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RB101(S), a dual inhibitor of enkephalinases does not induce antinociceptive tolerance, or cross-tolerance with morphine: a c-Fos study at the spinal level

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Abstract

In behavioural tests, RB101 (*N*-[(*S*)-2-benzyl-3[(*S*)(2-amino-4-methyl-thio)butyldithio]-1-oxopropyl]-L-phenylalanine benzyl ester), a mixed inhibitor of enkephalin-degrading enzymes, induces antinociceptive effects without producing tolerance, or cross-tolerance with morphine. In the present experiments, the acute or chronic effects of enantiomer RB101(S) were examined on the response of spinal cord neurons to nociceptive inflammatory stimulation (intraplantar injection of carrageenin) using c-Fos studies in awake rats. The number of c-Fos immunoreactive nuclei was evaluated in the lumbar spinal cord 90 min after carrageenin. c-Fos-immunoreactive nuclei were preferentially located in the superficial (I–II) and deep (V–VI) laminae of segments L4–L5 (areas containing numerous neurones responding exclusively, or not, to nociceptive stimuli). In the first experimental series, acute RB101(S) (30 mg/kg, i.v.), morphine (3 mg/kg, i.v.), or respective vehicles were injected in rats chronically treated with RB101(S) (160 mg/kg/day for 4 days, s.c.). In chronically treated RB101(S) rats, both acute RB101(S) and morphine reduced the total number of carrageenin-evoked c-Fos-immunoreactive nuclei. In the second experimental series, acute RB101(S) (30 mg/kg, i.v.) reduced the total number of carrageenin-evoked c-Fos-immunoreactive nuclei with similar magnitude in naive and in morphine-tolerant (100 mg/kg/day for 3 days, s.c.) rats. These data provide further evidence that different cellular mechanisms occurred after chronic stimulation of opioid receptors by morphine or endogenous enkephalins.

Keywords: c-Fos; Spinal Cord; RB101; Carrageenin; Tolerance; Enkephalin, endogenous

1. Introduction

Although opioid drugs such as morphine are widely used for the management of pain, their clinical usefulness is partially limited, especially in non-cancer pain patients, by the development of tolerance and dependence that occurs after repeated treatment. Antinociceptive morphine tolerance is indicated by a decreased efficacy of the drug with repeated administration and results in a need to increase the morphine dose in order to achieve the desired analgesic effect (Jaffe, 1990). However, the possibility of using increasing doses of morphine is limited by the occurrence of adverse side effects such as respiratory depression and constipation (references in Jaffe, 1990). Thus, there is considerable interest in the development of novel compounds that produce potent analgesia but devoid of major side effects. A possible strategy could be to potentiate the physiological pain control by protecting the endogenous opioid peptides from inactivation by the peptidases (for review, see Roques et al., 1993). Owing to the complementary roles of neutral endopeptidase and aminopeptidase N in enkephalin inactivation, dual inhibitors of these enzymes increase the half-life of the peptides. These inhibitors induce naloxone-reversible antinociceptive responses in all animal models of pain where morphine is active, without the main drawbacks of morphine (references in Roques et al., 1993).

Among several mixed inhibitors of neutral endopeptidase and aminopeptidase N, RB101 (*N*-[(*R*,*S*)-2-benzyl-3[(*S*)(2-amino-4-methyl-thio)butyldithio]-1-oxopropyl]-L-phenylalanine benzyl ester) has been shown to be able to

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cross the blood–brain barrier, eliciting antinociceptive effects in mice and rats after systemic administration (Fournié-Zaluski et al., 1992; Noble et al., 1992a; Noble and Roques, 1995). Besides, we have previously shown that intravenous RB101 dose-dependently reduced spinal carrageenin-evoked c-Fos protein expression (Honoré et al., 1997a; Le Guen et al., 1999b). Moreover, behavioural studies have shown that chronic treatment with RB101 does not induce tolerance, nor cross tolerance with morphine in the hot-plate test in mice (Noble et al., 1992b). The aim of the present study was to evaluate, on dorsal horn neurones involved in spinal nociceptive transmission, the eventual development of tolerance or cross-tolerance with morphine in rats chronically treated with enantiomer RB101(S). We have previously demonstrated that development of tolerance to morphine antinociception could be detected at the spinal cord level using c-Fos experiments (Le Guen et al., 1998). Moreover, in a previous study, we have confirmed that the isomer S of RB101 led to a dose-dependent reduction, naloxone-reversible, of the number of c-Fos-immunoreactive (c-Fos-IR) nuclei observed at the spinal cord level after the intraplantar injection of carrageenin (Le Guen et al., 2000a).

There is considerable evidence that the expression of the nuclear protein c Fos encoded by the immediate–early gene *c-fos* reflects the long-term intracellular changes associated with sustained nociceptive processing, especially at the spinal cord level. In addition, numerous studies have demonstrated that c-Fos protein expression provides an indirect marker of neuronal populations involved in nociceptive processes (see references in Chapman and Besson, 1997; Harris, 1998). The "c-Fos technique", based on the immunohistochemical investigation of c-Fos protein expression in the central nervous system, has been shown to be very suitable for analysis of the effects of various endogenous or exogenous substances involved in nociceptive processes, especially at the spinal cord dorsal horn level (see references in Chapman and Besson, 1997). Furthermore, this technique is sensitive enough to detect tolerance to morphine using a variety of peripheral noxious stimuli such as noxious heat (Abbadie et al., 1994), or intraplantar injection of carrageenin (Honoré et al., 1997b; Le Guen et al., 1998), and formalin (Rohde et al., 1997). This approach provides additional information on spinal nociceptive processes to those which can be obtained by behavioural and electrophysiological methods. It can be considered as a simultaneous high-resolution photographic image of the level of cellular activity of different neuronal populations of the spinal cord (superficial versus deep laminae), in the same animal, at a given time point.

