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Antinociceptive profile of the pseudopeptide B_2 bradykinin receptor antagonist NPC 18688 in mice

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1 The purpose of this study was to investigate the topical and systemic anti-hyperalgesic effect of the newly-developed pseudopeptide B_2 receptor antagonist, NPC 18688, in different models of nociception in mice.

2 Given systemically 30 min beforehand, NPC 18688 (10-300 nmol kg^{-1}, i.p.) caused no agonist effect, but produced a dose-related and significant inhibition of abdominal constrictions caused by intraperitoneal injection of acetic acid (0.6%), acetylcholine (ACh, 4.5 mg kg^{-1}) or kaolin (50 mg kg^{-1}). The calculated mean ID_{50}s and the percentages of maximal inhibitions (MI) for these effects were: 77, 34 and >300 nmol kg^{-1} and 65 ± 6, 70 ± 5 and 40 ± 3%, respectively. The anti-hyperalgesic effect of NPC 18688 (100 nmol kg^{-1}, i.p.) occurred rapidly (30 min) and lasted for at least 150 min. Hoe 140 (3-30 nmol kg^{-1}, i.p) given 30 min beforehand also inhibited, in a graded manner, acetic acid and ACh-induced writhing, with mean ID_{50}s and MI of 6 and 9 nmol kg^{-1} and 56 ± 7 and 62 ± 6%, respectively.

3 NPC 18688 (10-300 nmol kg^{-1}, i.p.) caused a graded inhibition of both phases of formalin (2.5%)-induced pain, its effects being more potent in relation to the second phase of the formalin test. The calculated mean ID_{50}s and the MI were >300 and 60 nmol kg^{-1} and 20 ± 3 and 60 ± 5% against the first and second phases of formalin-induced nociception, respectively. NPC 18688 at the same doses also inhibited, in a dose-related manner, formalin-induced paw oedema (MI of 35 ± 3%).

4 When injected locally in the mouse paw, NPC 18688 (2, 10 and 20 nmol/paw) had no agonist activity. However, when co-injected with formalin NPC 18688 (2-20 nmol/paw), it produced significant inhibition of both phases of formalin response, with MI of 40 ± 3 and 33 ± 2%, respectively. NPC 18688 at 10 nmol/paw also significantly inhibited formalin-induced paw oedema (25 ± 2%).

5 Given intraperitoneally, NPC 18688 (30-300 nmol kg^{-1}) determined a graded inhibition of the nociceptive response caused by intraplantar injection of capsaicin (1.6 μg/paw) (40 ± 2%). However, NPC 18688 (up to 300 nmol kg^{-1}, i.p.), given 30 min beforehand, had no significant analgesic effect when analyzed in the tail flick and in the hot plate pain models, nor did it change the performance of animals in the rota rod test.

6 The action of NPC 18688 was quite selective for the B_2 receptor, and like Hoe 140, (1 to 100 nmol kg^{-1}, i.p.) it caused graded inhibition of bradykinin (BK, 3 mol/paw)-induced increase in mouse paw volume, with mean ID_{50}s of 61 and 6 nmol kg^{-1}, respectively. In addition, at 100 nmol kg^{-1}, the dose at which NPC 18688 significantly antagonized BK (3 nmol)-mediated rat paw oedema in naive animals, it had no significant effect on des-Arg^9-BK (100 nmol/paw)-induced oedema in paws that had been desensitized to BK. NPC 18688 (210 nmol kg^{-1}), like Hoe 140 (230 nmol kg^{-1}) given s.c. 30 min beforehand, completely abolished BK (28 nmol)-induced hypotension, without affecting the fall of mean arterial blood pressure induced by i.v. injection of ACh (2 nmol kg^{-1}). Finally, NPC 18688 (1 μM) did not affect ACh-mediated contraction in the guinea-pig ileum or toad rectus abdominii *in vitro*.

7 These results demonstrate that the newly-developed and selective pseudopeptide B_2 receptor antagonist, NPC 18688, although less potent than the available second generation of B_2 peptide BK receptor antagonists, exhibits topical and long-lasting systemic anti-hyperalgesic properties when analysed in several models of nociception in mice, making it a useful tool for investigating the participation of BK and related kinins in physiological and pathological processes. Finally, this new class of selective pseudopeptide B_2 receptor antagonist may constitute a new strategy for developing the third generation of potent and long-lasting orally-active non-peptide BK antagonists, which may be useful for the management of clinical disorders involving BK and related kinins.

