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Intraplantar morphine depresses spinal c-Fos expression induced by carrageenin inflammation but not by noxious heat

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1 We have studied the effects of intraplantar administration of the same doses of morphine on intraplantar carrageenin (6 mg 150 μl^{-1} of saline) and noxious heat (52°C for 15 s) induced spinal c-Fos expression and inflammation.

2 Intraplantar carrageenin, in awake rats, induced numerous Fos-like immunoreactive (Fos-LI) neurones in the dorsal horn of L4-L5 lumbar segments of the spinal cord and extensive peripheral oedema. At 1 h 30 min, Fos-LI neurones were preferentially located in the superficial laminae (74±2%) whereas at 3 h, Fos-LI neurones were observed both in the superficial (45±2%) and deep (37±1%) laminae of the spinal dorsal horn.

3 Intraplantar morphine dose-dependently reduced c-Fos expression induced 1 h 30 min after carrageenin ($r=0.605$, $P<0.02$), these effects were completely blocked by intraplantar methiodide naloxone (20 μg) (121±22% of control carrageenin expression). The systemic injection of the highest dose of intraplantar morphine (50 μg) had no significant effect on the number of Fos-LI neurones (88±9% of control carrageenin expression). None of the drugs influenced unilateral peripheral oedema observed 1 h 30 min after carrageenin.

4 In the second series of experiments, intraplantar morphine dose-dependently reduced the number of superficial and deep Fos-LI neurones induced 3 h after carrageenin ($r=0.794$, $P<0.0004$ and $r=0.698$, $P<0.004$, respectively). Furthermore, the effects of the highest dose of intraplantar morphine were completely blocked by co-administration of intraplantar methiodide naloxone (20 μg).

5 In addition, intraplantar morphine dose-dependently reduced the ankle ($r=0.747$, $P<0.002$) and paw ($r=0.682$, $P<0.005$) oedema observed 3 h after carrageenin, with the effect of the highest dose of intraplantar morphine being completely blocked by co-administration of methiodide naloxone (98±4% and 102±8% of control paw and ankle oedema, respectively).

6 Brief noxious heat stimulation, in urethane anaesthetized rats, induced, 2 h after the stimulation, numerous Fos-LI neurones in the dorsal horn of L3-L4 lumbar segments of the spinal cord but no detectable peripheral oedema. Fos-LI neurones were preferentially located in superficial laminae (94±2%) of the spinal dorsal horn. None of the drugs influenced the noxious heat induced c-Fos expression.

7 Such results illustrate that peripheral effects of morphine preferentially occur during inflammatory states and outline the interest of extending clinical investigations of the possible use of local injection of morphine in various inflammatory pain states.

Keywords: Intraplantar morphine; c-Fos expression; nociception; carrageenin; noxious heat; anti-inflammatory effects

Introduction

Traditionally antinociceptive effects of opiates have been associated with activation of opioid receptors located in the central nervous system (Lim *et al.*, 1964; for recent review see Dickenson, 1994). However, there is accumulating experimental (Ferreira & Nakamura, 1979; Hargreaves *et al.*, 1988b; Levine & Taiwo, 1989; Ferreira *et al.*, 1990; Haley *et al.*, 1990; Parsons *et al.*, 1990; Joris *et al.*, 1987; 1990; Stein *et al.*, 1988a,b; 1989; 1990a; Kayser *et al.*, 1991; Hong & Abbott, 1995; Schäfer *et al.*, 1995) and clinical (Mays *et al.*, 1987; Posner *et al.*, 1990; Stein *et al.*, 1991; Heard *et al.*, 1992; Joshi *et al.*, 1992; 1993a,b; Khouri *et al.*, 1992; Allen *et al.*, 1993; McSwiney *et al.*, 1993; Dalsgaard *et al.*, 1994; see however Bullingham *et al.*, 1983; 1984; Raja *et al.*, 1986; Dahl *et al.*, 1988) evidence that, in particular under inflammatory conditions, exogenous opioids can also produce antinociceptive effects by interacting with peripheral opioid receptors (for review see Kayser & Guilbaud, 1994; Stein, 1993; 1994). This could be explained either by the fact that opioid receptors are present on sensory nerve terminals and that inflammatory processes increase their number and their axonal transport from the dorsal root ganglion cells to the periphery (see Laduron, 1984; Stein *et al.*, 1990b; Hassan *et al.*, 1993; Jeanjean *et al.*, 1994; Schäfer *et al.*, 1995) or more probably by a disruption of the perineurial barrier induced by the inflammation which leads to a better access of morphine to the peripheral opioid receptors (Antonijevic *et al.*, 1995). Indeed, in the rat, the effects of intraplantar injection of opioid agonists have been essentially detected in the inflamed paw following the peripheral injection of various agents such as Freund's adjuvant (Stein *et al.*, 1988a,b; 1989; Schäfer *et al.*, 1995), carrageenin or prostaglandins (Ferreira & Nakamura, 1979; Joris *et al.*, 1987; Hargreaves *et al.*, 1988b; Levine & Taiwo, 1989) and intraplantar injection of formalin (Haley *et al.*, 1990; Hong & Abbott, 1995). In contrast no or extremely weak antinociceptive effects were detected in non inflamed paw (Ferreira & Nakamura, 1979; Stein *et al.*, 1988a,b; 1989; Haley *et al.*, 1990). Furthermore, it has been shown that the peripheral action of opioid agonists does not take place when brief nociceptive stimulations are applied (Raja *et al.*, 1986; Senami *et al.*, 1986; Abbott, 1988; Levine & Taiwo, 1989).

