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Anti-exudative effects of opioid receptor agonists in a rat model of carrageenan-induced acute inflammation of the paw

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Abstract

We evaluated the anti-exudative effects (Evan's blue) of mu-, delta- and kappa-opioid receptor agonists in a rat model of carrageenan-induced acute inflammation. The contribution of different components was assessed after the administration of: cyclosporine A, capsaicin, 6-hydroxydopamine, compound 48/80, and specific histamine-receptor antagonists. The results show that the mu-opioid receptor agonists morphine and fentanyl and the delta-opioid receptor agonists DPDPE (enkephalin, [D-Pen^{2,5}]) and SNC 80 ((+)-4-[(αR)-α((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N* diethylbenzamide) decrease plasma extravasation in a dose-dependent manner, with a biphasic response. The effects were reversed by specific antagonists, and are predominantly mediated by peripheral opioid receptors. The integrity of sensory and sympathetic fibres is essential for the anti-exudative effects of fentanyl and DPDPE. Histamine and functional histamine H_2 and H_3 receptors are required for morphine and fentanyl (but not DPDPE) inhibition of plasma extravasation, suggesting different mechanism for mu- and delta-opioid receptor agonists. The present findings implicate multiple sites and mechanisms in the anti-exudative effects of exogenous opioids.

Keywords: Oedema; Histamine; Inflammation; Opioid receptor; Plasma extravasation

1. Introduction

Peripheral inflammation induces the local release of numerous chemical mediators that, among other effects, sensitise the peripheral terminals of primary afferents (nociceptors) inducing pain and hyperalgesia; these terminals also release neuropeptides (Richardson and Vasko, 2002) which participate in the local inflammatory response by inducing vasodilatation, plasma extravasation and oedema (Karimian and Ferrell, 1994; Amann et al., 1995; Siney and Brain, 1996). Inflammation-induced changes in vascular permeability (plasma extravasation and oedema) are the result of an overload of fluid in the endothelial cells that exceeds their absorptive capacity, and generate net fluid accumulation into the extravascular/interstitial compartment (McDonald et al., 1999).

Several studies have demonstrated that opioids, administered either locally or systemically during peripheral inflammation, have powerful antinociceptive (Stein, 1995; Planas et al., 2000) and anti-inflammatory effects, including anti-oedema (Joris et al., 1990, Binder et al., 2001) and anti-extravasation (Green and Levine, 1992; Taylor et al., 2000). These effects are induced by the activation of opioid receptors located in the central and peripheral nervous systems. In the periphery, opioid receptors are expressed on sensory fibres and on sympathetic postganglionic terminals, where they may participate in the modulation of nociceptive information under certain pathological conditions (Zhou et al., 1998). Opioid receptors are also constitutively expressed in non-neuronal sites including vascular endothelial cells

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(Cadet et al., 2000; Saeed et al., 2000), immune cells such as macrophages and lymphocytes (Gavériaux et al., 1995), and keratocytes (Bigliardi et al., 2002); the possible role/s of the extra-neuronal peripheral opioid receptors has not been fully established.

Although the effects of opioids on the inhibition of plasma extravasation have been previously reported by different groups (Barber, 1993; Taylor et al., 2000), the possible mechanisms involved and their anatomical localisation are not well characterised. In the present investigation we wanted to assess the type of opioid receptor implicated in the anti-exudative effects of opioids, as well as their likely location in peripheral sites/structures, which participate in the local inflammatory response. In our study we used a rat model of carrageenan-induced acute inflammation and evaluated the anti-exudative effects of mu-, delta- and kappa-opioid receptor agonists after the administration of Evan's blue. The contribution of the different structures that contain opioid receptors (Gavériaux et al., 1995; Stein, 1995; Cadet et al., 2000) was assessed after the administration of: cyclosporine A, an immunosuppressant (Stein et al., 1990); capsaicin and 6-hydroxydopamine (6-OHDA) which specifically damage sensory and sympathetic fibres, respectively (Lynn, 1990); compound 48/80 that induces mast cell depletion (Banks et al., 1990), and specific histamine-receptor antagonists (Ichinose et al., 1990; Yoshihara et al., 1995; Linardi et al., 2000). Our working hypothesis was that the selective impairment of structures/cells, which express opioid receptors in the periphery, would modify the anti-exudative effects of opioids and thus identify the structure/s responsible for the effect.

2. Methods

2.1. Animals

Male Sprague–Dawley rats weighing 150±10 g were used in all experiments. Animals were housed under 12 h light/dark conditions in a room with controlled temperature (22 °C) and humidity (60%). Rats had free access to food and water and were used after a minimum of 4 days acclimation to the housing conditions. All experiments were conducted between 09:00 and 15:00 h. The study protocol was approved by the Committee of Animal Use and Care of the University of Barcelona, in accordance with the International Association for the Study of Pain guidelines on ethical standards for investigation in animals.

2.2. Induction of the inflammatory response

Inflammation was induced by the subplantar (s.p.) injection of 0.5 mg of carrageenan in 0.05 ml saline, in the left hind paw (Winter et al., 1962), and the inflammatory response evaluated 3 h after the injection.

