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The contribution of peripheral bradykinin B_2 receptors to carrageenan-evoked oedema and spinal c-Fos expression in rats

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Abstract

Intraplantar co-injection of HOE140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin), a selective bradykinin B_2 receptor antagonist (0.1, 1 and 10 μg), with carrageenan dose-dependently ($r = 0.66$, $P < 0.01$) reduced the carrageenan-evoked total number of c-Fos protein-like immunoreactive (c-Fos-LI) neurones ($23 \pm 5\%$, $35 \pm 6\%$ and $50 \pm 5\%$ reduction; $P < 0.01$, $P < 0.001$ and $P < 0.001$, respectively). These reducing effects were dose-dependent for the number of c-Fos-LI neurones in both superficial ($r = 0.70$, $P < 0.01$) and deep ($r = 0.53$, $P < 0.05$) laminae. Intraplantar co-injection of HOE140 (0.1, 1 and 10 μg) with carrageenan significantly reduced the carrageenan-evoked paw ($25 \pm 7\%$, $41 \pm 6\%$ and $41 \pm 3\%$ reduction; $P < 0.001$ for all) and ankle ($46 \pm 6\%$, $61 \pm 5\%$ and $61 \pm 5\%$ reduction; $P < 0.001$ for all) oedema. Our results provide further evidence for the involvement of peripheral bradykinin B_2 receptors in carrageenan-induced inflammatory nociceptive transmission.

Keywords: Bradykinin; Carrageenan inflammation; c-Fos; Nociception; Spinal cord

1. Introduction

It is well established that inflammation is associated with hyperalgesia, which may have both peripheral and central origins (see references in Ferreira and Lorenzetti, 1995; Levine et al., 1993). Peripheral hypersensitivity of the nociceptors is considered to be an important factor in the development of inflammatory hyperalgesia (see Ferreira and Lorenzetti, 1995; Dray, 1995; Levine et al., 1993; Rang et al., 1991). Both basic (Hargreaves et al., 1988a; Taiwo et al., 1990; see Refs. in Kirchhoff et al., 1990; Dray, 1994) and clinical studies (Hargreaves et al., 1988a; Jensen et al., 1990) have implicated bradykinin as one of key mediators involved in the development of inflammation, and the associated nociception.

The established algesic action of bradykinin at the peripheral level (see Refs. in Bathon and Proud, 1991) has been shown to evolve from the activation of primary afferents (see references in Martin et al., 1987) via activation of bradykinin B_2 receptors (Steranka et al., 1988; Haley et al., 1989; Steranka and Burch, 1991; see Dray and Perkins, 1993). The recent autoradiographic study has indicated a B_2 subtype of bradykinin receptors throughout the stratum basale of the epidermis in normal human skin (Schremmer-Danninger et al., 1995).

The role of bradykinin in the carrageenan or formalin models of inflammatory nociception has been extensively investigated with HOE140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin), a selective bradykinin B_2 receptor antagonist (Bao et al., 1991; Hock et al., 1991; Lembeck et al., 1991; Wirth et al., 1991). Previous studies have demonstrated that intraplantar injection of HOE140 suppresses both the first and second phase of the behavioural response (Beresford and Birch, 1992; Correa and Calixto, 1993), and responses of deep dorsal horn neurones (Chapman and Dickenson, 1992) to the peripheral injection of formalin. Furthermore, the oedema (Wirth et al., 1991; Damas and Remacle-Volon, 1992) and hyperalgesia (Ferreira et al., 1993) associated with the intraplantar injection of carrageenan have been shown to be greatly reduced by HOE140. Thus there is strong evidence for a peripheral role of bradykinin B_2 receptors during inflammatory nociceptive processing.

In this study we have used the intraplantar injection of carrageenan (Winter et al., 1962) which induces an ipsilateral oedema characterised by sequential release of inflammatory mediators, including bradykinin (Di Rosa et al.,

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1971). Subcutaneous injection of carrageenan has been shown to lead to the sensitisation of unmyelinated mechano-heat sensitive nociceptors (Kocher et al., 1987), and polymodal nociceptors have an increased sensitivity to bradykinin during carrageenan inflammation (Kirchhoff et al., 1990). It has been demonstrated that the carrageenan-induced hyperalgesia (Hargreaves et al., 1988b; Iadarola et al., 1988; Joris et al., 1990) not only parallels the peripheral inflammation, but also the spinal expression of c-Fos (see references in Honoré et al., 1995), the nuclear protein product encoded by the immediate-early gene c-*fos* (for review, see Morgan and Curran, 1991; Hughes and Dragunow, 1995).