In the present study, the effects of acute RB101(S) (30 mg/kg, i.v.) were investigated on spinal c-Fos protein expression at 90 min after intraplantar injection of carrageenin (6 mg/150 μl of saline) in rats chronically pre-treated with RB101(S) (160 mg/kg/day for 4 days) or with morphine (100 mg/kg/day for 3 days). In addition, the effects of acute morphine (3 mg/kg, i.v.) were investigated on carrageenin-induced spinal c-Fos protein expression in rats chronically pre-treated with RB101(S) (160 mg/kg/day for 4 days). Aspects of these studies were presented at the annual meeting of the International Narcotics Research Conference in Seattle (USA, July 2000; Le Guen et al., 2000b).

2. Materials and methods

2.1. Animals

Experiments were performed on 75 adult male albino Sprague–Dawley rats (Charles River, France), ranging in weight from 175 to 200 g at the beginning of the experiments. They were housed six per cage in a room with controlled temperature (22 ± 1 °C) and a 12-h alternating light–dark cycle. Food and water were made available continuously. Animal experiments were carried out in accordance with the European Communities Council Directive (86/6609/EEC) as well as French law, and with the ethical guidelines of the International Association for the Study of Pain, for investigations of experimental pain in conscious animals (Zimmermann, 1983).

2.2. Drugs and chemicals

Morphine hydrochloride was obtained from Meram (France) and diluted in saline (0.9% NaCl). The mixed inhibitor of enkephalin-degrading-enzymes RB101(S) (*N*-[(*S*)-2-benzyl-3[(*S*)(2-amino-4-methyl-thio)butyldithio]-1-oxopropyl]-L-phenylalanine benzyl ester) was synthesised as previously described by Fournié-Zaluski et al. (1992), and was dissolved in a vehicle. The vehicle of RB101(S) was made by mixing ethanol (10%), Cremophor EL (10%, Sigma) and distilled water. Acute administration of drugs (RB101(S) or morphine) was performed intravenously (i.v., 1 ml/kg) into the lateral vein of the tail in awake rats. The choice of the isomer S of RB101 was based on previous behavioural results demonstrating that this isomer is more efficacious than the isomer R (F. Noble, personal communication). The doses of acute RB101(S) (30 mg/kg, i.v.) and morphine (3 mg/kg, i.v.) were selected from previous studies aimed at measuring their antinociceptive effects on carrageenin-induced spinal c-Fos protein expression (Honoré et al., 1995b; Le Guen et al., 1998, 2000a).

For the chronic pre-treatments, the drugs (RB101(S) or morphine) were incorporated into a sustained-release emulsion allowing a slow diffusion of compounds (Collier et al., 1972; Frederickson and Smits, 1973). The sustained-release preparation (vehicle) was made by mixing 50% (v/v) of saline, 42.5% (v/v) of light liquid paraffin oil (Sigma) and 7.5% (v/v) of an emulsifying agent, mannide monooleate (Arlacel A, Sigma), forming a thick white emulsion. The subcutaneous (s.c.) injections of emulsion (10 ml/kg) were performed in the neck of awake rats gently handled in a towel.

2.3. Inflammatory nociceptive stimulation

The awake rats received an intraplantar (i.pl.) injection of carrageenin (λ-carrageenin, type IV, Sigma; 6 mg/150 μl of saline) in the right hindpaw according to the method described by Winter et al. (1962). Rats were perfused 90 min after carrageenin, an optimal post-injection delay for c-Fos protein expression in numerous nuclei in the dorsal horn of the spinal cord (Honoré et al., 1995a). In addition, this delay corresponds to the peak effects of i.v. RB101 and morphine on carrageenin-evoked spinal c-Fos protein expression (Honoré et al., 1995b, 1997a). Peripheral oedema extent (paw and ankle diameters) was evaluated immediately before perfusion (for more details see methods in Buritova et al., 1996b).

2.4. Experimental design

The development of antinociceptive tolerance to morphine was previously described as the ineffectiveness of acute morphine (3 mg/kg, i.v.) to reduce significantly carrageenin-evoked spinal c-Fos protein expression (Le Guen et al., 1998; 1999a). In order to investigate the eventual development of antinociceptive tolerance to RB101(S) or its cross-tolerance with morphine, two experimental series were performed.

In the first experimental series ($n=40$ rats), the effects of acute RB101(S) or morphine on carrageenin-induced spinal c-Fos protein expression were gauged in chronically RB101(S) pre-treated rats. Rats were divided into six groups. Four groups received chronic pre-treatment with RB101(S) (160 mg/kg/day for 4 days, s.c.) incorporated into the sustained-release emulsion. Two naive groups received chronic pre-treatment with vehicle (sustained-release emulsion, 10 ml/kg/day for 4 days, s.c.). On day 5, the acute administration of drugs (RB101(S) or morphine) and the inflammatory nociceptive stimulation with i.pl. carrageenin were performed. Naive rats received an acute i.v. injection (1 ml/kg) of either vehicle of RB101(S) (naive-vehicle, $n=7$) or RB101(S) (30 mg/kg, naive-RB, $n=7$), 10 min before i.pl. injection of carrageenin. Chronic RB101(S) pre-treated rats received an acute i.v. injection (1 ml/kg) of either vehicle of RB101(S) (chronic RB-vehicle, $n=7$), RB101(S) (30 mg/kg, chronic RB-RB, $n=7$), saline (chronic RB-saline, $n=5$), or morphine (3 mg/kg, chronic RB-morphine, $n=7$), 10 min before i.pl. injection of carrageenin.

