the presence / absence of inflammation, non-CFA injected animals (instead of the contralateral paw) were used as control. In view of the fact that the % inhibition obtained experimentally in the presence of antagonists was at times very low, for the sake of simplicity, the inhibitory effects of morphine (ED$_{50}$’s) were normalized and considered to induce a 100% effect.
Molecular experiments: MOR mRNA was determined in the DRG. For each sample, DRG tissue from 3-4 animals was pooled for RNA isolation. For the receptor protein extraction, DRG tissue from 2-3 animals and plantar tissue (paw) from 1-2 animals was collected. In these experiments, the technician who performed the assays was blinded to the treatments received. Due to the small size of the tissues, we required approximately a total of 150 animals for these experiments. We could not utilize the same mice used in the behavioral tests, since the treatments (morphine, opioid antagonists) and the tests themselves, could interfere with the molecular determinations.

Drugs and reagents
Complete Freund’s adjuvant was obtained from Sigma Aldrich Co, St Louis, USA. Morphine-HCl and morphine base (for pellet preparation) were obtained from Alcaiber S.A., Spain. (-)-naloxone-HCl was purchased from Sigma Chemical Co, St Louis, USA, and naloxone methiodide from Research Biomedical Inc, USA. Drugs were dissolved in saline solution (ClNa 0.9%) 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 Biosystem, CA, USA.
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 when compared to 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 (non-injected with CFA), no significant changes were observed in any of the parameters when compared to the contralateral paw (Larson et al., 1986 and results 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 s.c. 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 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 7.3 ± 1.5 µg/ml) gradually decreasing thereafter. No significant
differences ($p > 0.05$, Student 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 s.c. 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 (Figure 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 anti-allodynic, effects of morphine were greater (approximately 2 times) in the inflamed than in the non-inflamed contralateral paw (control). Table 4 shows the ED$_{50}$’s of morphine obtained from the dose-response curves (see Methods) and the Emax 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 in the plantar and Randall-Selitto tests were shifted to the right in a parallel manner (Figure 1);
morphine ED$_{50}$'s 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), while 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.

**Anti-exudative effects of acute s.c. morphine during CFA-induced inflammation and morphine pellet implantation**

*Dose-response curves to the anti-exudative 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 biphasic response (ascending and descending slopes), with an Emax of 47.6 ± 4.3 %, obtained with a 10 mg/kg dose (Figure 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 anti-exudative effect of morphine in mice implanted with a morphine pellet.* Pellet implantation induced a rightward shift of the dose-response curve to the anti-exudative effects of morphine. The shape and Emax of the curve were similar to those obtained in naïve animals, and the calculated ED$_{50}$ of the ascending aspect of the curve (10.6 ± 0.3 mg/kg) was 4.2 times higher than in naïve animals (Figure 2, Tables 4 and 5). The results demonstrate that continuous exposure to morphine in the presence of CFA-inflammation induces tolerance to its anti-exudative effects.
Antagonism of the antinociceptive and anti-exudative 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 one individually, significantly decreased nociceptive thresholds in the plantar test by 7% and 9%, respectively, when compared to 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 naloxone (0.1 mg/kg) and naloxone-methiodide (0.3 mg/kg).* In these experiments we tested the ED$_{50}$'s 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, the effect of morphine was reduced to 28.2 % (Table 6). Naloxone-methiodide did not significantly alter the effects of morphine in the plantar and Von Frey tests, but 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 was unable to antagonize the anti-allodynic effects of morphine (Table 6).

*Antagonism of the anti-exudative effects of morphine by opioid-antagonists.* The anti-exudative effects of the ED$_{50}$'s of morphine were similarly decreased by naloxone and naloxone-methiodide (19.4 and 28.8 % effect), suggesting that peripheral MOR mediate this effect.
**MOR mRNA expression in the DRG.** MOR mRNA expression in the DRG was evaluated in the same experimental conditions than the nociceptive behavior: in untreated animals (control, 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 intra-plantar injection of CFA. The results show that MOR mRNA levels remained unaltered during the first 7 days after CFA (results 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 (Figure 3) in the presence of inflammation (CFA+MP).

**MOR protein expression in the DRG and the plantar tissue.** MOR protein expression levels were determined by western blot in the DRG and the plantar tissue in the same experimental conditions described above. No significant differences were found in MOR protein levels amongst experimental conditions, in the two tissues studied (Figure 4).
Discussion

The aim of our study was to determine if 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 non-functional 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 other's and our group in similar experimental conditions, and thus can be considered reproducible; on the other hand, most of the studies published in the literature that were used for comparison, did not follow strict randomization or blinding. The future systematic use of precise experimental designs that include blinding (whenever possible) and randomization in behavioral testing is essential to generate scientifically relevant data.