Numerous studies have used the immunohistochemical revelation of spinal c-Fos protein, an indirect marker of neurones implicated in the nociceptive transmission, to gauge the effect of analgesic and anti-inflammatory drugs in the various model of nociception (see Munglani and Hunt, 1995 and references therein). In our previous studies, we have used this approache to assess the effects of differents analgesic/anti-inflammatory drugs in the carrageenan model of inflammatory pain in rats (see Buritova et al., 1996a; Honoré et al., 1996 and references therein). In the present study, to assess the contribution of peripheral bradykinin B_2 receptors to carrageenan-evoked oedema and spinal c-Fos expression, we have used the peripherally co-administered HOE140 with carrageenan. We evaluated the effect of intraplantar HOE140, a bradykinin B_2 receptor antagonist, on carrageenan-induced peripheral oedema and c-Fos protein-like immunoreactivity (c-Fos-LI) in the dorsal horn of the spinal cord of the rat.

2. Material and methods

2.1. Experimental animals

Experiments were performed on 24 adult male albino Sprague-Dawley rats (Charles River, France; 20 carrageenan-stimulated and 4 non-stimulated rats), weighing 225–250 g. The ethical guidelines of the International Association for Study of Pain (IASP) for investigations of experimental pain in conscious animals were followed (Zimmermann, 1983).

2.2. Carrageenan-induced peripheral inflammation and oedema

Peripheral inflammation was induced by intraplantar injection of carrageenan (λ-carrageenan, Sigma) in the right hindpaw of non-anaesthetised rats according to the method described by Winter et al. (1962). Intraplantar injection of carrageenan (6 mg in 150 μl of saline (0.9% NaCl)) was administered subcutaneously with a 25 gauge needle. 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 (< 5 c-Fos-LI neurones per L4–L5 section) which was not significantly different from that observed in non-stimulated rats (Honoré et al., 1995).

Two measures of carrageenan peripheral oedema, ankle and paw diameters, were made using a calliper square at 3 h after carrageenan, immediately before perfusion. For comparison, ankle and paw diameters of carrageenan control (A_c, P_c, respectively; $n = 5$) and drug treated (A_t, P_t, respectively; $n = 15$) rats and non-stimulated rats (A_n, P_n, respectively; $n = 4$) were measured. The carrageenan-induced paw and ankle diameters were determined as the difference between the paw and ankle diameters of carrageenan-stimulated and non-stimulated rats. The effects of drugs were determined as percent changes of the carrageenan-induced paw and ankle diameter of drug-treated rats ($P_t - P_n$, $A_t - A_n$ respectively) as compared to the ankle and paw diameter of control carrageenan group of rats ($P_c - P_n$, $A_c - A_n$ respectively); the following formula 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 the carrageenan-induced peripheral oedema and spinal c-Fos expression were performed in the same rats, thus possible correlations between the parameters were determined.

2.3. Drug administration

In the present study, the effects of intraplantar injection of HOE140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin, Hoechst, Germany; dissolved in bidistilled water) on both the carrageenan-induced peripheral oedema and spinal c-Fos protein expression, 3 h after intraplantar injection of carrageenan, were studied. Intraplantar injections of HOE140 (0.1, 1 and 10 μg in 50 μl of bidistilled water; $n = 5$ for each group) were administered subcutaneously and simultaneously with carrageenan (in the same syringe) using a 25 gauge needle. Control carrageenan rats ($n = 5$) received an equal volume of intraplantar vehicle (50 μl of bidistilled water) simultaneously with carrageenan.