In the second experimental series ($n=35$ rats), the effects of acute RB101(S) on carrageenin-induced spinal c-Fos protein expression were investigated in chronic morphine pre-treated rats. Rats were divided into six groups. Four groups received chronic pre-treatment with morphine incorporated into the sustained-release emulsion (100 mg/kg/day for 3 days, s.c.). Two naive groups received chronic pre-treatment with vehicle (sustained-release emulsion, 10 ml/kg/day for 3 days, s.c.). On day 4, the acute administration of drugs (RB101(S) or morphine) and the inflammatory nociceptive stimulation with intraplantar carrageenin were performed. Naive rats received an acute i.v. injection (1 ml/kg) of either vehicle of RB101(S) (naive-vehicle, $n=6$) or RB101(S) (30 mg/kg, naive-RB, $n=6$), 10 min before i.pl. injection of carrageenin. Chronic morphine pre-treated rats received an acute i.v. injection (1 ml/kg) of either vehicle of RB101(S) (chronic morphine-vehicle, $n=6$), RB101(S) (30 mg/kg, chronic morphine-RB, $n=5$), saline (chronic morphine-saline, $n=5$), or morphine (3 mg/kg, chronic morphine-morphine, $n=7$), 10 min before i.pl. injection of carrageenin.

In these studies, carrageenin non-stimulated rats receiving acute or chronic administration of drugs (RB101(S) or morphine) were not included, since we have previously shown that, in these rats, spinal c-Fos protein expression was negligible (<5 c-Fos-immunoreactive nuclei per section of L4–L5 segments; Honoré et al., 1997a; Le Guen et al., 1998).

2.5. Immunohistochemistry for c-Fos protein

One hour and thirty minutes after i.pl. injection of carrageenin, rats were deeply anaesthetised with sodium pentobarbital (200 mg/kg, i.p., Sanofi) and transcardially perfused with 300 ml of heparinized (3000 U/l) phosphate-buffered saline (0.1 M, PBS, pH = 7.4), followed by 500 ml of 4% paraformaldehyde in phosphate buffer (0.1 M, PB, pH = 7.4). Spinal cords were removed, post-fixed for 4 h, and cryoprotected overnight in PB containing 30% sucrose at 4 °C. Lumbar segments were cut into 40 μm coronal sections on a microtome at −20 °C, and collected in PBS, in order, into multiwell trays, to be processed immunohistochemically as free-floating sections. To permit comparisons between groups, sections from the same experimental series were processed together. As described previously (Honoré et al., 1995a,b), sections were washed in PBS, and incubated for 30 min at room temperature in a solution containing 3% normal goat serum (NGST) in PBST (PBS with 0.3% Triton X-100) to block non-specific binding sites and facilitate tissue penetration. The sections were then incubated overnight at 4 °C in primary polyclonal rabbit c-Fos antiserum (1:30,000 dilution in NGST 1%, Tebu, SC-52, 2 mg/ml). This c-Fos antiserum recognises the N-terminal (residues 4–17) region of human c-Fos p62. Sections were then washed three times in PBS and incubated in biotinylated goat anti-rabbit Immunoglobulin G (Vector Laboratory, 1.5 mg/ml, 1:200) for 1 h at room temperature, washed twice in PBS and incubated for 1 h in avidin–biotin horseradish peroxidase complex (ABC) at room temperature. Finally, the sections were washed three times in PBS and developed with the peroxidase kit Vector® (VIP chromogen; Vector Labs, VSK 4600, 3 drops/10 ml PB 10 mM) for 5 min, and were then washed in PB to stop the staining reaction. Sections were mounted on gelatine-coated slides, air-dried, dehydrated through a graded alcohol

Table 1
Effects of acute RB101(S) (30 mg/kg, i.v.) or morphine (3 mg/kg, i.v.) on spinal c-Fos protein expression induced 1.5 h after intraplantar carrageenin in naive, chronic RB (160 mg/kg/day, 4 days), or chronic morphine (100 mg/kg/day, 3 days) pre-treated rats

Experimental groups	% Reduction of the number of c-Fos-IR nuclei		
	Total	Laminae I–II	Laminae V–VI
First experimental series			
Naive-RB	32 ± 3% c	24 ± 3% b	40 ± 5% c
Chronic RB-RB	26 ± 6% c	24 ± 6% b	33 ± 6% c
Chronic RB-morphine	35 ± 6% c	22 ± 10% a	46 ± 4% c
Second experimental series			
Naive-RB	40 ± 3% a	29 ± 5% (NS)	53 ± 7% b
Chronic morphine-RB	35 ± 6% b	21 ± 9% (NS)	61 ± 6% c

Results are expressed as percentages of reduction of carrageenin-induced spinal c-Fos protein expression (± S.E.M.) in the L4–L5 segments considering the total number of c-Fos-IR nuclei (Total) and their number in superficial (I–II) and deep (V–VI) laminae of the dorsal horn. Significance was noted as compared to the respective control groups (ANOVA, PLSD Fisher's post-hoc test; a = $P<0.05$, b = $P<0.01$, c = $P<0.001$, NS = non-significant).

series (70°, 95°, and 100°), xylene-treated and cover-slipped with Eukitt mounting medium. Controls of the staining were assessed by omission of either the primary or the secondary antibody; such a procedure failed to produce any staining.

2.6. Counting of c-Fos-immunoreactive nuclei

We have previously shown that the greatest number of c-Fos-immunoreactive nuclei is located in the L4–L5 segments after intraplantar injection of carrageenin (Honoré et al., 1995a). The sections of L4–L5 segments were examined under light field microscopy at × 125 to localise c-Fos-immunoreactive nuclei. Labeled nuclei were counted using a camera lucida attachment. To study the laminar distribution of c-Fos-immunoreactive nuclei, 4 regions were defined according to cytoarchitectonic organisation of the spinal cord (Rexed, 1952): superficial dorsal horn (laminae I–II), nucleus proprius (laminae III–IV), deep laminae of the dorsal horn (laminae V–VI), and ventral horn (laminae VII–X). For each rat, two parameters were considered: (1) the total number of c-Fos-immunoreactive nuclei in the grey matter for 10 most representative sections in the L4–L5 segments, and (2) the number of c-Fos-immunoreactive nuclei per specific laminar region of the spinal grey matter in these 10 sections. Results are expressed as mean ± standard error means (S.E.M.). All c-Fos-immunoreactive nuclei were counted without considering the intensity of the staining. The investigator responsible for plotting and counting the c-Fos-immunoreactive nuclei was blind to the experimental situation of each animal.