2.3. Assessment of the inflammatory response

Oedema was assessed by measuring the volume of the left hind paw (plethysmometer 7150, Ugo Basile, Italy) before (V_0) and 3 h after carrageenan (V_3). The increase in volume of the inflamed paw was determined by subtracting the volume measured before s.p. carrageenan from the observed value at 3 h, and expressed as a percentage:

$$\%\ \text{oedema} = (V_3 - V_0) \times 100/V_0.$$

Plasma extravasation was measured after the administration of 60 mg/kg of Evan's blue dye, which is a marker of protein leakage (Udaka et al., 1970). The dye was injected i.v. (in the tail) 15 min before the end of the experiments (2 h:45 min after carrageenan injection, Fig. 1). At this time, animals were anaesthetised with a mixture of i.m. ketamine (100 mg/kg) and xylazine (10 mg/kg), and sacrificed by decapitation. Hind paws (inflamed and non-inflamed) were divided into sections and incubated with formamide at 65 °C for at least 3 h. Samples were then filtered, and the extracted dye measured by spectrophotometry at 620 nm (SmartSpec3000, Bio-Rad Laboratories, Hercules, CA). The absorbance values were calculated from a calibration curve and expressed as μg dye/weight (g) of wet paw tissue. Percentage (%) of plasma extravasation was obtained as follows:

$$\frac{(\mu\text{g blue/g tissue inflamed paw}) - (\mu\text{g blue/g tissue non-inflamed paw})}{(\mu\text{g blue/g tissue non-inflamed paw})} \times 100$$

The effects of the different drugs and drug-combinations on the inflammatory response are expressed as % oedema and % plasma extravasation (mean±S.E.M). We used 6–8

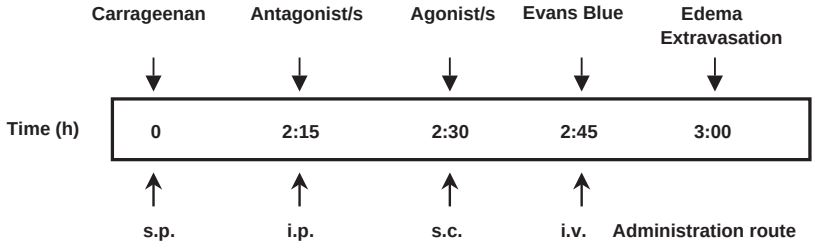

Fig. 1. Experimental design showing the sequence and route of administration of the different compounds used in the study. s.p.: subplantar; i.p.: intraperitoneal; s.c.: subcutaneous; i.v.: intravenous.

animals per dose, and a group of animals (n=10) injected with s.c. saline as controls.

2.4. Evaluation of the antinociceptive effects

The pain pressure threshold (PPT) was used to measure the antinociceptive effects of s.c. opioids. Pressure was applied to the dorsal surface of the hind paw by an automated gauge (Letica LI 7306; Ugo Basile, Italy) according to the method of Randall and Selitto (1957).

2.5. Immunohistochemistry

Under a dissecting microscope, the hind paw pads were carefully removed and stored in Zamboni's fixative solution for 24 h at 4 °C, and then cryoprotected in 0.1 M phosphate-buffered-saline (PBS; pH 7.4) containing 20% sucrose, and stored at 4 °C until further processing. Sections of tissue of 60 μm were obtained with a sliding cryotome (American Optical, Buffalo, NY) and washed in 0.1 M PBS with 0.3% Triton X-100 and 1% foetal calf serum for 1 h at 4 °C; then they were incubated overnight (at 4 °C) with one of the following specific antibodies: 1) a polyclonal rabbit anti-protein gene peptide 9.5 (PGP 9.5 at 1:1000) or 2) a polyclonal rabbit anti-calcitonin gene-related peptide (CGRP at 1:1000). After removal of the antibody, the sections were incubated with a second antibody, goat anti-rabbit cyacine 3.18 (1:200) overnight at 4 °C. After additional rinses in PBS–Triton X–foetal calf serum, the sections were adhered to gelatine-subbed cover slips, dehydrated in ethanol, cleared in methyl-salicylate and mounted in slides with DPX-medium (toluene, xilene and butylphthatale). Fluorescent samples were viewed with an epifluorescence-equipped microscope (Olympus BX-40, Tokyo, Japan). These experiments were repeated 5 times (n=5 animals for each, noninflamed controls and inflamed rat paws).

2.6. Groups of experiments

We performed the following groups of experiments.

1. Characterisation of the inflammatory response induced by the s.p. injection of carrageenan (oedema and plasma extravasation) (n=6–8).

2. Dose–response relationships on oedema and plasma extravasation were obtained for: morphine and fentanyl (mu-opioid receptor agonists), DPDPE and SNC 80 (delta-opioid receptor agonists) and U-50,488H (*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]benzeneazetamide) a kappa-opioid receptor agonist. Mean effective doses (ED_{20}, ED_{50}, ED_{80}) were obtained as a measure of potency. In all dose–response curves, 6–8 animals were tested per dose, and five or more points used to define a curve.

3. The reversibility of the effects induced by the opioid receptor agonists (oedema and extravasation) was established after the administration of antagonists (i.p.). We used naloxone (1 mg/kg), a non-selective opioid receptor antagonist, naltrindole (3 mg/kg) a delta-selective opioid receptor antagonist, and 3 mg/kg of MR-2266 ((−)-*a*-5,9-diethyl-2′-hydroxy-2-(3-furylmethyl)-6,7-benzomorphan) as a kappa-selective opioid receptor antagonist. The doses were selected on the basis of previous experiments performed by our group (Valle et al., 2001).

4. The peripheral component of the anti-extravasation effects was evaluated by two types of experiments: i) dose–response curves to PL017 ([*N*-MePhe^3, D-Pro^4]morphiceptin) a peripherally acting mu-opioid receptor agonist, and ii) the reversibility of the effects of *conventional* opioid receptor agonists (central and peripherally acting) by the i.p. administration of methyl-naloxone (Met-NX) or CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2), two peripherally acting mu-opioid receptor antagonists (non-selective and selective, respectively).

5. The site/s and possible mechanism/s implicated in the anti-exudative effects of mu and delta opioids were assessed after the s.c. administration of the ED_{80}'s of the agonists to animals treated with: Cyclosporine A (3 mg/kg i.p., for 5 days) (Stein et al., 1990). Capsaicin, which was administered s.c. under general anaesthesia (i.m. ketamine 100 mg/kg, plus i.m. xylazine 10 mg/kg), at doses of 30, 50 and 70 mg/kg on days 1, 2 and 3 before the experiment (Zhou et al., 1998). The peripheral postganglionic sympathetic fibres were damaged/depleted by the i.p. injection of 75 mg/kg of 6-OHDA each day, for three consecutive days (Zhang et al., 1998). The role of the histaminergic system was evaluated by the administration of compound 48/80 i.p.; the drug was given at a dose of 0.6 mg/kg twice daily for three consecutive days, followed by 1.2 mg/kg twice on the fourth day (Ohtsuki et al., 1985). In another series of experiments, we used the receptor-specific histamine receptor antagonists mepyramine (histamine H_1 receptor antagonist, 2.5 mg/kg; s.c.), cimetidine (histamine H_2 receptor antagonist, 50 mg/kg; s.c) and thioperamide (histamine H_3 receptor antagonist, 5 mg/kg; i.p.) (Ichinose et al., 1990; Linardi et al., 2000; Yoshihara et al., 1995).