Keywords: Bradykinin; B_1 and B_2 receptor; mouse and rat paw oedema; NPC 18688; pseudopeptide; anti-hyperalgesic action; formalin and capsaicin-induced pain; writhing test

Introduction

Kinins, including the nonapeptide bradykinin (BK) and the decapeptide kallidin (Lysyl-BK), are released from plasma and from most tissues by the action of kallikreins on kininogens following tissue damage or infection. There is now a great deal of evidence that kinins play an important role as vasoactive peptides which promote venular dilatation and increased vascular permeability, stimulating Aσ and C fibres in sensory neurones causing pain and hyperalgesia. In addition, it has been proposed that kinins may contribute to the pathogenesis of several inflammatory states (for review see: Lewis, 1970; Garcia Leme, 1978; Marceau *et al.*, 1983; Proud & Kaplan, 1988; Steranka & Burch, 1991; Dray & Perkins, 1993).

A considerable amount of evidence now suggests that most actions of kinins are mediated through two types of membrane

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receptor, designated B_1 and B_2. The B_1 receptors are predominantly found in certain rabbit tissues, and are expressed in pathological states after tissue injury or during *in vitro* incubation. Recently, a human B_1 receptor has been cloned from lung fibroblasts (Menke *et al.*, 1994). The B_1 receptors exhibit greater affinity for the kinin metabolites des-Arg^9-BK and des-Arg^{10}-kallidin than for BK. On the other hand, the B_2 kinin receptors are widely distributed through peripheral and central nervous systems and exhibit high affinity for BK and Lys-BK, appearing to mediate the majority of the known cellular and physiological kinin actions under normal conditions (Regoli & Barabé, 1980; Marceau & Regoli, 1991; Bhoola *et al.*, 1992; Farmer & Burch, 1992; Hall, 1992). Recently, the complementary DNA encoding of B_2 BK receptor, has been cloned in tissues of several animal species, including rat (McEachern *et al.*, 1991), man (Hess *et al.*, 1992; Eggerickx *et al.*, 1993; Powell *et al.*, 1993) and mouse (McIntyre *et al.*, 1993; Hess *et al.*, 1994). Thus, B_2 receptors belong to the seven transmembrane G-protein- coupled super family of receptors and their predicted amino acid sequences are highly conserved among the animal species studied.

Although in recent years several selective and highly potent B_2 BK receptor antagonists have been developed (Hock *et al.*, 1991; Kyle *et al.*, 1991a,b; Lembeck *et al.*, 1991; Kyle & Burch, 1992), these compounds are peptides, and most of them, despite their great potency and selectivity, present poor or non-existent activity when given systemically, because they are rapidly cleaved by the plasma peptidases. In addition, some peptide B_2 antagonists exhibit residual agonistic activity in certain models (Farmer & Burch, 1992; Hall, 1992). Although great efforts have been made in recent years to develop potent, selective and orally-active BK receptor antagonists, so far there are only functional studies reporting that some glycoside compounds isolated from the Brazilian plant *Mandevilla velutina*, given systemically, selectively antagonize most of the kinin actions in several pharmacological models (for review see: Calixto *et al.*, 1991). Very recently, Sawutz *et al.* (1994) reported that the nonpeptide compound, WIN 64338 inhibits, in a selective and competitive manner, ^{3}BK binding to B_2 receptors (K_i = 64 nM) in human IMR-90 cells, BK-stimulated $^{45}Ca^{2+}$ efflux from IMR-90 cells, and also BK-induced contraction in guinea-pig ileum. However, there is no evidence demonstrating systemic anti-BK effects of WIN 64338.

In this study, we describe the systemic and local anti-hyperalgesic properties of the newly-developed pseudopeptide B_2 receptor antagonist, NPC 18688, in several models of nociception in mice. We have also shown the selectivity of NPC 18688 at the B_2 receptor by analysing its *in vitro* and *in vivo* effects against des-Arg^9-BK- and acetylcholine-mediated responses.

Methods

Abdominal constriction response caused by intraperitoneal injection of acetic acid, acetylcholine or kaolin

Male Swiss mice (18–30 g), kept in a temperature-controlled environment (23 ± 2°C) with a 12 h light-dark cycle, were used. Food and water were freely available. The abdominal constrictions resulting from intraperitoneal injection of acetic acid (0.6%), acetylcholine (4.5 mg kg^{-1}), or kaolin suspension (50 mg kg^{-1}) consisting of a contraction of the abdominal muscle together with a stretching of hind limbs, were induced according to the procedures described previously (Collier *et al.*, 1968; Bentley *et al.*, 1981; Fujiyoshi *et al.*, 1990). Animals were pretreated with NPC 18688 (10 to 300 nmol kg^{-1}) 30 to 150 min before the irritant injection. Control animals received a similar volume of 0.9% NaCl (10 ml kg^{-1}). For the purpose of comparison, other groups of animals were treated with Hoe 140 (a selective B_2 receptor antagonist, 3 to 30 nmol kg^{-1}) 30 min before i.p. injections of acetic acid or acetylcholine. Animals were placed in separate boxes, and the number of abdominal constrictions was counted cumulatively over a period of 20 min for acetic acid and kaolin and 10 min for acetylcholine. The anti-hyperalgesic activity was expressed as the reduction of the number of abdominal constrictions between control animals (saline pretreated mice) and animals pretreated with NPC 18688 or with Hoe 140.

