Evidence for participation of B₁ and B₂ kinin receptors in formalin-induced nociceptive response in the mouse

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1 This study was designed to investigate the role of bradykinin (BK), as well as the subtype of BK receptors involved, in formalin-induced hindpaw pain in the mouse by use of selective B₁ and B₂ receptor antagonists. In addition, we have analysed whether or not BK may be involved in formalin-induced hindpaw oedema in the mouse.

2 The pretreatment of animals with captopril (2 and 5 mg kg⁻¹, s.c.) significantly increase the first and the second phases of formalin-induced pain.

3 Co-injection of the selective B₁ receptor antagonist des-Arg⁹[Leu⁸]-BK (0.2–0.4 nmol/paw), together with formalin, caused graded and similar inhibitions of both phases of formalin-induced pain. Similar results were obtained with the B₂ antagonists NPC 349 (D-Arg[Hyp³, Thi^{5,8}-D-Phe⁷]-BK) and NPC 567 (D-Arg[Hyp³, D-Phe⁷]-BK) (0.2 and 0.6 nmol/paw). Higher concentrations of these antagonists (1 nmol/paw) failed to antagonize formalin-induced pain.

4 The new potent and selective B₂ receptor antagonists, Hoe 140 (D-Arg[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK), NPC 17731 (D-Arg[Hyp³, *trans*-4-propoxy-D-proline (*trans*propyl)⁷, Oic⁸]-BK), and NPC 17761 (D-Arg[Hyp³, *trans*-4-propoxy-D-proline (*trans* thiophenyl)⁷, Oic⁸]-BK) (0.02 to 1.0 nmol/paw), also caused significant inhibitions of both phases of formalin-induced pain. When Hoe 140 was injected subcutaneously 30 min before formalin injection (9.9 and 99 nmol kg⁻¹), it significantly attenuated both phases of formalin-induced pain. The putative non-peptide BK antagonist, MV 8612 (1.6 to 9.6 nmol/paw), but not MV 8608 (5.5 to 33 nmol/paw), caused a graded inhibition of both phases of formalin-induced pain, being, however, more active against the first phase.

5 The pretreatment of animals with morphine (2.6 to 13 µmol kg⁻¹, s.c.) caused dose-dependent and equipotent inhibitions of both phases of formalin-induced pain. In contrast, indomethacin (2.7 to 27 µmol kg⁻¹) antagonized only the second phase of formalin-induced pain.

6 The B₂ receptor antagonists, Hoe 140, NPC 17731, NPC 17761, NPC 349 and NPC 567, all caused a significant inhibition of formalin-induced hindpaw oedema. A similar inhibition was also observed with indomethacin but not with captopril or morphine.

7 Our results provide strong evidence for the important role of endogenous BK, acting through both B₁ and B₂ receptors, in the genesis of both phases of formalin-induced persistent pain in the mouse. In addition, the current results also demonstrate that the inflammatory oedema associated with the later phase of formalin-induced pain seems to be mediated by endogenous BK, via activation of B₂ receptors.

Keywords: Formalin test; pain; oedema; kinins; B₁ and B₂ bradykinin antagonists; *Mandevilla velutina* compounds

Introduction

The nonapeptide bradykinin (BK) and its related kinins are generated in plasma and in several tissues following tissue damage or infection. Once released, BK may induce pain and is thought to be involved in many inflammatory states (for review see: Marceau *et al.*, 1983; Proud & Kaplan, 1988; Steranka & Burch, 1991). Exogenous BK produces pain by activating and/or sensitizing nociceptive afferent A-δ and C fibres (Collier & Lee, 1963; Franz & Mense, 1975; Mense, 1975; Besson & Chaouch, 1987; Szolcsányi, 1987).

Considerable biochemical and functional evidence indicates that the effects of BK result from stimulation of specific membrane receptors B₁ and/or B₂, widely distributed in the peripheral and central nervous system (for review see: Regoli & Barabé, 1980; Steranka *et al.*, 1988a,b; Burch *et al.*, 1990; Bathon & Proud, 1991; Farmer & Burch, 1992). With the development of more selective and competitive BK antagonists, many biochemical and pharmacological studies have suggested that kinins are involved in inflammatory pain, acting mainly by stimulation of B₂ receptors (for review see: Proud & Kaplan, 1988; Steranka *et al.*, 1988b; Steranka & Burch, 1991). Thus, BK and its related kinins may play an

important role as physiological mediators of pain and inflammatory hyperalgesia.

