Tolerance to the antinociceptive effects of peripherally administered opioids. Expression of β-arrestins

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Tolerance to peripheral antinociception after chronic exposure to systemic morphine was assessed in mice with chronic CFA-inflammation; cross-tolerance to locally administered μ, δ and κ-opioid agonists and levels of β-arrestins in the injured paw, were also evaluated. Tolerance was induced by the subcutaneous implantation of a 75 mg morphine-pellet, and antinociception evaluated with the Randall–Selitto test, 5 min after the subplantar injection of morphine, fentanyl, buprenorphine, DPDPE, U-50488H or CRF. Experiments were performed in the absence and presence of CFA-inflammation, in animals implanted with a morphine or placebo pellet. Beta-arrestin protein levels were determined by western blot. In mice without inflammation, subplantar opioids did not induce antinociception, while during CFA-inflammation, all drugs generated dose–response curves with an order of potency of: U-50488H < DPDPE < morphine < buprenorphine < fentanyl < CRF. During CFA-inflammation plus morphine-pellet, the potency of fentanyl decreased 1.25 times, while that of DPDPE, U-50488H and CRF diminished approximately 2.5–4.3 times. For each drug, the ratio between the ED50's in tolerant and naive animals, was significantly higher than 1 (except for buprenorphine and fentanyl), demonstrating partial cross-tolerance to systemic morphine. Inflammation induced a twofold increase in β-arrestin expression (p < 0.01), and the levels decreased after acute morphine exposure (p < 0.05). Tolerance did not alter β-arrestins, but partially prevented the increase induced by inflammation. The results suggest that peripheral β-arrestins could facilitate peripheral OR-desensitization and tolerance development. Clinically, the experiments could be useful to establish the effectiveness of local opioid administration in patients with musculoskeletal pain, chronically receiving morphine analgesia.

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Abbreviations: OR, opioid receptors; CFA, complete Freund’s adjuvant; PL, placebo pellet; MP, morphine pellet; MPE, maximal possible effect; ANOVA, analysis of variance; CRF, corticotrophin releasing factor; DPDPE, ([D-Pen(2),D-Pen(5)]-enkephalin hydrate; U-50488H, (trans-a)-3,4-dichloro-N-methyl-N-[2-1-pyrrolidinyl] cyclohexyl) benzeneacacetamide hydrochloride
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1. Introduction

Systemic opioids induce antinociception by binding to opioid receptors (OR) located at spinal, supra-spinal and peripheral sites (Pasternak, 2001). Opioids also induce multiple adverse effects, and the likelihood of obtaining effective analgesia by solely activating peripheral μ, δ and κ-OR (MOR, DOR and KOR, respectively) after the local administration of agonists, is still under active investigation. A major limitation to opioid analgesia is the development of tolerance, requiring continuous dose-escalation for effective analgesia, given that high doses of opioids often induce unacceptable adverse effects. The clinical observation that patients chronically treated with systemic morphine respond with less efficacy to therapeutic (low) doses of other μ-agonists, suggests partial cross-tolerance, and opioid switching is commonly used in the management of pain in cancer patients (Mercadante and Bruera, 2006). Cross-tolerance to the peripheral effects of opioids has been scarcely investigated.

Tolerance to the central effects of morphine is well documented, but controversy still exists regarding tolerance at peripheral OR (Fernández-Dueñas et al., 2007; Zöllner et al., 2008). Due the low density of OR in normal skin, the local injection of agonists does not induce antinociception (Stein et al., 1989). However during inflammation, peripheral OR are sensitized/up-regulated, and local or systemic administration of opioids induces peripheral analgesia (Stein et al., 2001). Immune cells at the inflamed site also express OR and endogenous opioids, physiologically contributing to peripheral antinociception (Janson and Stein, 2003). During CFA-inflammation in mice, we have recently demonstrated that chronic exposure to morphine induces tolerance to the anti-hyperalgesic effects of acute morphine administration. Moreover, during paw inflammation, the systemic administration of the peripherally acting opioid-antagonist naloxone-methiodide reversed approximately 64% of the effects of morphine on mechanical hyperalgesia (Fernández-Dueñas et al., 2007). These findings suggest that tolerance develops to the peripheral effects of opioids; however a definite demonstration of peripheral opioid tolerance was not possible in our previous study.