2.4. Immunohistochemistry

As previously described (Honoré et al., 1995), 3 h after intraplantar carrageenan, rats were deeply anaesthetised (pentobarbital, Sanofi; 55 mg/kg i.p.) and perfused intracardially with phosphate-buffered saline 0.1 M followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cord was then removed, postfixed for 4 h in the same fixative, and cryoprotected in 30% sucrose overnight. Frozen serial frontal sections (40 μm) of the lumbar L4–L5 segments were cut. Immunohistochemistry of the free-floating sections was performed with polyclonal antiserum, generated in rabbits, directed against the c-Fos protein (Oncogene Science, Ab-2 solution 0.1 mg/ml diluted 1:4000), using the conventional avidin-biotin-per-

oxydase complex method (Hsu et al., 1981). Finally, c-Fos protein-like immunoreactivity (i.e. labelled nuclei of c-Fos-LI neurones) was visualised 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 (Aldrich). The differentiation time in 70% alcohol was evaluated under the microscope. 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.

2.5. Counting of spinal c-Fos-LI neurones

At the lumbar spinal cord level, labelled nuclei of c-Fos-LI neurones were counted with a camera lucida attachment without considering the intensity of the staining. To study the distribution of c-Fos-LI neurones four regions were defined according to cytoarchitectonic criteria (laminae I–X; Molander et al., 1984): 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