2.7. Drugs

Carrageenan type IV lambda, Evan's blue dye, and Tween 80, were all purchased from Sigma Chemical (St. Louis, MO, USA), and NaCl from Merck (Farma-Química, S.A., Barcelona, Spain). The drugs used were: morphine hydrochloride (Alcaliber S.A., Madrid, Spain), fentanyl (Fentanest Roche®, Basel, Switzerland), PL017 (Peninsula Laboratories, San Carlos, CA, USA) and SNC 80 (Tocris, Biogen Cientifica S.L., Madrid, Spain). DPDPE, U50,488H, naloxone hydrochloride, methyl-naloxone, naltrindole hydrochloride and CTOP were also purchased from Sigma Chemical (St. Louis, MO, USA). MR-2266 was a generous gift from Boehringer-Ingelheim (Mannheim, Germany). All opioid receptor agonists were administered s.c. at the nape of the neck, 2 h 30 min after the injection of carrageenan,

and opioid receptor antagonists were given i.p. 15 min before the agonists (Fig. 1).

We also used: ketamine hydrochloride (Ketalar® Parke-Davis-Pfizer, NY, USA), xylazine (Rompun®, Bayer, Germany), cyclosporine A (Sandimmun A®, a gift from Novartis Laboratories), formamide (Merck Farma-Química, S.A., Spain). Capsaicin, compound 48/80, DMSO (dimethyl sulfoxide), mepyramine, cimetidine and thioperamide were purchased from Sigma Chemical (St. Louis, MO, USA). Triton X-100 and DPX were obtained from Fluka, Buchs, Switzerland.

For the immunohistochemistry experiments, the following antibodies were used: a polyclonal rabbit anti-protein gene peptide 9.5 (PGP 9.5 Ultraclone, Isle of Wight, England) or a polyclonal rabbit anti-calcitonin gene-related peptide (CGRP, Amersham Int., Arlington Heights, IL), and a goat anti-rabbit cyacine 3.18 second antibody (Jackson ImmunoResearch, West Grove, PA).

2.8. Statistical evaluation

Statistical analysis was performed with the SPSS program (v. 9.0, SPSS, Chicago, IL). Data are expressed as the mean values±S.E.M. The effects produced by different doses of mu-, delta- and kappa-opioid receptor agonists on the inhibition of plasma extravasation were adjusted to a biexponential equation, which provides two slopes (ascendant and descendent). Using the points that define the ascendant part of each dose–response curve, we plotted the results in a double reciprocal manner in order to calculate the ED (20, 50 and 80) values, as a measure of the potency (Tallarida and Murray, 1986).

We used the Student's *t* test to establish significant differences between two groups and when multiples groups were compared, we applied a one-way analysis of variance (ANOVA) followed by a Dunnett's C test, whenever applicable. In all instances, a value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Effects induced by the s.p. injection of carrageenan

The s.p. injection of carrageenan increased the volume (oedema) and plasma extravasation (Evan's blue) of the injected hind paw, which peaked 3 h after the injection (Fig. 2). At this time, both oedema and extravasation similarly increased by approximately two times in the inflamed paw (oedema: 108±4% and plasma extravasation: 95±3% increase). The variables followed a similar time-course, and declined 5 h after carrageenan; however at this time point, plasma extravasation was significantly lower (50±1%) than oedema (75±2%; $P<0.001$). On the basis of these results, all subsequent experiments were carried out 3 h after carrageenan administration.

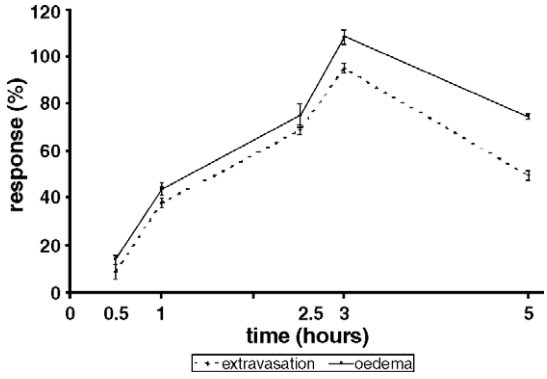

Fig. 2. Time course of oedema (paw volume) and plasma extravasation (Evan's blue) after the subplantar injection of carrageenan in the left hind paw. The results are expressed as percent (%) increase over pre-injection baseline values. Each point represents the mean value of 6–8 animals, and the vertical bars indicate the S.E.M.

3.2. Effects of mu-, delta- and kappa-opioid receptor agonists on oedema and plasma extravasation

In this model, we tested the effects of the s.c. administration of the following opioid receptor agonists on oedema and plasma extravasation: morphine (dose range 0.15–30 mg/kg) and fentanyl (0.01–0.7 mg/kg) as mu-opioid receptor agonists; DPDPE (0.01–1.5 mg/kg) and SNC 80 (0.1–15 mg/kg) as delta-opioid receptor agonists, and the kappa-opioid receptor agonist, U-50,488H (0.1–50 mg/kg). Morphine was the only opioid receptor agonist that induced a measurable anti-oedema effect with a maximal inhibition of 30±7%, obtained after the administration of doses between 0.7 and 10 mg/kg. The anti-oedema effect of morphine was not dose-related.