In our model (Larson et al., 1986), CFA injection induced a local inflammatory response with decreased nociceptive thresholds and increased plasma extravasation that was observed 4 hours after CFA and lasted 14 days. Most studies have shown that local inflammation increases opioid potency after intra-plantar injection (Stein et al., 1988; Perrot et al., 2001), while only a few observed increased morphine potency after systemic administration (Planas et al., 2000). In our study, morphine was injected subcutaneously, and we could demonstrate that its anti-hyperalgesic effects were significantly greater in the inflamed than in the contralateral paw, while the anti-allodynic effects were similar in both paws. Higher doses of morphine could not be used in these experiments due to hyper-motility that interferes with the behavioral testing (Zarrindast et al., 2001). This restriction partially explains the modest Emax
values obtained with the doses of morphine tested. However, even at these dose levels, we could clearly discriminate the effects of s.c. morphine in the different experimental conditions.

Doses of morphine that induced antinociception also produced inhibition of plasma extravasation, with a biphasic response that has been previously reported by our group and others (Stein et al., 2001b; 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 pA2 values were not obtained in order to avoid unnecessary use of animals. The antagonists alone did not affect mechanical thresholds, but showed a slight but significant pro-nociceptive effect in the plantar test, suggesting the release of endogenous opioids. Our experiments were performed 7 days after CFA, at what time opioid release could be of a lesser magnitude than in earlier phases of inflammation, when the pro-nociceptive effects of the antagonists have been demonstrated (Planas et al., 2000; Stein et al., 2001a).

In the absence of inflammation, naloxone-methiodide antagonized the effects of morphine in the Randall-Selitto but not in the plantar or Von Frey tests, suggesting that mechanical hyperalgesia has an important peripheral component. In CFA-treated mice, naloxone completely blocked the antinociceptive and anti-exudative effects of morphine; however, naloxone-methiodide significantly decreased the anti-hyperalgesic effects of morphine, but was unable to antagonize its anti-allodynic 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 anti-hyperalgesic 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-fibbers, and that MOR located in the central nervous system mediate the anti-allodynic effects. Allodynia may be mediated by different fibbers (Aβ) with diverse sensitivity to drugs (Ossipov et al., 1999); since these fibbers do not over-express MOR during inflammation, a central, but not peripheral mechanism could be postulated (Städer et al., 2002; Li and Zhao, 2003). During inflammation, pre-existent or newly synthesized MOR in the DRG of sensory neurons are 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 1-2 hours 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 or not, 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 / anti-exudative 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), which 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 (slow-release formulations, transdermal patches, other) capable to control pain in a continuous manner. Morphine plasma levels were
elevated during 3 days after pellet implantation, and 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 authors consider this period too short to obtain complete tolerance (Li and Clark, 2002), while 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 adaptative 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, while 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 anti-allodynic 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 anti-allodynic 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 investigators have reported that peripheral opioid-receptors do not become “tolerant” after continuous exposure to endogenous opioids released from immune cells (Stein et al.,
Our results clearly indicate that during CFA-inflammation, morphine tolerance develops both to the central and peripheral (antinociceptive and anti-exudative) 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 to the western blot.

In conclusion, our study shows that morphine tolerance to its antinociceptive and anti-exudative effects is enhanced during peripheral inflammation. The non-functional MOR up-regulation in morphine tolerant mice indicates that different regulatory mechanisms may be involved in the over-expression 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.
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References


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Footnotes

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Legends for figures

Fig. 1.- Morphine dose-response curves in the plantar (panel A), Randall-Selitto (B) and Von Frey tests (C).

Experiments were performed in naïve (continuous lines) and in mice implanted with a morphine-pellet (dotted lines) in the absence (squares) or presence (triangles) of inflammation. CTL: control without inflammation; CFA: inflammation; MP: morphine pellet. Each point represents the mean value of 6-8 animals and the vertical bars indicate the SEM. In each panel, the * indicate statistically significant differences between groups ($p < 0.05$, Student’s t-test and Student-Newman-Keuls).

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 6-8 animals, and the vertical bars indicate the SEM. For the ascending part of the curves, the * 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).

Fig. 3.- MOR mRNA expression in the DRG in the presence of peripheral inflammation and chronic exposure to morphine.

Experiments were performed in naïve and in mice implanted with a morphine pellet, in the absence or presence of inflammation. CTL: control without inflammation; CFA: inflammation; MP: morphine pellet. The results are expressed as mean values of three independent experiments (3-4 animals per sample) and show mRNA changes related to the control. Vertical bars indicate SEM, and the * designate significant differences between groups ($p < 0.05$; one way ANOVA, post hoc Student-Newman-Keuls).
Fig. 4.- MOR protein expression in DRG and plantar tissue in the presence of peripheral inflammation and chronic exposure to morphine.