The mechanisms involved in opioid-tolerance remain controversial. Chronic exposure to agonists induces a reduction in effector response (tolerance), attributed to receptor downregulation, desensitization or changes in gene expression (Martini and Whistler, 2007). Receptor phosphorylation, uncoupling with G-proteins and internalization have been implicated. After internalization, receptors can be degraded or recycled (re-sensitized), and internalization could be a way to reduce tolerance (Von Zastrow et al., 2003). Numerous studies suggest that β-arrestins 1 and 2 have a role in OR desensitization and internalization. Results obtained in mutant mice to β-arristin 2 exhibited increased sensitivity to the acute anti-nociceptive effects of morphine (Bohn et al., 1999) as well as decreased development of anti-nociceptive tolerance following prolonged administration of morphine (Bohn et al., 2000). These results suggest that arrestin-mediated desensitization (and presumably endocytosis) of OR is induced by morphine in vivo. However, the effects of chronic morphine on β-arrestins activity remain controversial: while some studies suggest that morphine induces desensitization/internalization via β-arrestin 2 recruitment (Zuo, 2005), others propose that a compensatory increase in the activity of adenyl cyclase isoenzymes in response to chronic opioid receptor stimulation (Nevo et al., 1998) or glial activation (Song and Zhao, 2001; Narita et al., 2006), are factors that may contribute to cellular opioid tolerance. The role of β-arrestins in peripheral tissues during chronic inflammatory pain has not been investigated.

The aims of our study were to: 1) assess the development of tolerance to peripheral antinociception after chronic exposure to systemic morphine, in a model of CFA-induced inflammation; 2) test cross-tolerance to locally administered MOR, DOR and KOR agonists and 3) determine the levels of β-arrestin 1 and 2 in the injured paw, in an attempt to detect changes that may correlate with the behavioral development of peripheral antinociceptive tolerance. These experiments could be useful to establish the effectiveness of local opioid administration in patients chronically treated with morphine, and to describe the role of peripheral β-arrestins in morphine tolerance.

2. Results

2.1. Antinociceptive effects of subplantar opioids. Cross tolerance to morphine

In the absence of inflammation (PL and MP groups), none of the drugs induced antinociception when injected into the paw. However, during CFA-inflammation (CFA+PL and CFA+MP groups), dose–response curves could be generated when testing antinociception in the inflamed paw (Figs. 1 and 2). High doses of the drugs were not utilized to avoid systemic effects.

Table 1 shows the calculated ED50’s obtained during CFA inflammation, in mice implanted with a placebo (CFA+PL) or morphine pellet (CFA+MP). The table also displays the estimated Emax values obtained from the dose–response relationships. In naïve animals (CFA+PL) the Emax for morphine, buprenorphine, DPDP, U-50488H and CRF attained values of 48 to 64%, while for fentanyl the Emax value was 88%. Estimated Emax values were approximately between 39 and 50% in CFA+MP animals.

In the CFA+PL group (Table 1), the ratio between the ED50 of subplantar morphine and the ED50 of each one of the study drugs, indicates their relative potency. CRF showed the highest relative potency, followed by fentanyl, buprenorphine, DPDP and U-50488H (p<0.05 when compared to morphine).

In the CFA+MP group, morphine administered at a dose range of 50–100 μg did not generate a dose response curve; higher doses of morphine were not tested to avoid systemic effects. Dose–response curves to all other opioids, except to buprenorphine, were shifted to the right (Figs. 1 and 2), and the ED50’s significantly increased (p<0.01, Student’s t-test) demonstrating peripheral tolerance. For each drug, the ratio between the ED50 in mice implanted with a morphine pellet
(CFA+MP) and the ED\textsubscript{50} with placebo (CFA+PL) pellet, indicates the extent of cross-tolerance to morphine (ratio >1). At the doses tested (1–5 μg) subplantar buprenorphine did not induce systemic withdrawal symptoms, and its calculated ED\textsubscript{50} remained unaltered in morphine-tolerant mice (CFA+MP group). In the same experimental conditions, the potency of fentanyl decreased 1.25 times, while the potencies of DPDPE, U-50488H and CRF decreased approximately 2.5–4.3 times (Table 1).

These results show that inflammation is required to attain opioid-induced peripheral antinociception, and that morphine tolerance develops to its peripheral effects.