Formalin-induced pain

Male Swiss mice (25–30 g) were used. The procedure was similar to that described previously (Corrêa & Calixto, 1993). Animals from the same strain were slightly anaesthetized with ether, except when used to analyse the first phase of formalin-induced pain, and 20 µl 2.5% formalin (0.92% formaldehyde) made up in phosphate-buffer solution (PBS; concentration: NaCl 137 mM, KCl 2.7 mM and phosphate buffer 10 mM) was injected under the paw surface of the right hindpaw. Two mice (control and treated) were observed simultaneously from 0 to 30 min following formalin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of pain. The first phase of nociceptive response normally peaked 5 min after formalin injection and the second phase 15–30 min after formalin injection, representing the tonic and inflammatory pain responses, respectively (Hunskaar & Hole, 1987). Animals were treated with NPC 18688 (10 to 300 nmol kg^{-1}) intraperitoneally 30 min beforehand or locally (2–20 nmol/paw) together with formalin injection, respectively. Control animals received only the vehicle used to dilute NPC 18688 (0.9% of NaCl solution, 10 ml kg^{-1} i.p. or 20 µl/paw of PBS). Following intraplantar injection of formalin, the animals were immediately placed into a glass cylinder of 20 cm in diameter, and the time spent licking the injected paw was determined.

To investigate whether the antinociceptive activity of NPC 18688 in the formalin-induced pain was associated with anti-oedematogenic activity, we measured the oedema by comparing the difference in weight of the formalin-treated paw and the weight of the control paw (PBS treated 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.

Capsaicin-induced pain

In an attempt to provide more direct evidence concerning the action of NPC 18688 on the neurogenic pain, we also investigated whether this compound antagonized capsaicin-induced pain in the mouse paw. Male Swiss mice (25–30 g) were used. Before testing, the animals were placed individually in transparent glass cylinders of 20 cm in diameter, serving as observation chambers. Following the adaptation period, 20 µl of capsaicin (1.6 µg/paw made in PBS) was injected under the skin of the dorsal surface of the right hindpaw with a microsyringe having a gauge 26 needle. The contralateral paw received a similar volume of PBS. The procedure used was similar to that described previously (Sakurada *et al.*, 1992; 1993) with minor modifications. Mice were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of pain. Animals were treated with NPC 18688 (30 to 300 nmol kg^{-1}) intraperitoneally 30 min before capsaicin injection. Control animals received a similar volume of 0.9% NaCl solution (10 ml kg^{-1}).

Hot-plate test

Male Swiss mice (25–30 g) were used. The hot-plate test was used to measure response latencies according to the method described by Eddy & Leimback (1953), with minor modifications. In these experiments the hot-plate (Ugo Basile, Model-DS 37) was maintained at 56 ± 1°C. The control group of an-

imals (saline treated, 10 ml kg^{-1}, i.p.), or mice pretreated with NPC 18688 (300 nmol kg^{-1}, i.p.) 30 min beforehand were placed in a glass cylinder of 24 cm diameter on the heated surface. Another group of animals, which was used as positive control, was treated with morphine (2.6–26 μmol kg^{-1}, s.c., 30 min earlier). The time(s) between placement and shaking, licking of the paws or jumping was recorded as response latency. An automatic 20 s cut-off was used to prevent tissue damage. Each animal was tested twice before administration of drugs in order to obtain a baseline.

Tail-flick test

Male Swiss mice (25–30 g) were used. A radiant heat tail-flick analgesiometer was used to measure response latencies as described by D'Amour & Smith (1941), with minor modifications. Animals responded to a focused heat-stimulus by flicking or removing their tail, exposing a photocell in the apparatus immediately below the tail. The reaction time was recorded for control mice or for animals pretreated with NPC 18688 (300 nmol kg^{-1}, i.p.) 30 min before or with morphine (2.6–26 μmol kg^{-1}) given 30 min beforehand. An automatic 20 s cut-off was used to minimize tissue damage. Animals were selected 24 h earlier on the basis of their reactivity in the test. Each animal was tested twice before administration of its respective drug in order to determine the baseline.

Rota-rod test

In order to evaluate possible nonspecific muscle relaxant or sedative effects of NPC 18688, the mice were tested on the rota-rod. Male Swiss mice (25–30 g) were used. The apparatus consisted of a bar, (diameter 2.5 cm) subdivided into six compartments by disks 25 cm in diameter (Duham & Miya, 1957). The bar rotated at a constant speed of 14 rev min^{-1}. The animals were selected 24 h previously by eliminating those mice which did not remain on the bar for two consecutive periods of 60 s. Animals were treated with 0.9% NaCl (10 ml kg^{-1} i.p.) or with NPC 18688 (300 nmol kg^{-1}, i.p.) 30 min before being tested. Results are expressed as the time(s) which animals managed to remain on the rota-rod; 60 s was the cut-off time used.