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The aim of this study was to investigate whether intraplantar morphine could influence c-Fos expression, at the spinal level, which is one of the long term intracellular events (for review see Morgan, 1991; Morgan & Curran, 1995) which is used as an indirect marker of nociceptive processes (see references in Zieglgansberger & Tölle, 1993; Abbadie *et al.*, 1994b). It must be noted that preadministered intravenous, intracerebroventricular or subcutaneous morphine has been shown to depress c-Fos expression in the dorsal horn of the spinal cord induced by various types of peripheral nociceptive stimulations such as intraplantar injection of formalin (Presley *et al.*, 1990; Gogas *et al.*, 1991; Jasmin *et al.*, 1994), intraplantar injection of carrageenin (Chapman *et al.*, 1995; Honoré *et al.*, 1995d,e), intraperitoneal injection of acetic acid (Hammond *et al.*, 1992), noxious heat (Abbadie *et al.*, 1994c; Tölle *et al.*, 1990; 1991; 1994a,b), noxious cold (Abbadie *et al.*, 1994a), noxious mechanical stimulation (Abbadie & Besson, 1993) and chemical stimulation of the meninges (Nozaki *et al.*, 1992).

The effects of various doses of intraplantar morphine were tested on c-Fos expression induced by either carrageenin inflammation or a single brief noxious heat stimulus. Both types of stimulation have been shown to induce c-Fos expression in the dorsal horn of the spinal cord which was dose-dependently depressed by pretreatment with systemic morphine (see above). Furthermore we have performed antagonist reversal studies, with the intraplantar injection of the opioid antagonist methiodide naloxone which does not cross the blood brain barrier (Iorio & Frigeni, 1984; Milne *et al.*, 1990), thus determining the selectivity of these effects. In addition, in order to confirm that intraplantar morphine, even for the highest dose, produced its effect, if any, via a peripheral site of action, an equal dose of morphine was administered intravenously as a control. Part of this work has been presented previously as an abstract (Honoré *et al.*, 1995b).

Methods

Experimental animals

Experiments were performed on 89 adult male albino Sprague-Dawley rats (Charles River, France), weighing 225–250 g. Guidelines on ethical standards for investigations of experimental pain in conscious animals were followed (Zimmermann, 1983). Rats were kept in an animal room at a constant temperature of 22°C, with a 12 h alternating light-dark cycle.

In the first series of experiments, the effects of intraplantar injection of morphine (morphine hydrochloride, injectable solution, 10 mg ml^{-1}, Meram, diluted in normal saline), methiodide naloxone (Research Biochemicals International, dissolved in saline) and the co-administration of morphine and methiodide naloxone on c-Fos expression induced 1 h 30 min after carrageenin were studied. The duration of 1 h 30 min was chosen because we have shown that the peak effect of intravenous morphine occurs on c-Fos expression induced 1 h 30 min after intraplantar carrageenin (Honoré *et al.*, 1995d,e). Intraplantar injection of morphine (10, 25 or 50 μg 50 μl^{-1}, in saline, $n=5$ for each group) was administered at the same time as intraplantar injection of carrageenin (6 mg 150 μl^{-1}, in saline). Intraplantar injection of methiodide naloxone (20 μg 50 μl^{-1}, in saline, $n=5$) was administered at the same time as intraplantar injection of carrageenin. Intraplantar injection of morphine (50 μg 50 μl^{-1}) and methiodide naloxone (20 μg 50 μl^{-1}) was administered at the same time as intraplantar injection of carrageenin ($n=5$). A group of rats received the highest dose of morphine in intravenous route of administration, 10 min prior to intraplantar injection of carrageenin ($n=5$). A control group of carrageenin-stimulated rats received an equal volume of intraplantar saline at the same time as intraplantar injection of carrageenin ($n=5$).

In the second series of experiments, the effects of intraplantar injection of morphine, methiodide naloxone and the co-administration of morphine and methiodide naloxone on c-Fos expression induced 3 h after intraplantar injection of carrageenin were studied. The duration of 3 h was chosen because we have shown that 3 h after intraplantar carrageenin injection, a maximal number of Fos-LI neurones were observed in both superficial and deep laminae of the dorsal horn of the spinal cord (Honoré *et al.*, 1995a,b). In addition, maximal oedema and mechanical and thermal hyperalgesia were observed at this time point (Kayser & Guilbaud, 1987; Hargreaves *et al.*, 1988a; Iadarola *et al.*, 1988; Joris *et al.*, 1990). Intraplantar injection of morphine (10, 25 or 50 μg 50 μl^{-1}, in saline, $n=5$ for each group) was administered at the same time as intraplantar injection of carrageenin (6 mg 150 μl^{-1}, in saline). Intraplantar injection of methiodide naloxone (20 μg 50 μl^{-1}, in saline, $n=5$) was administered at the same time as intraplantar injection of carrageenin. Intraplantar injection of morphine (50 μg 50 μl^{-1}) and methiodide naloxone (20 μg 50 μl^{-1}) was administered at the same time as intraplantar injection of carrageenin ($n=5$). A control group of carrageenin-stimulated rats received an equal volume of intraplantar saline at the same time as intraplantar carrageenin ($n=5$). In this experimental series, we have not investigated the effect of the highest dose of morphine injected systemically on c-Fos expression induced 3 h after intraplantar carrageenin since we have already demonstrated that an equally low dose of intravenous morphine does not influence c-Fos expression induced 3 h after intraplantar carrageenin (Chapman *et al.*, 1995).

