

Antiexudative Effects of Opioids and Expression of κ - and δ -Opioid Receptors during Intestinal Inflammation in Mice: Involvement of Nitric Oxide

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Received July 4, 2005; accepted September 15, 2005

ABSTRACT

The study evaluates the effects of κ - (KOR), δ - (DOR), and μ -opioid receptor (MOR) agonists on the inhibition of plasma extravasation during acute and chronic intestinal inflammation in mice. The antiexudative effects of KOR and DOR agonists in animals treated with nitric oxide synthase (NOS) inhibitors and their protein levels in the gut (whole jejunum and mucosa) and spinal cord of mice with chronic intestinal inflammation were also measured. Inflammation was induced by the intragastric administration of one (acute) or two (chronic) doses of croton oil. Plasma extravasation was measured using Evans blue and protein levels by Western blot and immunoprecipitation. Plasma extravasation was significantly increased 2.7 times during chronic inflammation. The potency of the KOR agonist *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolydiny)cyclohexyl]benzeneazetamine (U50,488H) inhibiting plasma extravasation was enhanced 26.3 times during chronic compared with acute

inflammation. [D-Pen²,D-Pen⁵]-Enkephalin (DPDPE) (a DOR agonist) was also 11.8 times more potent during chronic inflammation, whereas the antiexudative effects of fentanyl (a MOR agonist) were not significantly altered. Receptor-specific antagonists reversed the effects. Protein levels of KOR and DOR in the whole jejunum and mucosa were significantly increased after chronic inflammation. Treatment with NOS inhibitors *N*^ω-nitro-L-arginine methyl ester or L-*N*⁶-(1-iminoethyl)-lysine hydrochloride diminished plasma extravasation and inhibited the increased antiexudative effects of U50,488H and DPDPE during chronic intestinal inflammation. The data show that the enhanced antiexudative effects of KOR and DOR agonists could be related to an increased expression of KOR and DOR in the gut and that the release of nitric oxide may play a role augmenting the effects of opioids during chronic inflammation.

Peripheral inflammation induces the local release of numerous chemical mediators that, among other effects, sensitize the peripheral terminals of primary afferents inducing pain and hyperalgesia; these terminals also release neuropeptides (Richardson and Vasko, 2002) that participate in the local inflammatory response by inducing vasodilatation,

plasma extravasation, and edema (Karimian and Ferrell, 1994; Amann et al., 1995; Siney and Brain, 1996). Opioids reduce plasma extravasation induced by peripheral inflammation by binding to specific opioid receptors located in the central and peripheral nervous systems (Taylor et al., 2000; Romero et al., 2005).

Opioid receptors are found in the central and peripheral nervous system as well as in non-neuronal sites such as the vascular endothelium and immune cells (Mansour et al., 1994; Cadet et al., 2000; Saeed et al., 2000; Tomassini et al., 2003). In the gut, opioid receptors are present in the myenteric and submucosal plexuses as well as in epithelial cells (Lang et al., 1996; Bagnol et al., 1997; Pol et al., 2001) and modulate several intestinal functions such as motility and

This work was supported by grants from Comisión Interministerial de Ciencia y Tecnológica (SAF2003-02578), Fondo de Investigaciones Sanitarias (C03/06 and PI030245) (Madrid, Spain), and Generalitat de Catalunya (2001SRG00409) (Barcelona, Spain). Part of these results was presented as a communication to the 34th International Narcotics Research Conference, Perpignan, France (July 2003).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.105.091991.

ABBREVIATIONS: MOR, μ -opioid receptor(s); DOR, δ -opioid receptor(s); KOR, κ -opioid receptor(s); NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; EB, Evans blue; EU, extravasation unit(s); PBS, phosphate-buffered saline; U50,488H, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolydiny)cyclohexyl]benzeneazetamine; DPDPE, [D-Pen^{2,5}]-enkephalin; nor-BNI, nor-binaltorphimine dihydrochloride; L-NAME, *N*^ω-nitro-L-arginine methyl ester; L-NIL, L-*N*⁶-(1-iminoethyl)-lysine; D-NAME, *N*^ω-nitro-D-arginine methyl ester; ANOVA, analysis of variance.

secretion under certain pathological conditions (Valle et al., 2000; Pol and Puig, 2004). Intestinal inflammation increased the transcription and expression of MOR located in the myenteric plexus and is responsible for the enhanced antitransit effects of MOR agonists in these experimental conditions (Pol et al., 2001). Inflammation of the gut also increased transcription and protein levels of DOR in the myenteric and submucosal plexuses, a fact that could explain the increased effects of DOR agonists on the inhibition of gastrointestinal transit and intestinal permeability (Pol et al., 2003). Although the expression of KOR in the submucosal plexus-mucosa of mice was also enhanced after chronic inflammation (Pol et al., 2003), a definite role for this receptor and its precise location in the mucosal layer have not been established.

Nitric oxide produced by the three nitric-oxide synthase (NOS) isoforms—neuronal NOS, inducible (iNOS), and endothelial (eNOS)—is as a neurotransmitter in the central and peripheral nervous systems. Evidence indicates that nitric oxide is involved in the behavioral and antinociceptive effects of opioids (Nozaki-Taguchi and Yamamoto, 1998; Manzanedo et al., 2004; Tasatargil and Sadan, 2004). Nitric oxide is also implicated in several pathophysiological processes such as inflammation and regulation of gene expression. Thus, we have recently demonstrated that nitric oxide derived from iNOS was implicated in the enhanced antitransit effects of morphine as well as in the enhanced transcription of MOR gene observed during intestinal inflammation (Pol et al., 2005). In contrast, nitric oxide suppressed the transcription of KOR gene in stem cells cultures (Park et al., 2002). No studies have been carried out to evaluate the effects of nitric oxide in the antiexudative effects of KOR and DOR agonists during intestinal inflammation.

In a model of intestinal inflammation induced by croton oil, the aims of the present investigation were to evaluate 1) the effects of specific opioid receptor agonists on plasma extravasation during acute and chronic intestinal inflammation; 2) the contribution of endogenous opioid peptides released during inflammation in plasma extravasation; 3) the role of nitric oxide in the enhanced effects of KOR and DOR agonists during chronic inflammation; and 4) the expression of KOR and DOR in the intestine (whole jejunum and dissected mucosal) and spinal cord of animals with and without chronic intestinal inflammation.

Materials and Methods

Animals. Male Swiss CD-1 mice, weighing 25 to 30 g, were used in all experiments. Mice were housed under 12-h/12-h light/dark conditions in a room with controlled temperature (22°C) and humidity (66%). Animals had free access to food and water and were used after a minimum of 4 days acclimatization to the housing conditions. All experiments were conducted between 9:00 AM and 6:00 PM. The study protocol was approved by the local committee of animal use and care of our Institution, in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Intestinal Inflammation. Two types of intestinal inflammation (acute and chronic) were used in our study. Acute inflammation was induced by the intragastric administration of a single dose (0.05 ml) of croton oil (Sigma-Aldrich, St. Louis, MO) diluted in olive oil (1:1); control animals received the same volume of intragastric saline. Mice in the chronic treatment group were gavaged with a second dose of

croton oil or saline (0.05 ml) 24 h after the first dose (Fig. 1A). In both instances, mice were fasted for 18 h before croton oil or saline administration, except for free access to water, which was available for the duration of the study. In the acute treatment group, plasma extravasation was evaluated 3 h 10 min after croton oil or saline, whereas in the chronic treatment experiments plasma extravasation was measured 96 h 10 min after the first dose of croton oil. These time points were selected based on previous studies from our laboratory, demonstrating that they were the times of maximal epithelial injury in both models of intestinal inflammation (Puig and Pol, 1998).