Each one of the mu- and delta-opioid receptor agonists tested induced a dose-related inhibition of plasma extravasation, with a characteristic biphasic response (Fig. 3A). The experimental results obtained with each drug were adjusted to a biexponential equation, which provides two slopes. Using the points that define the ascendant part of the dose–response curves, we obtained the effective doses (EDs) at different levels of effect, according to the method of Tallarida and Murray (1986).

Table 1 shows the ED values (20, 50 and 80) of the mu- and delta-opioid receptor agonists tested. The four lines had comparable Emax values (99–120%) and slopes (between 52 and 65). At the level of the ED_{50}'s, the relative potencies (determined as the ratio of the ED_{50} of morphine and each one of the opioid receptor agonists) showed that fentanyl and DPDPE were approximately 18 and 7 times more potent than morphine, while SNC 80 was about two times less potent.

No dose-related inhibition of plasma extravasation could be obtained after the administration of the kappa-opioid receptor agonist (U-50,488H); this drug induced a maximal inhibition of extravasation of 36–40% when administered at doses between 0.5 and 10 mg/kg. When higher doses were

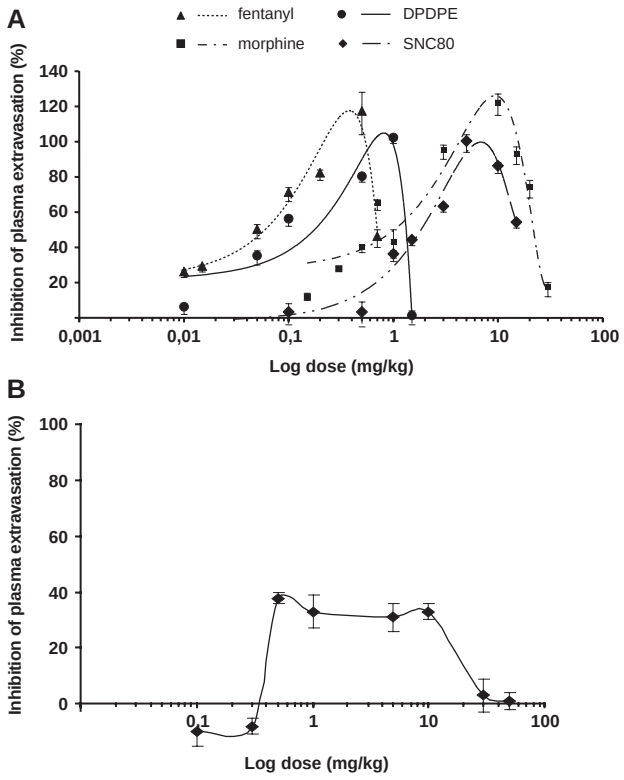

Fig. 3. (A) Effects of the administration of mu- and delta-opioid receptor agonists on plasma extravasation evaluated after the i.v. administration of Evan's blue. Biphasic log dose–response curves obtained after the administration of morphine (squares), fentanyl (triangles), DPDPE (dots) and SNC 80 (diamonds). The experimental results obtained with each opioid receptor agonist have been adjusted to a biexponential equation (see Methods). (B) Effects of the administration of U-50,488H (a kappa-opioid receptor agonist) on the inhibition of plasma extravasation. Each point represents the mean value obtained from 6–8 animals, and the vertical bars indicate the S.E.M.

tested (20 and 50 mg/kg) a biphasic response was also observed (Fig. 3B).

3.3. Reversibility of the effects induced by mu-, delta- and kappa-opioid receptor agonists by the administration of specific antagonists

To perform these experiments, we first tested the effects of the different opioid receptor antagonists administered individually on plasma extravasation. We used naloxone (a non-selective opioid receptor antagonist), naltrindole (delta-opioid receptor antagonist) and MR-2266 (kappa-opioid receptor antagonist); the doses were selected on the basis of previous studies performed by our group (Planas et al., 2000; Valle et al., 2001). In our experimental conditions, the administration of 1 mg/kg naloxone (i.p.) increased carrageenan-induced extravasation by 35±10% ($P<0.05$, when compared to saline), while i.p. naltrindole (3 mg/kg) increased basal extravasation by 48±14%; however, the kappa-opioid receptor antagonist, MR-2266 at a dose of 3 mg/kg, did not have a significant effect (Table 2).

Next we tested the reversibility of the effects of the ED_{80} values of the mu- and delta-opioid receptor agonists (Table 1), after the administration of naloxone (1 mg/kg) or naltrindole (3 mg/kg). For the kappa-opioid receptor agonist U-50,488H, we assessed the reversibility of a dose of 0.5 mg/kg (which produced a maximal inhibition of 40%), by MR-2266 (3 mg/kg). The results show that the effects of all opioid receptor agonists were completely reversed by the administration of specific antagonists, demonstrating the opioid nature of the effects (Table 2). We also observed that the effects of morphine, but not those of fentanyl, were completely reversed by naltrindole (data not shown).

Table 1
Potency (ED_{20}, ED_{50} and ED_{80}) in mg/kg of the mu- and delta-opioid receptor agonists on the inhibition of plasma extravasation

Agonist	ED_{20}	ED_{50}	ED_{80}
mu			
Morphine	0.22±0.02	0.71±0.10	1.92±0.37
Fentanyl	0.01±0.001	0.04±0.007	0.18±0.06
delta			
DPDPE	0.01±0.005	0.10±0.01	0.36±0.04
SNC 80	0.58±0.02	1.70±0.10	5.03±0.49

The data is shown as mean values±S.E.M. of 6–8 animals per dose.