In the third series of experiments, the effects of intraplantar injection of morphine, methiodide naloxone and the co-administration of morphine and methiodide naloxone on c-Fos expression induced 2 h after noxious thermal stimulation (one stimulation of 52°C for 15 s) were studied. We have chosen to observed c-Fos expression 2 h after noxious heat stimulation because we have previously shown that at this time point after noxious heat we had numerous Fos-LI neurones in the dorsal horn of the rat spinal cord (Abbadie *et al.*, 1994c) and in addition intravenous morphine is more efficacious on c-Fos expression observed a short time after stimulation (Honoré *et al.*, 1995d,e). In this experimental series, for ethical reasons, rats were deeply anaesthetized with urethane (1500 mg kg^{-1}, i.p.; ethyl carbamate, Prolabo), and the right hind paw was dipped in water (52°C) for 15 s. Intraplantar injection of morphine (50 μg 50 μl^{-1}, in saline, $n=4$) was administered 5 min before the stimulation. Intraplantar injection of methiodide naloxone (20 μg 50 μl^{-1}, in saline, $n=5$) was administered 5 min before the stimulation. Intraplantar injection of morphine (50 μg 50 μl) and methiodide naloxone (20 μg 50 μl^{-1}) was administered 5 min before the stimulation ($n=5$). A group of rats received the highest dose of morphine by intravenous route of administration, 5 min before the stimulation ($n=5$). A control group of heat-stimulated rats received an equal volume of intraplantar saline 5 min before the stimulation ($n=5$).

Immunohistochemistry

At 1 h 30 min or 3 h after intraplantar carrageenin injection and 2 h after noxious thermal stimulation, the animals were deeply anaesthetized with pentobarbitone (55 mg kg^{-1}. i.p.; Sanofi) and perfused intracardially with 200 ml of phosphate buffered saline 0.1 M (PBS) followed by 500 ml of 4% para formaldehyde in 0.1 M phosphate buffer (PB). The spinal cord was then removed and postfixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose in PB. Frontal frozen sections, 40 μm thick, were cut and collected in PB to be processed immunohistochemically as free floating sections.

The serial sections from the lumbar segment were immunostained for c-Fos-like protein according to the avidin-biotin-peroxidase method (Hsu *et al.*, 1981). The tissue sec-

tions were incubated for 30 min at room temperature in the blocking solution of 3% normal goat serum in phosphate buffer 0.1 M + saline 0.9% (PBS) with 0.3% Triton-X (NGST) and were then incubated overnight at 4°C in the primary antiserum directed against the c-Fos protein. The c-Fos antibody (Oncogene Science Inc.; Ab-2 solution, 0.1 mg ml^{-1}; diluted at 1:4000) is a rabbit polyclonal antibody directed against residues 4–17 of the N-terminal region of the peptide. The incubated sections were washed 3 times in 1% NGST and incubated in biotinylated goat anti-rabbit Ig G for 1 h at room temperature, then washed twice in 1% NGST and incubated for 1 h in Avidin-Biotin-Peroxidase complex (Vectastain, Vector Laboratories). Finally, the sections were washed 3 times in PBS and developed in 1-naphtol ammonium carbonate solution (89.5 ml 0.1 M PB, 10 ml ammonium carbonate (1% in distilled water), 0.5 ml 1-naphtol (N-199-2 Aldrich, 10% in absolute alcohol) and 0.1 ml hydrogen peroxide) for 5 min, and were washed 3 times in PB to stop the staining reaction. The sections were mounted on gelatine-subbed slides and air dried for the stain to be intensified and made alcohol resistant through basic dye enhancement in 0.025% crystal violet (42555 Aldrich) in PB for 3 min. After 2 short PB rinses to take off the excess stain, sections were differentiated in 70% alcohol and the differentiation time was evaluated under the microscope. After being air dried, the slides were coverslipped. To test the specificity of the primary antibody, controls were performed; preabsorption with the corresponding synthetic peptide or omission of any stage in the protocol abolished the staining.

Counting of c-Fos labelled neurones

Tissue sections were first examined using darkfield microscopy to determine the segmental level according to Molander *et al.* (1984), as well as the gray matter landmarks. The sections were then examined under lightfield microscopy at X10 to localise c-Fos positive cells. Labelled nuclei were counted using a camera lucida attachment. To study the laminar distribution 4 regions were defined: superficial dorsal horn (laminae I-II; superficial), nucleus proprius (laminae III-IV; nucleus proprius), neck of the dorsal horn (laminae V–VI; neck) and the ventral gray (laminae VII-X; ventral).

We have previously shown that the most numerous c-Fos positive neurones were localised in the L4–L5 segments after carrageenin and in the L3–L4 after thermal stimulation, so for all the pharmacological studies, for each rat, two sets of analyses were made: (1) the total number of Fos-LI neurones in the gray matter for 10 sections through L4–L5 or L3–L4 segments depending of the stimulation, and (2) the number of Fos-LI neurones per specific defined region of the spinal gray matter in these 10 sections.

Evaluation of inflammation

In order to assess the level of the peripheral inflammation, we considered two inflammatory parameters at the time the animals were killed, the diameters of both the ankle and paw, ipsilateral and contralateral to the stimulation, measured with a calliper square.

Statistical tests

Statistical analysis was performed to compare the total number of Fos-LI neurones, using 1-way analysis of variance for the different groups of animals, and 2-way analysis of variance for the different groups of animals and the laminar region. To compare the ankle or the paw diameters we used 1-way analysis of variance for the different groups of animals. For multiple comparisons, the Fisher's PLSD (Protected Least Significant Difference) test was used. Linear regression (R) has been used for dose-dependent effects of morphine on a single parameter. Correlation coefficients (r) were used for correlation between the effects of morphine on c-Fos and oedema. The investigator responsible for plotting and counting the Fos-LI neurones was blind to the experimental situation of each animal.

Results

Effects of intraplantar morphine on carrageenin induced Fos-LI neurones and peripheral oedema

After intraplantar carrageenin, Fos-LI nuclei, which were stained to a variable degree, were located in the ipsilateral dorsal horn of the spinal cord. All Fos-LI nuclei were analysed without considering the intensity of the staining. In agreement with previous studies of carrageenin evoked c-Fos expression (Honoré *et al.*, 1995a,b), Fos-LI neurones were observed in lumbar segments L2–L6, with maximal labelling in segments L4 and L5.