The figure shows a representative western blot experiment. Samples were obtained from naïve and mice implanted with a morphine pellet, in the absence or presence of inflammation. CTL: control without inflammation; CFA: inflammation; MP: morphine pellet. In each lane, total protein pooled form 2-3 animals (DRG) or 1-2 animals (paw) were used. β-Actin was utilized as a loading control. The experiments were repeated three times.
Table 1.- Local inflammatory reaction induced by the intra-plantar injection of CFA.

<table>
<thead>
<tr>
<th>Time</th>
<th>Basal</th>
<th>4 hours</th>
<th>4 days</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter paw (cm)</td>
<td>3.32 ± 0.02</td>
<td>4.16 ± 0.02</td>
<td>4.23 ± 0.05</td>
<td>4.22 ± 0.01</td>
<td>4.19 ± 0.03</td>
</tr>
<tr>
<td>Evan’s blue (AU/g)</td>
<td>1.15 ± 0.14</td>
<td>2.83 ± 0.31</td>
<td>2.07 ± 0.24</td>
<td>1.94 ± 0.13</td>
<td>1.66 ± 0.12</td>
</tr>
<tr>
<td>Plantar test (s)</td>
<td>7.32 ± 0.22</td>
<td>3.75 ± 0.16</td>
<td>3.68 ± 0.25</td>
<td>3.52 ± 0.12</td>
<td>3.95 ± 0.16</td>
</tr>
<tr>
<td>Randall and Selitto (g)</td>
<td>152 ± 4.2</td>
<td>70.5 ± 4.8</td>
<td>67.5 ± 5.7</td>
<td>65.7 ± 4.2</td>
<td>76.2 ± 4.9</td>
</tr>
<tr>
<td>Von Frey (AUC)</td>
<td>3399 ± 58</td>
<td>3527 ± 51</td>
<td>3582 ± 23</td>
<td>3612 ± 17</td>
<td>3481 ± 30</td>
</tr>
</tbody>
</table>

Results are shown as mean values of 6-8 animals ± SEM. For each test, the different letters (a, b, c) indicate significant differences between times of evaluation (basal, 4h, 4, 7 and 14 d), while the same letter indicates that the values were analogous ($p < 0.05$; one way ANOVA followed by Student-Newman-Keuls test).
Table 2.- Baseline behavioural values in mice implanted with a placebo or a morphine pellet, with and without CFA-inflammation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>NO INFLAMMATION</th>
<th>CFA-INFLAMMATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellet</td>
<td>Placebo</td>
<td>Morphine</td>
</tr>
<tr>
<td>Plantar test (s)</td>
<td>7.28 ± 0.14</td>
<td>8.55 ± 0.19</td>
</tr>
<tr>
<td>Randall-Selitto (g)</td>
<td>157.5 ± 5.7</td>
<td>162.5 ± 4.9</td>
</tr>
<tr>
<td>Von Frey (AUC)</td>
<td>3449 ± 25</td>
<td>3325 ± 70</td>
</tr>
<tr>
<td>Evan’s blue (AU/g)</td>
<td>1.09 ± 0.07</td>
<td>1.21 ± 0.06</td>
</tr>
</tbody>
</table>

Results are shown as mean values ± SEM of 6-8 animals per experimental condition. Experiments were performed 7 days after CFA and 3 days after pellet implantation. For each test, different letters (a, b, c) indicate significant differences between groups, while 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pellet</th>
<th>Day 0: CFA injection</th>
<th>Day 4: Pellet implantation</th>
<th>Day 7: Behavioral testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>Placebo</td>
<td>27.6 ± 0.4 a</td>
<td>29.1 ± 0.4 a</td>
<td>30.3 ± 0.5 a</td>
</tr>
<tr>
<td></td>
<td>Morphine</td>
<td>27.7 ± 0.4 a</td>
<td>29.4 ± 0.3 a</td>
<td>26.7 ± 0.5 b</td>
</tr>
<tr>
<td>Temperature (ºC)</td>
<td>Placebo</td>
<td>37.2 ± 0.2 a</td>
<td>37.4 ± 0.1 a</td>
<td>37.3 ± 0.1 a</td>
</tr>
<tr>
<td></td>
<td>Morphine</td>
<td>37.3 ± 0.1 a</td>
<td>37.3 ± 0.2 a</td>
<td>36.6 ± 0.2 b</td>
</tr>
</tbody>
</table>

Results are expressed as mean values ± SEM of 6-8 mice. Both parameters were evaluated in the same animals. For each parameter and time of evaluation, different letters (a, b) indicate significant differences between animals implanted with placebo or morphine pellets, while the same letter indicates that the values were analogous (p < 0.05; one way ANOVA followed by Student’s-Newman-Keuls test).
Table 4.- ED₅₀’s and Emax values of s.c. morphine (mg/kg) in the different experimental conditions.