2.2. Evaluation of the effects of opioids after systemic administration

To rule out systemic effects of the opioids after subplantar administration, we tested nociceptive thresholds in CFA+PL animals, 5 min after the subcutaneous (s.c.) administration of the highest doses of each drug used in the previous experiments (Figs. 1 and 2). The doses used were: morphine 100 μg (corresponding to 4 mg/kg s.c.); fentanyl 1.4 μg (0.056 mg/kg s.c.); buprenorphine 7 μg (0.28 mg/kg s.c.); DPDPE 300 μg (12 mg/kg s.c.); U-50488H 300 μg (12 mg/kg s.c.) and CRF 7 ng (0.28 μg/kg s.c.). In these experimental conditions, we did not observe an effect with any of the study drugs in any of the hindpaws (data not shown). The results suggest that 5 min after subcutaneous administration, systemic absorption

Fig. 1 – Antinociceptive dose–response relationships to subplantar morphine, fentanyl or buprenorphine, in the different experimental conditions. Experiments were performed in naïve (squares) and morphine-pellet implanted (stars) mice, in the presence of inflammation (empty triangles) or inflammation plus morphine-pellet (filled triangles). PL, placebo pellet without inflammation (control); CFA: inflammation; MP: morphine pellet. Each point represents the mean value of 6–8 animals and the vertical bars indicate the S.E.M. % MPE, percent of maximal possible effect.

Fig. 2 – Antinociceptive dose–response relationships to subplantar DPDPE, U-50488H or CRF, in the different experimental conditions. Experiments were performed in naïve (squares) and morphine-pellet implanted (stars) mice, in the presence of inflammation (empty triangles) or inflammation plus morphine-pellet (filled triangles). PL, placebo pellet without inflammation (control); CFA: inflammation; MP: morphine pellet. Each point represents the mean value of 6–8 animals and the vertical bars indicate the S.E.M. % MPE, percent of maximal possible effect.
Table 1 - Antinociceptive potency of the different drugs (ED$_{50}$’s in μg) during CFA-induced inflammation, in placebo (PL) and morphine pellet-implanted (MP) animals

<table>
<thead>
<tr>
<th>Drugs</th>
<th>ED$_{50}$ (μg) CFA+PL</th>
<th>ED$_{50}$ (μg) CFA+MP</th>
<th>Ratio CFA+MP/ED$_{50}$ CFA+PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine, MS</td>
<td>16.3±2.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(E$_{max}$ %)</td>
<td>(64.55±0.7)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ratio MS/MS</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fentanyl, FE</td>
<td>0.2±0.02</td>
<td>0.25±0.01</td>
<td>1.25</td>
</tr>
<tr>
<td>(E$_{max}$ %)</td>
<td>(87.8±5.3)</td>
<td>(47.7±1.5)</td>
<td></td>
</tr>
<tr>
<td>Ratio MS/PE</td>
<td>81.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Buprenorphine, BP</td>
<td>1.1±0.1</td>
<td>1.08±0.07</td>
<td>1</td>
</tr>
<tr>
<td>(E$_{max}$ %)</td>
<td>(51.9±3.1)</td>
<td>(39.3±2.2)</td>
<td></td>
</tr>
<tr>
<td>Ratio MS/BP</td>
<td>14.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DPDPE, DP</td>
<td>28.1±1.0</td>
<td>121.1±3.2**</td>
<td>4.3</td>
</tr>
<tr>
<td>(E$_{max}$ %)</td>
<td>(54.3±1.4)</td>
<td>(50.9±1.6)</td>
<td></td>
</tr>
<tr>
<td>Ratio MS/DP</td>
<td>0.58</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>U-50488H, U50</td>
<td>39.9±9.0</td>
<td>115.0±11.72**</td>
<td>2.9</td>
</tr>
<tr>
<td>(E$_{max}$ %)</td>
<td>(48.56±4.4)</td>
<td>(46.32±7.67)</td>
<td></td>
</tr>
<tr>
<td>Ratio MS/U50</td>
<td>0.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CRF</td>
<td>0.0008±0.042</td>
<td>0.002±0.02</td>
<td>2.5</td>
</tr>
<tr>
<td>(E$_{max}$ %)</td>
<td>(60.0±2.1)</td>
<td>(40.1±4.5)</td>
<td></td>
</tr>
<tr>
<td>Ratio MS/CRF</td>
<td>20375</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

In the CFA+PL group, the potency of morphine (MS) is significantly lower than fentanyl, buprenorphine and CRF, while it is higher than DPDPE and U-50488H (p<0.05, one way ANOVA). The ratio between the ED$_{50}$’s of morphine (MS) and the ED$_{50}$ of each treatment, indicates their relative potencies, while the ratio between the ED$_{50}$’s with (MP) and without (PL) morphine pellet indicates the extent of cross-tolerance to morphine (ratio>1). For each drug, * indicates p<0.01, when comparing the ED$_{50}$’s from the CFA+PL and CFA+MP groups (Student’s t-test).