Fos expression and peripheral oedema induced at 1 h 30 min after carrageenin The total number of Fos-LI neurones observed 1 h 30 min after intraplantar injection of carrageenin was 76±2 Fos-LI neurones per section, in segments L4–L5. The Fos-LI neurones were preferentially located in the superficial laminae (I–II) of the dorsal horn (74±2%) while fewer neurones were observed in the nucleus proprius (1±1%), the deep laminae (V–VI; 20±3%) and the ventral horn (4±1%). Intraplantar injection or morphine (10 μg, 25 μg and 50 μg) dose-dependently reduced the number of superficial Fos-LI neurones ($r=0.605$, $P<0.02$, Figures 1a and 2a; for percentages see Table 1). In addition, the highest dose of intraplantar morphine (50 μg) had no significant reductory effect on the number of Fos-LI neurones when given by intravenous route of administration (see Figure 2a and Table 1). Intraplantar injection of methiodide naloxone (20 μg), which had no effect when administered alone (109±12% of control carrageenin expression), completely blocked the effects of intraplantar morphine (50 μg) on the number of superficial Fos-LI neurones (no difference with the control group, see Figures 1a and 2a and Table 1).

The unilateral peripheral oedema associated with carrageenin was extensive, both the paw and ankle diameters of the injected hind paw were increased (185±7% and 121±3% paw and ankle diameters of non-stimulated rats) whereas contralateral hindpaw was unaffected (97±2% and 94±1% of control non-stimulated rats respectively). Neither morphine, methiodide naloxone nor the co-administration of morphine and methiodide naloxone influenced the carrageenin-induced peripheral oedema.

Fos expression and peripheral oedema induced 3 h after carrageenin The total number of Fos-LI neurones observed 3 h after intraplantar injection of carrageenin was 241±8 Fos-LI neurones per section, in segments L4–L5. Fos-LI neurones were almost equally distributed in the superficial laminae (I–II) and deep laminae (V–VI) of the dorsal horn (45±2% and 37±1% respectively). The number of Fos-LI neurones in the ventral horn was moderate (12±2%), while very few neurones in laminae III and IV expressed c-Fos (5±1%).

Intraplantar injection of morphine dose-dependently reduced the number of superficial and deep Fos-LI neurones ($r=0.794$, $P<0.0004$ and $r=0.698$, $P<0.004$, respectively, (Figures 1b and 2b, and Table 1). However, all doses of intraplantar morphine studied had a tendency towards more marked effects on the number of superficial, as compared to the number of deep, Fos-LI neurones ($P<0.02$, for 10 μg of intraplantar, see Figure 2b and Table 1). Intraplantar injection of methiodide naloxone (20 μg), slightly but significantly, decreased the number of deep Fos-LI neurones (13±4% reduction of control carrageenin expression, $P<0.05$). Co-administration of intraplantar methiodide naloxone (20 μg) plus intraplantar morphine (50 μg) totally blocked the effects of morphine on the total number of Fos-LI neurones induced