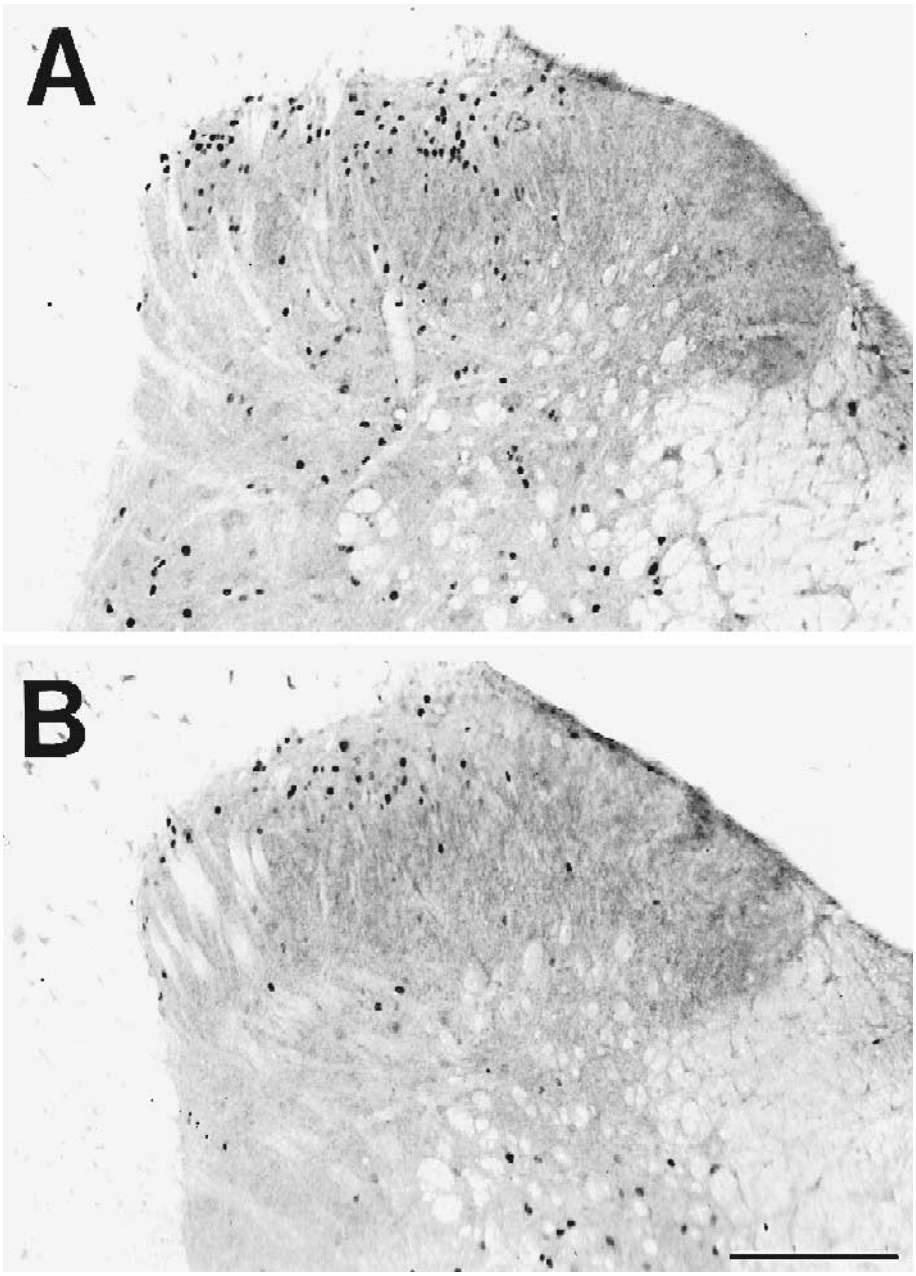

Fig. 1. Effects of the highest dose of intraplantar HOE140 (10 μg) on the carrageenan-induced expression of c-Fos protein, 3 h after intraplantar co-injection of carrageenan and HOE140 in rats. Each microphotograph represents an individual example including nuclei of c-Fos-LI neurones in the spinal dorsal horn per section (40 μm) of segment L4–L5 of the spinal cord; scale bar: 200 μm. A: Intraplantar carrageenan + bidistilled water (control group). B: Intraplantar carrageenan + HOE140 (10 μg in 50 μl of bidistilled water).

shown, the most numerous c-Fos-LI neurones were localised in the L4–L5 segments (Honoré et al., 1995), so for the pharmacological study of the effects of HOE140, for each rat, two counts were made: (1) the total number of c-Fos-LI neurones in the grey matter for 10 sections through L4–L5 segments, and (2) the number of Fos-LI neurones per specific defined region of the spinal grey matter in these 10 sections. Plotting and counting the c-Fos-LI neurones was performed blind to the experimental condition of each rat.

2.6. Statistical tests

Statistical analyses of variance (ANOVA) were performed using the Fisher's protected least squares difference (Fisher's PLSD) test for multiple comparisons. The dose-dependent effects of HOE140 on both the number of c-Fos-LI neurones and the inflammatory oedema (paw and ankle diameter) and possible correlations between the parameters were determined using a simple regression and a correlation coefficient, respectively. The estimation of the ED_{50} was performed using the graphic extrapolation, i.e., from the graph, we have considered the dose of HOE140 producing 50% reduction of the total number of c-Fos-LI neurones.

3. Results

3.1. Carrageenan-induced c-Fos protein expression in the rat lumbar spinal cord

Intraplantar injection of carrageenan evoked a high level of spinal c-Fos expression which was maximal in the ipsilateral dorsal horn of segments L4–L5 of the spinal cord. The total number of spinal Fos-LI neurones was 167 ± 5 per section, in segments L4–L5, following intraplantar co-administration of carrageenan with bidistilled water in the control group. c-Fos-LI neurones were predominantly and similarly located in laminae I–II and V–VI ($39 \pm 2\%$ and $39 \pm 1\%$ of the total number of c-Fos-LI neurones per section, respectively) of the dorsal horn of the spinal cord (Fig. 1). The number of c-Fos-LI neurones in the ventral horn was moderate ($17 \pm 1\%$) and very few c-Fos-LI neurones were present in laminae III–IV ($5 \pm 1\%$); Fig. 1. The number of c-Fos-LI neurones in the contralateral lumbar spinal cord was negligible (less than four c-Fos-LI neurones per section).

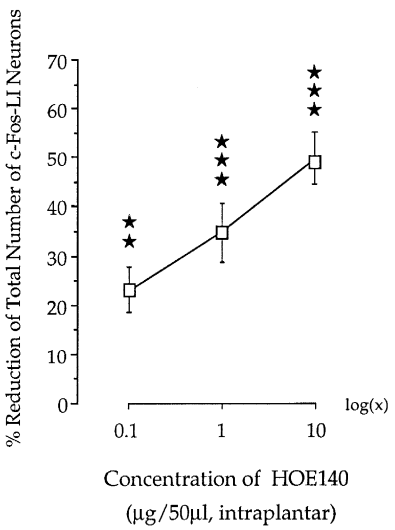

Fig. 2. The effects of intraplantar co-injection of HOE140 (0.1, 1 and 10 μg in 50 μl of bidistilled water; $n = 5$ for each group) with carrageenan on the total number of c-Fos-LI neurones in the L4–L5 segments of spinal cord, 3 h after co-injection. Results are expressed as percent reduction of the control total number of carrageenan-induced c-Fos-LI neurones (±S.E.M.). Significance as compared to the carrageenan control group was determined using ANOVA and Fisher's PLSD test (** $P < 0.01$, *** $P < 0.001$).