Plasma Extravasation. The extravasation of plasma proteins to the small intestine was assessed with Evans Blue (EB) dye using a technique adapted from Udaka et al. (1970). At 3 or 96 h after the first dose of croton oil (for the acute and chronic inflammation, respectively), animals were briefly anesthetized with halothane and injected intravenously with 50 mg/kg EB (85 μ l) to allow subsequent quantification of plasma extravasation. For each animal, a sterile insulin syringe coupled to a 0.33×12.7 -mm needle was used. Animals were sacrificed by cervical dislocation 10 min times after EB administration, and the small intestine was carefully removed, washed with saline, and the first 24 cm from the pyloric valve was reserved. EB was extracted by incubation of this fragment in 6 ml of

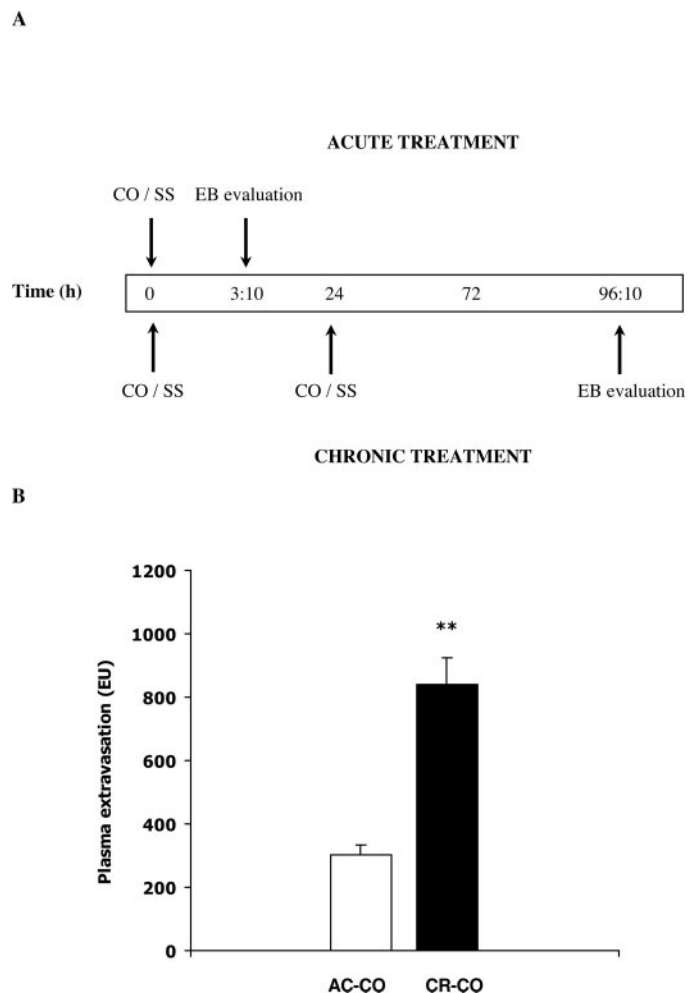


Fig. 1. A, experimental design showing the sequence of croton oil (CO) or saline (SS) administration and the time (h) of evaluation of Evans blue (EB) in acute and chronic treatment groups. B, plasma EU values in animals with acute (AC-CO) or chronic (CR-CO) intestinal inflammation. Each column represented the mean values \pm S.E.M from 10 animals. **, significant differences compared with animals with acute inflammation ($P < 0.01$; Student's t test).

formamide at 60°C for 24 h. The EB extracted was quantified by spectrophotometry (SmartSpec3000; Bio-Rad, Hercules, CA) at 620 nm and expressed in extravasation units (EU). One EU was defined as 0.001 units of absorbance (Ohishi and Odagiri, 1984). Plasma extravasation, defined as the increase in plasma protein due to inflammation, was obtained by subtracting from the EB values obtained in the gut of animals with acute or chronic inflammation the corresponding EB values of control animals (without inflammation) using the following equation: plasma extravasation (EU) = EB inflamed intestine – EB control intestine.

Tissue Isolation. Small intestine (jejunum) and the thoracic section of the spinal cord from animals with and without chronic intestinal inflammation were excised, placed in sterile Microfuge tubes, snap-frozen in liquid nitrogen, and stored at –80°C until assay. The dissection of the mucosa of the gut was performed by placing segments of jejunum in ice-chilled phosphate-buffered saline (PBS). Then, the gut was opened longitudinally to expose the mucosal side, which was subsequently pinned to a silicone elastomer-coated Petri dish. The mucosal layer was separated from the remaining layers by performing a careful superficial scraping with a glass slide, and samples from four animals were pooled into one experimental sample. Then, samples were frozen in liquid nitrogen and stored at –80°C. All dissections were performed under a stereomicroscope at 4°C.

The present experiments were performed on the jejunum because we have previously demonstrated that the greatest morphological inflammatory changes after croton oil treatment were observed in this intestinal segment (Puig and Pol, 1998). To confirm that mucosal samples extracted from the jejunal section of the gut contained only mucosa (without submucosal plexus), we performed a histological examination. Samples previously fixed with 4% paraformaldehyde for 24 h were embedded in paraffin, and longitudinal and radial sections 5 μ m in thickness were obtained with a sliding microtome. Dewaxed sections were stained with hematoxylin and eosin and examined by optical microscopy (data not shown).

Protein Extraction. Samples from whole jejunum, mucosa, and the thoracic section of the spinal cord were solubilized in a buffer containing 62.5 mM Tris-HCl, 2.3% SDS, 10% glycerol, and 5% β -mercaptoethanol, adjusted to a pH 6.8. After 3-h incubation at room temperature, the samples were boiled for 5 min and stored at –20°C until use. The protein concentration of the samples was determined by using the method of Bradford (1976). In these experiments, a similar efficiency in protein extraction was obtained between controls and inflamed tissues.

Western Immunoassay. To determine the expression of KOR, 150 (jejunum), 200 (mucosa), and 5 μ g (spinal cord) of total protein per lane were used to perform the SDS-polyacrylamide gel electrophoresis (10% acrylamide gel) at 125 V during 3 h (Hoeffer miniVE, electrophoresis unit; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The resolved proteins were transferred (Hoeffer miniVE mini-Trans-Blot electrophoretic transfer cell; Bio-Rad) to nitrocellulose membranes (Hybond-ECL; GE Healthcare) by the application of 100 V (200–300 mA) for 2 h at 4°C. Membranes were first blocked with nonfat dry milk (5%) and Tween 20 (0.1%) in PBS overnight at 4°C, and after five washes with Tween 20 (0.1%) in PBS, they were incubated with anti-KOR antibody (BioSource LabClinics, Barcelona, Spain) diluted 1:250 (whole jejunum and mucosa) and 1:500 (spinal cord) in 0.5% nonfat dry milk in PBS for 3 h at room temperature and overnight at 4°C. After removal of the antibody, membranes were washed with PBS and then incubated with a goat anti-rabbit secondary antibody conjugated with peroxidase at a 1:2000 dilution (AP132P, conjugated with horseradish peroxidase; Chemicon International, Temecula, CA) for 1 h 30 min at room temperature. The secondary antiserum was removed, and the membranes were washed again and incubated with a solution that contains luminol (ChemiLucent Western blot detection system 2600; Chemicon International) for 5 min at room temperature and detected by chemiluminescence. Negative controls were performed for the

Western blot assay, in which all components were included except the first antibody. Immunoblots were quantified by digitizing the images and measuring the integrated density of the immunoreactive bands by the Diversity database program 2.1.1 (Bio-Rad).