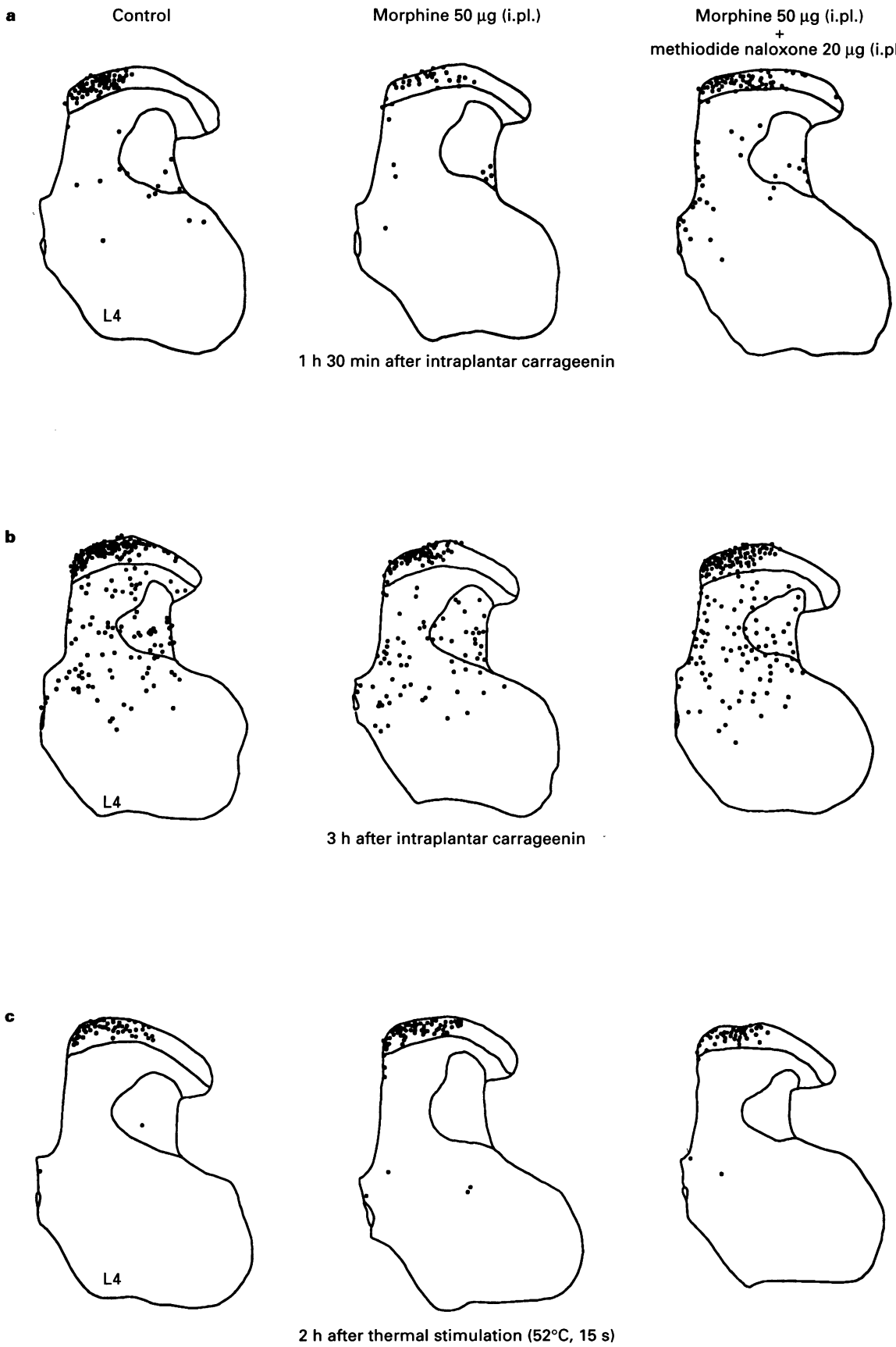

Figure 1 The effects of intraplantar morphine (50 μg 50 μl^{-1}) and co-administration of intraplantar morphine (50 μg 50 μl^{-1}) and methiodide naloxone (20 μg 50 μl^{-1}) on c-Fos expression induced at 1 h 30 min (a), 3 h (b) after intraplantar carrageenin and 2 h after noxious thermal stimulation (c). Each illustration includes all labelled neurones in one 40 μm section; each dot represents one labelled neurone. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined.

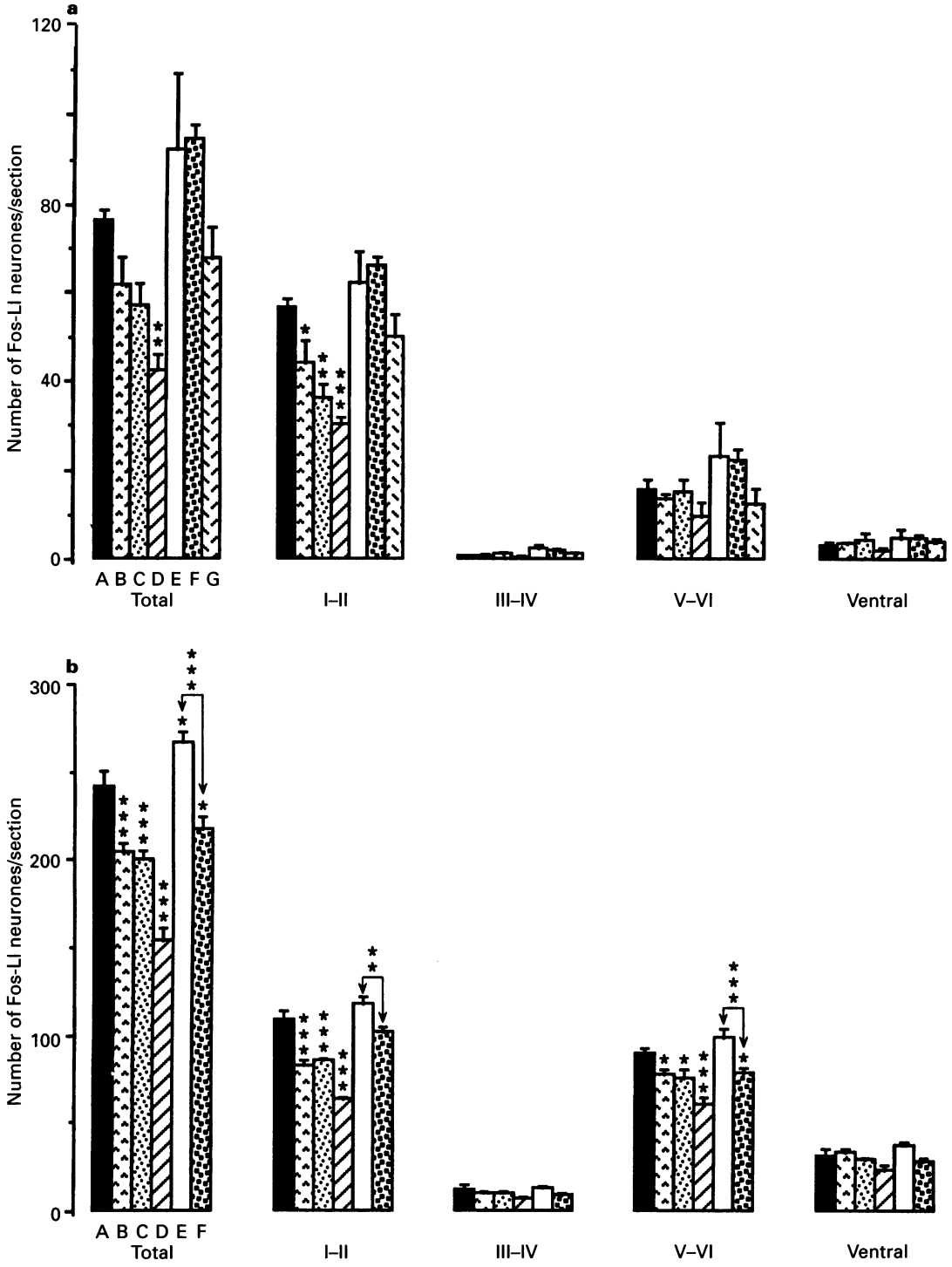

Figure 2 The effects of intraplantar morphine (10, 25 or 50 μg 50 μl^{-1}), intraplantar methiodide naloxone (20 μg 50 μl^{-1}), co-administration of intraplantar morphine (50 μg 50 μl^{-1}) and methiodide naloxone (20 μg 50 μl^{-1}) and systemic morphine (50 μg) on c-Fos expression induced at 1 h 30 min (a) and 3 h (b) after intraplantar carrageenin. Results are expressed as number of Fos-LI neurones per section ± s.e.mean for the total number of Fos-LI neurones in the dorsal horn (Total) and for the laminar distribution, in L4–L5 segments. Significant differences between groups were performed with ANOVA, PLSD Fisher's test. Significant differences between pharmacological groups are indicated by arrows ($^{\circ}P<0.05$; $^{\circ\circ}P<0.01$; $^{\circ\circ\circ}P<0.001$). Each column represents: (A) control carrageenin, (B) morphine 10 μg i.pl., (C) morphine 25 μg i.p.l., (D) morphine 50 μg i.pl., (E) morphine 50 μg i.pl. + methiodide naloxone 20 μg i.p.l., (F) methiodide naloxone 20 μg i.pl., (G) morphine 50 μg i.v.