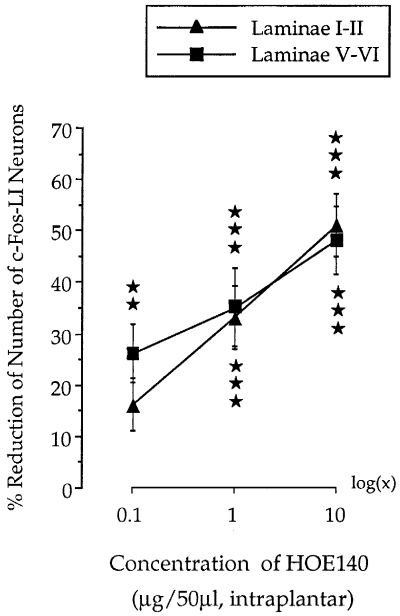

Fig. 3. Effects of intraplantar co-injection of HOE140 (0.1, 1 and 10 μg in 50 μl of bidistilled water; $n = 5$ for each group) with carrageenan on the number of superficial (laminae I–II; triangles) and deep (laminae V–VI; squares) c-Fos-LI neurones in the L4–L5 segments, 3 h after co-injection. Results are expressed as percent reduction of the control number of carrageenan-induced c-Fos-LI neurones for each region (±S.E.M.). Significance as compared to the carrageenan control group was determined using ANOVA and Fisher's PLSD test (** $P < 0.01$, *** $P < 0.001$).

3.2. The effect of peripheral admininstration of HOE140 on the spinal c-Fos protein expression

Intraplantar co-injection of HOE140 with carrageenan reduced the level of spinal c-Fos protein expression at 3 h after co-injection (for the effect of the highest dose (10 μg) of HOE140 see Fig. 1). These effects were significant when considering the total number of c-Fos-LI neurones in segments L4–L5 ($F(3,16) = 19.25$; $P < 0.001$), and their laminar distribution ($F(3,64) = 37.61$; $P < 0.001$). Intraplantar HOE140 (0.1, 1 and 10 μg) dose-dependently ($r^2 = 0.434$; $P < 0.01$) reduced the total number of c-Fos-

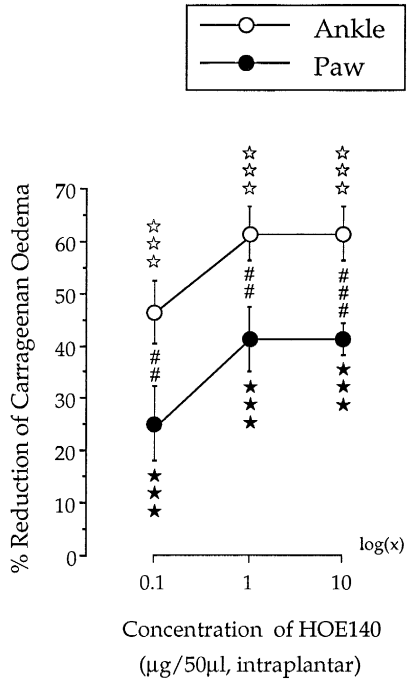

Fig. 4. Effects of intraplantar co-injection of HOE140 (0.1, 1 and 10 μg in 50 μl of bidistilled water; $n = 5$ for each group) with carrageenan on the paw (black circles) and ankle (open circles) diameter, 3 h after co-injection. Results are expressed as percent reduction of the control carrageenan-induced oedema for both paw and ankle (±S.E.M.). Significance as compared to the carrageenan control group was determined using ANOVA and Fisher's PLSD test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Statistical comparisons between the effects of HOE140 on the carrageenan-induced paw versus ankle oedema were performed using ANOVA and Fisher's PLSD test ($^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$).