Selectivity of NPC 18688 for B_2 receptor

Rat and mouse hindpaw oedema To assess whether NPC 18688 has selectivity for B_2 receptors, we investigated the effect of NPC 18688 (3 to 100 nmol kg^{-1}, i.p.), given 30 min prior to intraplantar injection of BK (3 nmol/paw) or des-Arg^9-BK (100 nmol/paw)-induced increase of paw volume, in naive or in BK (10 nmol/paw) desensitized paws, respectively. The procedure used was essentially similar to that described by Campos & Calixto (1995). Briefly, experiments were conducted on non-fasted male Wistar mice (25–30 g) or rats (100–120 g) kept in a room controlled for temperature (22±2°C) and illumination (12 h on and 12 h off). All animals were pretreated with captopril (5 mg kg^{-1}, s.c.) and with cyproheptadine (20 mg kg^{-1}, i.p.) 1 and 0.5 h prior to any given experiment to prevent BK degradation and release of histamine and/or 5-hydroxytryptamine (5-HT), respectively (Steranka & Burch, 1991; Corrêa & Calixto, 1993). For the purpose of comparison, we also tested the effect of the selective B_2 receptor antagonist, Hoe 140 (3 to 100 nmol kg^{-1}, i.p.), given 0.5 h beforehand, on BK (3 nmol)-induced hindpaw oedema. Under ether anaesthesia animals received 0.1 ml (rats) or 0.05 ml (mice) intraplantar injections in one hindpaw of BK (3 nmol/paw) or des-Arg^9-BK (100 nmol/paw) dissolved in PBS. The contralateral paw received 0.1 ml of PBS and was used as a control. Oedema was measured by use of a plethysmometer (Ugo Basile) at several time points after peptide injections. Oedema is expressed in ml as the difference between the test and control paws.

Blood pressure

Adult male Wistar rats (280–300 g) were used. Animals were anaesthetized with urethane (1.5 g kg^{-1}, i.p.). The trachea was cannulated to permit spontaneous breathing. Polyethylene catheters (PE 20 tubing) were inserted into the right common carotid artery for recording blood pressure and into the right femoral vein for drug administration. The arterial blood pressure was recorded by use of a Gould P23 transducer coupled to a Beckman polygraph. The NPC 18688 (210 nmol kg^{-1}) and Hoe 140 (230 nmol kg^{-1}) were administered s.c. 30 min prior to intravenous injection of BK (28 nmol kg^{-1}) or acetylcholine (2 nmol kg^{-1}).

Guinea-pig ileum Guinea-pigs of either sex (300–350 g) were killed by cervical dislocation and were exsanguinated. The abdominal cavity was exposed and the ileum isolated. The terminal ileum (about 10 cm) was discarded. Preparations of 15–20 mm, were set up in a 5 ml organ bath containing Krebs solution pH 7.4 at 37°C, continuously gassed with 5% of CO_2 and 95% of O_2, under a load of 1 g as described previously (Medeiros & Calixto, 1993). Isotonic contractions were recorded by means of a light lever (six fold amplification), writing on a kymograph. A stabilization period of at least 60 min was allowed before drug addition. Following the equilibration period, complete cumulative concentration-response curves for acetylcholine (1 nM to 100 μM) were obtained in the absence (control) or in the presence of NPC 18688 (1 μM) incubated with the tissues 10 min before the addition of acetylcholine. The first curve obtained in the absence of NPC 18688 was taken as 100% value and the subsequent response was calculated as a function of this value. In order to correct for any spontaneous change in the sensitivity of the tissues, control experiments were carried out in the presence of the vehicle used to dilute NPC 18688 (0.9% of NaCl solution).

Toad rectus abdominii The rectus abdominii muscles (2 per animal) were removed, stripped of adherent fascia, and set up in a 5 ml organ bath containing Ringer solution at 24±2°C, under a 1 g load, continuously oxygenated with air. The Ringer solution had the following composition (mM): NaCl 111.2, KCl 1.9, $MgCl_2$ 0.59, $CaCl_2$ 0.65 and glucose 5.5. Isotonic contractions were recorded on a kymograph (six fold of amplification). After 60 min of equilibration, cumulative concentration-responses were obtained to acetylcholine (0.2 μM–300 μM) in the absence or in the presence of NPC 18688 (1 μM) added to the preparations 10 min beforehand. The first concentration-response curve for acetylcholine obtained in the absence of NPC 18688 was taken as 100%, and the subsequent response to acetylcholine obtained in presence of NPC 18688 was determined as a function of the first value. Control experiments were carried out in presence of 0.9% NaCl solution.