Table 2
Reversibility of the anti-extravasation effects of mu-, delta- and kappa-opioid receptor agonists, by the administration of antagonists

Agonist	Antagonist	Plasma extravasation (%)
Saline	Saline	95±7
mu	mu	
saline	naloxone	130±10[a]
morphine	saline	18±5[c]
morphine	naloxone	111±11
fentanyl	saline	15±5[c]
fentanyl	naloxone	97±8
delta	delta	
saline	naltrindole	143±14[b]
DPDPE	saline	18±6[c]
DPDPE	naltrindole	93±7
SNC 80	saline	14±10[c]
SNC 80	naltrindole	117±6
kappa	kappa	
saline	MR-2266	94±9
U-50,488H	saline	38±5[b]
U-50,488H	MR-2266	96±4

Results are expressed as mean values±S.E.M. of 6–8 animals per dose. The letters a, b and c indicate $P<0.05$, $P<0.01$ and $P<0.001$ respectively, when compared to saline–saline (control) treated animals (one-way ANOVA, Dunnett's C-test). For the mu- and delta-opioid agonists, we tested the ED_{80} values (mg/kg) as shown in Table 1, while for the kappa-opioid agonist (U-50,488H) we tested the reversibility of a dose of 0.5 mg/kg which produced the maximal inhibitory effect (40%). The doses of the antagonists utilized in these experiments were: naloxone (1 mg/kg), naltrindole (3 mg/kg) and MR-2266 (3 mg/kg).

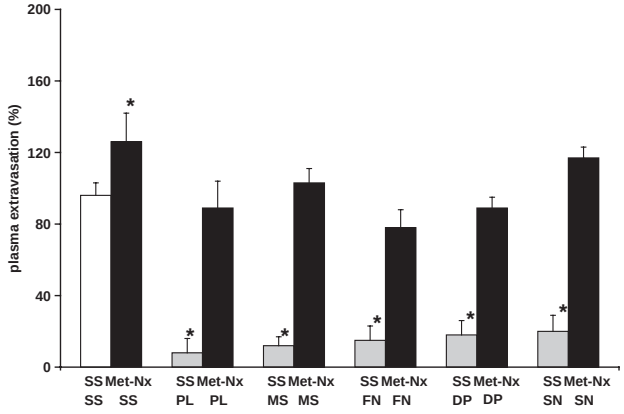

Fig. 4. Reversibility of the anti-exudative effects of mu- and delta-opioid receptor agonists by *N*-methyl-naloxone. In these experiments the ED_{80} values of each agonist were tested in the presence of saline (SS) or 1 mg/kg of *N*-methyl-naloxone (Met-Nx). Each column represents the mean value of 6–8 animals and the vertical bars indicate the S.E.M. The * indicates a $P<0.05$ when compared to the control group which did not receive any drug (SS/SS; one-way ANOVA, Dunnett's *C*-test). PL: PL-017 (0.36 mg/kg); MS: morphine (1.92 mg/kg); FN: fentanyl (0.18 mg/kg); DP: DPDPE (0.36 mg/kg) and SN: SNC80 (5.03 mg/kg).

3.4. Effects of peripherally acting opioids on the inhibition of plasma extravasation

The administration of PL017 (a peripherally acting mu-opioid receptor agonist) induced a biphasic dose–response curve, similar to those obtained with morphine or fentanyl. The ascending portion of the curve had a slope of 62.3±3.2 and an Emax of 118±10, while the calculated ED values were: ED_{20}=0.03±0.003, ED_{50}=0.11±0.02 and ED_{80}= 0.36±0.08 mg/kg. Thus at the 50% level of effect, PL017 was approximately 6.5 times more potent that morphine on the inhibition of plasma extravasation. The effects of the ED_{80} value of PL017 were completely antagonised by two peripherally acting mu-opioid receptor antagonists, administered i.p., each at a dose of 1 mg/kg: CTOP (mu-selective) and *N*-methyl-naloxone (Met-NX, non-selective). Moreover the administration of Met-NX alone, induced a 30±10% increase in plasma extravasation (Fig. 4) which was statistically significant when compared to the control values (SS-SS).

Due to the difficulty in obtaining receptor-selective peripherally acting delta and kappa opioids (agonists and antagonists), the peripheral effects of conventional mu- and delta-opioid receptor agonists (which have central and peripheral effects) were evaluated by the administration of Met-NX. The inhibitory effects of the ED_{80}'s of morphine and fentanyl (mu-opioid receptor agonists), as well as those of DPDPE and SNC80 (delta-opioid receptor agonists) were completely reversed by Met-Nx (Fig. 4). The results indicate that the effects on plasma extravasation induced by the administration of mu- or delta-opioid receptor agonists are predominantly mediated by interaction with peripheral opioid receptors.

3.5. Effects of mu and delta opioids on plasma extravasation in animals pretreated with cyclosporine A

The administration of cyclosporine A (see Methods) did not alter % baseline plasma extravasation, which was 114±7% and 80±17% in controls and cyclosporine A-treated animals, respectively (n=6 rats/group). In these animals, the administration of the ED_{80}'s of morphine (1.92 mg/kg), fentanyl (0.18 mg/kg) or DPDPE (0.36 mg/kg), induced inhibitory responses, which were not significantly different from the responses obtained in saline-treated animals.

3.6. Effects of the pretreatment with capsaicin on the inhibition of plasma extravasation induced by mu and delta opioids

The administration of capsaicin for three days (see Methods) induced a marked decrease in the density of sensory fibres in the inflamed paw, demonstrated by immunohistochemistry (Fig. 5). In order to confirm the effectiveness of the treatment with capsaicin, we tested these animals for mechanical hyperalgesia using a modified Randall and Selitto test (1957). The results show that in animals pretreated with vehicle (saline) for 3