LI neurones (23 ± 5%, $P < 0.01$, 35 ± 6% and 50 ± 5% reduction of the control total number of c-Fos-LI neurones, $P < 0.001$ for both, respectively; Fig. 2). Laminar analysis revealed that HOE140 (0.1, 1 and 10 μg) significantly reduced the number of c-Fos-LI neurones in the deep laminae (26 ± 6%, $P < 0.01$, 35 ± 7% and 48 ± 7% reduction of the control number, $P < 0.001$ for both, respectively; Fig. 3). Only the two higher doses of intraplantar HOE140 (1 and 10 μg) significantly reduced the number of c-Fos-LI neurones in the superficial laminae (33 ± 6% and 51 ± 6% reduction of the control number, respectively; $P < 0.001$ for both; Fig. 3). Overall, the effects of intraplantar HOE140 were dose-dependent for both superficial ($r = 0.70$, $P < 0.01$) and deep ($r = 0.53$, $P < 0.05$) laminae of the dorsal horn in segments L4–L5. Finally, there was no significant difference between the effect of HOE140 on the number of superficial and deep c-Fos-LI neurones (Fig. 3).

3.3. The effect of peripheral admininstration of HOE140 on the carrageenan-induced ankle and paw oedema

Three hours after intraplantar injection of carrageenan both the ipsilateral paw and ankle diameters were significantly increased as compared to non-stimulated rats (169 ± 8% and 88 ± 4% increase, respectively). An inflammatory oedema was not exhibited in the contralateral hindpaw.

Intraplantar HOE140 (0.1, 1 and 10 μg), injected simultaneously with intraplantar carrageenan, significantly reduced both the paw (25 ± 7%, 41 ± 6% and 41 ± 3% reduction of control carrageenan-induced paw diameters, respectively; $P < 0.001$ for all doses) and ankle (46 ± 6%, 61 ± 5% and 61 ± 5% reduction of control carrageenan-induced ankle diameters, respectively; $P < 0.001$ for all doses) oedema (Fig. 4). The effects of HOE140 were greater on the ankle as compared to the paw oedema (Fig. 4). In addition, there was a tendency to the plateau of the effect of the higher doses of HOE140 on both the paw and ankle oedema (Fig. 4). The effects of intraplantar HOE140 on the carrageenan-induced spinal c-Fos expression and the paw and ankle oedema were not correlated.

4. Discussion

Three hours after intraplantar injection of carrageenan into the hindpaw of non-anaesthetised rats, peripheral oedema and an associated spinal c-Fos expression in the ipsilateral dorsal horn of the lumbar spinal cord were observed. In accordance with our previous studies (Honoré et al., 1995; Buritova et al., 1996a and Refs. therein), the control level of carrageenan-induced c-Fos protein expression was maximal in the L4–L5 segments corresponding to the hindlimb dermatomes, and preferentially located in the medial part of the dorsal horn, which contains the somatotopic projection from the hindlimb foot (Molander and Grant, 1985, 1986). Carrageenan-induced c-Fos-LI neurones were predominantly located in the superficial (I–II) and deep (V–VI) laminae of the dorsal horn of the spinal cord. This localisation was in concordance with the spinal areas containing neurones activated by noxious stimuli driven by C- and Aδ-fibres (see Besson and Chaouch, 1987; Willis and Coggeshall, 1991), and neurones expressing the c-Fos protein after the electrical activation of C- and Aδ-fibres, but not Aβ-fibres (Herdegen et al., 1991).

Intraplantar co-administration of HOE140, a selective bradykinin B_2 receptor antagonist, with carrageenan dose-dependently reduces carrageenan-evoked spinal c-Fos protein expression in both the superficial and deep laminae of the dorsal horn of the spinal cord. The estimated ED_{50} for effects of HOE140 on the number of c-Fos-LI neurones was 10 μg. In addition, HOE140 attenuated the extent of the peripheral oedema; however, this effect had a tendency to plateau with the higher concentrations of HOE140 studied. Consequently, the effects of HOE140 on carrageenan-evoked spinal c-Fos protein expression and peripheral oedema were not correlated.

