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Interactions between NMDA- and prostaglandin receptor-mediated events in a model of inflammatory nociception

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Abstract

Preadministered niflumic acid, a nonsteroidal anti-inflammatory drug (1, 3 and 9 mg/kg i.v.), dose-relatedly reduced carrageenan-evoked spinal c-Fos expression and the peripheral ankle oedema, with the highest dose reducing in parallel both parameters (55 ± 3% reduction of carrageenan c-Fos expression, 57 ± 13% reduction of carrageenan-evoked ankle oedema, respectively, $P < 0.001$ for both). Co-administration of low doses of niflumic acid and (+)-HA966, a low-efficacy partial agonist at the glycine site of the NMDA receptor (1 mg/kg i.v. + 2.5 mg/kg s.c., respectively) significantly reduced spinal c-Fos expression, this effect was significantly different from the lack of effect of niflumic acid alone or (+)-HA966 alone on spinal c-Fos expression ($P < 0.01$ for both drugs). Co-administered niflumic acid and (+)-HA966 did not influence the peripheral carrageenan-evoked oedema. Spinal interactions between prostaglandin- and NMDA receptor-mediated events during inflammatory nociceptive transmission are discussed.

Keywords: Carrageenan; c-Fos; Inflammatory pain; Niflumic acid; (+)-HA966; Spinal cord; (Rat)

1. Introduction

Many studies have provided evidence for the contribution of NMDA receptor-mediated events to the generation of central hyperalgesia in animal models (for review, see Dickenson, 1994) and clinical trials (Price et al., 1994) of pain. NMDA receptor antagonists have been demonstrated to reduce the induction and maintenance of central hypersensitivity, in animal models of inflammatory pain, in particular to reduce the second phase of the formalin response (Haley et al., 1990; Dickenson and Aydar, 1991; Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992; Kristensen et al., 1993; Vaccarino et al., 1993; Hunter and Singh, 1994; Millan and Seguin, 1993, 1994), the hyperalgesia associated with Freund's adjuvant (Ren et al., 1992a; Ren and Dubner, 1993), carrageenan-evoked inflammation (Ren et al., 1992b; Yamamoto et al., 1993; Eisenberg et al., 1994; Laird et al., 1994) as well as the facilitated flexor reflex (Woolf and Thompson, 1991; Xu et al., 1992). In addition, NMDA receptor antagonists reduce C fiber-evoked windup (Dickenson and Sullivan, 1990; Dickenson and Aydar, 1991) and the temporal summation of second pain, a psycho-physical correlate of windup in clinical trials (Price et al., 1994). The potential clinical use of NMDA receptor antagonists is evident, but unfortunately limited by various side-effects (Kemp and Leeson, 1993). Thus, to reduce such side-effect liability there is considerable interest in the study of low-dose combinations of NMDA receptor antagonists with agents acting at different receptor systems (Chapman and Dickenson, 1992; Chapman et al., 1995a).

Recent studies have provided evidence for a relationship between spinal excitatory amino acid systems and prostaglandins. Prolonged afferent inputs, such as those arising during the biphasic response to formalin, have been shown to result in the biphasic release of spinal glutamate and prostaglandins, both of which were attenuated by cyclo-oxygenase inhibitors (Malmberg and Yaksh, 1995). Furthermore, thermal hyperalgesia evoked by intrathecal administration of NMDA, AMPA or substance P has been

Abbreviations: ANOVA, analysis of variance; c-Fos-LI, c-Fos protein-like immunoreactivity, c-Fos protein-like immunoreactive; Fisher's PLSD test, Fisher's protected least squares difference test; NMDA, *N*-methyl-D-aspartate; NSAID, non-steroid anti-inflammatory drug; PBS, phosphate-buffered saline; PB, phosphate buffer

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shown to be attenuated not only by the corresponding antagonists but also cyclo-oxygenase inhibition (Malmberg and Yaksh, 1992a). These previous findings are intriguing and, therefore, it is important to establish the contribution of ongoing physiological NMDA–cyclo-oxygenase interactions under conditions of prolonged nociceptive transmission. In an attempt to address this issue, we have studied the effect of co-administration of low doses of a NSAID, niflumic acid, and (+)-HA966 in the carrageenan model of inflammatory pain (Winter et al., 1962). (+)-HA966 is a low-efficacy partial agonist at the modulatory strychnine-insensitive glycine site of NMDA receptor complex, which acts as a functional NMDA receptor antagonist (Singh et al., 1990; see references in Kemp and Leeson, 1993).

Intraplantar injection of carrageenan is associated with a peripheral inflammation (see references in Kocher et al., 1987), a heat and mechanical hyperalgesia (Kayser and Guilbaud, 1987; Hargreaves et al., 1988; Iadarola et al., 1988; Joris et al., 1990) and spinal c-Fos protein expression (Noguchi et al., 1991, 1992; Honoré et al., 1995a). For a recent review of the mechanisms of c-fos gene induction and c-Fos protein expression, see Hughes and Dragunow (1995). Previously, we have demonstrated that (+)-HA966 (Chapman et al., 1995b) and NSAIDs, including indomethacin (Honoré et al., 1995a), diclofenac (Buritova et al., 1995a), piroxicam (Buritova et al., 1995b) and aspirin (Honoré et al., 1995c), dose-relatedly reduce carrageenan-evoked c-Fos expression in the dorsal horn of the rat lumbar spinal cord.