2.7. Data analyses

One-way analysis of variance (ANOVA) was conducted using computer software (Statview for Macintosh) for comparison across the experimental conditions considering the total number of spinal c-Fos-immunoreactive nuclei in the ipsilateral side to the i.pl. injection of carrageenin, and the number in each laminar region. When a significant difference among the treatments was obtained in the ANOVA, the Fisher's PLSD (protected least significant difference) post-hoc test was applied to define which group contributed to these differences. Significance was accepted with $P<0.05$.

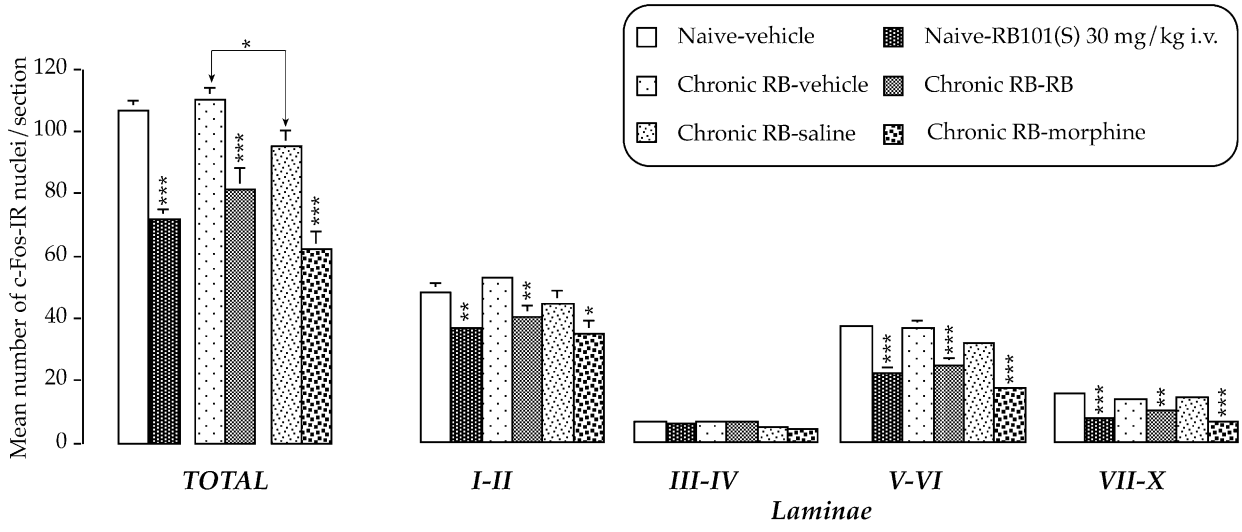

Fig. 1. Effects of acute RB101(S) (30 mg/kg, i.v.) or morphine (3 mg/kg, i.v.) on the number of c-Fos-immunoreactive nuclei/section (c-Fos-IR nuclei/section), 90 min after intraplantar injection of carrageenin (6 mg/150 μl of saline), in naive and chronic RB pre-treated rats (160 mg/kg/day for 4 days). Results are expressed as mean values (± S.E.M.) for the total number of carrageenin-evoked c-Fos-immunoreactive nuclei per section in the L4–L5 segments of the spinal cord (Total) and their number in laminar regions (laminae I–II, III–IV, V–VI, and VII–X). Significant differences between groups were performed with ANOVA, PLSD Fisher's test (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to respective control groups).

3. Results

3.1. Carrageenin-evoked spinal c-Fos protein expression

In all pharmacological groups performed in the two experimental series, 1.5 h after i.pl. carrageenin, c-Fos-immunoreactive nuclei were located in the ipsilateral dorsal horn of the spinal cord. The number of c-Fos-immunoreactive nuclei in the contralateral dorsal horn was extremely low and not significantly different from the low number of spinal c-Fos-immunoreactive nuclei in non-stimulated rats (data not shown). The total numbers of carrageenin-evoked c-Fos-immunoreactive nuclei observed in the two control naive groups were different in the two experimental series (106 ± 3 and 42 ± 3 c-Fos-immunoreactive nuclei/section in the first and second experimental series, respectively). Irrespective of this difference, for both series of experiments, the carrageenin-evoked c-Fos-immunoreactive nuclei were preferentially located in the superficial (I–II, about 45% of the total number of c-Fos-immunoreactive nuclei) and the deep (V–VI, about 35% of the total number of c-Fos-immunoreactive nuclei) laminae of the dorsal horn, while fewer nuclei were observed in the nucleus proprius (III–IV, about 5% of the total number of c-Fos-immunor-