Table 2 – Expression of β-arrestin 1 and 2 in the inflamed paw, after the subcutaneous injection of morphine or saline (SS)

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>β-arrestin 1</th>
<th>β-arrestin 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>100±6.0a</td>
<td>100±4.7a</td>
</tr>
<tr>
<td>Morphone</td>
<td>100±4.8a</td>
<td>100±4.5a</td>
</tr>
<tr>
<td>CFA+PL</td>
<td>226±8.7b*</td>
<td>176±7.8b*</td>
</tr>
<tr>
<td>CFA+MP</td>
<td>152±6.3c</td>
<td>138±5.7c</td>
</tr>
</tbody>
</table>

Results are expressed as mean values±SEM of % optical density (OD) versus control.

For each experimental condition, different letters (a,b,c) indicate significant differences (p<0.05, Student’s t-test) between groups (PL, MP, CFA+PL and CFA+MP). The * indicate a p<0.05 when comparing arrestin levels after a challenge with SS or morphine. No significant differences were observed between PL and MP. PL: placebo pellet (control); MP, morphine pellet; CFA+PL, inflammation induced by Complete Freund’s Adjuvant. We used 4 animals per experimental condition and each experiment was repeated 4–5 times.

2.3. Expression of β-arrestin 1 and 2 in the tissue of the paw in the different experimental conditions

Levels of β-arrestin 1 and 2 were assessed in all the study groups, 60 min after a challenge with subcutaneous saline (SS) or morphine (MS); β-arrestins were determined by western blot. In Fig. 3 we show immunoblots of β-arrestin 1 and 2 after a morphine challenge (MS), in the different experimental conditions. CFA inflammation (CFA+PL) induced over a twofold increase in β-arrestin 1/2 expression (p<0.001, Table 2, Fig. 3), that was slightly but significantly decreased (approximately by 20%) after a challenge with subcutaneous morphine (p<0.05). Chronic exposure to morphine (MP) did not alter β-arrestin 1 or 2 expression when compared to naive animals (PL), and a morphine challenge did not induce any changes (Table 2). In the presence of inflammation plus chronic morphine (CFA+MP) β-arrestin expression increased approximately 1.5 and 1.3 times for β-arrestin 1 and 2, respectively, when compared to MP or PL and a morphine challenge did not induce additional changes, suggesting that morphine tolerance (MP) partially prevented the increase in β-arrestin induced by CFA.

These results show that inflammation, but not tolerance, significantly increases β-arrestin 1 and 2 expression, and that the increase is of a lesser magnitude in animals receiving acute or chronic (MP) morphine.

![Fig. 3 - Expression of β-arrestin 1 and 2 in the soft tissue of the paw, in the different experimental conditions. β-arrestins 1 and 2 were determined by western blot, in naive (PL), morphine-pellet implanted (MP), in the presence of inflammation (CFA+PL), or inflammation plus morphine-pellet (CFA+MP) mice. PL: placebo pellet without inflammation (control); MP: morphine pellet; CFA: inflammation. For each condition, animals were randomly assigned to one group that received a subcutaneous injection of morphine (MS, challenge). In each lane, total protein pooled from four animals (paw) was used. β-actin was used as a loading control.](image)
3. Discussion

The present investigation shows that during inflammation, intense tolerance to morphine develops at peripheral OR, and partial cross-tolerance occurs to several peripherally administered opioids.