In the first part of this study, we have evaluated the effect of systemic preadministration of varying doses of niflumic acid on carrageenan-induced c-Fos expression, in the dorsal horn of the spinal cord and on the peripheral oedema in the rat. In the second part of this study, we have investigated the effect of co-administration of subthreshold doses of niflumic acid and (+)-HA966 on carrageenan-induced spinal c-Fos expression and peripheral oedema. A subthreshold concentration of niflumic acid, which had no effect on the peripheral signs of inflammation, was selected from the first part of the study and the subthreshold concentration of (+)-HA966 was previously ascertained under the same paradigm (Chapman et al., 1995b, and references therein).

2. Materials and methods

2.1. Experimental animals

Two experiments were performed on 44 adult male albino Sprague-Dawley rats (40 carrageenan-stimulated and 4 nonstimulated rats, 225–250 g; Charles River, France). The ethical guidelines of IASP for investigations of experimental pain in conscious animals were followed (Zimmermann, 1983).

2.2. Model of peripheral inflammation

Peripheral inflammation was induced by intraplantar injection of carrageenan (λ-carrageenan, Sigma; 6 mg/150 μl saline (0.9% NaCl)) in the right hindpaw of nonanaesthetized rats according to the method described by Winter et al. (1962). Rats were perfused 3 h after intraplantar carrageenan, when the number of c-Fos-LI neurons in the dorsal horn of the lumbar spinal cord is maximal (Honoré et al., 1995a). Carrageenan-nonstimulated rats were perfused with the other rats. In the present study, control rats receiving an intraplantar injection of saline were not included since we have previously shown negligible spinal c-Fos expression after intraplantar saline ($n < 5$ c-Fos-LI neurons/section L4–L5), which was not significantly different to spinal c-Fos expression in nonstimulated rats (Honoré et al., 1995a). In addition, a combination of s.c. bi-distilled water and i.v. vehicle (0.5% (v/v) NaOH 10 M in bi-distilled water) did not induce spinal c-Fos expression significantly different to that observed in nonstimulated rats ($n < 5$ c-Fos-LI neurons/section L4–L5; previous nonpublished data), thus, with consideration of this finding, nonstimulated rats receiving vehicles were not included in this study.

2.3. Drug administration

In the first part of this study, the effects of niflumic acid (UPSA, France; dissolved in a vehicle composed 0.5% (v/v) NaOH 10 M in bi-distilled water) on the number of c-Fos-LI neurons, in the dorsal horn of the rat spinal cord, 3 h after intraplantar carrageenan was studied. Niflumic acid (1, 3 and 9 mg/kg, $n = 5$ each group; vol. 0.25 ml) was injected i.v. into the rat tail vein 25 min prior to intraplantar carrageenan. Control carrageenan rats ($n = 5$) received an equal volume of i.v. vehicle (0.5% (v/v) NaOH 10 M in bi-distilled water; 0.25 ml) under the same experimental conditions. I.v. injections into the tail vein of conscious rats were made using a cylindrical rodent restrainer (Harvard Apparatus, Ealing, France) where the rats were fixed for 1 min during the manipulation.

In the second part of this study, the effects of a low dose of niflumic acid (1 mg/kg i.v., $n = 5$; vol. 0.25 ml) vs. a low dose of (+)-HA966 (2.5 mg/kg s.c., $n = 5$; vol. 0.25 ml) vs. co-administration of niflumic acid 1 mg/kg and (+)-HA966 2.5 mg/kg s.c. ($n = 5$; vol. 0.25 ml) on the number of c-Fos-LI neurons in the dorsal horn of the spinal cord, at 3 h after intraplantar carrageenan, was studied. (+)-HA966 ((+)-(3*R*)-3-amino-1-hydroxy-pyrrolidin-2-one, Tocris Cookson, UK; dose of 2.5 mg/kg, dissolved in bi-distilled water) was injected s.c. into the scruff of the rat neck 30 min prior to intraplantar carrageenan. Control rats ($n = 5$) received a combination of s.c. bi-distilled water (0.25 ml) and i.v. vehicle (0.5% (v/v) NaOH 10 M in bi-distilled water; 0.25 ml) 30 and 25 min prior to intraplantar carrageenan, respectively.

2.4. Inflammatory oedema

We considered two indicators of peripheral oedema: paw diameter, the measure of the oedema of the site of inflammatory stimulation with intraplantar injection of carrageenan; and ankle diameter, the measure of the extension of the oedema. For each rat, one measure of both paw and ankle diameters was performed with a calliper square at 3 h after carrageenan, immediately before perfusion. Carrageenan-enhanced paw and ankle diameters of the control group of rats (P_c and A_c, respectively; $n = 10$) and the drug-treated rats (P_t and A_t, respectively; $n = 30$) were measured. For comparison, paw and ankle diameters (P_n and A_n, respectively) of nonstimulated rats were measured ($n = 4$). The carrageenan-induced paw and ankle diameters were determined as the difference between the paw and ankle diameters of carrageenan- stimulated and -nonstimulated rats. The effects of drugs were determined as percentage changes of the carrageenan-induced paw and ankle diameter of drug-treated rats ($P_t - P_n$ and $A_t - A_n$, respectively) as compared with the ankle and paw diameter of control carrageenan group of rats ($P_c - P_n$ and $A_c - A_n$, respectively); the following formulas for the paw diameter: $((P_t - P_n)/(P_c - P_n)) \times 100$; and for the ankle diameter: $((A_t - A_n)/(A_c - A_n)) \times 100$ were used. Studies of carrageenan-evoked spinal c-Fos-LI neurons and peripheral oedema were performed in the same rats, thus, possible correlations between the parameters were determined.