3 h after intraplantar injection of carrageenin (110 ± 3%, $P<0.02$ of control carrageenin expression, see Figures 1b and 2b, and Table 1). These effects were observed on both superficial and deep Fos-LI neurones (see Figure 2b and Table 1).

The unilateral peripheral oedema associated with intraplantar injection of carrageenin was extensive, both the paw and ankle diameters of the injected hind paw were increased (307 ± 8% and 203 ± 6% of paw and ankle diameters of non-stimulated rats) whereas the contralateral hindpaw was unaffected (103 ± 2% and 98 ± 1% of control non-stimulated rats respectively). Intraplantar injection of morphine dose-dependently reduced the ankle ($r=0.747$, $P<0.002$) and paw ($r=0.682$, $P<0.005$) diameters, with the effect of the highest dose of intraplantar morphine being completely blocked by co-

Table 1 The effects of intraplantar morphine (10, 25 or 50 μg 50 μl^{-1}), intraplantar methiodide naloxone (20 μg 50 μl^{-1}), co-administration of intraplantar morphine (50 μg 50 μl^{-1}) and methiodide naloxone (20 μg 50 μl^{-1}) and systemic morphine (50 μg) on c-Fos expression induced 1 h 30 min and 3 h after intraplantar carageenin

		1 h 30 min		*3 h*		
Drugs	*Dose* (μg)	*Total number* (%)	*Superficial laminae* (%)	*Total number* (%)	*Superficial laminae* (%)	*Deep laminae* (%)
Morphine	10 (i.pl.)	81±8	77±9*	84±2***	75±3***	87±3*
	25 (i.pl.)	74±6	63±5**	82±2***	78±2***	84±5*
	50 (i.pl).	55±4**	53±2***	63±3***	58±1***	67±4***
Morphine+ Meth Nx	50 (i.pl.) 20 (i.pl.)	121±22	109±12	110±3*	108±4	110±5
Meth Nx	20 (i.pl.)	124±4	116±3	90±3*	93±3	87±4*
Morphine	50 (i.v.)	88±9	88±9	–	–	– –

The results are expressed as percentage of the control carrageenin group value±s.e.mean. Significance as compared to the control carrageenin group were performed by ANOVA, PLSD Fisher's test, (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

administration of methiodide naloxone (98±4% and 102±8% of control paw and ankle diameters, respectively), which alone had no effect on the peripheral oedema (92±4% and 87±2% of control paw and ankle diameters, respectively). However, there was no correlation between the effects of intraplantar injection of morphine on the number of Fos-LI neurones and on the peripheral oedema ($r=0.555$, $P=0.3$ and $r=0.432$, $P=0.1$ for the ankle and the paw diameters, respectively).

Effects of intraplantar morphine on Fos-LI neurones induced by noxious thermal stimulation

After noxious thermal stimulation, Fos-LI nuclei, which were stained to variable degree, were located in the ipsilateral dorsal horn of the spinal cord. All Fos-LI nuclei were analysed without considering the intensity of the staining. In agreement with a previous study on noxious heat evoked c-Fos expression (Abbadie *et al.*, 1994a), Fos-LI neurones were observed in lumbar segments L2–L6, with maximal labelling in segments L3 and L4.

The total number of Fos-LI neurones observed 2 h after noxious heat was 46±1 Fos-LI neurones per section, in segments L3–L4. The Fos-LI neurones were preferentially located in the superficial laminae (I–II) of the dorsal horn (94±2%) while fewer neurones were observed in the nucleus proprius (2±1%), the deep laminae (V–VI; 2±1%) and the ventral horn (1%). Neither intraplantar morphine (50 μg), intravenous morphine (50 μg), intraplantar methiodide naloxone (20 μg) or the co-administration of intraplantar morphine (50 μg) and methiodide naloxone (20 μg) influenced the noxious heat induced c-Fos expression (see Figure 1c and Table 2). Furthermore, no peripheral inflammation was observed after such a single noxious heat stimulation of brief duration (15 s).

Table 2 The effects of intraplantar morphine (50 μg 50 μl^{-1}), intraplantar methiodide naloxone (20 μg 50μl^{-1}), co-administration of intraplantar morphine (50 μg 50μl^{-1}) and methiodide naloxone (20 μg 50μl^{-1}) and systemic morphine (50 μg) on c-Fos expression induced 2 h after noxious thermal stimulation (52°C, 15 s)

Drugs	*Dose* (μg)	*Total number* (%)	*Superficial laminae* (%)
Morphine	50 (i.pl.)	97±2	97±3
Meth Nx	20 (i.pl)	95±4	96±4
Morphine + Meth Nx	50 (i.pl.) 20 (i.pl.)	93±1	95±2
Morphine	50 (i.v.)	95±2	97±2

The results are expressed as percentage of the control carrageenin group value±s.e.mean.