Immunoprecipitation. Since DOR are expressed at low levels in mouse intestine, especially in the mucosa, we measured their expression by using an immunoprecipitation assay according to the procedure used in our laboratory (Pol et al., 2003). Briefly, immunoprecipitation was performed in all samples (whole jejunum, mucosa, and thoracic spinal cord) of animals with and without chronic intestinal inflammation. In the immunoprecipitation, 25 μ g of the anti-DOR polyclonal antibody against sequences in the N terminus of the DOR protein (Chemicon International) was incubated with 200 μ l of resuspended protein A-Sepharose (GE Healthcare) for 1 h at 4°C. Afterward, samples were centrifuged at 1000 rpm for 5 min at 4°C, and pellets were washed four times with PBS-1% bovine serum albumin. Immunocomplexes were obtained by incubation of 100 μ l of the protein A-Sepharose linked to the antibody with 60 μ g of tissue protein for whole jejunum, 80 μ g for mucosa, and 40 μ g for thoracic spinal cord or PBS (as a control without protein) overnight at 4°C. After washing the pellets four times with PBS buffer, they were resuspended in 50 μ l of Laemmli SDS buffer and heated at 100°C for 5 min. After Western blot confirmation that immunoprecipitated samples contained specific proteins for DOR antibody (Pol et al., 2003), 15 μ l of each sample was separated on a 10% SDS-polyacrylamide gel electrophoresis at 100 V during 4 h (Hoeffer miniVE, electrophoresis unit; GE Healthcare). Finally, proteins were detected by silver staining, images were digitalized, and the intensity of the bands measured by using the Diversity database program 2.1.1.

Experimental Design. The plasma extravasation values were determined by measuring the EB content in the small gut at 3 h 10 min and at 96 h 10 min after the administration of the first dose of vehicle or croton oil, in controls and mice with acute and chronic intestinal inflammation, respectively.

The effects of s.c. administration of KOR U50,488H, DOR [D-Pen²,D-Pen⁵]-enkephalin (DPDPE), and MOR agonist fentanyl on the inhibition of plasma extravasation in mice with acute or chronic intestinal inflammation were also evaluated. According to their maximal concentration in plasma, the effects of opioids were measured at 30 min (U50,488H or DPDPE) and at 20 min (fentanyl) after their administration (Valle et al., 2001).

The opioid nature of the inhibitory effects of opioid agonists on plasma extravasation during intestinal inflammation was tested by evaluating their effects in animals cotreated with a specific opioid receptor antagonist. The doses of the agonists used were those that produced an inhibitory effect of 70 and 80% of the maximal observed effect in animals with acute or chronic intestinal inflammation, respectively. In animals with acute inflammation, we used 5 mg/kg U50,488H or DPDPE and 0.075 mg/kg fentanyl; and in animals with chronic inflammation, we used 0.3 mg/kg U50,488H, 0.5 mg/kg DPDPE, and 0.075 mg/kg fentanyl. The doses of the antagonists tested were 10 mg/kg nor-binaltorphimine dihydrochloride (nor-BNI), a KOR antagonist; 3 mg/kg naltrindole, a DOR antagonist; and 0.1 mg/kg naloxone, a MOR antagonist at this dose. These doses were selected on the basis of previous studies performed in our laboratory (Puig and Pol, 1998; Valle et al., 2001); all antagonists were administered i.p. 15 min after the administration of the agonist. The role of endogenous opioid system on plasma extravasation was studied by evaluating the effects of these three opioid receptor-specific antagonists administered alone, in animals with acute or chronic intestinal inflammation.

The role of nitric oxide on plasma extravasation produced by chronic inflammation was evaluated by the quantification of the EB from animals treated with three consecutive doses (10 mg/kg) of a nonspecific NOS inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME), or a specific iNOS inhibitor, L-N^ω-(1-iminoethyl)-lysine hydrochloride (L-NIL). NOS inhibitors were injected intraperitoneally starting the next day after the second dose of croton oil. The same

experiments were performed in animals treated with *N*^ω-nitro-D-arginine methyl ester (D-NAME), an inactive isomer of NAME. In another group of experiments, we measured the antiexudative effects of a fixed dose of the KOR U50,488H (0.3 mg/kg) or DOR DPDPE (0.5 mg/kg) agonists in animals with chronic inflammation treated with L-NAME, L-NIL, or D-NAME. Protein levels of KOR and DOR in the whole jejunum, mucosa, and thoracic spinal cord from controls and animals with chronic inflammation were also measured by using Western blot and immunoprecipitation, respectively.

Drugs. We used U50,488H, DPDPE, fentanyl, nor-BNI, naltrindole, and naloxone. All of these drugs were acquired from Sigma-Aldrich, with the exception of fentanyl (Syntex Latino, Madrid, Spain). L-NAME and L-NIL were purchased from Tocris Cookson Inc. (Ellisville, MO) and D-NAME from Sigma-Aldrich. Drugs were dissolved in distilled water and injected in a volume of 10 ml/kg. Opioid agonists were injected s.c., whereas antagonists were administered i.p. For each group treated with a drug, the respective control group received the same volume of saline.

Statistics. The inhibitory effects of the opioid receptor agonists are expressed as the percentage of inhibition of plasma extravasation in drug-treated animals (test) compared with the mean plasma extravasation measured in the corresponding group of control mice ($n = 6-8$) using the following equation: % inhibition of plasma extravasation = [(control - test)/(control)] × 100.

Data are expressed as a mean ± S.E.M. All statistical calculations were performed with the statistical program SPSS 11.5 (SPSS Inc., Chicago, IL). The ED₅₀ ± S.E.M. (dose that produced a 50% of the maximal effect) values were determined by linear regression analysis of dose-response relationships based on at least six to eight animals per dose. Statistical analysis for significant differences between two groups was obtained by Student's *t* test. When multiple groups were compared, one-way analysis of variance (ANOVA) was used, followed by the Student-Newman-Keuls test, whenever applicable. A value of $P < 0.05$ was considered significant.

Results

Plasma Extravasation in Animals with Intestinal Inflammation. The EB intestinal content was assessed in controls and in animals with acute and chronic intestinal inflammation induced by croton oil. In these experiments, the EB intestinal content was evaluated at 3 h 10 min (acute treatment) and at 96 h 10 min (chronic treatment) after the administration of the first dose of vehicle or croton oil. The EB contents in the gut were 585.6 ± 22.1 and 588 ± 20.2 EU for acute and chronic vehicle treated mice, respectively, and 889.9 ± 30.8 EU and 1423.6 ± 85.1 EU for acute and chronic croton oil-treated animals, respectively. Plasma extravasation was obtained by subtracting from the EB values obtained in the gut of animals with acute or chronic inflammation the corresponding EB values of control animals (without inflammation). These values are represented in Fig. 1B, showing that chronic inflammation induces a 2.7-fold increase in plasma extravasation compared with animals with acute inflammation ($P < 0.001$; Student's *t* test).