Drugs

The drugs used were: NPC 18688 (Figure 1) (synthesized at Scios Nova, Baltimore, U.S.A.), formalin, acetic acid, acetylcholine chloride, bradykinin (Sigma Chemical Company, St. Louis, U.S.A.), kaolin (Wako Pure Chemical Industries, Ltd., kindly supplied by Dr T. Fujiyoshi, Kitasato University, Minato-Ku, Tokyo, Japan), capsaicin (Calbiochem, San Diego, California, U.S.A.), des-Arg^9-BK (Peninsula, Belmonte, CA, U.S.A.) and morphine hydrochloride (Merck, Germany). Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK was kindly supplied by Hoechst (Frankfurt Main, Germany). All other reagents used were of a high grade of purity. The stock solution for NPC 18688 and Hoe 140 (1 mM), kept in siliconized plastic tubes, were maintained in the freezer at −18°C. The other drugs were prepared just before use in 0.9% w/v of NaCl solution, except capsaicin, which was dissolved in ethanol. The final concentration of ethanol did not exceed 5% and did not cause any effect *per se*.

Statisitical analysis

The results are presented as mean ± s.e.mean, except the ID_{50}s (i.e. the dose of NPC 18688 or Hoe 140 reducing response by 50% relative to control value) and the EC_{50} (i.e. the concentration causing half-maximum contractile response) in individual experiments which are reported as geometric means accompanied by their respective 95% confidence limits. The statistical significance between groups was analysed either by means of analysis of variance followed by Dunnett's multiple comparison test or by unpaired t test when appropriate. P values less than 0.05 were considered as indicative of significance. The ID_{50}s or the EC_{50}s were estimated for individual experiments by the use of graphical interpolation.

Results

Intraperitoneal injection of NPC 18688 (10 to 300 nmol kg^{-1}) had no agonist activity (results not shown). Results in Figure 2 show that pretreatment of animals with intraperitoneal doses of NPC 18688 (10 to 300 nmol kg^{-1}) 30 min beforehand caused a graded and significant anti-hyperalgesic effect against acetic acid, acetylcholine or kaolin-induced writhing responses in mice. The estimated mean ID_{50}s (and their 95% confidence limits) and the maximal inhibitions of writhing responses caused by NPC 18688 were: 77.3 (44.4–134.3), 34.5 (19.6–60.6) and >300 nmol kg^{-1} and 65 ± 6; 70 ± 5 and 40 ± 3%, against acetic acid, acetylcholine or kaolin, respectively. Figure 3 shows the time course of the anti-hyperalgesic response of NPC 18688 (100 nmol kg^{-1} i.p.) against acetic acid-induced writhing response. The anti-hyperalgesic action of NPC 18688 appeared within the first 30 min following its intraperitoneal injection and lasted for at least 150 min. Similarly, i.p. administration of Hoe 140 (3 to 30 nmol kg^{-1}) caused a dose-related inhibition of acetic acid and acetylcholine-induced abdominal constrictions (results not shown, $n = 6$ to 8 in each group). The calculated mean ID_{50}s and the maximal inhibition for these effects were: 6.0 (3.6–10.0) and 9.6 (7.4–12.4) nmol kg^{-1} and 56 ± 7 and 62 ± 6, respectively.

NPC 18688 (10 to 100 nmol kg^{-1}, i.p.), administered 30 min beforehand, also antagonized in a dose-related manner the second phase of formalin-induced nociception and, to a lesser extent, the neurogenic component (first phase) of the formalin response (Figures 4a and b). The mean ID_{50}s and maximal inhibitions of formalin-induced pain caused by NPC 18688 were: >300 and 60.4 (44.4–81.6) nmol kg^{-1} and 20 ± 3 and 60 ± 5%, against the first and second phases, respectively. In addition, NPC 18688 at the same doses also caused a dose-related and significant inhibition of the oedema response associated with the second phase of the formalin-induced algesic response, with maximal inhibition of 30 ± 2% (Figure 4c).

When injected locally in association with formalin, NPC 18688 (2 to 20 nmol/paw) had no agonist effect, but caused a significant inhibition of both phases of formalin-induced pain (Figure 5a and b). The maximal inhibition of the responses (mean ± s.e.mean) were 40 ± 3 and 33 ± 2%, respectively. NPC 18688 (10 nmol/paw) also antagonized partially, but sig-

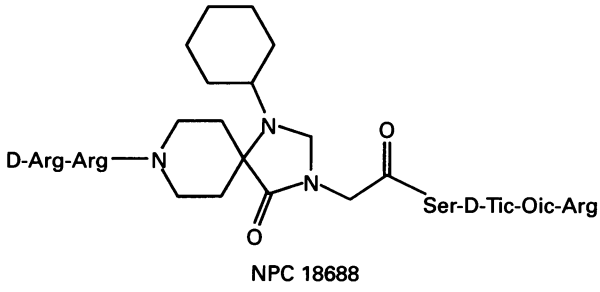

Figure 1 Molecular structure of NPC 18688.