2.5. Immunohistochemistry

As previously described (Chapman et al., 1995c; Honoré et al., 1995a), rats were deeply anesthetized (pentobarbital, 55 mg/kg i.p.; Sanofi) and perfused intracardially with the PBS 0.1 M followed by 4% paraformaldehyde in 0.1 M PB. The spinal cord was then removed and post-fixed for 4 h in the same fixative and cryoprotected by 30% sucrose in PB for 16 h. Frozen serial frontal sections (40 μm) of the lumbar enlargement (from L2 to L6) were cut and collected in PB. Immunohistochemistry of the free floating sections was performed with polyclonal antiserum, generated in rabbits and directed against the c-Fos protein (Oncogene Science, Ab-2 solution 0.1 mg/ml diluted 1 : 4000), using the conventional avidin-biotin-peroxydase complex method (Hsu et al., 1981). Finally, the c-Fos-LI were visualized by 1-naphthol ammonium carbonate solution for 5 min (Menétrey et al., 1992). The sections were mounted on gelatin-subbed slides and air dried for the stain to be intensified by 0.025% Crystal violet (42555 Aldrich) in bi-distilled water. 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. As immunochemistry of different experiments might vary, the spinal cord sections of rats from the same experiment were immunoreacted at the same time, to justify the use of statistical tests.

2.6. Counting of spinal c-Fos-LI neurons

As previously described (Buritova et al., in press; Honoré et al., 1995a), labeled c-Fos-LI neurons in the rat lumbar spinal cord were counted with a camera lucida attachment through 4 defined regions: superficial laminae (laminae I–II), nucleus proprius (laminae III–IV) and neck (laminae V–VI) of dorsal horn and, in addition, the ventral horn (laminae VII–X; ventral). As previously shown, the most numerous c-Fos-LI neurons were localised in the L4–L5 segments (Honoré et al., 1995a), so for the pharmacological study of the tested drugs, for each rat, two counts were made: (1) the total number of c-Fos-LI neurons in the grey matter for 10 sections through L4–L5 segments; and (2) in these 10 sections the number of c-Fos-LI neurons/4 defined regions. The investigator responsible for plotting and counting the c-Fos-LI neurons was blind to the experimental condition of each rat.

2.7. Statistical tests

Statistical analysis was performed using ANOVA and the Fisher's protected least squares difference test for multiple comparisons. The dose-dependent effects of niflumic acid on both the number of c-Fos-LI neurons and the peripheral inflammatory oedema (enhanced paw and ankle diameter) and possible correlations between the parameters were determined using a simple regression and correlation coefficient, respectively.

3. Results

3.1. c-Fos-LI neurons in the lumbar spinal cord at 3 h after intraplantar carrageenan

Intraplantar carrageenan evoked a significantly high level of expression of c-Fos-LI in segments L4–L5 of the spinal cord ipsilateral to the carrageenan inflammation in non-anaesthetised freely moving rats. The number of c-Fos-LI neurons of control carrageenan groups with vehicles, i.v. vehicle for first experiment and the combination of i.v. vehicle and s.c. bi-distilled water for second experiment, were greatly increased at 3 h after carrageenan (97 ± 6 and 145 ± 6 c-Fos-LI neurons/section in segments L4–L5, respectively; Fig. 1A, Fig. 2, Table 1). Although the spinal c-Fos-LI of the control carrageenan groups were significantly different for two experimental series ($P < 0.05$), in both control carrageenan groups, c-Fos-LI neurons were predominantly located in the laminae I–II (46 ± 1 and 54 ± 3 c-Fos-LI neurons/L4–L5 section, respectively) and V–VI (32 ± 3 and 61 ± 4 c-Fos-LI neurons/L4–L5 section, respectively) of the dorsal

horn of the spinal cord. The number of c-Fos-LI neurons in the ventral horn was moderate (15 ± 2 and 24 ± 1 c-Fos-LI neurons/L4–L5 section, respectively). Very few c-Fos-LI neurons were present in nucleus proprius (laminae III–IV; $n < 7$ c-Fos-LI neurons/L4–L5 section); the number of c-Fos-LI neurons in the contralateral spinal cord was negligible ($n < 5$ c-Fos-LI neurons/section).

3.2. Effects of niflumic acid on carrageenan-evoked spinal c-Fos expression

The effects of i.v. preadministered niflumic acid (1, 3 and 9 mg/kg) on the number of c-Fos-LI neurons were dose-dependent ($r^2 = 0.797$, $P < 0.001$) and significant, when considering both the total number of c-Fos-LI neurons in segments L4–L5 ($F(3,16) = 22.99$; $P < 0.001$) and their laminar distribution ($F(3,64) = 45.00$; $P < 0.001$). The higher doses of niflumic acid (3 and 9 mg/kg i.v.) significantly decreased the total number of c-Fos-LI neurons in segments L4–L5 at 3 h after intraplantar carrageenan (28 ± 6 and 55 ± 3% reduction of the control total number of carrageenan-evoked c-Fos-LI neurons, $P < 0.01$ and $P < 0.001$, respectively; Figs. 1 and 2).