Effects of Opioids on Plasma Extravasation in Animals with Acute or Chronic Inflammation. The inhibitory effects of U50,488H (a selective KOR agonist), DPDPE (a selective DOR agonist), and fentanyl (a selective MOR agonist) on plasma extravasation were evaluated in animals with acute and chronic inflammation.

The s.c. administration of U50,488H produced a dose-related inhibition of plasma extravasation in both acute and chronic croton oil-treated animals (Fig. 2A). In both groups, dose-response curves were parallel without significant differ-

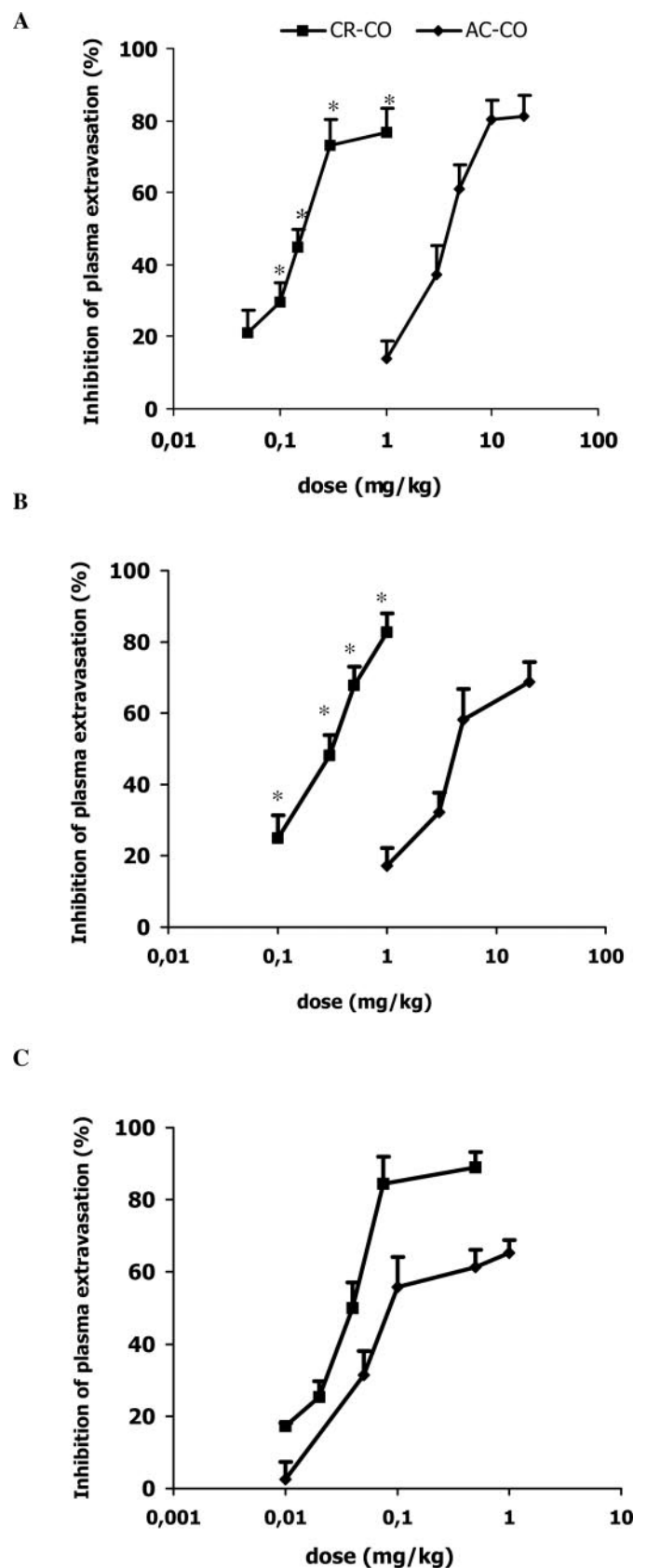


Fig. 2. Dose-related inhibition of plasma extravasation induced by U50,488H (A), DPDPE (B), and fentanyl (C) in animals with acute (AC-CO) or chronic intestinal inflammation (CR-CO). Each point represents the mean ± S.E.M. from six to eight mice. *, significant differences compared with animals with acute inflammation ($P < 0.05$; Student's *t* test).

ences in their slopes (acute, 67.6 ± 1.3 and chronic, 67.5 ± 8.9) and in their E_{\max} values (90% in acute and 85% in chronic). During chronic inflammation, the dose-response curve to U50,488H was shifted to the left demonstrating an increase in the κ -agonist inhibitory effect. The ED_{50} values obtained from each curve (as a measure of the potency) showed that the potency of U50,488H-inhibiting plasma extravasation increased 26.3 times during chronic inflammation (Table 1).

The DOR agonist DPDPE also induced a dose-related inhibition of plasma extravasation in both experimental conditions (Fig. 2B). In animals with chronic inflammation, the dose-response curve to DPDPE was also shifted to the left. No significant differences in the slopes of the curves were observed between acute or chronic inflammation (54.0 ± 3.2 for acute and 58.9 ± 4.4 for chronic), and the E_{\max} values were not significantly different among themselves (range 80–95%). The analysis of the ED_{50} values (Table 1) showed that DPDPE was 11.8 times more potent in chronic than in acute croton oil-treated animals ($P < 0.05$; Student's *t* test).

Similarly, the administration of fentanyl produced a dose-related inhibition of plasma extravasation in acute and chronic croton oil-treated mice (Fig. 2C). In this case, the slope of the curve and the maximal effects obtained after chronic inflammation (76.5 ± 1.5 and 89%) were significantly higher than those obtained in acute inflammation (51.2 ± 3.2 and 65.3%; $P < 0.05$ Student's *t* test). Although the ED_{50} value of fentanyl diminished 1.5 times in animals with chronic inflammation, the statistical analysis of the data did not show significant differences between both groups of study (Table 1).

Antagonism of the Inhibitory Effects of KOR, DOR, and MOR Agonists by Specific Antagonists. We first evaluated the effects on plasma extravasation produced by the administration of specific antagonists alone in animals with acute and chronic inflammation. During acute inflammation, the administration of specific antagonists significantly increased plasma extravasation ($P < 0.05$; Student-Newman-Keuls test), and the observed values were 525.3 ± 49 EU for nor-BNI, 788.1 ± 77.3 EU for naltrindole, 604.4 ± 64.1 EU for naloxone, and 304.3 ± 30.8 EU for the control group. The maximum increase in plasma extravasation was observed after naltrindole administration (159%), although the administration of KOR and MOR antagonists alone also increased plasma extravasation in a 73 and 98%, respectively. In contrast, the systemic administration of each of the three specific antagonists did not alter the plasma extravasation values obtained in chronic croton oil-treated animals.

To evaluate the specificity of the observed responses in the

presence of intestinal inflammation, the effects of KOR, DOR, and MOR agonists were assessed after the administration of specific-opioid receptor antagonists. The results show that during inflammation, the inhibitory effects of all agonists were completely reversed by their respective antagonists, demonstrating the opioid nature of the results (Table 2). However, whereas during acute inflammation the plasma extravasation values obtained in animals treated with the specific agonist and antagonist together were significantly higher than those obtained in basal conditions ($P < 0.05$; Student-Newman-Keuls test), this effect did not occur after chronic inflammation.