Formalin induces a long lasting nociceptive response in the mouse paw, and this effect has been widely used to model persistent tonic pain of moderate intensity which involves chemical irritation, some tissue damage, and formation of oedema due to release of inflammatory mediators (Hunskar *et al.*, 1985; Hunskar & Hole, 1987; Murray *et al.*, 1988). This nociceptive model usually involves two distinct phases: an early transient phase, which occurs in the first 5 min and a late tonic phase, evident 15 to 30 min after injection. It has been proposed that the early phase reflects direct stimulation of nociceptors, while the late phase may be associated with release of inflammatory mediators (Dubuisson & Dennis, 1977; Hunskar *et al.*, 1985; 1986; Rosland *et al.*, 1990). In addition, formalin-induced persistent pain is thought to resemble clinical pain due to its tonic nature (Dennis & Melzack, 1979; Abbott *et al.*, 1982; Abbott & Franklin, 1986).

A recent electrophysiological study suggested that BK may be involved in the second phase of formalin-induced tonic pain in the mouse (Haley *et al.*, 1989). As the nociceptive response to formalin was markedly inhibited by the NPC 349 (D-Arg[Hyp³, Thi^{5,8}, D-Phe⁷]-BK), but not by the selective B₁ antagonist des-Arg⁹[Leu⁸]-BK, the authors concluded that

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activation of B₂ receptors may underlie formalin-induced pain. In contrast, Shibata *et al.* (1989) reported that the first and second phases of pain elicited by formalin in the mouse were significantly antagonized by the B₁ selective antagonist des-Arg⁹[Leu⁸]-BK.

In the present study we have attempted to examine further the possible participation of BK in formalin-induced peripheral persistent pain in mice, and to characterize the subtype of BK receptors involved, by use of selective and competitive B₁ and B₂ receptor antagonists. In addition, we have also investigated whether BK is involved in formalin-induced hindpaw oedema in mice.

Methods

Formalin-induced pain

Male Swiss mice (25–35 g) were housed at 22–24°C under a 12 h light/12 h dark cycle and were given access to water and purina chow *ad libitum*. The procedure used was essentially similar to that described by Hunskaar & Hole (1987) and Murray *et al.* (1988), with minor modifications. Briefly, animals of the same strain were lightly anaesthetized with ether, except when used to analyse the first phase of formalin-induced pain, and 20 µl of 2.5% formalin solution (0.92% of formaldehyde), made up in PBS (phosphate-buffered solution containing: NaCl 137 mM; KCl 2.7 mM and phosphate buffer 10 mM), was injected s.c. under the plantar surface of the left hindpaw with a Hamilton microsyringe. Animals were acclimatized to the laboratory for at least 24 h before the experiments. Usually, two mice (control and treated) were observed simultaneously for 0 to 30 min following formalin injection. All experiments were carried out during the light period at 23 ± 2°C (Rosland *et al.*, 1990).

The amount of time (s) that animals spent licking the injected paw was timed with a chronometer and was considered as an index of pain. The initial nociceptive response normally peaked about 5 min after formalin injection (first phase), and was followed by a second peak that occurred 15 to 30 min after formalin injection (second phase). This later phase was usually accompanied by inflammation due to release of inflammatory mediators (Hunskaar & Hole, 1987). To avoid the influences of pharmacokinetics in the action of the drugs studied, different groups of animals were used to analyse each phase of formalin-induced pain. The animals were treated topically (in association with formalin) or by the s.c. route (0.1 ml 10 g⁻¹ weight) with several selective B₁ and B₂ antagonists, or with functional BK antagonists isolated from the rhizome of the plant *Mandevilla velutina* (Calixto *et al.*, 1988). For the purpose of comparison in a separate set of experiments, the animals were treated with morphine (2.6–13 µmol kg⁻¹, s.c.) or with indomethacin (2.7–27 µmol/paw, i.p.), 30 and 60 min before formalin injection, respectively. Other groups of animals were treated with captopril (2 and 5 mg kg⁻¹, s.c.) 2 h before subplantar injection of formalin. Control animals received only the vehicle used to dilute these drugs (PBS or NaCl solution containing 5% w/v NaHCO₃; 0.1 ml 10 g⁻¹ weight). Following intraplantar injection of formalin, the animals were immediately placed into the glass cylinder 20 cm in diameter and the time spent licking the injected paw was determined.