Opioids induce peripheral antinociception in different models of inflammatory pain (Stein, 1993; Zhang et al., 1998a; Fernández-Dueñas et al., 2007), but the mechanisms are not fully understood, although sensitization/up-regulation of peripheral OR seem to be involved (Stein et al., 2001). Our results support previous studies demonstrating that peripheral opioid-analgesia is attained only in the presence of inflammation (Janson and Stein, 2003). In our model, all opioids tested induced antinociception when injected in the inflamed but not in the contralateral paw, suggesting the sensitization of peripheral MOR, DOR, and KOR. The relative potencies of the different opioids show that peripherally injected DOR and KOR agonists have a similar potency than morphine, while the potencies of CRF, fentanyl and buprenorphine are considerably higher. In our study, CRF had the highest potency; this drug induces analgesia by releasing endogenous opioids from immune cells recruited during inflammation, that subsequently bind to OR on sensory terminals (Schäfer et al., 1994).

The experiments show that endogenously released opioids are more potent than synthetic drugs inducing local antinociception in mice, and that there is cross-tolerance between morphine and endogenous opioid peptides.

During inflammation (CFA+PL group), the \( E_{\text{max}} \) values derived from the dose-response curves indicate that fentanyl behaves as a full opioid-agonist and has a higher efficacy (\( E_{\text{max}} \)) than morphine, buprenorphine, DPDPE, U-50488H or CRF on peripheral OR. Even if the limitations of the route of administration precluded the use of higher doses of opioids, all curves were constructed from at least four points, and thus we think that the \( E_{\text{max}} \) and ED\(_{50}\)’s values obtained during inflammation (CFA+PL groups) are reasonably accurate. Furthermore, the curves clearly allow the distinction between the effects of the different drugs in the inflamed paw of naive (CFA+PL) and tolerant animals (CFA+MP).

The present results demonstrate that chronic exposure to morphine also induces tolerance to the peripheral antinociceptive effects of morphine. In a previous paper from our group, we showed development of systemic tolerance after the subcutaneous implantation of a 75 mg morphine pellet (Fernández-Dueñas et al., 2007) during CFA-inflammation; in these experimental conditions, the ED\(_{50}\) of acute subcutaneous morphine decreased approximately 5 times, demonstrating tolerance to morphine-induced antinociception. In the present report, we have used the same model and nociceptive test, in order to evaluate tolerance to antinociception after the subplantar administration of MOR, DOR, and KOR agonists. Our results show that in animals with inflammation implanted with a morphine pellet (CFA+MP), the local administration of morphine (dose range 50–100 \( \mu \)g), did not induce antinociception, illustrating the development of intense tolerance; higher doses were not tested to avoid systemic effects. However, Zöllner et al (2008) were unable to observe acute peripheral tolerance in the rat. The discrepancy seems to be related to the different experimental conditions: In our study, we attempted to closely reproduce in mice, the clinical situation where patients with chronic musculoskeletal pain (replicated in our experiments by CFA-induced chronic inflammation) receive chronic opioids for analgesia (morphine pellet implantation in mice). Thus, in our model, animals had a well established/characterized chronic inflammation before starting the treatment with opioids. In Zöllner’s paper, morphine administration and CFA inflammation were initiated simultaneously, a fact that would mimic a clinical situation where an acute inflammatory injury (acute CFA administration in the rat) is concomitantly treated with high doses of morphine. The different experimental conditions could explain the disparity in the results. Moreover, when morphine and CFA are injected simultaneously, it is possible that the anti-inflammatory effects of morphine (Romero et al., 2005) would modify the progression of acute to chronic inflammation, and thus alter the signaling pathways conducting to the development of chronic opioid-tolerance.

To assess peripheral cross-tolerance to morphine, we obtained dose–response curves to the various opioids in the different experimental conditions (PL, MP, CFA+PL and CFA+MP). Since we tested the same doses of opioids in naive (PL) and tolerant animals (MP), the curves obtained in the latter could be considered incomplete, and the efficacy \( E_{\text{max}} \) of the different opioids in these groups (CFA+MP) is only approximate. Thus, from the present experiments a decreased efficacy of s.p. opioids in tolerant animals cannot be excluded. All dose–response curves were shifted to the right showing partial cross-tolerance to systemic morphine. For each drug, we calculated the ratio between the ED\(_{50}\)’s obtained in tolerant and naive animals, attempting to roughly quantify the degree of cross-tolerance. Even if these ratios have no absolute value, they suggest a lesser degree of morphine cross-tolerance with buprenorphine or fentanyl, than with DPDPE, U-50488H or CRF. These findings agree with the clinical observation that patients tolerant to morphine respond to fentanyl analgesia (Mercadante and Bruera, 2006). The mechanisms involved in cross-tolerance could be related to receptor subtypes or splice variants of the MOR (Pasternak, 2005), together with changes in the transduction mechanisms induced by the different OR agonists.