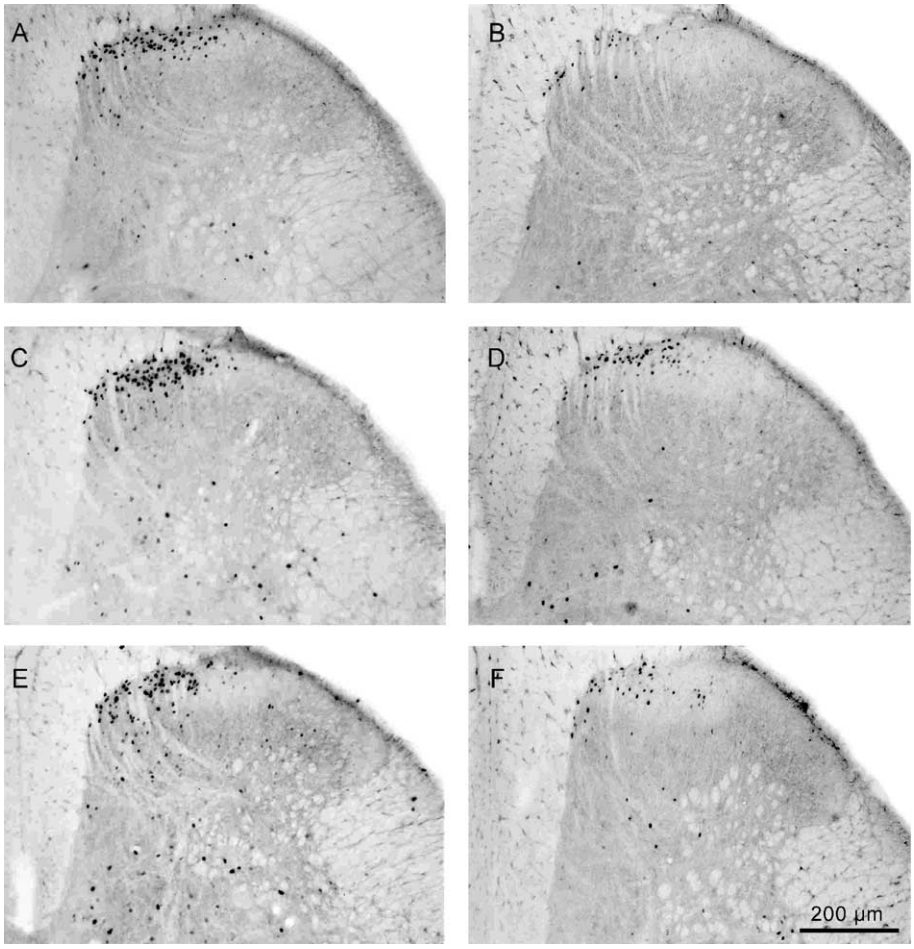

Fig. 2. Photomicrographs illustrating carrageenin-evoked c-Fos protein immunoreactivity in frontal sections (40 μm) of the dorsal horn at the level of L4–L5 segments. Six experimental situations are represented from the first experimental series. (A) Naive-vehicle group of rats receiving chronic pre-treatment with vehicle (sustained-released emulsion, 10 ml/kg/day for 4 days, s.c.), and acute injection of the RB101(S) vehicle (1 ml/kg, i.v., 10 min before i.pl. carrageenin) on day 5. (B) Naive-RB group of rats receiving chronic pre-treatment with vehicle (sustained-released emulsion, 10 ml/kg/day for 4 days, s.c.), and acute injection of RB101(S) (30 mg/kg, i.v., 10 min before i.pl. carrageenin) on day 5. (C) Chronic RB-vehicle group of rats receiving chronic pre-treatment with RB101(S) (160 mg/kg/day for 4 days, s.c. incorporated into a sustained-released emulsion), and acute injection of the RB101(S) vehicle (1 ml/kg, i.v., 10 min before i.pl. carrageenin) on day 5. (D) Chronic RB-RB group of rats receiving chronic pre-treatment with RB101(S) (160 mg/kg/day for 4 days, s.c. incorporated into a sustained-released emulsion), and acute injection of RB101(S) (30 mg/kg, i.v., 10 min before i.pl. carrageenin) on day 5. (E) chronic RB-saline group of rats receiving chronic pre-treatment with RB101(S) (160 mg/kg/day for 4 days, s.c. incorporated into a sustained-released emulsion), and acute injection of saline (1 ml/kg, i.v., 10 min before i.pl. carrageenin) on day 5. (F) Chronic RB-morphine group of rats receiving chronic pre-treatment with RB101(S) (160 mg/kg/day for 4 days, s.c. incorporated into a sustained-released emulsion), and acute injection of morphine (3 mg/kg, i.v., 10 min before i.pl. carrageenin) on day 5. Scale bar: 200 μm.

eactive nuclei) and the ventral horn (about 15% of the total number of c-Fos-immunoreactive nuclei).

3.2. Does antinociceptive tolerance develop after chronic administration of RB101(S)?

In naive rats receiving daily pre-treatment with vehicle, acute pre-administration of RB101(S) (30 mg/kg, i.v.) significantly reduced the total number of carrageenin-evoked c-Fos-immunoreactive nuclei ($32 \pm 3\%$ of reduction, $P < 0.0001$; Table 1 and Figs. 1 and 2A–B). The depressive effects of acute RB101(S) were still pronounced in the group that received chronic RB101(S) pre-treatment (160 mg/kg/day for 4 days, $26 \pm 6\%$ of reduction, $P < 0.0001$; Table 1 and Figs. 1 and 2C–D). Statistical analyses revealed that the effects of acute RB101(S) in naive or chronic RB pre-treated rats have the same magnitude. The effects of acute RB101(S) (30 mg/kg, i.v.) on the number of carrageenin-evoked c-Fos-immunoreactive nuclei were predominant in the deep dorsal horn ($40 \pm 5\%$ and $33 \pm 6\%$ of reduction in naive and chronic RB pre-treated rats, respectively, $P < 0.0001$ for both; Table 1 and Fig. 1). In the superficial laminae, acute RB101(S) produced respective reductions of the number of carrageenin-evoked c-Fos-immunoreactive nuclei of $24 \pm 3\%$ ($P < 0.01$) and $24 \pm 6\%$ ($P < 0.01$) in naive and chronic RB pre-treated rats (Table 1 and Fig. 1).