Effect of bradykinin antagonists on formalin-induced hindpaw oedema in the mouse

We measured the oedema by comparing the difference in weight of the formalin-treated paw and the weight of the control paw (untreated paw). For this purpose, the animals were killed 30 min after formalin injection by cervical dislocation, and the paw was cut at the knee joint and weighed on an analytical balance.

Statistical analysis

The results are presented as the mean ± s.e.mean, and statistical significance of differences between groups was analysed by means of analysis of variance followed by Dunnett's test or by the unpaired *t* test where indicated. *P* values less than 0.05, were considered as indicative of significance. When possible, the ID₅₀ or IC₅₀ (i.e. the dose or concentrations of drugs that reduced formalin pain by 50% relative to control values), were estimated from individual experiments by using the least squares method in a computer programme, produced in our laboratory (Armando Dettmer).

Drugs

The following drugs were used: des-Arg⁹[Leu⁸]-BK, captopril, indomethacin (from Sigma Chemical Company, St. Louis, U.S.A.), formalin, morphine sulphate (Merck, Germany), NPC 349 (D-Arg-[Hyp³, Thi^{5,8}, D-Phe⁷]-BK), NPC 567 (D-Arg-[Hyp³, D-Phe⁷]-BK) (Peninsula Laboratories, U.S.A.), Hoe 140 (D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK) was kindly supplied by the Department of Pharma Synthesis, Hoechst, Frankfurt Main, Germany; NPC 17731 [D-Arg⁹, Hyp³, trans-4-propoxy-D-proline (trans propyl)⁷, Oic⁸]-BK and NPC 17761 [D-Arg⁹, Hyp³, trans-4-propoxy-D-proline (trans thiophenyl)⁷, Oic⁸]-BK were kindly supplied by Nova Pharmaceutical Corporation, Baltimore, U.S.A. The *Mandevilla velutina* compounds (MV 8608, molecular weight of 326 and MV 8612, molecular weight of 1182) were obtained in our laboratory as described previously (Calixto *et al.*, 1988). The stock solution for all peptides used was prepared in PBS (1–10 mM) and kept in siliconized plastic tubes, maintained in a freezer at –18°C. The other drugs were prepared just before use in 0.9% w/v of NaCl solution, except indomethacin (NaCl solution plus 5% w/v of NaHCO₃) and *M. velutina* compounds (absolute ethanol). The final ethanol concentration did not exceed 5%.

Results

Effects of drugs on formalin-induced pain

Figure 1 (a and b) shows that the pretreatment of animals with captopril (2 and 5 mg kg⁻¹, s.c.) 2 h beforehand caused a significant enhancement of both the first and the second phases of formalin-induced pain. Figure 2 (a and b) illustrates that the co-injection of the selective B₁ receptor

Figure 1 Effect of pretreatment with subcutaneous captopril on the formalin-induced pain in mice. In this and subsequent figures the total time (s, mean ± s.e.mean) spent licking the hindpaw was measured in the first (0–5 min, a) and the second phase (15–30 min, b), after intradermal injection of formalin in the hindpaw. Each column represents the mean with s.e.mean of 5 to 8 animals. C indicates the control animals (injected with the vehicle) and the asterisks denote the significance levels. Significantly different from controls: **P* < 0.05; ***P* < 0.01.

antagonist des-Arg⁹-[Leu⁸]-BK (0.2 and 0.4 nmol/paw), together with formalin, caused a graded and similar degree of inhibition of the first and the second phases of the formalin-induced pain (maximal inhibition of about 40%). Nevertheless, at a higher concentration (0.6 nmol/paw) its analgesic effect against both phases of formalin-induced pain was absent. Very similar results were obtained when animals were pretreated with the B₂ antagonists NPC 349 (0.2 and 0.6 nmol/paw) and NPC 567 (0.2 and 0.6 nmol/paw) (Figure 3). Both BK antagonists consistently inhibited both phases of formalin-induced pain (maximum inhibition of about 45 to 50%). However, higher concentrations of these antagonists (1 nmol/paw) failed to antagonize the formalin-induced analgesia (Figure 3).