In many systems, decreased responsiveness to opioid agonists has been correlated to receptor desensitization (Marie et al., 2006). Studies in vitro show that desensitization results from a series of events including receptor phosphorylation/uncoupling from G-proteins, and receptor internalization, a process involving G-protein receptor kinases (GRKs) and \( \beta \)-arrestins. Morphine has been reported to be less efficient than other opioid agonists inducing receptor phosphorylation, \( \beta \)-arrestin recruitment and MOR internalization (Cheng et al., 1998; Whistler and von Zastrow, 1998; Zhang et al., 1998b; Lowe et al., 2002; Bohn et al., 2004). After opioid administration, receptor desensitization can induce compensatory mechanisms that results in the activation or inhibition of intracellular pathways (Reiter and Lefkowitz, 2006; De Wiere et al., 2007; Premont and Gainetdinov, 2007). The involvement of \( \beta \)-arrestins in the development of morphine tolerance in vivo has been investigated in knockout
mice for β-arrestin 2, where tolerance was decreased and/or delayed in thermal nociceptive assays (Bohn et al., 1999, 2000, 2002). In our study, the acute and/or chronic morphine administration to control animals (no inflammation, PL or MP groups) did not alter β-arrestin expression. Several in vivo studies have assessed β-arrestin levels after acute or chronic exposure to morphine, with contradictory results. A small increase in β-arrestin 2 in the locus caeruleus (Terwilliger et al., 1994), but not in other regions of the brain, has been reported in morphine-tolerant rats. However, using in-situ hybridization, Fan et al. (2003) observed a significant decrease in β-arrestin mRNA in several areas of the brain (including the locus caeruleus), after morphine administration. Moreover, Narita et al. (2006) have shown that chronic exposure to morphine induces antinociceptive tolerance without modifying β-arrestin levels in the spinal cord, a finding that agrees with our present results. In the same paper, Narita et al. observed an increase of glial fibrillary acidic protein (GFAP) expression, suggesting that morphine tolerance may be unrelated to β-arrestin expression in the spinal cord.

During inflammation (CFA + PL group), a two-fold increase of β-arrestins in the injured paw could be demonstrated, while after an acute morphine challenge, we observed a decrease in β-arrestin levels in the injured tissue. Although neural and non-neural β-arrestin expression could not be discriminated in the present study, the substantial increase observed in the inflamed tissue would suggest a relevant role of β-arrestins originated from inflammatory cells. It has been suggested that β-arrestins could act as signaling scaffolds in chemotaxis, antiapoptotic signaling, cell proliferation, and increased gene expression during inflammation (Lefkowitz and Shenoy, 2005; Fan et al., 2007). In fact, GRKs and β-arrestins seem to play an important role in inflammatory processes, mainly on the regulation of chemokine responsiveness (Barlic et al., 2000; Su et al., 2005; Vroon et al., 2006). It could be hypothesized that the increase in β-arrestin 1/2 reported in our study could contribute to the sensitization of peripheral opioid receptors and peripheral antinociception observed during inflammation. However, other studies have reported that knockout mice for β-arrestin 2 exhibit enhanced antinociception (Bohn et al., 1999), while rats overexpressing the same protein in the periaqueductal gray had attenuated morphine antinociception (Jiang et al., 2006). We have not a logical explanation for the discrepancies, other than the possible different function/s of β-arrestins in peripheral injured tissues.

In conclusion, the present data show that during inflammation, tolerance develops to the antinociceptive effects of peripherally administered opioids. Partial cross-tolerance to systemic morphine after the peripherally administered DOR and KOR, but not MOR agonists, was also demonstrated. The increase in β-arrestins 1/2 observed in the injured paw during inflammation, could facilitate OR internalization/recycling explaining the enhanced response to opioids observed during inflammation. This increase however, was partially blocked by acute and chronic exposure to morphine suggesting that in the presence of inflammation, the morphine-mediated decrease in peripheral β-arrestin levels could partially impair the internalization/recycling of peripheral desensitized OR, contributing the development of tolerance.