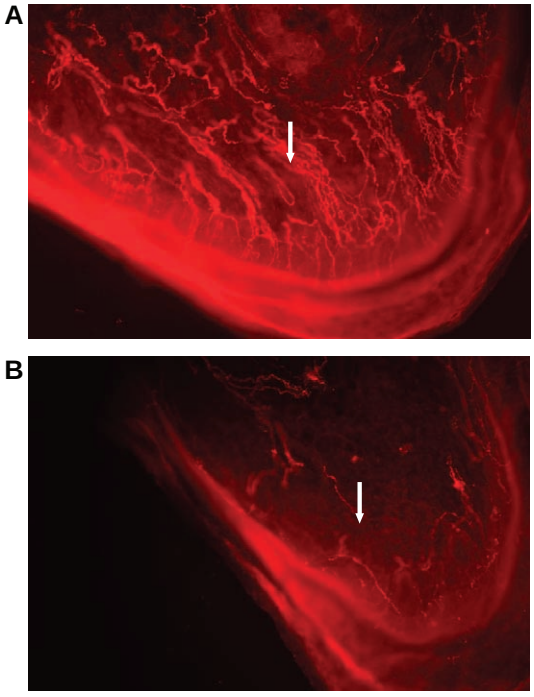

Fig. 5. Immunohistochemistry experiments performed with anti-PGP 9.5 and anti-CGRP antibodies showing the density of sensory fibres stained in red. The figure shows a representative experiment of tangential sections of the soft tissue taken from the plantar aspect of the rat paw, obtained after carrageenan-induced inflammation. Control animals (A), and rats (B) that received capsaicin for a period of 3 days; the arrows indicate sensory fibres. A decreased density of sensory fibres is observed in capsaicin-treated animals (magnification: ×400).

Table 3
Effects of capsaicin, 6-OHDA and 48/80 pretreatment on the percent (%) inhibition of plasma extravasation induced by an ED_{80} value of morphine, fentanyl or DPDPE

Treatment	Vehicle	Capsaicin	6-OHDA	48/80
Morphine	85±6[a]	96±4[a]	0.4±0.003[b]	2.5±6[b]
Fentanyl	82±10[a]	6±8[b]	3±6[b]	45±17[c]
DPDPE	81±10[a]	63±9[b]	32±12[c]	74±14[a]

Results are expressed as mean values±S.E.M. of 6–8 animals per group. One-way ANOVA was used to establish differences between the inhibitory effects of each agonist (morphine, fentanyl or DPDPE) in the different experimental conditions (treatments: vehicle, capsaicin, 6-OHDA, 48/80). For each drug, the same letter (placed next to the numeric value) indicates that there are no significant differences between treatments, while different letters (a, b, c) show significant differences with a $P<0.05$ (one-way ANOVA, Dunnett's C-test).

days, PPT values in the inflamed paw (203±8 g) were significantly lower than in the noninflamed paw (301±9 g; $P<0.05$, Student's t test), demonstrating that carrageenan induces mechanical hyperalgesia. However, in capsaicin-treated animals, PPT values were slightly but significantly increased in the noninflamed paw (332±7 g), but greatly enhanced in the inflamed paw (640±14 g) when compared to the respective controls ($P<0.05$, Student's t test). Thus pretreatment with capsaicin had a significant anti-hyperalgesic effect both in the inflamed and noninflamed paws.

Plasma extravasation was not altered in the inflamed paw of animals that received capsaicin, when compared to vehicle-treated animals (vehicle 105±15% and capsaicin 110±12%). In these animals, we evaluated the effects of the ED_{80}'s of morphine, fentanyl and DPDPE on plasma extravasation. The results show (Table 3) that the inhibitory effects of morphine are unaltered by pretreatment with capsaicin; however, in the same experimental conditions, the effects of fentanyl were completely abolished and those of DPDPE decreased by approximately 22%.

3.7. Inhibitory effects of morphine, fentanyl and DPDPE on plasma extravasation in animals treated with 6-OHDA

The selective damage of sympathetic postganglionic fibres by 6-OHDA, induced a significant decrease in plasma extravasation when compared to untreated animals. Percent extravasation was 110±15% in controls and 52±10% in 6-OHDA-treated animals ($P<0.001$, Student's t test). In these experimental conditions, the inhibitory effects of the ED_{80}'s of morphine, fentanyl and DPDPE were significantly decreased ($P<0.05$). The results show (Table 3) that whereas the anti-extravasation effects of morphine and fentanyl were abolished after 6-OHDA, the inhibitory effects of DPDPE were reduced by approximately 60% when compared to animals receiving vehicle.

3.8. Effects of the administration of the compound 48/80 on the inhibition of plasma extravasation induced by morphine, fentanyl and DPDPE

Pretreatment with the compound 48/80 that depletes histamine from mast cells, induced a 11.5±1% increase in plasma extravasation, which was not statistically significant from non-treated rats. In these animals, we tested the effects of the ED_{80}'s of morphine, fentanyl and DPDPE and observed that the inhibitory effect of morphine was completely abolished. The effect of fentanyl was significantly decreased (by approximately 45%), while that of DPDPE was unaltered (Table 3; $P<0.05$). Thus, mast cell integrity (and probably histamine release) seems to be required for the anti-extravasation effects of morphine, and in a lesser degree fentanyl.

3.9. Effects of the administration of histamine receptor antagonists on the inhibition of plasma extravasation induced by morphine and fentanyl

The unexpected findings obtained with compound 48/80 on the anti-extravasation effects of morphine and fentanyl, were further investigated by the administration of receptor-specific histamine receptor antagonists. We hypothesised that the local release of histamine from mast cells and its

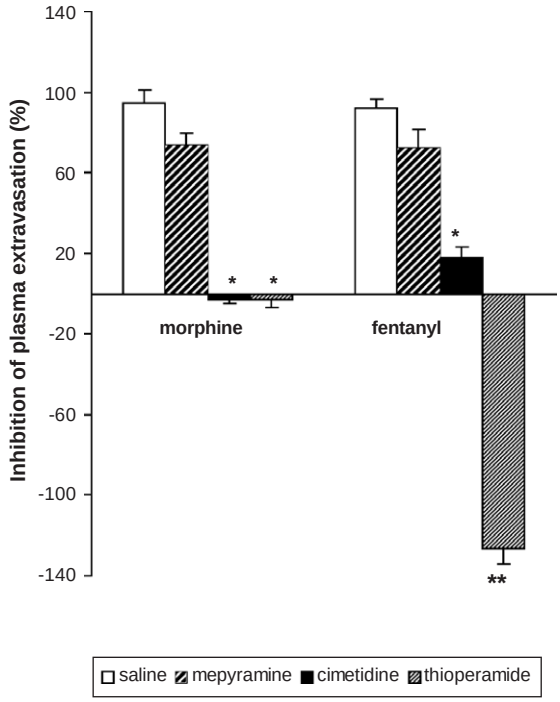

Fig. 6. Inhibitory effects of the ED_{80}'s of morphine (1.92 mg/kg) and fentanyl (0.18 mg/kg) on plasma extravasation in animals treated with i.p. saline, mepyramine (2.5 mg/kg), cimetidine (50 mg/kg) or thioperamide (5 mg/kg). Each column represents the mean value of 6–8 animals and the vertical bars indicate the S.E.M. For each drug (morphine, fentanyl) *$P<0.01$ and **$P<0.001$ indicate the level of significance when compared to the group that received saline (one-way ANOVA, Dunnett's C-test).