The new generation of selective B₂ receptor antagonists, NPC 17731 (0.02 to 0.2 nmol/paw) and 17761 (0.1 to 1 nmol/paw), also caused a significant inhibition of both phases of the formalin-induced nociceptive response (Figure 4). NPC 17731 was more potent than NPC 17761, and their

antinociceptive effects were not dose-dependent. At higher concentrations NPC 17731 (0.2 and 0.4 nmol/paw) did not cause any significant analgesic effect (Figure 4). The maximal inhibition produced by NPC 17731 and NPC 17761 was about 40–45%, against the first and the second phase of formalin-induced pain. Figure 5 shows that Hoe 140, a selective B₂ antagonist (0.19 to 1.9 nmol/paw), caused discrete, but significant inhibition of both phases of the formalin-induced persistent nociceptive response (maximal inhibition

Figure 2 Effect of intradermal injection of the selective B₁ antagonist, des-Arg⁹-[Leu⁸]-bradykinin, on formalin-induced pain in mice. Each column represents the mean with s.e.mean of 5 to 8 animals. C indicates control animals. Significantly different from controls: **P* < 0.05; ***P* < 0.01.

Figure 3 Effect of intradermal injection of B₂ bradykinin receptor antagonists, NPC 349 (upper panel) and NPC 567 (lower panel), in formalin induced pain in mice. Each column represents the mean with s.e.mean of 5 to 7 animals. C indicates control animals. Significantly different from controls; **P* < 0.05; ***P* < 0.01.

Figure 4 Effect of intradermal injection of B₂ bradykinin receptor antagonists NPC 17731 (upper panel) and NPC 17761 (lower panel) on formalin-induced pain in the mice. Each column represents the mean with s.e.mean of 5 to 8 animals. C indicates control animals. Significantly different from controls; **P* < 0.05; ***P* < 0.01.

Figure 5 Effect of intradermal (upper panel) and subcutaneous (lower panel) injection of B₂ bradykinin receptor antagonist Hoe 140 on the formalin-induced pain in the mice. Each column represents the mean with s.e.mean of 5 to 9 animals. C indicates control animals. Significantly different from controls; **P* < 0.05; ***P* < 0.01.

of about 40%). On the other hand, when Hoe 140 was administered subcutaneously 30 min before formalin injection (9.9 to 99 nmol kg⁻¹) it was markedly more effective in inhibiting the second phase of formalin-induced pain (maximal inhibition of 50%), than the first phase of formalin-induced pain. The nonpeptide BK antagonist isolated from *M. velutina*, compound MV 8612 (1.6 to 9.6 nmol/paw) caused graded and potent inhibitions of both phases of formalin-induced pain, being however much more effective against the first phase, with maximal inhibitions of 50 and 20%, respectively (Figure 6). Compound MV 8608 (5.5 to 33 nmol/paw) was completely ineffective against both phases of the formalin-induced nociceptive response (Figure 6).

The pretreatment of mice with morphine (2.6–13 µmol/kg, s.c.) caused dose-dependent and equipotent inhibition of both phases of formalin-induced pain, with an ID₅₀ of about 3.5 µmol kg⁻¹ (against both phases) and caused about 90% to 100% inhibition of formalin-induced pain (Figure 7). In contrast, indomethacin (2.7 to 27 µmol kg⁻¹, i.p.) dose-dependently antagonized only the second phase of formalin-induced pain, with an ID₅₀ of about 10 µmol kg⁻¹ (Figure 7).