Our results with HOE140 are in keeping with previous studies of both the anti-inflammatory (Wirth et al., 1991;

Damas and Remacle-Volon, 1992) and antinociceptive (Ferreira et al., 1993) effect of systemic HOE140 in the carrageenan model of inflammation in rats. As discussed in Section 1, the antinociceptive effect of subcutaneous HOE140 using various different models of inflammation has been demonstrated. More specifically our results are in agreement with, and extend, previous behavioural (Beresford and Birch, 1992) and electrophysiological (Chapman and Dickenson, 1992) studies demonstrating that the intraplantar administration of HOE140 suppresses both first and second phase responses to the peripheral injection of formalin.

From previous studies and our results it can be concluded that bradykinin has a peripheral role via activation of B_2 receptors during inflammatory nociceptive processing. However, the B_2 receptor contribution may occur predominantly during the initial phase of inflammation since HOE140 is ineffective during persistent inflammatory hyperalgesia in rats, and a predominant B_1 receptor involvement has been suggested during persistent inflammatory condition (Perkins et al., 1993; Dray and Perkins, 1993). Furthermore it has been demonstrated that bradykinin-induced mechanical hyperalgesia during arthritis is mediated by activation of both B_1 and B_2 receptors whereas only B_2 receptors contribute to hyperalgesia in normal rats (Khasar et al., 1995). Overall, it has been suggested that B_2 and B_1 receptors play an important role in the initiation and in the maintenance of inflammation, respectively (Dray and Perkins, 1993).

In addition to bradykinin, prostaglandins also play a pivotal role during inflammation, with bradykinin initiating the generation of the prostaglandins (Lembeck et al., 1976), and in turn prostaglandins sensitising the fine peripheral afferent endings to activation by bradykinin (Ferreira, 1972; Ferreira et al., 1973; see Dray, 1994). Similar antinociceptive effects of HOE140 and cyclo-oxygenase inhibitors in the abdominal constriction response induced by acetic acid and kaolin have been demonstrated (Heapy et al., 1993). With consideration of these studies it is interesting to note the comparable anti-inflammatory/antinociceptive effects of intraplantar HOE140 and systemically administered cyclooxygenase inhibitors such as a selective cyclooxygenase-2 inhibitor (Buritova et al., 1996b), and non-selective cyclooxygenase-1 and cyclooxygenase-2 inhibitors as various non-steroidal anti-inflammatory drugs (NSAIDs, see references in Buritova et al., 1996a) tested under similar experimental conditions. In these previous studies the effects of cyclooxygenase inhibitors on spinal c-Fos expression have been correlated with their effects on peripheral oedema. In contrast, in the present study the effects of the higher doses of HOE140 on the peripheral oedema, but not the spinal c-Fos protein expression, had a tendence to plateau, and the effects at the peripheral level and the spinal level were not correlated. This dissociation of the peripheral anti-inflammatory versus the antinociceptive effect of HOE140, but not the cyclooxygenase inhibitors (NSAIDs), is not so surprising when considering their different mechanisms of action. Bradykinin has been shown to directly activate the fine primary afferent endings, and therefore antagonism of the bradykinin B_2 receptors by HOE140 presumably reduces the direct activation of the nociceptors by bradykinin, irrespective of the extent of the inflammation. In contrast, the prostaglandins appear to play a greater role in the maintenance of the inflammation and sensitising the peripheral nociceptors, but less so in the direct activation of the nociceptors. The differences between the effects of cyclooxygenase inhibitors (NSAIDs) and HOE140 may be also due to the different administrations: systemic versus intraplantar. In our experimental conditions, the central site of action of the systemically administered cyclooxygenase inhibitors (Malmberg and Yaksh, 1992, 1994) has not been excluded, whereas the action of intraplantar administration of low doses of HOE140 rests limited at peripheral level.

The present study shows that peripheral bradykinin B_2 receptors antagonism reduce both the carrageenan-induced peripheral oedema and consequent nociceptive transmission at the spinal cord level, as shown by the reduction of carrageenan-induced spinal c-Fos expression. In conclusion, our results provide further evidence for the involvement of the peripheral bradykinin B_2 receptors in the inflammatory pain processing due to intraplantar injection of carrageenan.

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