The Role of Nitric Oxide on Plasma Extravasation during Intestinal Inflammation. The possible participation of nitric oxide in the increased plasma extravasation observed during chronic intestinal inflammation was evaluated by using NOS inhibitors. In these animals, the administration of L-NIL or L-NAME diminished plasma extravasation by 23 and 35%, respectively ($P < 0.05$; one-way ANOVA; Student-Newman-Keuls test). In contrast, the administration of D-NAME did not alter the plasma extravasation compared with control animals (Fig. 3A).

Antiexudative Effects of KOR and DOR Agonists during Chronic Inflammation in Animals Treated with NOS Inhibitors. The possible involvement of nitric oxide in the enhanced inhibitory effects of KOR and DOR agonists (26.3 and 11.8 times) during chronic inflammation was evaluated. Thus, the antiexudative effects produced by 0.3 mg/kg U50,488H and 0.5 mg/kg DPDPE in animals with chronic intestinal inflammation treated with L-NAME, L-NIL, or D-NAME were measured. The results show a significant decrease in the inhibitory effects of U50,488H ($P < 0.05$; one-way ANOVA, Student-Newman-Keuls test; Fig. 3B) and DPDPE ($P < 0.05$; one-way ANOVA; Student-Newman-Keuls test; Fig. 3C) in animals treated with L-NAME or L-NIL. For both opioid receptor agonists, similar reductions in their effects are produced by the unspecific and the specific iNOS inhibitors: 45 to 52% for U50,488H and 44 to 43% for DPDPE. The administration of D-NAME did not alter the antiexudative effects produced by KOR or DOR agonists during chronic inflammation (Fig. 3, B and C).

Expression of KOR in the Whole Jejunum, Mucosa, and Thoracic Spinal Cord of Mice with Chronic Inflammation. Our objective was to evaluate whether there was a correlation between the antiexudative effect of

TABLE 1

ED_{50} values (milligrams per kilogram) of U50,488H, DPDPE, and fentanyl on the inhibition of plasma extravasation during acute and chronic intestinal inflammation

For each agonist, the ratio of the ED_{50} values between acute and chronic inflammation is also indicated. Results are expressed as a mean values \pm S.E.M. of six to eight animals per dose. For each agonist, asterisk (*) indicates significant differences compared with animals with acute intestinal inflammation ($P < 0.05$; Student's *t* test).

Agonist	Acute Inflammation	Chronic Inflammation	Ratio (Acute/Chronic)
U50,488H	3.16 ± 0.3	$0.12 \pm 0.09^*$	26.3
DPDPE	2.95 ± 0.20	$0.25 \pm 0.03^*$	11.8
Fentanyl	0.042 ± 0.03	0.028 ± 0.01	1.5

TABLE 2

Reversal of the effects of KOR, DOR, and MOR agonists on plasma extravasation (EU) induced by acute or chronic inflammation by the administration of specific antagonists

The doses of the agonists tested for acute and chronic inflammation were 5 and 0.3 mg/kg U50,488H, 5 and 0.5 mg/kg DPDPE, and 0.075 and 0.075 mg/kg fentanyl, respectively. The doses of the specific antagonists used were 10 mg/kg nor-BNI, 3 mg/kg naltrindole, and 0.1 mg/kg naloxone. Results are expressed as a mean values \pm S.E.M. of six to eight animals.

Agonist	Antagonist	Acute Inflammation	Chronic Inflammation
Vehicle	Saline	304.3 ± 30.8	838.0 ± 85.1
U50,488H	Saline	$118.9 \pm 29.8^*$	$227.2 \pm 61.8^*$
U50,488H	nor-BNI	$777.6 \pm 53.4^*$	809.5 ± 104.4
DPDPE	Saline	$127.5 \pm 36.1^*$	$271.7 \pm 46.3^*$
DPDPE	Naltrindole	$827.4 \pm 49.9^*$	974.2 ± 125.2
Fentanyl	Saline	$120.2 \pm 25.2^*$	$130.7 \pm 56.7^*$
Fentanyl	Naloxone	$525.4 \pm 34.2^*$	878.7 ± 93.3

* Significant differences compared with vehicle-saline (control) treated animals ($P < 0.05$; one-way ANOVA; Student-Newman-Keuls test).

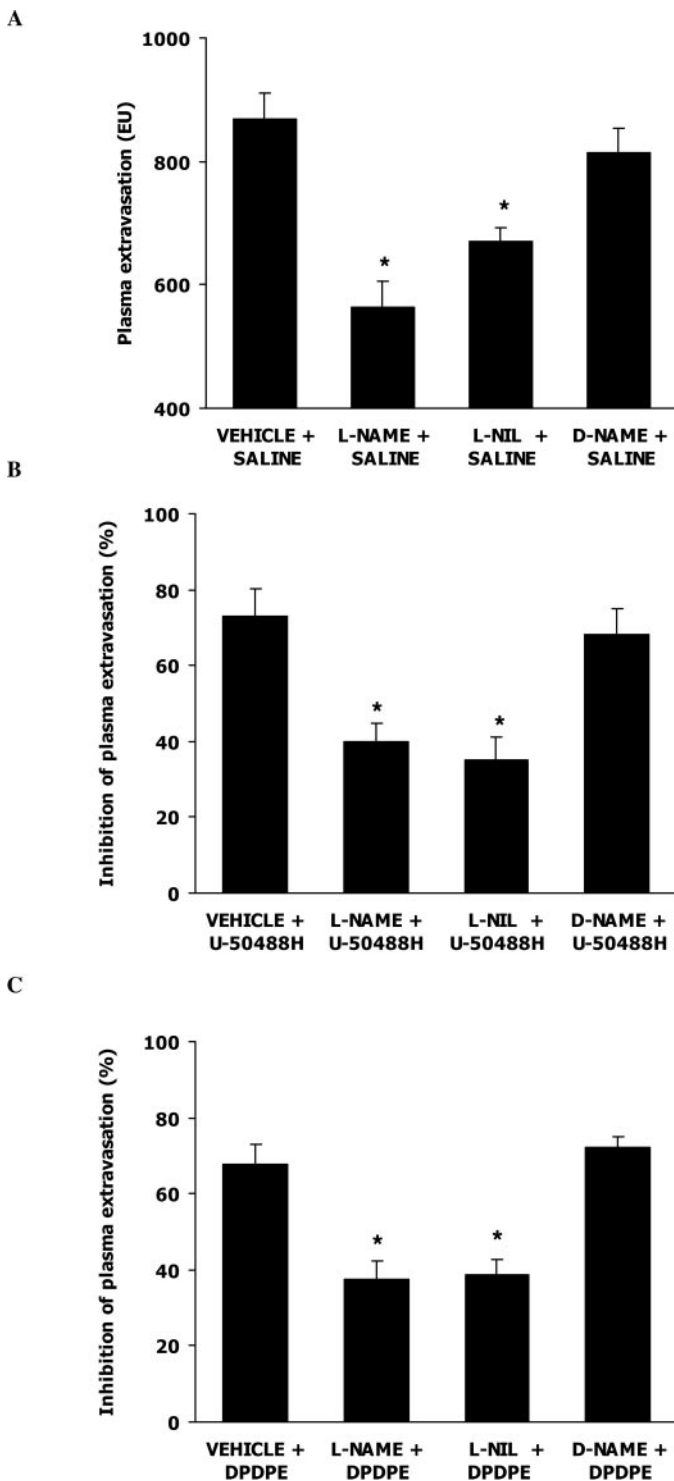


Fig. 3. A, plasma extravasation (EU) from animals with chronic intestinal inflammation treated with vehicle, L-NAME, L-NIL, or D-NAME. *, significant differences compared with animals treated with vehicle ($P < 0.05$; one-way ANOVA; Student-Newman-Keuls test). B, inhibition of plasma extravasation induced by the s.c. administration of U50,488H in croton oil-treated animals, cotreated with vehicle, L-NAME, L-NIL, or D-NAME. *, significant differences compared with animals treated with vehicle plus U50,488H ($P < 0.05$; one-way ANOVA; Student-Newman-Keuls test). C, inhibition of plasma extravasation induced by the s.c. administration of DPDPE in croton oil-treated animals, cotreated with vehicle, L-NAME, L-NIL, or D-NAME. *, significant differences compared with animals treated with vehicle plus DPDPE ($P < 0.05$; one-way ANOVA; Student-Newman-Keuls test). In each panel, columns represent the mean \pm S.E.M. from six to eight animals.