### 4. Experimental procedures

#### 4.1. Animals

Male Swiss CD1 mice weighing 25–30 g used for all experiments, were purchased from Charles River Laboratories (France). The 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. Mice were housed under a 12 h dark/12 h light cycle in a room with controlled temperature (22 °C) and relative humidity (60%). 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 conducted between 9.00 AM and 5.00 PM. During the study, animal welfare (feeding, posture, grooming, motor activity) was verified daily. Mice were used only once, and were sacrificed immediately after the experiments by cervical dislocation.

#### 4.2. Paw inflammation

We used the model previously reported by our group (Fernández-Dueñas et al., 2007). In brief, under halothane anesthesia, mice received a single intra-plantar injection of 30 mg of Complete Freund’s Adjuvant (CFA: 0.1% Mycobacterium tuberculosis) in 30 μl of saline in the right hindpaw, according to the method of Larson et al. (1986). Animals develop a local inflammatory reaction that remains confined to the injected paw and persists for 14 days; in the present investigation, experiments were performed 7 days after CFA. Intra-plantar saline was not injected to control animals, since we have shown that induces a slight but significant inflammatory reaction (Franas et al., 1995).

#### 4.3. Morphine-pellet implanted mice

Morphine tolerance was induced by the subcutaneous implantation of a 75-mg morphine pellet, while control animals received an inert placebo pellet. Under halothane anesthesia, a small skin pocket was dissected in the animal’s back where the pellet was inserted; afterwards the skin was closed with surgical sutures. All the experiments were performed three days after the pellet. During inflammation, pellets were implanted four days after CFA, and experiments performed on day 7. The 75 mg the morphine pellet induces high plasma levels of morphine during the first three days after implantation (7–8 μg/ml), and development of tolerance to the antinociceptive, anti-extravasation (Fernández-Dueñas et al., 2007) and anti-intestinal transit effects of morphine (Pol and Puig, 1997).

#### 4.4. Behavioral testing

Mechanical nociceptive thresholds were evaluated in the Randall–Selit test using an Analgesy-Meter (Ugo Basile, Comerio, Italy), as previously described (Fernández-Dueñas et al., 2007). Mice were gently held, and incremental pressure (cut-off 250 g) was applied to the dorsal surface of the hindpaw. The pressure required to elicit paw withdrawal or
paw-pressure threshold (PPT) was then determined. The mean of three consecutive measurements separated by a period of 1 min was used. Experimental drugs were always administered in the subplantar tissue of the right hindpaw (30 μl, s.p.) or subcutaneously (250 μl, s.c.) at the nape of the neck; in all instances antinociception was assessed 5 min afterwards. For subplantar administration, animals were briefly restrained in a polyethylene holder that permitted access to the hindpaws. Baseline paw withdrawal thresholds were obtained immediately before the administration of the drugs and the mean baseline values were: PL (placebo pellet, control)=153±0.69 g; MP (morphine pellet)=144±2.56 g; CFA+PL=63.29±2.55 g and CFA+MP=70.97±1.84 g. Baseline thresholds in the PL and MP groups, and those in the CFA and CFA+MP groups were not significantly different; this allowed the comparison of the results in the inflamed (CFA+PL versus CFA+MP) and non-inflamed (PL versus MP) groups.

Drug-induced antinociception is expressed as the percentage of maximal possible effect (% MPE), calculated according to:

\[
\%\text{MPE} = \frac{\text{drug} - \text{baseline}}{\text{cut-off} - \text{baseline}} \times 100
\]

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

4.5. Western blotting

After sacrifice, the soft tissue of the paw was carefully dissected and frozen in liquid nitrogen. Tissues were homogenized in phosphate buffered saline (PBS), 2% sodium dodecylsulfate (SDS) and protease and phosphatase inhibitors (Boehringer Mannhein, Germany). Total protein concentration was determined by spectrophotometry using the bichinchoninic acid method. For the western analysis, protein samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresed (100–150 V for three and a half hours), and transferred to polyvinylidene difluoride (PVDF) filters (Millipore Corp., Bedford, MA) (100 V for 2 h), using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad, Hercules, CA). Parallel gels were stained with Comassie Blue to verify loading, sample integrity, and protein separation. Similar transfer was ascertained by cutting the lower portion of the blot and staining for total protein with Amido Black.