<table>
<thead>
<tr>
<th>TEST</th>
<th>CTL</th>
<th>CFA</th>
<th>MP</th>
<th>CFA + MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randall-Sellito</td>
<td>ED₅₀</td>
<td>12.8 ± 0.2</td>
<td>6.3 ± 0.3 b</td>
<td>34.5 ± 0.6 c</td>
</tr>
<tr>
<td></td>
<td>Emax</td>
<td>51.7 ± 5.1</td>
<td>66.3 ± 2.8 b</td>
<td>33.1 ± 5.1 c</td>
</tr>
<tr>
<td>Plantar</td>
<td>ED₅₀</td>
<td>4.9 ± 0.2 a</td>
<td>2.7 ± 0.1 b</td>
<td>21.5 ± 0.4 c</td>
</tr>
<tr>
<td></td>
<td>Emax</td>
<td>22.1 ± 3.1 a</td>
<td>40.2 ± 1.7 b</td>
<td>29.7 ± 4.4 a</td>
</tr>
<tr>
<td>Von Frey</td>
<td>ED₅₀</td>
<td>4.9 ± 0.1 a</td>
<td>5.1 ± 0.2 a</td>
<td>no effect</td>
</tr>
<tr>
<td></td>
<td>Emax</td>
<td>25.8 ± 2.7 a</td>
<td>22.8 ± 1.8 a</td>
<td>5.5 ± 1.9 b</td>
</tr>
<tr>
<td>Extravasation</td>
<td>ED₅₀</td>
<td>-</td>
<td>2.5 ± 0.1 a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Emax</td>
<td>-</td>
<td>47.6 ± 4.3 a</td>
<td>-</td>
</tr>
</tbody>
</table>

Results (ED₅₀ and Emax values) are expressed as mean values ± SEM. CTL: control without inflammation; CFA: inflammation; MP: morphine pellet. For each test and parameter (ED₅₀, Emax), different letters (a, b, c) indicate statistically significant differences between treatment groups, while 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).
Table 5.- Changes in the potency of morphine in the different tests and experimental conditions.

<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>1</td>
<td>2.03 (↓)</td>
<td>2.69 (↑)</td>
<td>2.58 (↑)</td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>-</td>
<td>1</td>
<td>5.48 (↑)</td>
<td>5.25 (↑)</td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.04</td>
</tr>
<tr>
<td>Plantar test</td>
<td>CTL</td>
<td>1</td>
<td>1.81 (↓)</td>
<td>4.38 (↑)</td>
<td>4.04 (↑)</td>
</tr>
<tr>
<td></td>
<td>CFA</td>
<td>-</td>
<td>1</td>
<td>7.96 (↑)</td>
<td>7.33 (↑)</td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.08</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>-</td>
<td>1</td>
<td>-</td>
<td>4.24 (↑)</td>
</tr>
</tbody>
</table>

The table shows the ratios of the ED$_{50}$'s of morphine derived from the dose-response curves, in the different experimental conditions (Figure 1 and 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 6.- Inhibitory effects of the ED$_{50}$’s of morphine in control conditions (saline), and after the s.c. administration of naloxone (0.1 mg/kg) or naloxone methiodide (0.3 mg/kg).

<table>
<thead>
<tr>
<th>TEST</th>
<th>Plantar test</th>
<th>Randall-Selitto</th>
<th>Von Frey</th>
<th>Extra-vasation</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 ± 2.9$^a$</td>
<td>100 ± 5.7$^a$</td>
<td>100 ± 2.7$^a$</td>
<td>100 ± 3.0$^a$</td>
</tr>
<tr>
<td>% effect morphine + naloxone</td>
<td>0.0 ± 2.8$^b$</td>
<td>3.4 ± 1.9$^b$</td>
<td>28.2 ± 3.6$^b$</td>
<td>9.0 ± 2.1$^b$</td>
</tr>
<tr>
<td>% effect morphine + naloxone methiodide</td>
<td>70.5 ± 3.1$^a$</td>
<td>38.8 ± 2.5$^c$</td>
<td>31.2 ± 3.8$^b$</td>
<td>35.7 ± 2.0$^c$</td>
</tr>
</tbody>
</table>

In these experiments we tested the ED$_{50}$’s of morphine obtained in the different experimental conditions (Table 4). Non-CFA injected animals were used as control. Since the % 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$_{50}$’s), and considered to induce a 100% effect. For each test and experimental condition (CTL, CFA) the results are shown as the mean values of 6-8 animals ± SEM. Different letters (a, b, c) 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).
Figure 4

A) DRG

Anti-MOR

70 Kda

Anti-β-actin

42 Kda

CTL  CFA  MP  CFA + MP

B) Paw

Anti-MOR

70 Kda

Anti-β-actin

42 Kda

CTL  CFA  MP  CFA + MP