Discussion

In this article, we present evidence that intraplantar morphine dose-dependently reduces the number of Fos-LI neurones induced 1 h 30 min after intraplantar carrageenin, without influencing the peripheral oedema. Increasing the duration of the inflammatory stimulation to 3 h, we have shown that intraplantar morphine dose-dependently reduces the number of Fos-LI neurones induced by intraplantar carrageenin and also the peripheral oedema. This raised the question whether these effects of intraplantar morphine on both inflammatory aspect and spinal c-Fos expression reflect a peripheral and/or central site of action. To assess this possibility, we have studied the effect of intravenous administration of morphine, at concentration which is comparable to the maximal concentration of morphine injected at the peripheral site and we have shown that this dose of intravenous morphine had no effect on both inflammatory parameters and spinal c-Fos expression. In addition, the effects of intraplantar morphine were completely blocked by co-administration of intraplantar methiodide naloxone, an opioid receptor antagonist which does not cross the blood brain barrier (Iorio *et al.*, 1984; Milne *et al.*, 1990). In the third part of our experiment, we have shown that intraplantar morphine does not influence the number of Fos-LI neurones induced by an acute noxious heat stimulation.

At 1 h 30 min after intraplantar carrageenin, spinal c-Fos expression was essentially located in the superficial laminae of the dorsal horn of L4–L5 segments of the spinal cord, whereas 3 h after intraplantar carrageenin, the number of Fos-LI neurones was increased, both in the superficial and deep laminae of the spinal dorsal horn, in good agreement with previous studies using the same stimulation (Draisci & Iadarola, 1989; Noguchi *et al.*, 1991, 1992; Honoré *et al.*, 1995a,d,e). In addition, intraplantar carrageenin was associated with peripheral inflammation, including oedema, and we have previously shown that c-Fos expression is well correlated with the development of peripheral oedema associated with carrageenin inflammation (Honoré *et al.*, 1995d,e). In the third part of our study, performed in anaesthetized rats, numerous Fos-LI neurones were observed essentially in the superficial laminae of the dorsal horn of L3–L4 segments of the spinal cord 2 h after noxious heat stimulation (see Abbadie *et al.*, 1994c) and no detectable peripheral oedema was observed. In all experiments, the number of Fos-LI neurones in the contralateral dorsal horn was not significantly different from the well-established low number of spinal Fos-LI neurones in non-stimulated rats, <5 Fos-LI neurones per section (see Abbadie & Besson, 1992).

Pretreatment with intraplantar administration of morphine dose-dependently reduced the spinal c-Fos expression observed at 1 h 30 min or 3 h after intraplantar carrageenin. These effects are essentially due to a peripheral site of action since they were completely blocked by intraplantar methiodide naloxone, and the highest dose of intraplantar morphine had no effect

when injected systemically. Thus, it seems to us really interesting to compare the results of the present study with those that we have previously obtained with intravenous morphine on carrageenin induced spinal c-Fos expression. The effects of intraplantar morphine (50 μg) are pronounced since at 1 h 30 min they are comparable to those obtained with 3 mg kg^{-1} intravenous morphine (superficial laminae: 47±2% and 58±3% reduction of carrageenin induced c-Fos expression, for intraplantar and intravenous morphine respectively, see Honoré *et al.*, 1995d,e). More interestingly, it must be emphasized that the effects of intraplantar morphine are longer lasting than those of intravenous morphine. Indeed, 3 h after intraplantar carrageenin injection, intraplantar morphine (50 μg) was more efficient (superficial laminae: 42±1% reduction; deep laminae: 33±4% reduction of carrageenin induced Fos expression) at decreasing carrageenin induced spinal c-Fos expression than intravenous (3 mg kg^{-1}) morphine (superficial laminae: 29±5% reduction; deep laminae: 29±6% reduction of carrageenin induced c-Fos expression, see Chapman *et al.*, 1995). Overall, this seems to indicate that intraplantar morphine has a longer duration of action than intravenous morphine, these observations being in good agreement with clinical observations (see Stein, 1993; 1994). Several explanations could be advanced to explain such effects including a more direct access to peripheral opioid receptors and a very limited diffusion to the general circulation resulting in a strong reduction of the liver degrading processing. In addition, since there is evidence for an increased axonal transport of opioid receptors during inflammation (Hassan *et al.*, 1993; Jeanjean *et al.*, 1994) and that antinociceptive effects of intraplantar opioids are pronounced during inflammation but hardly demonstrated under non-inflamed conditions (see references in Kayser & Guibaud, 1994; Stein 1993; 1994), these long-lasting effects may possibly result from an increase in the number of opioid binding sites in inflamed tissue. However, this possibility is unlikely since the axonal fluxes of thin primary afferent C fibres are extremely slow (Tavitian *et al.*, 1986) and not likely to increase, 3 h after carrageenin injection, the number of peripheral opioid receptors. Recently, Antonijevic *et al.* (1995) have demonstrated that during the early stage of Freund's adjuvant induced inflammation, perineurial leakage and peripheral opioid antinociception occur simultaneously and that both can be mimicked by the administration of hyperosmolar solution in normal tissue, which results in a disruption of the perineurial barrier. It is most probable that such a mechanism takes place during carrageenin inflammation and is responsible for the large effects of intraplantar morphine on carrageenin-induced spinal c-Fos expression that we observed in the present experiment.

In any case, our data suggest that the peripheral effects of morphine following systemic administration of this compound play a major role in the antinociceptive effect of this compound, since long lasting effects have also been obtained after its intrathecal or intracerebroventricular administration of the same doses we used in the present study (for review see Yaksh *et al.*, 1988). Taken together, these results suggest that local administration of morphine, i.e. injecting the drug at the periphery, intrathecally or into supraspinal structures (periaqueducal grey matter or ventromedial medulla), is more efficient than systemic morphine.