U50,488H and the protein levels of KOR in the whole intestine (jejunum), mucosa, and the thoracic section of the spinal cord obtained from animals with and without chronic intestinal inflammation. Figure 4 shows the results of a representative immunoblot experiment, obtained in samples of whole jejunum (A), mucosa (B), and thoracic spinal cord (C) from controls (SS) and animals with chronic intestinal inflammation (CR-CO). The resulting immunoblots exhibited a band at approximately 49.3 kDa. This band size is consistent with the results obtained by other investigators, which describe molecular masses of 43 to 57 kDa for the KOR (Joshi et al., 2000; Cichewicz et al., 2001; Fan et al., 2003). In the gut (whole jejunum and mucosa), KOR occurs as a doublet (40.7 kDa), which probably indicates the presence of two subtypes of KOR proteins in this tissue (Joshi et al., 2000). The upper band of the doublet (49.3 kDa) was used for quantification. The densitometric analysis of the data showed a significant increase in KOR immunoreactivity in the whole intestine and mucosal samples from animals with intestinal inflammation compared with their respective controls ($P < 0.05$ and $P < 0.01$; Student's t test). In contrast, nonsignificant differences between groups could be observed in the spinal cord samples (Fig. 4D).

Immunoblot experiments from pooled samples were repeated four times in each of the controls ($n = 4$) and in animals with intestinal inflammation ($n = 4$). In all experimental conditions, immunoreactivity was abolished when experiments were performed in the absence of first or secondary antibody.

Expression of DOR in the Whole Jejunum, Mucosa, and Thoracic Spinal Cord of Mice with Chronic Inflammation. In these experiments, we also investigated whether the enhanced antiexudative effects produced by the DOR agonist during chronic inflammation were associated with an increase in the expression of DOR protein. Figure 5 shows a gel electrophoresis of DOR protein from a representative experiment, obtained in samples of whole intestine (A), mucosa (B), and thoracic spinal cord (C) from animals with (CR-CO) and without (SS) chronic intestinal inflammation. The gels show a band at approximately 67 kDa corresponding to the DOR protein. This band size is consistent with the results obtained by ours and other investigators, which describe molecular masses of 43 to 125 kDa for the DOR (Cichewicz et al., 2001; Cahill et al., 2003; Pol et al., 2003). The densitometric analysis of the data showed that inflammation induces a significant increase in DOR protein expression in the whole jejunum and mucosal samples ($P < 0.01$ and $P < 0.05$; Student's t test) but not the thoracic spinal cord samples (Fig. 5D). Immunoprecipitated experiments from pooled samples were repeated four times in each control ($n = 4$) and in animals with intestinal inflammation ($n = 4$).

Discussion

In this work, we show that the administration of specific KOR, DOR, and MOR agonists reduces plasma extravasation during acute and chronic intestinal inflammation induced by croton oil. A relevant increase in the potencies of KOR (26.3 times) and DOR (11.8 times) agonists inhibiting plasma extravasation during chronic inflammation was also observed. The results suggest that the increased levels of KOR as well as DOR in the gut, and nitric oxide originating from iNOS