binding to neuronal and non-neuronal receptors could alter the anti-extravasation effects of mu opioids. Therefore, we evaluated the anti-exudative effects of the ED_{80}'s of morphine and fentanyl in the presence of mepyramine (histamine H_1 receptor antagonist), cimetidine (histamine H_2 receptor antagonist) or thioperamide (histamine H_3 receptor antagonist). The doses of the antagonists were those that have been proven to antagonise the effects of histamine on the different receptors, in rats (Ichinose et al., 1990; Linardi et al., 2000; Yoshihara et al., 1995).

At the doses tested, none of the histamine receptor antagonists by themselves had an effect on plasma extravasation. The inhibitory effects of morphine (ED_{80}) were completely abolished by cimetidine (50 mg/kg) and thioperamide (5 mg/kg), but unaltered by mepyramine (2.5 mg/kg) ($P<0.05$ when compared to saline). Similarly, mepyramine did not modify the effects of fentanyl (ED_{80}), which again were reversed by cimetidine (Fig. 6). However, the administration of fentanyl 15 min after thioperamide (see Methods) significantly increased plasma extravasation by approximately 2.2 times ($P<0.05$). This unexpected interaction is under investigation by our group. These results suggest that the presence of histamine and the availability of histamine H_2 and H_3 receptors are required in order to observe the effects of mu-opioid receptor agonists on plasma extravasation.

4. Discussion

In the present study we have evaluated the effects of mu, delta and kappa opioids on oedema and plasma extravasation in a rat model of carrageenan-induced acute inflammation. In this model, the subplantar injection of carrageenan induces oedema, plasma extravasation and hyperalgesia (Planas et al., 1995, 2000), which peaks approximately 3 h after carrageenan, and then progressively declines over a period of 5 h. Oedema receded slower than extravasation, a fact that could be related to the limitations of plethysmography; however, Evan's blue accurately measures plasma extravasation in the inflamed paw (Udaka et al., 1970).

In our experimental conditions, none of the opioid receptor agonists tested had an anti-oedema effect except morphine, which induced a 30% inhibition regardless of the dose administered (range of 0.7–10 mg/kg), a finding that has been previously reported by other investigators (Joris et al., 1990; Alebouyeh et al., 2002; Amann et al., 2002). We could not demonstrate an anti-oedema effect of U-50,488H (a kappa-opioid) but the effects of kappa-opioid receptor agonists on oedema are controversial (Barber, 1993; Catheline et al., 1999; Machelska et al., 1999), a fact that is likely related to the different experimental conditions used, mainly the type of inflammation. During peripheral inflammation kappa-opioid receptors are found on immune (Suzuki et al., 2001) and neural cells, and the type of inflammation (neurogenic or not) may influence or even determine the observed effects after the administration of kappa-opioid receptor agonists.

A number of groups have previously reported dose-related inhibitions of plasma extravasation induced by mu- and delta-opioid receptor agonists (Green and Levine, 1992; Barber, 1993; Taylor et al., 2000), as well as a biphasic (bell-shaped) response produced by morphine (Lei and Rogers, 1999). However, this is the first report that demonstrates a biphasic effect of delta- and kappa-opioid receptor agonists on plasma extravasation during acute inflammation, although a biphasic response has been observed for other effects of opioids including thermoregulation (Salmi et al., 2003), locomotor activity (Kuzmin et al., 2000), neurotransmitter release (Maisonneuve et al., 2001) and T-lymphocyte stabilisation/destabilisation (Donahoe et al., 2001). The mechanism/s involved in this type of response are unknown at present. However the work of Lei and Rogers (1999) suggests that opioid receptors located on sensory fibres and immune cells could be selectively activated by low (neuronal) or high (non-neuronal) doses of mu- and delta-opioid receptor agonists, and as a consequence, a decrease or an increase in plasma extravasation would occur, explaining the biphasic response. Other likely explanations could be related to the activation of different opioid receptor subtypes, or to high- and low-affinity states for the same receptor (Pasternak, 2001a).

In our experiments we calculated the EDs of the agonists on the basis of the ascending portion of the bell shaped curves, since these doses are roughly the same than those that induce antinociception in the same model (Planas et al., 2000). The contribution of peripheral opioid receptors in the modulation of plasma extravasation was demonstrated by the similarity of effects induced by conventional (with central and peripheral action) and peripherally acting agonists, and by the reversibility of the effects of conventional opioids by peripherally acting antagonists. These results are supported by the fact that the local administration of mu- and delta-opioid receptor agonists, at doses that show no systemic effect, decrease plasma extravasation during peripheral inflammation (Hong and Abbott, 1995).

Taking the ED_{50} of morphine as a standard, we could demonstrate that fentanyl (mu-opioid receptor agonist) and DPDPE ($delta_1$ opioid receptor agonist) were 17 and 7 times more potent on the inhibition of plasma extravasation, while SNC 80 (a $delta_{1-2}$ opioid receptor agonist) was about two times less potent than morphine. The results suggest that mu- and delta-opioid receptors are involved in the inhibitory control of plasma extravasation during peripheral inflammation; the view is supported by the significant increase in extravasation observed after the administration of naloxone (non-selective opioid receptor antagonist) and naltrindole (delta-opioid receptor antagonist), but not MR-2266 (kappa). On the basis of these findings we hypothesise that carrageenan-induced inflammation produces a local release of endogenous mu and delta opioids from non-neural cells

(beta-endorphin, enkephalins), that bind to peripheral opioid receptors (neuronal and non-neuronal) and modulate plasma extravasation in the rat paw.