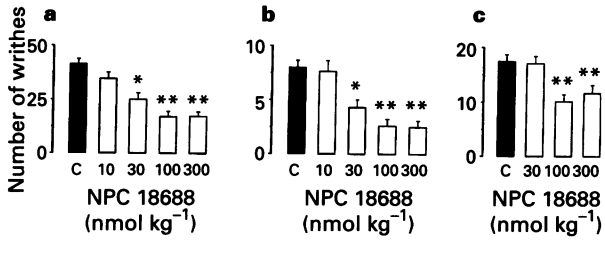

Figure 2 Effect of systemic treatment of animals with NPC 18688 on the writhing responses induced by intraperitoneal injection of (a) acetic acid, (b) acetylcholine and (c) kaolin in mice. The total number of writhes (mean ± s.e.mean) was measured during the first 20 min (acetic acid and kaolin) or 10 min (acetylcholine) following intraperitoneal injection of the irritants. Each column represents the mean with s.e. mean of 6 to 10 animals. The solid columns indicate the control values (animals injected with the vehicle) and the asterisks denote the significance levels. Significantly different from control groups: $*P < 0.05$; $**P < 0.01$.

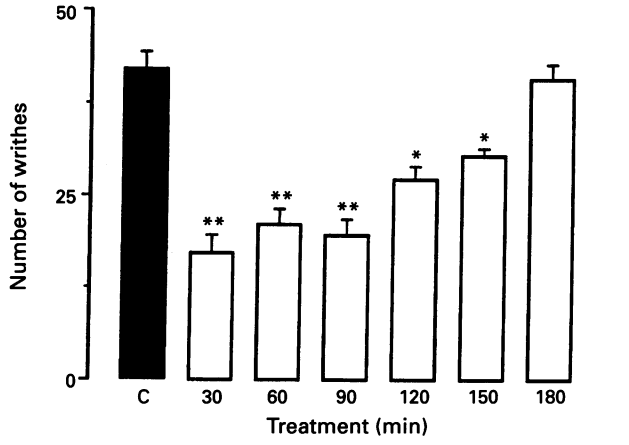

Figure 3 Time-course for the antinociceptive effect caused by systemic treatment of animals with NPC 18688 on the writhing responses induced by injection of acetic acid in mice. The total number of writhes (mean ± s.e.mean) was measured during the first 20 min after injection of acetic acid. Each column represents the mean with s.e. mean of 6 animals. The solid column indicates the control value (animals injected with the vehicle) and the asterisks denote the significance levels. Significantly different from controls: $*P < 0.05$; $**P < 0.01$.

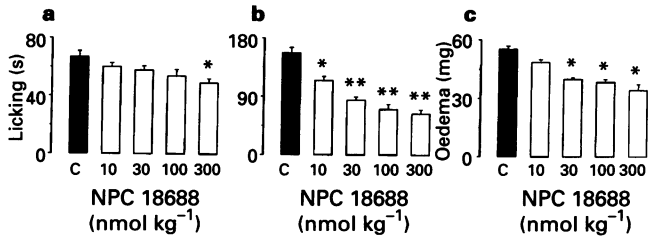

Figure 4 Effect of systemic treatment of animals with NPC 18688 on formalin-induced nociception (a and b) and formalin-induced paw oedema (c) in mice. The total time (mean ± s.e.mean) spent licking the hindpaw was measured in the first (0–5 min, a) and the second phase (15–30 min, b), after intradermal injection of formalin in the hindpaw. Each column represents the mean with s.e. mean of 5 to 8 animals. The solid columns represent the control values (animals injected with the vehicle) and the asterisks denote the significance levels. Significantly different from controls: $*P < 0.05$; $**P < 0.01$.

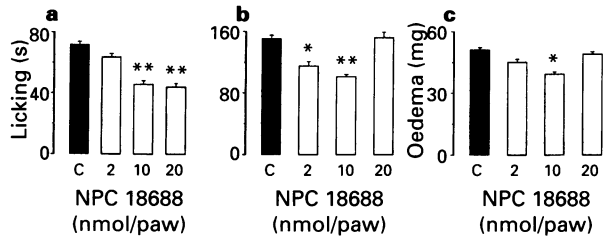

Figure 5 Effect of topical injection of the pseudopeptide B_2 receptor antagonist, NPC 18688, on formalin-induced nociception (a and b) and formalin-induced paw oedema (c) in mice. The total time (mean ± s.e.mean) spent licking the hindpaw was measured in the first (0–5 min, a) and the second phase (15–30 min, b) after intradermal injection of formalin in the hindpaw. Each column represents the mean with s.e. mean of 5 to 8 animals. The solid columns indicate the control values (animals injected with the vehicle) and the asterisks denote the significance levels. Significantly different from control groups: $*P<0.05$; $**P<0.01$.