Laminar analysis revealed that the higher doses of preadministered niflumic acid (3 and 9 mg/kg i.v.) significantly reduced the number of c-Fos-LI neurons in the deep laminae (37 ± 7 and 64 ± 4% reduction of control carrageenan c-Fos-LI, $P < 0.01$ and $P < 0.001$, respectively) of the dorsal horn (Fig. 3). In contrast, only the highest dose (9 mg/kg) of niflumic acid had a significant effect on the number of superficial c-Fos-LI neurons (38 ± 5% reduction of control carrageenan c-Fos-LI, $P < 0.001$). However, the effects of niflumic acid were dose-dependent for both the superficial and deep laminae ($r^2 = 0.708$ and $r^2 = 0.706$, respectively, $P < 0.001$ for both). Furthermore, both 3 and 9 mg/kg niflumic acid had a significantly stronger effect on the number of deep as compared with superficial c-Fos-LI neurons ($P < 0.05$ and $P < 0.01$, respectively; Fig. 3).

3.3. Effects of various doses of niflumic acid on the carrageenan-evoked oedema

3 h after intraplantar carrageenan both the ankle and paw diameters were significantly increased as compared with nonstimulated rats (Fig. 4). Inflammatory oedema

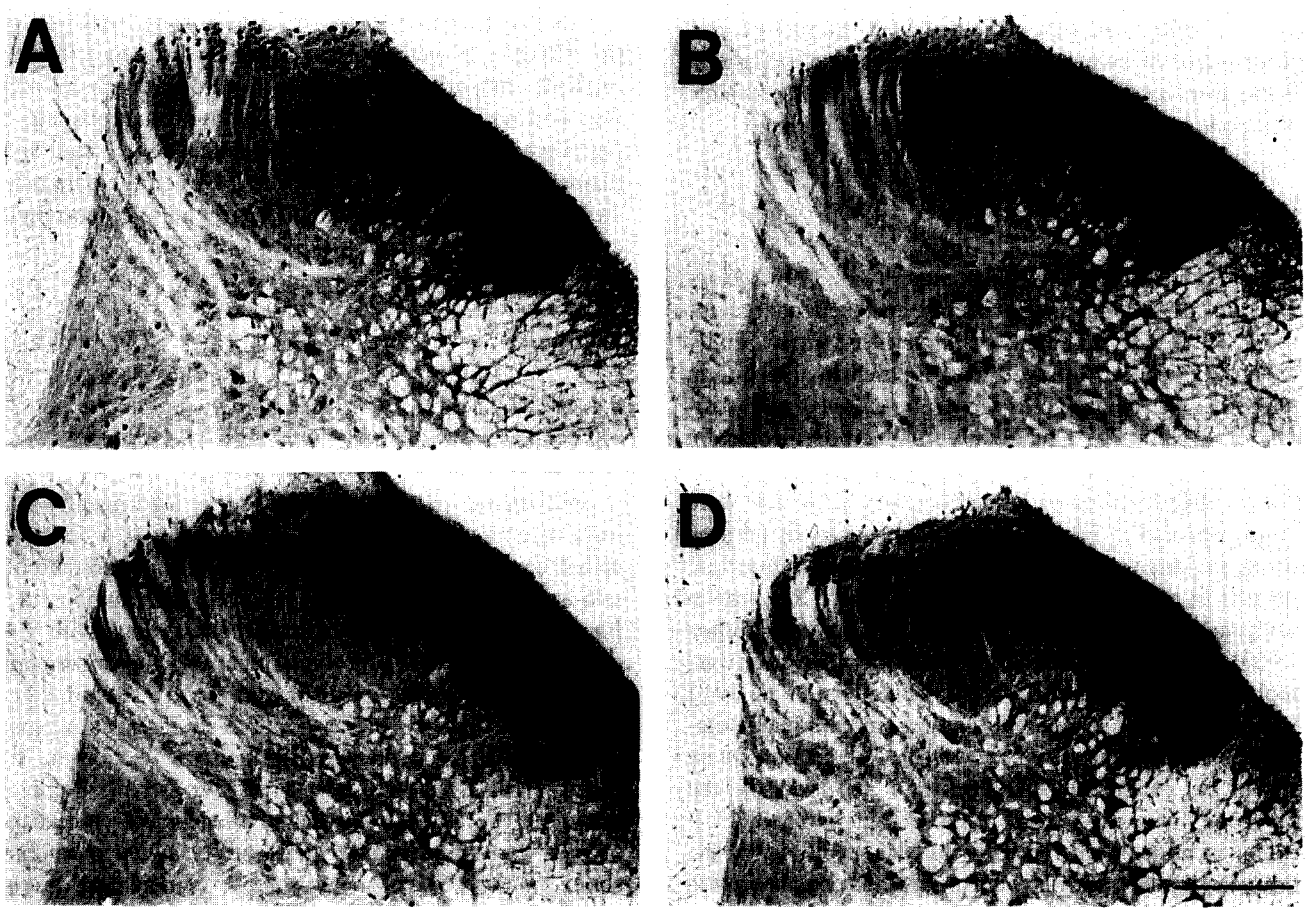

Fig. 1. Microphotographs of individual sections (40 μ) of the L4–L5 segments of the rat spinal cord showing the effect of preadministered niflumic acid (1, 3 and 9 mg/kg i.v.) on the number of spinal c-Fos-LI neurons, 3 h after intraplantar carrageenan (6 mg/150 μl saline). Scale bar: 200 μm. (A) Carrageenan + vehicle i.v. (control). (B) Carrageenan + niflumic acid 1 mg/kg i.v. (C) Carrageenan + niflumic acid 3 mg/kg i.v. (D) Carrageenan + niflumic acid 9 mg/kg i.v.