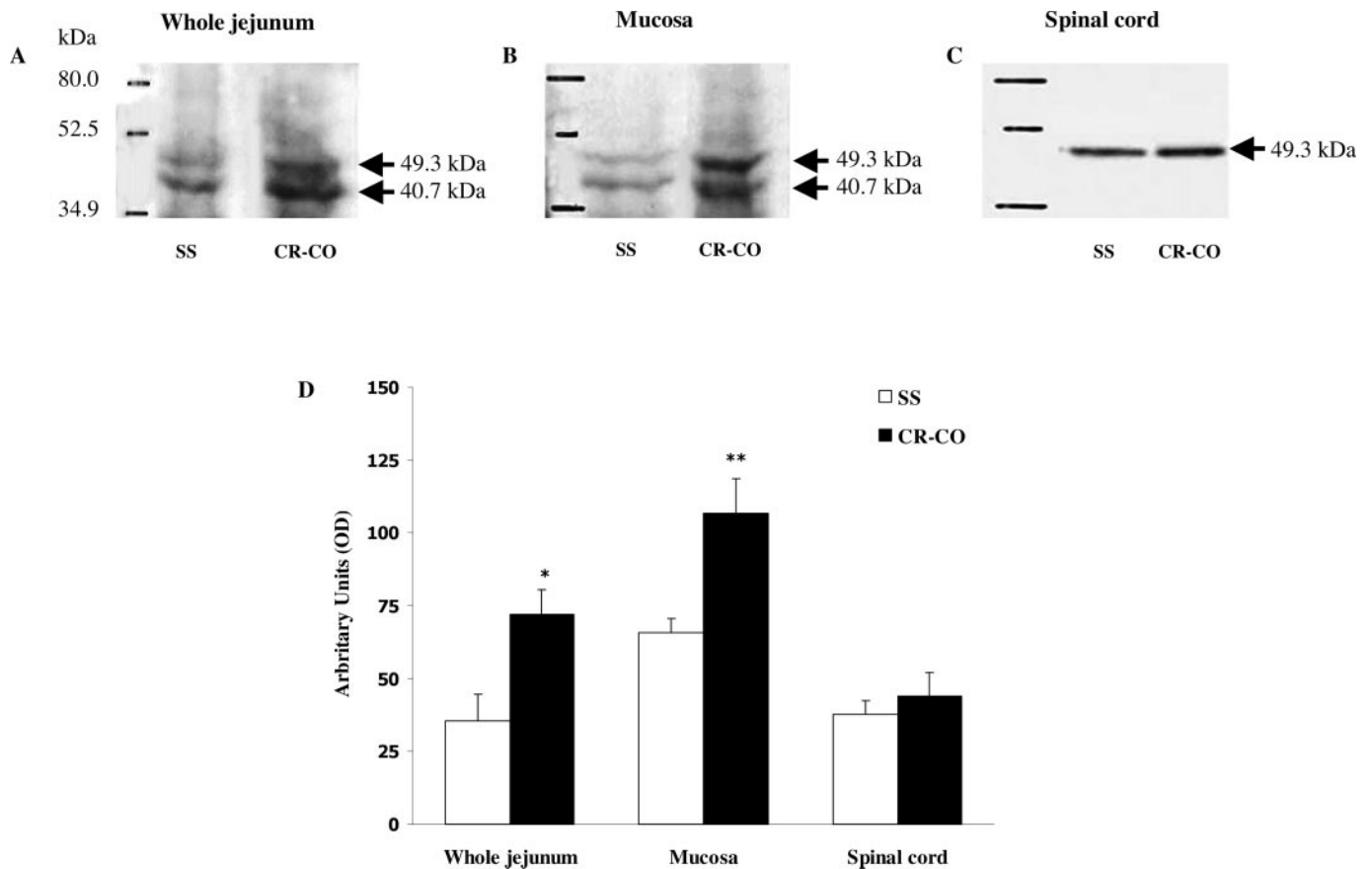


Fig. 4. Immunoblots of a representative experiment with KOR antibody of samples from whole jejunum (A) mucosa (B), and spinal cord samples (C) of mice with (CR-CO) and without intestinal inflammation (SS). Two immunoreactive bands of about 49.3- and 40.7 kDa were detected in the intestine (whole jejunum and mucosa), and a unique band of approximately 49.3 kDa was detected in the spinal cord (arrows). In all instances, immunoreactivity was abolished when staining was performed without the primary antibody. The molecular mass standards used were bovine serum albumin (80 kDa), ovalbumin (52.5 kDa), and carbonic anhydrase (34.9 kDa) from Bio-Rad. D, graphical representation of the optical density (OD) of the bands of KOR in the whole jejunum, mucosa, and spinal cord from animals with chronic (CR-CO) and without intestinal inflammation (SS). For each tissue, asterisk indicates significant differences compared CR-CO with SS animals (*, $P < 0.05$ and **, $P < 0.01$; Student's t test). Each column represents the mean \pm S.E.M. from four experiments.

could be responsible for the increased potency of KOR and DOR agonists during chronic inflammation.

Evans blue, which has the property to form a complex with the plasmatic proteins, was used as a marker of the vascular permeability produced by croton oil in the intestine. A positive correlation between the intensity of inflammation and plasma extravasation values were observed in our results. Animals with chronic inflammation presented higher plasma extravasation values (2.7 times) than those obtained during acute inflammation, supporting previous findings from our laboratory (Puig and Pol, 1998; Valle et al., 2001). In addition, our results also show that nitric oxide released during inflammation is implicated in the increased plasma extravasation induced by chronic inflammation. That is, treatment with an unspecific or a specific iNOS inhibitor both reduced the increased plasma extravasation induced by croton oil, probably as a consequence of their anti-inflammatory properties in this intestinal inflammatory model (Pol et al., 2005). This effect could not be mimicked by the administration of the inactive stereoisomer D-NAME. In accordance with our findings, the administration of selective neuronal and inducible NOS inhibitors inhibited plasma extravasation induced by other inflammatory agents (Laszlo et al., 1994; Evans and Whittle, 2003; Li et al., 2005). However, the different reduc-

tion in plasma extravasation produced by L-NAME (35%) or L-NIL (23%) administration suggests that the increased plasma extravasation induced by croton oil could be mediated by nitric oxide derived from several NOS isoenzymes. Thus, other sources of nitric oxide such the endothelial and/or neuronal NOS could be also implicated. However, taking into account that the expression of eNOS was significantly increased during acute, but not chronic inflammation, and that the eNOS-derived nitric oxide is critical for the increased vascular permeability only during acute inflammation, we did not attempt to evaluate the specific effects of this NOS isoform in our experiments (Bucci et al., 2005).

The systemic administration of specific KOR, DOR, or MOR agonists inhibited plasma extravasation induced by croton oil in a dose-related manner. In accordance with our results, plasma extravasation induced by antidromic electric stimulation or bradykinin administration was dose dependently inhibited by the administration of selective KOR agonists (Green and Levine, 1992; Barber, 1993). However, no dose-related inhibition of plasma extravasation was induced by U50,488H in a model of carrageenan-induced acute inflammation of the paw (Romero et al., 2005) or in the formalin test (Hong and Abbott, 1995). The administration of the MOR agonist fentanyl also produced a dose-related inhibition