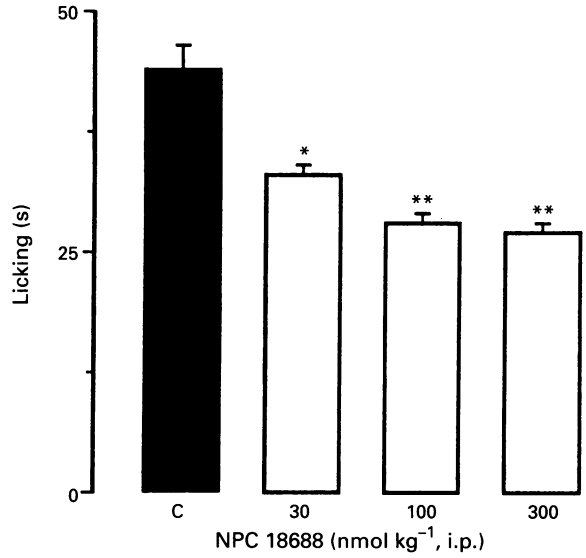

Figure 6 Effect of systemic treatment of animals with the B_2 bradykinin pseudopeptide receptor antagonist, NPC 18688, on capsaicin-induced pain in mice. The total time (mean ± s.e.mean) spent licking the hindpaw was measured after intradermal injection of capsaicin in the hindpaw. Each column represents the mean with s.e. mean of 5 to 9 animals. The solid column indicates control values (animals injected with the vehicle) and the asterisks denote the significance levels. Significantly different from control groups: $*P<0.05$; $**P<0.01$.

nificantly, the paw oedema associated with the second phase of formalin-induced pain (Figure 5c). Figure 6 shows that NPC 18688 (30 to 300 nmol kg^{-1}, i.p.), given 30 min beforehand, inhibited in a significant manner, capsaicin-mediated neurogenic pain in the mouse paw, with an $ID_{50}>$ of 300 nmol/paw and maximal inhibition (mean ± s.e.mean) of 40 ± 2%.

When analysed either in the tail-flick test or in the hot-plate model, NPC 18688 (up to 300 nmol kg^{-1}, i.p.) was virtually ineffective, (tail-flick saline-treated mice 5.7 ± 0.5 s versus 5.8 ± 0.5 s in NPC 18688-treated animals; hot-plate, control response of 7.5 ± 0.6 s and 6.8 ± 0.4 s in NPC 18688 pretreated animals; $n=5$ to 6 animals in each group). In the same experimental conditions, morphine (2.6–26 μmol kg^{-1}, s.c.) given 30 min beforehand caused a marked and significant increase of the latency responses in both models (results not shown). In the case of NPC 18688 (300 nmol kg^{-1}, i.p.) given 30 min beforehand (the higher dose used) failed to interfere with the motor response of animals as revealed in the rota-rod test (control response 52.5 ± 1.6 s versus 54.5 ± 2 s in animals pretreated with NPC 18688, $n=6$ in each group).

Both NPC 18688 and Hoe 140 (each 100 nmol kg^{-1}, i.p.) given 30 min before consistently antagonized BK (3 nmol/paw)-mediated increase in rat paw volume (percentage inhibition, mean ± s.e.mean) of 48 ± 3, 47 ± 4, 50 ± 4, 52 ± 5, 48 ± 4 and 49 ± 5, 60 ± 5, 50 ± 4, 44 ± 3 and 38 ± 4, at 10, 20, 30, 60 and 120 min after BK injection, respectively, $P<0.05$, $n=4-6$ in each group). Both NPC 18688 (10 to 100 nmol kg^{-1}) and Hoe 140 (3 to 30 nmol kg^{-1}) produced dose-related and long-lasting (up to 120 min) inhibition of BK (3 nmol)-induced oedema in the mouse paw. The calculated mean ID_{50}s and the inhibition at the higher dose tested (100 and 30 nmol kg^{-1}, respectively) were: 61.3 (45.4–79.3) and 6.1 (3.4–8.3) nmol kg^{-1} and 63 ± 3% and 52 ± 3%, respectively. In contrast, at the same dose NPC 18688 did not significantly affect des-Arg^9-BK (100 nmol/paw)-mediated rat paw oedema in BK-desensitized paws (control response of 0.32 ± 0.02 ml versus 0.32 ± 0.03 ml in rats pretreated with NPC 18688, analysed at the peak of the oedema at 20 min, $n=4$).