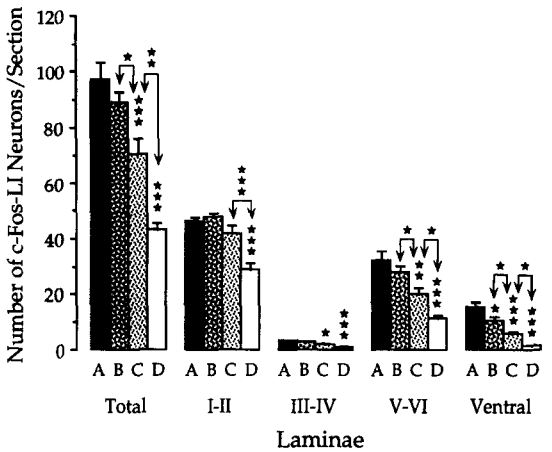

Fig. 2. The effects of niflumic acid (B, C and D; 1, 3 and 9 mg/kg i.v., respectively) on the number of c-Fos-LI neurons in the L4–L5 segments of the rat spinal cord, 3 h after intraplantar carrageenan. Results are expressed as mean number of c-Fos-LI neurons (±SEM), per L4–L5 segments (total) and per laminar region (laminae I–II, III–IV, V–VI, ventral). Significance as compared with control carrageenan group with vehicle (A) was performed using ANOVA and Fisher's PLSD test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

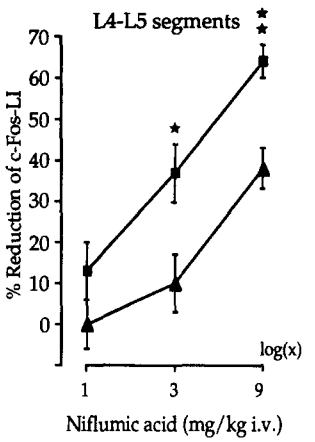

Fig. 3. Comparison of the effect of niflumic acid (1, 3 and 9 mg/kg i.v.) on the number of superficial (laminae I–II; triangles) and deep (laminae V–VI; squares) c-Fos-LI neurons in the L4–L5 segments. Results are expressed as percentage reduction of the control number of carrageenan-evoked c-Fos-LI neurons for each region (±SEM). Statistical comparisons between the effects of niflumic acid on the c-Fos-LI neurons in the superficial vs. deep laminae of the dorsal horn of the L4–L5 segments were performed using ANOVA and Fisher's PLSD test (* $P < 0.05$, ** $P < 0.01$).

was not exhibited in the hindpaw contralateral to carrageenan intraplantar injection.

Preadministered niflumic acid (1, 3 and 9 mg/kg i.v.) dose-dependently reduced both the carrageenan-enhanced ankle and paw diameters ($r^2 = 0.494$ and $r^2 = 0.499$, respectively, $P < 0.01$ for both diameters; Fig. 4). The higher doses of niflumic acid (3 and 9 mg/kg i.v.) significantly reduced both the ankle (33 ± 9 and 57 ± 13% reduction of the control carrageenan ankle diameters, respectively, $P < 0.001$ for both doses) and paw (12 ± 5 and 27 ± 5% reduction of the control carrageenan paw diameters, $P < 0.05$ and $P < 0.001$, respectively) oedema. Furthermore, the effects of niflumic acid on the carrageenan-evoked spinal c-Fos expression and both the paw and ankle oedema were positively correlated ($r = 0.721$, $0.331 < r < 0.901$; $P < 0.05$ and $r = 0.699$, $0.291 < r < 0.892$; $P < 0.01$, respectively; Fig. 5).

3.4. Effects of co-administered niflumic acid and (+)-HA966 on carrageenan-evoked spinal c-Fos expression

Co-administered niflumic acid and (+)-HA966 (1 mg/kg i.v. + 2.5 mg/kg s.c., respectively) significantly decreased the total number of c-Fos-LI neurons in L4–L5 segments at 3 h after intraplantar carrageenan (38 ± 8% reduction of control carrageenan c-Fos expression, $P < 0.01$; Table 1). This attenuating effect of the niflumic acid/(+)-HA966 combination was also significantly different from the lack of effect of niflumic acid alone (1 mg/kg i.v.) or (+)-HA966 alone (2.5 mg/kg s.c.) on spinal c-Fos expression ($P < 0.01$ for both; Table 1).

Laminar analysis revealed that co-administered niflumic acid/(+)-HA966 (1 mg/kg i.v. + 2.5 mg/kg s.c., respectively) significantly reduced the number of c-Fos-LI neurons in the superficial (34 ± 8% reduction as compared

Table 1
The effect of niflumic acid alone, (+)-HA966 alone and co-administered niflumic acid/(+)-HA966 on the number of c-Fos-LI neurons in the superficial laminae (laminae I–II), deep laminae (laminae V–VI) and in all laminae (Total), of the L4–L5 segments of the rat spinal cord, and on the enhanced ankle and paw diameter following intraplantar injection of carrageenan.