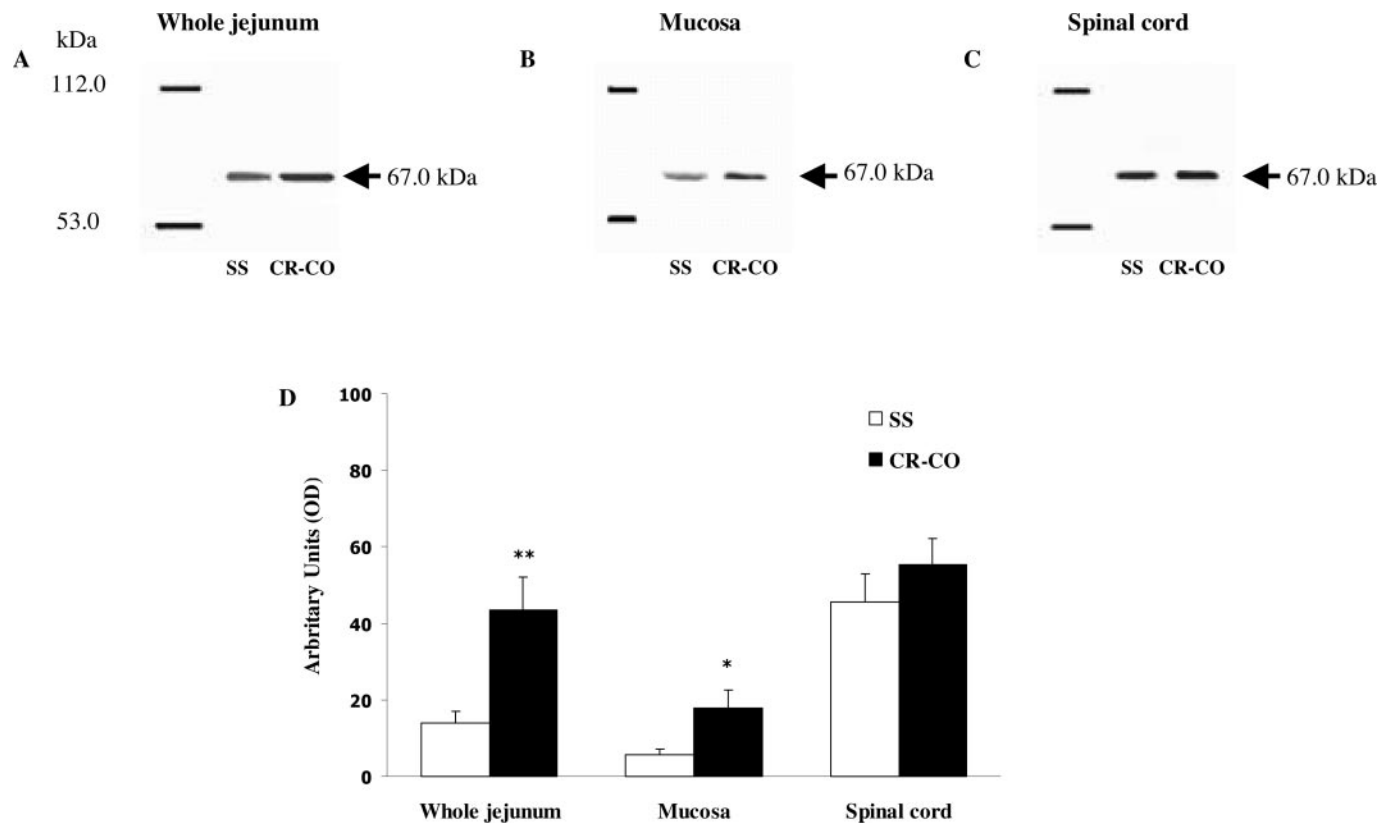


Fig. 5. Gel electrophoresis of DOR proteins from a representative experiment performed by the immunoprecipitation of DOR antibody with protein samples from whole jejunum (A) mucosa (B), and spinal cord samples (C) of mice with (CR-CO) and without intestinal inflammation (SS). For each tissue, lane 1 is the molecular mass marker, and lanes 2 and 3 are samples obtained from saline and croton oil-treated animals. Equal amounts of samples (15 μ l/lane) from saline and croton oil-treated animals were loaded into the gels. Each gel shows one band at approximately 67 kDa corresponding to the DOR protein. The molecular mass standards used were bovine serum albumin (112 kDa) and ovalbumin (53 kDa) from Bio-Rad. D, graphical representation of the OD of the bands of DOR in the whole jejunum, mucosa, and spinal cord samples from CR-CO animals with chronic inflammation and SS animals without intestinal inflammation. For each tissue, asterisk indicates significant differences for CR-CO compared with SS animals (*, $P < 0.05$ and **, $P < 0.01$; Student's *t* test). Each column represents the mean \pm S.E.M. from four experiments.

of plasma extravasation in the gut, as reported by several groups in other models of peripheral inflammation (Barber, 1993; Hong and Abbott, 1995; Taylor et al., 2000; Romero et al., 2005). Although, in a neurogenic inflammation induced by bradykinin, [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (a MOR agonist) was ineffective at inhibiting plasma extravasation. These findings indicate that the antiexudative effects produced by KOR and MOR agonists differ in the different models of plasma extravasation. Furthermore, and similar with what occurs in other tissues, DOR agonists also inhibited plasma extravasation induced by croton oil, in a dose-related manner (Green and Levine, 1992; Hong and Abbott, 1995; Romero et al., 2005).

The potency of KOR and DOR agonists inhibiting plasma extravasation increased between 26 and 11 times during chronic inflammation, whereas the potency of fentanyl (a MOR agonist) remained unaltered. This is the first time that a relevant role for KOR and DOR modulating plasma extravasation during intestinal inflammation has been suggested. Previously, we have demonstrated that during inflammation MOR agonists mainly modulate intestinal motility, and DOR were mainly responsible for the control of intestinal secretion (Pol and Puig, 2004). Therefore, the control of the different intestinal functions in the inflamed gut seems to be regulated by different types of opioid receptors.

The specificity of the effects produced by the opioid recep-

tor agonists in animals with acute or chronic inflammation was demonstrated by their complete reversibility by specific opioid receptor antagonists. The administration of these antagonists alone significantly increased plasma extravasation in mice with acute but not with chronic inflammation. The maximal increase in plasma extravasation during acute inflammation was observed after naltrindole administration (159%), suggesting that an important release of enkephalins occurred in these experimental conditions. The administration of KOR and MOR antagonists alone also increased plasma extravasation in a similar manner (73 and 98%, respectively), suggesting a possible release of β -endorphin and dynorphin. We have not a clear explanation for the fact that the administration of antagonists alone did not alter intestinal extravasation in animals with chronic inflammation. We hypothesize that a substantial liberation of endogenous opioid peptides probably occurs during the first hours after croton oil-induced intestinal inflammation and that the pharmacological approach used in this work did not permit to detect minor changes in the release of endogenous opioid peptides that may occur during chronic inflammation.

In the gut, KOR, DOR, and MOR are widely distributed in the myenteric and submucous plexuses (Bagnol et al., 1997; Pol et al., 2001, 2003; Townsend and Brown, 2002), whereas lower densities of MOR and DOR have been also demonstrated to be present in enterocytes (Lang et al., 1996; Nano

et al., 2000). In this work, we show that besides of DOR, KOR are also present in the mucosa, and their levels increased during chronic intestinal inflammation. These results expanded our previous findings, showing that chronic intestinal inflammation enhances the protein levels of KOR and DOR in the submucosal section of the gut and corroborated the absence of changes in their protein levels in the spinal cord of these animals (Pol et al., 2003). In agreement with our results, Sengupta et al. (1999) have demonstrated that the antinociceptive effects of KOR were increased in rats with inflammation of the colon, suggesting an up-regulation of this receptor in the inflamed gut. The enhanced protein levels of KOR and DOR in the mucosa of the inflamed intestine could probably be responsible for the enhanced effects of both agonists on the inhibition of plasma extravasation after croton oil-induced chronic intestinal inflammation in mice. In contrast, the absence of changes in the protein levels of MOR in the submucous plexus plus mucosa section of the gut after chronic inflammation could explain the lack of increased potency of fentanyl inhibiting plasma extravasation in these experimental conditions (Pol et al., 2001). Conversely to what occurs with the MOR and DOR, KOR mRNA levels in the gut were not altered by intestinal inflammation, suggesting that post-transcriptional and/or post-translational changes of the KOR gene could be responsible for the increased potency of KOR agonists during intestinal chronic inflammation (Pol et al., 2003). However, other factors, such as the increased nitric oxide in the gut of animals with chronic inflammation, may also contribute to enhance the effects of KOR and DOR agonists in this model (Pol et al., 2005).

The role of nitric oxide enhancing the effects of KOR and DOR agonists during chronic intestinal inflammation was evaluated by measuring the antiexudative effects of U50,488H and DPDPE in animals treated with NOS inhibitors. The reduction in the antiexudative effects produced by KOR and DOR agonists in animals treated with L-NAME or L-NIL suggests that nitric oxide may play a role mediating the enhanced effects of U50,488H and DPDPE during chronic inflammation. For each agonist, the fact that treatment with the specific or the nonspecific iNOS inhibitor similarly reduced their effects suggests, that nitric oxide synthesized by iNOS could be mainly responsible for the increased antiexudative effects of U50,488H and DPDPE during chronic inflammation. This hypothesis is supported by the 10-fold increase in the iNOS mRNA levels in the gut of mice with chronic croton oil-induced intestinal inflammation (Pol et al., 2005). In agreement with the present results, several reports have shown that during inflammation, the antinociceptive effects of opioids are produced through the nitric oxide-cGMP pathway (Nozaki-Taguchi and Yamamoto, 1998; Tasatargil and Sadan, 2004). In addition, using knockout mice, we have recently demonstrated that the absence of iNOS gene abolishes the increased antitransit effects produced by morphine during chronic inflammation, supporting the fact that nitric oxide derivate from iNOS is implicated in the enhanced effects of opioids during intestinal inflammation (Pol et al., 2005). However, further studies are required to determine the specific mechanisms by which nitric oxide modulates the effects of opioids after chronic inflammation.

In summary, our results show that the enhanced inhibitory effects of U50,488H and DPDPE on plasma extravasation during chronic intestinal inflammation could be related to

the increased levels of KOR and DOR proteins in the gut and/or to the increased levels of nitric oxide derived from the overexpression of iNOS during inflammation.

Acknowledgments

We thank Drs. Francesc Alameda and Teresa Baró (Department of Pathology, Hospital del Mar, Barcelona, Spain) for help in the histological examination of the intestinal sections.

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