Acute pre-administration of morphine (3 mg/kg, i.v.) significantly decreased the total number of carrageenin-evoked c-Fos-immunoreactive nuclei in chronic RB pre-treated rats ($35 \pm 6\%$ of reduction, $P < 0.0001$; Table 1 and Figs. 1 and 2E–F). The depressive effects of acute morphine on the number of carrageenin-evoked c-Fos-immunoreactive nuclei were predominant in the deep dorsal horn ($46 \pm 4\%$ of reduction, $P < 0.0001$; Table 1). In the superficial laminae, acute morphine produced $22 \pm 10\%$ of reduction of the number of carrageenin-evoked c-Fos-immunoreactive nuclei ($P < 0.05$; Table 1 and Fig. 1).

There was a slight increase in carrageenin-evoked spinal c-Fos protein expression in chronic RB pre-treated rats receiving acute vehicle injection compared to those receiving acute saline (about 14% of increase, $P < 0.05$; Fig. 1). However, the number of carrageenin-evoked c-Fos-immunoreactive nuclei in these two groups did not differ significantly with that in the control naive-vehicle group.

3.3. Does acute RB101(S) still decrease carrageenin-evoked spinal c-Fos protein expression in morphine-tolerant rats?

In the second experimental series, although the number of carrageenin-evoked c-Fos-immunoreactive nuclei was lower than that in the first one (Fig. 3), the effects of acute pre-administration of RB101(S) (30 mg/kg, i.v.) in naive rats receiving daily pre-treatment with vehicle ($40 \pm 3\%$ of reduction of the total number of carrageenin-evoked c-Fos-immunoreactive nuclei, $P < 0.05$; Table 1) were highly comparable to those described in the first experimental series ($32 \pm 3\%$ of reduction of the total number of carrageenin-evoked c-Fos-immunoreactive nuclei, $P < 0.001$; Table 1). The depressive effects of acute RB101(S) were still pronounced in the group receiving chronic morphine pre-treatment (100 mg/kg/day for 3 days, $35 \pm 6\%$ of reduction of the total number of carrageenin-evoked c-Fos-immunoreactive nuclei, $P < 0.01$; Table 1 and Fig. 3).

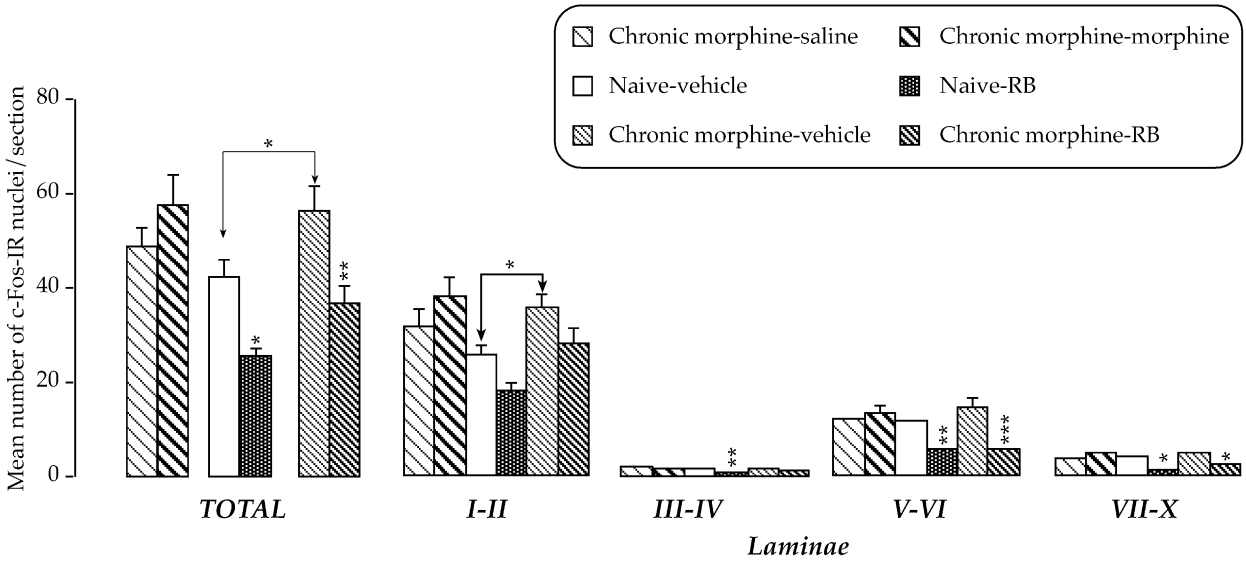

Fig. 3. Effects of acute RB101(S) (30 mg/kg, i.v.) or morphine (3 mg/kg, i.v.) on the number of c-Fos-immunoreactive nuclei/section (c-Fos-IR nuclei/section), 90 min after intraplantar injection of carrageenin (6 mg/150 μl of saline) in naive or chronic morphine pre-treated rats (100 mg/kg/day for 3 days). Results are expressed as mean values (± S.E.M.) for the total number of carrageenin-evoked c-Fos-immunoreactive nuclei per section in the L4–L5 segments of the spinal cord (Total) and their number in laminar regions (laminae I–II, III–IV, V–VI, and VII–X). Significant differences between groups were performed with ANOVA, PLSD Fisher's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to respective control groups).

Statistical analyses revealed that the effects of acute RB101 (S) in naive or chronic morphine pre-treated rats have the same magnitude. The effects of acute RB101(S) (30 mg/kg, i.v.) on the number of carrageenin-evoked c-Fos-immunoreactive nuclei were predominant in the deep dorsal horn ($53 \pm 7\%$ and $61 \pm 6\%$ of reduction in naive and chronic morphine pre-treated rats, $P < 0.01$ and $P < 0.001$, respectively; Table 1). In the superficial laminae, acute RB101(S) produced respective reductions of the number of carrageenin-evoked c-Fos-immunoreactive nuclei of $29 \pm 5\%$ ($P > 0.05$) and $21 \pm 9\%$ ($P > 0.05$) in naive and chronic morphine pre-treated rats (Table 1 and Fig. 3).