Drugs	Dose (mg/kg)	Number of spinal c-Fos-LI neurons			Diameter of inflammation (cm)	
		Total	Laminae I–II	Laminae V–VI	Ankle	Paw
Control	–	145 ± 6	54 ± 3	61 ± 4	1.36 ± 0.02	1.28 ± 0.04
Niflumic acid (i.v.)	1.0	136 ± 12	52 ± 4	57 ± 7	1.28 ± 0.03	1.15 ± 0.09 *
(+)-HA966 (s.c.)	2.5	135 ± 9	51 ± 2	57 ± 5	1.34 ± 0.06	1.28 ± 0.04
Niflumic acid (i.v.) + (+)-HA966 (s.c.)	1.0 ± 2.5	90 ± 12 **,oo,##	35 ± 4 **,oo,##	37 ± 5 **,o,#	1.31 ± 0.03	1.24 ± 0.04

Results are expressed as mean number of c-Fos-LI neurons for each region (±SEM) and as mean ankle and paw diameters (±SEM). Significance of the effects of all drugs as compared with the control carrageenan values, were performed using ANOVA and Fisher's PLSD test (* $P < 0.05$, ** $P < 0.01$). Significance of the effects of co-administered niflumic acid/(+)-HA966 were performed using ANOVA and Fisher's PLSD test (° $P < 0.05$, °° $P < 0.001$ as compared with the effects of niflumic acid alone; # $P < 0.05$, ## $P < 0.001$ as compared to the effects of (+)-HA966 alone).

with control carrageenan c-Fos expression, $P < 0.01$) and deep ($39 \pm 9\%$ reduction of control carrageenan c-Fos expression, $P < 0.01$) laminae of the dorsal horn (Table 1). Neither preadministered niflumic acid alone or (+)-HA966 alone influenced the number of spinal c-Fos-LI neurons induced at 3 h after intraplantar carrageenan (Table 1). The effect of co-administered niflumic acid/(+)-HA966 was also significant, as compared with the effect of niflumic acid alone and (+)-HA966 alone, for both the superficial (I–II; $P < 0.01$ for both drugs) and deep (V–VI; $P < 0.05$ for both drugs) laminae. Co-administerd niflumic acid/(+)-HA966 had similar effects on the number of deep, as compared with superficial, c-Fos-LI neurons.

3.5. Co-administered niflumic acid/(+)-HA966 does not influence the carrageenan-evoked oedema

In the second study, preadministration of the low dose of niflumic acid alone (1 mg/kg i.v.) had a weak effect on the carrageenan paw oedema ($16 \pm 11\%$ reduction of control carrageenan paw diameters, $P < 0.05$; Table 1). Preadministration of the low dose of (+)-HA966 alone (2.5 mg/kg s.c.) had no effect on either the carrageenan-evoked ankle or paw oedema. Co-administered niflumic acid/(+)-HA966 (1 mg/kg i.v. + 2.5 mg/kg s.c.) did not influence the carrageenan-evoked ankle or paw oedema (Table 1).

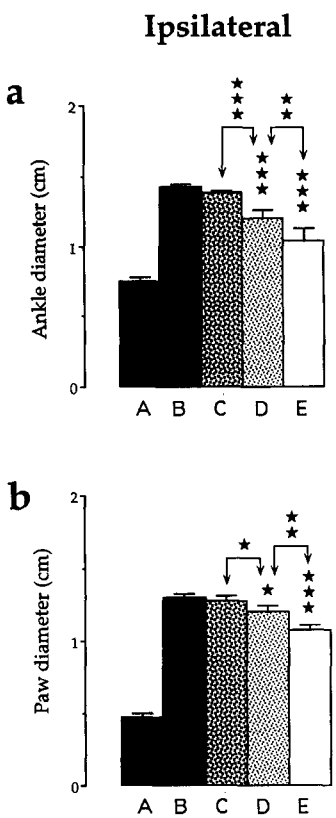

Fig. 4. The effect of niflumic acid (C, D and E; 1, 3 and 9 mg/kg i.v., respectively) on the carrageenan-enhanced paw and ankle diameters, compared with the control carrageenan rats (B; carrageenan with i.v. saline control group for drug administration) and a nonstimulated rats (A). Results are expressed as the absolute mean ankle (panel a) and paw (panel b) diameter (±SEM). Significance as compared with the control carrageenan paw and ankle oedema (B) was performed using ANOVA and Fisher's PLSD test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

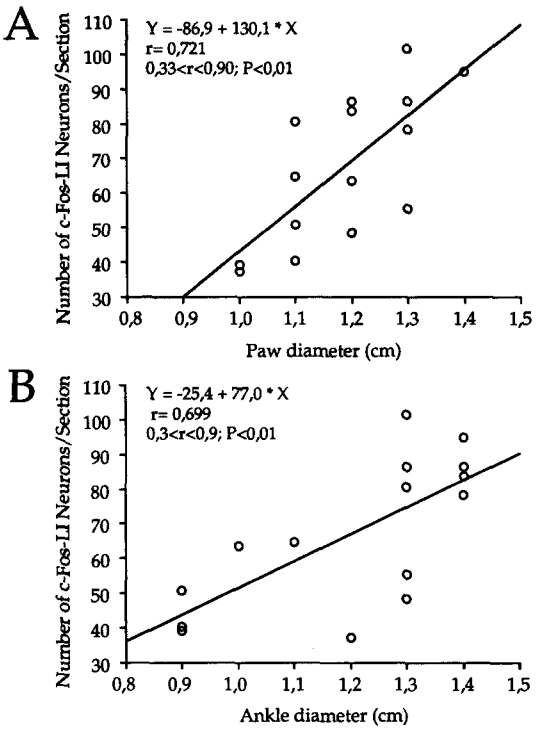

Fig. 5. Correlations between the total number of spinal c-Fos-LI neurons and paw (A) and ankle (B) diameter (cm), 3 h after intraplantar injection of carrageenan. Statistical tests of individual results for each rat were performed using a simple regression ($P < 0.01$).