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\]

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

4.6. Statistical evaluation

Data are expressed as group means±S.E.M. The ED_{50} values were determined by linear regression analysis of the dose–response curves [GraphPad Prism 4 software]. In the present study the ED_{50} is defined as the dose that produces a 50% of the maximal effect (E_{max}) obtained from the double reciprocal plot. The ratio between the ED_{50} of morphine and that of the other drugs was used to establish the relative potencies. For each drug, the extent of cross-tolerance to morphine was estimated by the ratio between the ED_{50}'s obtained in mice implanted with a placebo and a morphine pellet (ratio>1). Statistical analysis for significant differences between two groups was obtained by the Student’s t-test, and significant differences between multiple groups by two-way ANOVA followed by a post-hoc Games-Howell test (SPSS version 11.5; SPSS Inc, Chicago, IL). A p<0.05 was considered statistically significant.

4.7. Groups of experiments

4.7.1. Behavioral experiments

In the first series of experiments, antinociception was assessed 5 min after the subplantar (s.p.) injection of morphine, fentanyl, buprenorphine, DPDPE, U-50488H or corticotrophin releasing factor (CRF). For the sake of simplicity, these drugs will be globally designated as opioids, since the peripheral injection of CRF in the injured tissue, induces the release of endogenous opioids from immune cells (Cabot, 2001). Dose–response relationships were assessed in the following experimental conditions: mice without inflammation implanted with a placebo (control group, PL) or morphine pellet (MP) to induce opioid tolerance. Paw inflammation in animals receiving a placebo (inflammation group, CFA+PL) or morphine pellet (CFA+MP). For the generation of the dose–response curves, we used 6–8 animals per dose, and 3–6 points per curve. The dose range tested for each drug was as follows: morphine (5–100 μg), fentanyl (0.1–1.4 μg), buprenorphine (1–7 μg), DPDPE (20–300 μg), U-50488H (20–300 μg) and CRF (0.5–7 ng). High doses of opioids were not used in order to avoid systemic effects (number of mice used to in these experiments was approximately 420).
In another group of experiments we assessed if the highest doses of opioids used to generate the dose–response curves would increase nociceptive thresholds in the Randall and Selitto test, when administered subcutaneously. These experiments were performed in CFA-induced inflammation (CFA+Pl). Antinociception/anti-hyperalgesia was assessed in both hindpaws, 5 min after injection, at the same time point than after subplantar administration (number of mice used was 36).

To perform the experiments, animals were randomly allocated in cages according to the drugs to be administered. The investigator performing the nociceptive test was blinded to the treatments (number of mice used was 36). The investigator performing the nociceptive test was blinded to the treatments (number of mice used was 36).

Western blot experiments were performed in the four experimental conditions: PL, MP, CFA+Pl and CFA+MP. For each condition, animals were randomly assigned to one of two groups that received in a blinded manner, a subcutaneous injection of saline (SS) or a morphine challenge. We injected 10 mg/kg of morphine in PL and CFA mice, and 30 mg/kg in the MP and CFA+MP groups; the doses of morphine were selected on the basis of previous experiments performed by our group with the same experimental model (Fernández-Dueñas et al., 2007). Sixty min after morphine or saline injection, animals were sacrificed by cervical dislocation, and the soft tissue of the paw dissected for the determination of β-arrestins 1 and 2.

We used 4 animals per experimental condition (we have four experimental conditions, see above) and each experiment was repeated 4–5 times. The investigator performing the western blot assay was blinded to the treatments (number of mice used was approximately 20).

### 4.8. Drugs and chemicals

Complete Freund's Adjuvant (CFA) was purchased from Sigma Aldrich Co, St Louis, USA. Morphine HCl and morphine base (for pellet preparation) were obtained from Alcaliber S.A., (Spain). Fentanyl NaCl was from Kern Pharma (Spain), and buprenorphine from Schering-Plough (Spain). DPDP (δ-Pen (2),δ-Pen(5))-enkephalin hydrate, U-50,488H (trans-α-3,4-dichloro-N-methyl-N-[2-1-pyrrolidinyl] cyclohexyl) benzeneaceta midine hydrochloride, and CRF (corticotrophin releasing factor) were purchased from Sigma-Aldrich (Spain). All drugs were dissolved in 0.9% NaCl. Other reagents were from: protease inhibitor (Boehringer Mannheim, Germany); phosphatase inhibitor Cocktail Set (Calbiochem, Germany); goat serum (Sigma); ABC kits (Vector, Burligame, CA, USA).

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