There was a slight increase in carrageenin-evoked spinal c-Fos protein expression in chronic morphine pre-treated rats receiving acute vehicle compared to naive rats receiving acute vehicle (about 25% of increase, $P < 0.05$; Fig. 3). However, the number of carrageenin-evoked c-Fos-immunoreactive nuclei in these two groups was not significantly different in comparison with that in the chronic morphine–saline group.

3.4. Inflammatory parameters

Ninety minutes after i.pl. carrageenin, ipsilateral peripheral oedema was developed. The diameters of both the paw and ankle of the carrageenin-injected hindpaw were increased in comparison to paw and ankle diameters of non-stimulated rats (about 113% and 14% of increases for the paw and ankle diameters, respectively), whereas the contralateral hindpaw was not significantly affected.

In the first experimental series, acute RB101(S) (30 mg/kg, i.v.) slightly reduced paw and ankle diameters of the carrageenin-injected hindpaw, both in naive ($16 \pm 5\%$ and $14 \pm 3\%$ of reduction of carrageenin-enhanced paw and ankle diameters, respectively, $P < 0.01$ for both), and in chronic RB pre-treated rats ($12 \pm 3\%$ and $4 \pm 3\%$ of reduction of carrageenin-enhanced paw and ankle diameters, $P < 0.01$ and $P > 0.05$, respectively). However, the latter effects of acute RB101(S) were not retrieved in the second experimental series, neither in naive nor in chronic morphine pre-treated rats. In addition, acute morphine (3 mg/kg, i.v.) slightly reduced paw and ankle diameters of the carrageenin-injected hindpaw in chronic RB pre-treated rats ($18 \pm 4\%$ and $11 \pm 3\%$ of reduction of carrageenin-enhanced paw and ankle diameters, $P < 0.001$ and $P < 0.01$, respectively).

4. Discussion

In the present study, we have clearly demonstrated that RB101(S) (*N*-[(*S*)-2-benzyl-3[(*S*)(2-amino-4-methyl-thio)-butyldithio]-1-oxopropyl]-L-phenylalanine benzyl ester), a mixed inhibitor of enkephalin-catabolizing enzymes (Fournié-Zaluski et al., 1992; Roques et al., 1993), does not induce antinociceptive tolerance, nor cross-tolerance with morphine in the carrageenin model of inflammatory nociception. These studies based on the spinal c-Fos protein expression not only complement previous behavioural studies (hot-plate test in mice; Noble et al., 1992b), but offer several advantages: (1) to be able to focus on the central nervous system (CNS), especially on the dorsal horn level of the spinal cord, a major site for the integration of nociceptive messages prior to their transmission to the brain (references in Besson and Chaouch, 1987; Willis and Coggeshall, 1991); (2) to visualise simultaneously drug effects on different neuronal populations at the spinal cord level (superficial versus deep laminae dorsal horn neurones), the majority of them receiving noxious inputs from the periphery; (3) to study simultaneously drug-effects in the CNS and in the periphery (for example, effects on the peripheral oedema). The “ c-Fos technique ” has been shown to be particularly useful for the study of the pharmacology of pain transmission, especially at the spinal cord level (references in Chapman and Besson, 1997), and could be used for in vivo experiments in awake animals. Furthermore, this technique is able to detect tolerance phenomenon as demonstrated by the lack of effects of acute morphine to decrease either noxious heat-evoked (Abbadie et al., 1994), carrageenin-evoked (Honoré et al., 1997b; Le Guen et al., 1998) or formalin-evoked (Rohde et al., 1997) spinal c-Fos protein expression in morphine tolerant rats.

In good agreement with our previous studies (see Honoré et al., 1997a and references therein), 1.5 h after intraplantar injection of carrageenin, 80% of the total number of c-Fos-immunoreactive nuclei were observed in the superficial laminae (I–II) and deep laminae (V–VI) of the dorsal horn of the spinal cord. This laminar distribution corresponds to spinal areas, which contain numerous neurones responding exclusively, or not to nociceptive stimuli (Besson and Chaouch, 1987; Willis and Coggeshall, 1991). The control level of carrageenin-evoked spinal c-Fos protein expression in naive groups was different for the two experimental series; however, comparisons of data between these two series of experiments were not performed. Such variations in the number of carrageenin-evoked spinal c-Fos-immunoreactive nuclei are presumably due to the inherent variability among experimental series associated with this experimental approach (see Buritova et al., 1996a). Overall, in the same experimental series, the data were very homogenous, allowing the statistical analysis of the drug effects.

Notwithstanding this difference of carrageenin-evoked c-Fos protein labeling in naive rats, acute pre-administration of RB101(S) (30 mg/kg, i.v.) significantly reduced the number of carrageenin-evoked c-Fos-immunoreactive nuclei. This reduction was remarkably similar over the two experimental series, where the depressive effects of acute RB101(S) on the total number of carrageenin-evoked c-Fos-immunoreactive nuclei were, respectively, 32% and 40% of reduction. These results are in good agreement with previous studies demonstrating that systemic RB101(R/S) (Honoré et al., 1997a) or RB101(S) (Le Guen et al., 2000a) reduced dose-dependently carrageenin-evoked spinal c-Fos

protein expression. Moreover, these results extend previous studies which have demonstrated that systemic enkephalin-catabolizing enzymes inhibitors, such as kelatorphan (Tölle et al., 1994) and RB101 (Abbadie et al., 1994), dose-dependently reduced noxious heat-evoked spinal c-Fos protein expression, in a naloxone reversible manner. Furthermore, our results are reminiscent of behavioural studies, which have shown that various enkephalin-catabolizing enzymes inhibitors exert antinociceptive effects in animals, and electrophysiological studies showing clear depressive effects of these compounds on responses of deep dorsal horn neurones to peripheral noxious stimulation (see references in Roques et al., 1993).