4. Discussion

In the present study, intraplantar injection of carrageenan into the hindpaw of nonanaesthetised rats resulted in the development of a peripheral oedema and an associated spinal c-Fos expression. In accordance with our previous studies (Honoré et al., 1995a, b, c; Buritova et al., 1995a, b, in press; Chapman et al., 1995a, b), spinal c-Fos expression, induced by intraplantar injection of carrageenan, was maximal in the L4–L5 segments of lumbar spinal cord, corresponding to the hindlimb dermatomes (Molander and Grant, 1986). In good agreement with our previous studies, carrageenan-evoked c-Fos-LI neurons were essentially located in the superficial (I–II) and deep (V–VI) laminae of the dorsal horn of the spinal cord. The control level of carrageenan-evoked spinal c-Fos expression was different for the two experimental series (niflumic acid vs. co-administration of niflumic acid/(+)-HA966 series), however, comparisons between the two series of experiments were not made. Such differences between the

two experimental series are likely to arise from the inherent variability of immunohistochemistry for different experimental series. Irrespective of this difference, for both series of experiments, c-Fos expression was predominantly located in the superficial (I–II) and deep (V–VI) laminae of the dorsal horn, thus, corresponding to spinal areas driven by noxious stimuli (see references in Besson and Chaouch, 1987, and Willis and Coggeshall, 1991). Interestingly, it has previously been shown that electrical activation of C and Ad fibers, but not Ab fibers, evokes spinal c-Fos expression in the superficial and deep laminae of the dorsal horn (Herdegen et al., 1991). We have previously shown that intraplantar injection of saline results in negligible spinal c-Fos expression ($n < 5$ c-Fos-LI neurons/section), which is similar to that observed in nonstimulated rats (Honoré et al., 1995a).

The first part of this study demonstrated that i.v. preadministered niflumic acid, a NSAID, dose-dependently reduces the number of carrageenan-evoked c-Fos-LI neurons in the superficial and deeper laminae of the dorsal horn of segments L4–L5 of the spinal cord. Thus, niflumic acid reduced c-Fos expression in areas of the dorsal horn previously shown to contain neurons activated by noxious stimulation (see above). Higher doses of niflumic acid produced a significantly more pronounced reduction of the number of deep, as compared with superficial c-Fos-LI neurons. The efficacy of niflumic acid was similar to that of other NSAIDs tested under similar conditions (Honoré et al., 1995a, c; Buritova et al., 1995a, b). The effect of niflumic acid on carrageenan-evoked spinal c-Fos expression was correlated with a reduced carrageenan-evoked paw and ankle oedema. This correlation suggests a peripheral anti-inflammatory action of niflumic acid and, thus, a subsequent reduction of nociceptive inputs to the spinal cord and an attenuation of spinal c-Fos expression. However, with consideration of the mounting evidence for a spinal role of prostaglandins (see Introduction; Malmberg and Yaksh, 1992b, 1994a, b) a central site of action of niflumic acid cannot be excluded.

Overall our results are in agreement with and extend previous studies of the anti-inflammatory/analgesic effects of niflumic acid in the carrageenan model of inflammation (Flower et al., 1972) and clinical trials of inflammatory processing (Gulati et al., 1975; Dömötör, 1979; Bienvenu et al., 1985). Niflumic acid, a member of the fenamate family of NSAIDs (see references in Insel, 1991), is used for the treatment of long-term inflammatory pain states associated with rheumatoid arthritis and osteoarthritis (Tilve et al., 1976). However, the clinical use of fenamates is somewhat limited because of the gastro-intestinal and other side-effects (e.g. Wolfe et al., 1976, and Boyle et al., 1976; see references in Insel, 1991). Thus, in the present study, we have used a low dose of niflumic acid in combination with the NMDA receptor antagonist, (+)-HA966, to investigate therapeutic effects, with a reduced possible side-effect liability of both drugs. Recent behavioral studies have demonstrated that s.c. (+)-HA966, which crosses the blood-brain barrier (see references in Kemp and Leeson, 1993), dose-dependently inhibited the later phase of the licking and biting response to an intraplantar injection of formalin in rats (Hunter and Singh, 1994) and mice (Millan and Seguin, 1993, 1994), in the absence of motor side-effects.

The second part of our study clearly demonstrates that co-administration of low doses of niflumic acid and (+)-HA966 significantly decreases carrageenan-evoked spinal c-Fos expression and that this effect of the co-administration was significantly different to the effect of either drug given alone. Co-administered niflumic acid/(+)-HA966 had similar effects on the number of deep, as compared with superficial, c-Fos-LI neurons. With consideration of our present study and a previous study of (+)-HA966 (Chapman et al., 1995b) under the same experimental paradigm, we can compare the effect of co-administered niflumic acid/(+)-HA966 (1 mg/kg niflumic acid + 2.5 mg/kg (+)-HA966) to the effects of higher doses of both drugs tested separately (9 mg/kg niflumic acid alone or 10 mg/kg (+)-HA966 alone). As previously demonstrated (Chapman et al., 1995b), a high concentration of (+)-HA966 (10 mg/kg) significantly reduces the number of c-Fos-LI neurons in the deep laminae whereas lower doses of (+)-HA966 (0.5 and 2.5 mg/kg) did not significantly influence either the number of superficial or deep c-Fos-LI neurons. Overall, the effects of co-administered low dose of niflumic acid and (+)-HA966 is comparable to the effects of high dose of niflumic acid alone on the carrageenan-induced spinal c-Fos expression in both the superficial and deep laminae.