Effects of drugs on formalin-induced paw oedema

The results summarized in Table 1 show that the selective B₁ receptor antagonist, des-Arg⁹-[Leu⁸]-BK, did not significantly inhibit formalin-induced hindpaw oedema. The B₂ antagonists NPC 349 and NPC 567 caused a discrete but significant inhibition of formalin-induced oedema (Table 1). However, the most potent and selective B₂ receptor antagonists, NPC 17731, NPC 17761 and Hoe 140 were all very effective and significantly inhibited formalin-induced paw oedema. Similar inhibition of formalin-induced paw oedema was observed in animals pretreated with indomethacin (Table 1). In contrast, *M. velutina* compounds, captopril and morphine all failed to inhibit significantly formalin-induced oedema (results not shown).

Figure 6 Effect of intradermal injection of compounds from *Mandevilla velutina* MV 8612 (upper panel) and MV 8608 (lower panel) on the formalin-induced pain in the mice. Each column represents the mean with s.e.mean of 5 to 10 animals. C indicates control animals. Significantly different from controls; * $P < 0.05$; ** $P < 0.01$.

Figure 7 Effect of pretreatment with morphine given subcutaneously (upper panel) or with indomethacin given intraperitoneally (lower panel) on the formalin-induced pain in the mice. Each column represents the mean with s.e.mean of 5 to 12 animals. C indicates control animals. Significantly different from controls; * $P < 0.05$; ** $P < 0.01$.

Discussion

The present study clearly demonstrates that selective and competitive BK receptor antagonists caused a pronounced antinociceptive effect in a model of persistent pain. This strongly supports the view that endogenous BK plays an important role in the genesis of both the first and second phases of the peripheral nociceptive response caused by intradermal injection of formalin in the mouse hindpaw. In addition, our results also indicate that both BK receptor subtypes, B₁ and B₂, are involved in both phases of formalin-induced tonic nociceptive response in mice. These views are substantiated by the fact that the selective B₁ receptor antagonist, des-Arg⁹-[Leu⁸]-BK, as well as the most potent and selective B₂ receptor antagonists, including NPC 349, NPC 567 (Burch *et al.*, 1990; Bathon & Proud, 1991), Hoe 140 (Hook *et al.*, 1991; Lembeck *et al.*, 1991), NPC 17761 and NPC 17731 (Kyle *et al.*, 1991a,b; Kyle & Burch, 1992), when co-injected with formalin or given subcutaneously, caused a pronounced inhibition of both phases of the pain caused in mice by formalin. Similar inhibition has been previously observed with selective B₂ antagonists, including Hoe 140 (Chapman & Dickenson, 1992), NPC 349 (Haley *et al.*, 1989), and with the selective B₁ receptor antagonist, des-Arg⁹-[Leu⁸]-BK (Shibata *et al.*, 1989). Interestingly, higher doses of most of the BK antagonists, failed to affect formalin-induced pain. These data are consistent with the view that BK antagonists may exhibit agonist activity (Steranka *et al.*, 1988a,b; 1989; Kindsen-Mells & Klement, 1992; Farmer & Burch, 1992). However, our results agree well with previously reported data, indicating that neither of the selective BK receptor antagonists was able to abolish completely the formalin-induced nociceptive response. These findings are consistent with the notion that other inflammatory mediators besides BK also participate in this response (Shibata *et al.*, 1986; 1989; Haley *et al.*, 1989; Dray & Dickenson, 1991; Murray *et al.*, 1991; Moore *et al.*, 1991). Another clear piece of evidence support-

Table 1 Effect of several peptide bradykinin receptor antagonists on formalin-induced oedema in the mice paw