In the present study, tolerance phenomenon was observed following chronic morphine pre-treatment, while acute RB101(S) (30 mg/kg, i.v.) was still able to decrease carrageenin-evoked spinal c-Fos protein expression in chronic RB pre-treated (160 mg/kg/day, 4 days) rats, with the same magnitude as that in vehicle pre-treated rats (see Table 1). In both naive and chronic RB pre-treated rats, acute RB101(S) had a greater effect on carrageenin-evoked c-Fos protein expression in deep laminae as compared to that in the superficial laminae. These results are in accordance with previous studies showing a greater depressing effect of morphine on c-Fos protein expression, at 2 h after intraplantar carrageenin, in deep laminae than that in superficial laminae (Presley et al., 1990; Honoré et al., 1995b). The lack of tolerance observed after chronic administration of RB101(S) is in accordance with previous behavioural studies (Noble et al., 1992b; Valverde et al., 1995). As already suggested by these authors, endogenous enkephalins protected from their enzymatic degradation by RB101, would be expected to act only upon pathways recruited by the nociceptive stimulus, and not upon all opioid receptors in the CNS, as the case with exogenous opioids such as morphine. Thus, this fact might limit opioid receptor occupation by endogenous opioids peptides (Meucci et al., 1989; Ruiz-Gayo et al., 1992), and therefore minimise receptor desensitisation, which thought to occur after the ubiquitous and massive stimulation of opioid receptor by chronic morphine treatment (references in Law et al., 2000). Moreover, difference in intracellular trafficking as a function of both the nature of the receptor and the ligand may account for the different consequences of long-term adaptation during sustained treatment with morphine or endogenous enkephalins (references in Akil et al., 1998). For example, Keith et al. (1998) showed that enkephalins cause rapid internalisation of the mu opioid receptor, while morphine does not. However, these results observed in carrageenin model of inflammatory nociception may appear contradictory with several data on model of stress-induced analgesia, which is thought to be mediated by endogenous enkephalins (Akil et al., 1986; Yamada and Nabeshima, 1995). Indeed, for example, it has been shown that tolerance develops to food deprivation-induced analgesia (Davidson et al., 1992) or swim-induced antinociception (Christie et al., 1982; Panocka et al., 1991) after these chronic stress exposures (see also Lewis et al., 1981; Miczek and Winslow, 1987). However, it is extremely difficult to compare the above-mentioned drastic modifications to the inflammation induced in the present experiment, which is more reminiscent of the clinical situation.

Besides, in the second part of the present study, the lack of cross-tolerance between RB101(S) and morphine was observed. Indeed, acute morphine significantly decreased carrageenin-induced spinal c-Fos protein expression in chronic RB pre-treated rats. Moreover, even though we did not re-evaluate the effects of acute morphine in naive rats in the present study, the effects of acute morphine in chronic RB pre-treated rats are very similar to those previously observed in the same experimental conditions (see Honoré et al., 1997b). On the other hand, acute RB101(S) significantly decreased carrageenin-induced spinal c-Fos protein expression in morphine tolerant rats. These depressive effects were similar to those in naive rats (Table 1). These results are in good agreement with previous behavioural data (Noble et al., 1992b). Several hypotheses have been put forward by these authors to explain the lack of cross-tolerance between morphine and endogenous enkephalins (for more details, see Roques et al., 1993). First, because of their higher intrinsic efficacy, enkephalins need to occupy fewer opioid receptors than does morphine to give the same pharmacological responses (Noble and Roques, 1995). Second, the action of morphine and enkephalins on different putative opioid receptor subtypes (references in Sanchez-Blazquez et al., 1999; Pan et al., 2000) and their different desensitisation after chronic stimulation may account for the lack of cross-tolerance between morphine and endogenous enkephalins. A third hypothesis was linked to the release of anti-opiate peptides, such as cholecystokinin (CCK), which occurs during chronic opioid treatment (references in Stanfa et al., 1994; Harrison et al., 1998). Thus, it may be conceivable that morphine and enkephalins do not induce the same release of these peptides. However, this last hypothesis seems unlikely, since a profound potentialisation of the antinociceptive effects of both morphine and RB101 with concurrent administration of CCK_B (CCK_2) receptor antagonists has been demonstrated (Valverde et al., 1994, 1995; Chapman et al., 1995; Honoré et al., 1997a).

Surprisingly, in the first experimental series, RB101(S) and morphine had a slight anti-inflammatory effect. By contrast, these effects were not retrieved in the second experimental series, nor in previous experiments (Honoré et al., 1997a; Le Guen et al., 1999b; 2000a). It must be underlined that these effects are negligible in comparison to the pronounced effects of anti-inflammatory drugs in the same experimental conditions of carrageenin-evoked inflammatory nociception (see references in Buritova and Besson, 1999). Overall, without excluding a peripheral effect, these results are in good favour of the fact that the effects of endogenous enkephalins, protected of their deg-

radation by inhibitors of catabolizing enzymes, are mainly due to a spinal and/or supraspinal site of action. These effects could result from a direct action of enkephalins at the spinal cord level, and this action could be pre- or post-synaptic (references in Stanfa and Dickenson, 1994), but also from an indirect action by reinforcement of inhibitory descending system (see references in Basbaum and Fields, 1984; Gogas et al., 1996; see however Bouhassira et al., 1988). However, we cannot totally exclude a peripheral site of action, since the antinociceptive effects of systemic RB101 on responses to noxious pressure of rat hindpaw in rats with peripheral inflammation are partially blocked by the administration of methylnaloxonium, an opioid receptor antagonist which does not cross the blood–brain barrier (Maldonado et al., 1994).

In conclusion, these studies, based on "Fos imaging" of spinal dorsal horn neurones involved in nociceptive processes, in awake freely moving rats, provide further evidence that no tolerance, nor cross-tolerance with morphine, develops to the chronic administration of RB101(S), a mixed inhibitor of enkephalin-catabolizing enzymes. These results could be of clinical relevance for long-term treatment of patients suffering from chronic non-malignant pain.

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