Co-administered niflumic acid/(+)-HA966 did not influence the peripheral carrageenan-evoked oedema and, although a peripheral side of action can not be excluded, our results suggest a predominant spinal site of action of co-administered (+)-HA966/niflumic acid on carrageenan-evoked c-Fos expression. The lack of effect of the low dose of (+)-HA966 alone was in concordance with our previous study demonstrating that the same dose of (+)-HA966 (2.5 mg/kg) and also higher dose of (+)-HA966 (10 mg/kg) had no effect on the carrageenan-induced peripheral oedema (Chapman et al., 1995b) and another study showing that higher dose of (+)-HA966 (30 mg/kg) does not influence the carrageenan-induced paw oedema (Hunter and Singh, 1994).

Our results are in keeping with those of previous studies (Malmberg and Yaksh, 1992a, b) illustrating that under conditions of prolonged inflammation that there is a spinal interaction between prostaglandin- and NMDA receptor-mediated events. Although the mechanism of the interaction between the two receptor systems is not clear, it is possible that it is a consequence of common intracellular endpoints or spatial interaction. The origin of spinal prostaglandins is not well-established, however, release into the extracellular space may result from not only

neuronal but also glial structures (Marriott et al., 1990; for review, see Barres, 1991). Increased levels of prostaglandins have been shown to be present in the perfusate of the spinal cord upon hindlimb stimulation (Ramwell et al., 1966) and following noxious peripheral thermal stimulation (Coderre et al., 1990). More recently, direct evidence for the spinal release of prostaglandins following peripheral injection of formalin in the rat has been reported (Malmberg and Yaksh, 1995). Furthermore, it has been demonstrated that spinal administration of NMDA evokes prostaglandin E_2 release into the spinal cord, which is blocked by intrathecal cyclo-oxygenase inhibitor (Sorkin, 1993). Finally, intrathecal administration of prostaglandins has been shown to induce allodynia via NMDA receptor- and nitric oxide-mediated pathways (Minami et al., 1994, 1995).

In general, direct NMDA receptor-mediated events are considered to be postsynaptic to the primary afferent ending. NMDA receptor activation results in an increase of intracellular calcium (MacDermott et al., 1986), which activates phospholipases and nitric oxide synthase. Subsequently, NMDA receptor activation of phospholipases results in an increased level of free arachidonic acid (Dumuis et al., 1988; Pellerin and Wolfe, 1991), which may then be converted by cyclooxygenase enzyme to the prostanoids, including the prostaglandins. Since the generation and release of spinal prostaglandin appears to be mediated, at least in part, by NMDA receptor activation, which is frequency-dependent, requiring a certain level of postsynaptic activation prior to the necessary removal of the magnesium block of the NMDA receptor (see references in Dickenson, 1994), it can be assumed that spinal prostaglandin release requires a certain level of repetitive stimulation of the fine primary afferent (Malmberg and Yaksh, 1992a).

Although to our knowledge the location of prostaglandin receptors is not well-established, there is evidence for prostaglandin E_2-binding sites in laminae I–II of the dorsal horn (Matsumura et al., 1992), an area of fine primary afferent termination (Willis and Coggeshall, 1991). Furthermore, there is evidence that prostaglandins enhance depolarisation-evoked release of substance P and calcitonin gene-related peptide from fine primary afferent terminals (Nicol et al., 1992; Vasko et al., 1993; Hingtgen et al., 1995) and increase the spinal release of glutamate and aspartate (Malmberg et al., in press). In addition, prostaglandin-evoked increase of calcitonin gene-related peptide (CGRP) release has been demonstrated from spinal cord slices (Andreeva and Rang, 1993). This action of prostaglandin on neuropeptide release from sensory neurons has been shown to be mediated via activation of the cAMP transduction cascade (Hingtgen et al., 1995). Thus, there is strong evidence that spinal prostaglandins enhance fine primary afferent transmitter release and, thus, may modify postsynaptic neuronal responses to excitatory C-fiber inputs arising from periphery. Thus, it is feasible that during periods of prolonged afferent input that spinal prostaglandins augment the level of primary afferent transmitter release and consequently reduce the latency of NMDA receptor activation. Therefore, the enhanced attenuating effect of co-administered NSAID and NMDA receptor antagonism, on noxiously evoked c-Fos expression, may result from this dual site of action at both pre- and postsynaptic sites. However, it is worth considering that an in vitro study has shown that niflumic acid may interact directly at the polyamine modulatory site of the NMDA receptors (Lerma and Martin Del Rio, 1992). Thus, a direct interaction between niflumic acid and (+)-HA966 at the NMDA receptor level can not be excluded.

In conclusion, from the present study it appears that concurrent NMDA receptor antagonism and prostaglandin synthase inhibition reduces nociceptive transmission at the spinal level, as shown by the reduction of carrageenan-induced spinal c-Fos expression. Our results suggest that therapy with a combination of low doses of (+)-HA966 and niflumic acid may produce enhanced antinociceptive effects and possible beneficial pain relief with a reduced side-effect liability.

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