Group	Dose		Oedema (mg)	% Inhibition
	Systemic (nmol kg ⁻¹)	Local (nmol/paw)		
Control	—	—	63 ± 4	—
Des-Arg ⁹ -[Leu ⁸]-BK	—	0.2	57 ± 3	10
	—	0.4	52 ± 3	18
	—	0.6	63 ± 5	00
Hoe 140	9.9	—	67 ± 3	00
D-Arg-[Hyp ³ ,Thi ⁵ ,D-Thi ⁷ ,Oic ⁸]-BK	49.5	—	48 ± 3	26*
	99.0	—	56 ± 4	11
	—	0.1	52 ± 5	21*
NPC 567	—	0.99	63 ± 5	00
	—	0.2	58 ± 2	08
	—	0.4	47 ± 2	26*
D-Arg-[Hyp ³ ,D-Phe ⁷]-BK	—	1.0	55 ± 4	12
	—	0.2	53 ± 2	16
	—	0.6	49 ± 3	22*
NPC 349	—	1.0	54 ± 5	15
	—	0.02	53 ± 2	16
	—	0.1	39 ± 3	38**
Arg-[Hyp ³ ,Thi ^{5,8} ,D-Phe ⁷]-BK	—	0.2	42 ± 3	33**
	—	0.4	53 ± 2	16
	—	0.1	63 ± 3	00
NPC 17731	—	0.2	46 ± 1	28**
	—	0.6	53 ± 1	16
	—	—	66 ± 5	00
D-[Arg ⁰ ,Hyp ³ ,trans-4-propoxy-proline-(transpropyl) ⁷ ,Oic ⁸]-BK	2.7	—	56 ± 2	14*
	8.4	—	49 ± 3	25**
	27.0	—	—	—

^aμmol kg⁻¹, i.p.

Each group represents the mean of 5 to 12 animals. Oedema was measured 30 min after formalin injection.

Significantly different from controls: **P* < 0.05; ***P* < 0.01.

ting a role of BK in formalin-induced tonic pain was the significant potentiation of formalin-induced licking by systemic captopril, a kininase II inhibitor (Ondetti *et al.*, 1977). However, captopril failed to affect the paw oedema associated with late phase of formalin-induced pain. Thus, we cannot rule out the possibility that the potentiation caused by captopril in formalin-induced pain may be, at least in part, secondary to the fall in blood pressure. The potent antinociceptive effect caused by morphine against both phases of formalin-induced pain, and the inhibition of the second phase of the formalin-induced pain by indomethacin are in good agreement with previous evidence reported in the literature (Hunskar *et al.*, 1985; Murray *et al.*, 1988; Shibata *et al.*, 1989; Dray & Dickenson, 1991; Moore *et al.*, 1991; Oluyomi *et al.*, 1992; Chapman & Dickenson, 1992).

An interesting aspect investigated in the present study was whether the oedema associated with the later phase of formalin-induced pain, was also mediated by BK. Our results clearly show that the most potent and selective B₂ receptor antagonists such as Hoe 140, NPC 17731 and NPC 17761, and to a lesser extent the less potent B₂ antagonists such as NPC 349 and NPC 567, but not the selective B₁ antagonist, des-Arg⁹[Leu⁸]-BK, were found to be effective in antagonizing formalin-induced hindpaw oedema. This supports the view that only B₂ receptors appear to be involved in the formalin-induced inflammatory response. Our results are in agreement with a great deal of experimental evidence that suggests the involvement of the B₂ but not B₁ receptors in many experimental inflammation models (see for review: Marceau *et al.*,

1983; Proud & Kaplan, 1988; Burch *et al.*, 1990; Bathon & Proud, 1991; Steranka & Burch, 1991).

Concerning the potent antinociceptive effect of the functional BK antagonist isolated from *Mandevilla velutina*, compound MV 8612 but not MV 8608 was found very active in inhibiting both phases of formalin-induced pain, being more effective against the first phase of the formalin-induced pain. These findings provide additional evidence supporting our previous view that this compound may interfere with some stage of BK action (Calixto *et al.*, 1985; 1987; 1988; 1991).

In conclusion, our current data show that the selective and competitive BK receptor antagonists caused a pronounced antinociceptive effect against formalin-induced persistent pain in mice, thus providing strong evidence supporting the notion that endogenous BK, acting through B₁ and B₂ receptors, contributes to the genesis of both phases of peripheral nociceptive response. In addition, these results also demonstrate that the inflammatory response associated with the late phase of formalin-induced pain in mice seems to be mediated, at least in part, by endogenous BK via stimulation of the B₂ receptors.

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