In contrast, pretreatment with intraplantar administration of the highest dose of morphine did not influence the number of spinal Fos-LI neurones observed 2 h after noxious heat stimulation. The present results are in good agreement with previous behavioral studies which have shown that intraplantar opioid agonists have antinociceptive effects in a model of inflammatory nociception but not when inflammation is absent (see Introduction). Indeed, in the rat, the effects of intraplantar injection of opioid agonists have been essentially detected in the inflamed paw following the peripheral injection of various agents such as Freund's adjuvant (Stein *et al.*, 1988a,b; 1989; Schäfer *et al.*, 1995), carrageenin or prostaglandins (Ferreira & Nakamura, 1979; Joris *et al.*, 1987; Hargreaves *et al.*, 1988b; Levine & Taiwo, 1989) and intraplantar injection of formalin (Haley *et al.*, 1990; Hong & Abbott, 1995). In contrast no or extremely weak antinociceptive effects were detected in non-inflamed paw (Ferreira & Nakamura, 1979; Stein *et al.*, 1988a,b; 1989; Haley *et al.*, 1990). Furthermore, it has been shown that systemic (Raja *et al.*, 1986) and local (Senami *et al.*, 1986) administration of a μ opioid receptor agonist did not influence the responses of C-fibre nociceptors to brief noxious heat stimulation. This is in good agreement with the fact that intradermal injection of morphine did not significantly affect the mechanical nociceptive threshold in normal rats but produced dose-dependent inhibition of prostaglandin E_2-induced hyperalgesia (Levine & Taiwo, 1989). Overall, we think that in the present study, the lack of effects on intraplantar morphine on noxious heat induced spinal c-Fos expression is essentially due to the absence of peripheral inflammation following the single brief heat stimulation we used. In the present study, the presence of urethane anaesthesia, which could eventually depress the responsiveness of dorsal horn neurones to peripheral stimulation, is certainly not implicated in the lack of effect of intraplantar morphine in the latter experimental conditions, since we have previously shown that intravenous morphine was very efficacious at reducing noxious heat-induced c-Fos expression in rats under the same anaesthesia (Abbadie *et al.*, 1994c). In this latter study the effects of morphine are clearly dose-dependent and blocked by naloxone. Our present results, based on intraplantar administration of a relatively high dose of morphine (up to 50 μg) suggest that the effects of intravenous morphine on noxiously evoked spinal c-Fos expression in normal rat were essentially due to a direct spinal effect and/or to an indirect effect via activating spinal descending systems (see references in Basbaum & Besson, 1991).

With a 3 h inflammatory stimulation, intraplantar morphine, in addition to reducing spinal c-Fos expression, dose-dependently reduced the extent of the peripheral oedema and this effect was completely blocked by methiodide naloxone. Our results are in full agreement with previous studies demonstrating the anti-inflammatory effect (reduction of the oedema and plasma extravasation) of morphine during intraplantar carrageenin inflammation (Hargreaves *et al.*, 1988b, see also Joris *et al.*, 1990; Gavalas *et al.*, 1994). These anti-inflammatory effects could be related to the presence of opioid binding sites on immune cells and to the opioid-mediated modulation of several of their functions (Sibinga & Goldstein, 1988). However, in the present experiment, the rapid onset of antinociceptive effects of morphine (47±2% reduction of the number of superficial Fos-LI neurones induced 1 h 30 min after intraplantar carrageenin) suggest that local antinociceptive effects of opioids could not be exclusively mediated indirectly through these mechanisms. This assertion is in good agreement with the fact that, 1 h 30 min after the injection of a large dose of carrageenin (6 mg 150 μl^{-1}), intraplantar morphine decreases c-Fos expression without influencing peripheral oedema. In addition, despite the fact that 3 h after carrageenin injection, intraplantar morphine dose-dependently reduced peripheral oedema, there was no correlation between these effects and those observed on the number of Fos-LI neurones. Taken together, these results suggest that, in addition to its anti-inflammatory effects via immune cells, intraplantar morphine could more directly modulate the excitability of nociceptors.

In favour of this hypothesis, Stein *et al.* (1990b) have demonstrated immuno-cytochemically the presence of opioid receptors, which are synthesized in dorsal root ganglia (for review see Mansour *et al.*, 1995), on thinly myelinated and unmyelinated cutaneous nerves of the paw (see also Hassan *et al.*, 1993). As already discussed by Stein (1993, 1994), following the occupation of peripheral neuronal opioid receptors by an opioid agonist, an antinociceptive effect may be produced by several mechanisms: a decrease of the excitability of the nociceptive terminal, a blockade of the propagation of action potentials, but also an inhibition of the peripheral release of

excitatory, pro-inflammatory neuropeptides due to antidromic activation of primary afferents (see references in Stein, 1993; 1994). In favour of this latter hypothesis, opiates have been found to inhibit the release of bradykinin in inflamed tissue in a model of carrageenin inflammation (Wells *et al.*, 1989) and they also inhibit the release of substance P in the intra-articular space of the cat knee induced by electrical antidromic activation of the sciatic nerve or by local administration of capsaicin (Yaksh, 1988). This latter hypothesis is pertinent since carrageenin-evoked inflammation has a significant neurogenic component (Joris *et al.*, 1987).

Overall, our results demonstrated for the first time, that intraplantar morphine reduces c-Fos expression induced in the nuclei of dorsal horn neurones of the spinal cord by carrageenin inflammation, but not c-Fos expression induced by acute noxious heat stimulation. In addition, we have observed anti-inflammatory effects of intraplantar morphine. Furthermore, we have clearly demonstrated that these effects are opioid receptor mediated and strictly peripheral since there was an effect after local administration but not after systemic administration of an equal dose of morphine. In addition, the effects of intraplantar morphine were dose-dependent and blocked by intraplantar methiodide naloxone. Such results confirm that peripheral effects of morphine preferentially occur during inflammatory states and strongly support the use of peripheral opioids in clinical inflammatory pain states. Furthermore, these results extend the evidence towards the necessity of persisting in the search for novel analgesics as potent as opioids but acting essentially at the periphery so decreasing the risk of opioid side-effects.

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