The role of the immune system in the anti-exudative effects of exogenously administered mu and delta opioids was investigated after the administration of cyclosporine A. This compound induces cellular immunosuppression by inhibiting the activation and proliferation of T- and B-lymphocytes, macrophages and other immunocytes at the site of inflammation. As a consequence, the number of opioid receptors located in immune cells is also reduced (Stein et al., 1990; Schäfer et al., 1994). Based on the behavioural experiments, we could conclude that opioid receptors in immune cells do not contribute to the inhibitory effects of low doses of exogenous opioids on plasma extravasation. However, the efficacy of the cyclosporine treatment on the immune cells and the density of opioid receptors in these cells (in the paw) were not tested, and thus these results cannot be considered conclusive.

The contribution of mu- and delta-opioid receptors located at other peripheral anatomical sites (nociceptors, sympathetic fibres, mast cells) was assessed using different pharmacological tools. To evaluate the role of the primary afferent fibres we used animals pretreated with capsaicin, which resulted in a major loss of C-fibres and a significant anti-hyperalgesic effect, thus confirming the efficacy of the treatment (Zhou et al., 1998). Interestingly, no changes in baseline plasma extravasation were observed after capsaicin, suggesting that capsaicin-sensitive fibres do not play an important role in carrageenan-induced plasma extravasation. Similar results were reported when evaluating the volume (oedema) of the inflamed paw, which was also unaffected by the administration of capsaicin (Zhou et al., 1998).

An unexplained finding is that while the inhibition of extravasation induced by morphine is unaltered by capsaicin, fentanyl effects are completely abolished, suggesting different sites and mechanisms of action for the two mu-opioid receptor agonists. We are not aware of similar reports in the literature suggesting different mechanisms of action of morphine and fentanyl on the inhibition of plasma extravasation. However, it has been shown that the same drugs induce dissimilar patterns of antinociception in various animal models as well as in man, a fact that has been explained by binding to different sub-types of mu-opioid receptors (Pasternak, 2001b) or to mu/delta-opioid receptor heterodimers (Gomes et al., 2004). The latter is supported by the complete reversal of the effects of morphine (but not fentanyl) by naltrindole.

The anti-exudative response to the delta-opioid receptor agonist DPDPE was decreased in capsaicin-treated animals, thus opioid receptors located in sensory fibres seem to participate in the inhibitory effects of fentanyl and DPDPE on plasma extravasation.

Pretreatment with 6-OHDA induced a 52% decrease in plasma extravasation, demonstrating that the integrity of sympathetic fibres is required for extravasation during acute inflammation (Coderre et al., 1989). In these animals, mu-opioid receptor agonists had no anti-exudative effect, while the effects of DPDPE were greatly and significantly reduced. These experiments show that both mu- and delta-opioid receptors, present on postganglionic sympathetic fibres are involved in the anti-extravasation effects of exogenous opioids. A possibility that was not explored in our study is that norepinephrine present in sympathetic fibres (Levine et al., 1986) may be required for the anti-extravasation effects of mu- and delta-opioid receptor agonists.

In our model, pretreatment with compound 48/80 or with specific histamine receptor antagonists did not alter plasma extravasation, indicating that histamine is not the primary pro-inflammatory agent implicated in the exudative response induced by carrageenan. Similarly, histamine does not seem to be involved in the modulation of nociception, since the administration of different receptor-specific histamine receptor antagonists alone does not alter nociceptive responses in mice (Suh et al., 1999).

However, our results show that the presence of histamine is required for the anti-exudative effects of morphine and in a lesser degree of fentanyl. Treatment with 48/80 not only reduces histamine in mast cells, but also other mediators such as cytokines and chemokines. The results also show that the anti-extravasation effects of delta-opioid receptor agonists are unaltered by 48/80, thus histamine (or cytokines) are not required for delta-opioid receptor activation by specific agonists. Blocking histamine H_2 and H_3 (but not H_1) receptors abolished the anti-exudative effects of morphine and fentanyl; moreover, the simultaneous administration of fentanyl and thioperamide (histamine H_3 receptor antagonist) enhanced plasma extravasation by approximately 2 times. At present we have no explanation for the interaction between these two drugs, a finding that is under active investigation by our group. The results obtained when blocking histamine receptors or depleting mast cells reflect the complex mechanism and interactions involved in the anti-exudative effects of opioids during acute peripheral inflammation. The role of histamine receptors in opioid-induced antinociception has been investigated by several groups (Suzuki et al., 1994; Owen et al., 1994; Suh et al., 1999); the reported results show that all three histamine receptor antagonists attenuate the antinociceptive effects of morphine. Thus suggesting that histamine H_1 receptors may be involved in nociception but not in plasma extravasation.

In summary, our results show that during carrageenan-induced inflammation, mu- and delta-opioid receptor agonists decrease plasma extravasation in a dose-dependent manner, with a characteristic bell-shaped curve. The inhibitory effects are reversed by specific antagonists, and are mediated by binding (mainly) to peripheral opioid receptors. The likely location of these receptors in the inflamed tissues was investigated using capsaicin, 6-OHDA, 48/80 and receptor-specific anti-histaminergic drugs. The

results indicate that the integrity of sensory and sympathetic fibres is needed for fentanyl and DPDPE to induce an inhibition of plasma extravasation. Moreover, the presence of histamine, probably acting through histamine H_2 and H_3 receptors, is required for the anti-exudative effects of morphine and fentanyl, but not of DPDPE. The results suggest that different mechanisms may be involved in the anti-exudative effects of morphine and fentanyl. These findings reflect the complex role of endogenous opioids and their receptors during peripheral inflammation, and suggest that multiple sites and mechanisms may be involved in the anti-exudative effects of exogenous opioids.

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