NPC 18688 (210 nmol kg^{-1}, s.c.), like Hoe 140 (230 nmol kg^{-1}, s.c.) given 30 min beforehand abolished BK (28 nmol kg)-induced hypotension in rats (from −27 ± 5 mmHg in control animals to 0 in animals treated with NPC 18688 or Hoe 140, respectively ($n=4$) $P<0.01$). However, at the same doses, both NPC and Hoe 140 did not interfere significantly with the hypotension caused by i.v. injection of acetylcholine (2 nmol kg^{-1}) (control response −23 ± 4 mmHg versus 25 ± 3 and 26 ± 3 mmHg in animals treated with NPC 18688 and Hoe 140, respectively, $n=4$).

When tested *in vitro*, NPC 18688 up to 1 μM did not significantly affect acetylcholine-mediated contractions either in the guinea-pig ileum or in the toad rectus abdominii (results not shown, $n=6$ to 7 experiments in each group). The calculated mean EC_{50}s for acetylcholine were: guinea-pig ileum, control response of 8.0 (5.3–12.2) nM versus 7.9 (6.0–10.4) nM in the presence of NPC 18688; toad rectus abdominii, 6.0 (3.5–10.5) μM versus 9.5 (5.5–14.7) μM in the presence of NPC 18688.

Discussion

NPC 18688 is a recently-described pseudopeptide antagonist of the human B_2 receptor (Chakraverty *et al.*, 1994). It was designed on the basis of structural insights gathered from three-dimensional models of the receptor and conformationally-constrained peptide antagonists (Kyle, 1994). The structure of NPC 18688 is shown in Figure 1. The current results demonstrate that NPC 18688, the first representative of a novel class of B_2 receptor antagonists, produces topical and systemic long-lasting anti-hyperalgesic action as revealed by its ability to antagonize acetic acid, acetylcholine, kaolin, formalin and capsaicin-induced nociception in mice. Importantly, in contrast to results with some selective peptide BK receptor antagonists (Steranka *et al.*, 1998a,b; Kindgen-Milles & Klement, 1992; Corrêa & Calixto, 1993), NPC 18688 did not itself display any partial or total agonist activity, either when injected topically or when given systemically to mice.

Although NPC 18688 was about 4 to 12 fold less potent, but was more effective than some selective peptide B_1 and B_2 BK receptor antagonists (Haley *et al.*, 1989; Shibata *et al.*, 1989; Chapman & Dickenson, 1992; Corrêa & Calixto, 1993, and present study), it antagonized, in a dose-related manner, both the neurogenic and the inflammatory pain response caused by intraplantar injection of formalin and also, as reported for Hoe 140 (Heapy *et al.*, 1993, and present study), the abdominal constrictions caused by several irritants in mice. In addition, NPC 18688, when given systemically, and, to a lesser extent, when co-injected locally together with formalin, caused a pronounced inhibition of formalin-mediated paw oedema associated with the later phase of the pain response. These data support further our previous notion (Corrêa & Calixto, 1993)

that kinins acting through B_2 receptors are implicated in both phases of the pain response as well as in the oedema formation associated with the later phase of the formalin test in the mouse. Interestingly, as reported previously for the second generation of highly potent selective B_2 receptor antagonists, Hoe 140, NPC 17731 and NPC 17761 (Corrêa & Calixto, 1993), the pseudopeptide B_2 receptor antagonist, NPC 18688, did not completely abolish either phase of the formalin-mediated response or the inflammatory response associated with the later response of this model. These views are consistent with the previous notion that other inflammatory mediators besides kinins are probably involved in formalin-induced pain and inflammatory response (Shibata *et al.*, 1986; 1989; Haley *et al.*, 1989; Dray & Dickenson, 1991; Moore *et al.*, 1991; Murray *et al.*, 1991).

The anti-hyperalgesic effect of NPC 18688 reported in the present study seems to be associated with its selective antagonist effect towards the B_2 kinin receptor subtype, as shown by the fact that the pseudopeptide bradykinin receptor antagonist, like the selective peptide B_2 receptor antagonist Hoe 140 (Lembeck *et al.*, 1991; Hock *et al.*, 1991, and present study), given systemically, consistently antagonized BK-induced paw oedema in rats and mice with long duration of action. The reason why NPC 18688 was more efficacious against acetylcholine than acetic acid or kaolin-mediated abdominal constrictions still remains unclear. However, this fact is not associated with the anti-cholinoceptor action of NPC 18688 acting either at muscarinic or nicotinic receptors, because it had no significant effect against acetylcholine-induced contraction of the guinea-pig ileum and toad rectus abdominii *in vitro*. In addition, NPC 18688 did not affect the fall of mean arterial blood pressure induced by i.v. injections of acetylcholine in rats, in conditions where, like Hoe 140, it completely abolished the hypotensive response caused by BK. These results suggest that kinins acting at B_2 receptors have a role in acetic acid and acetylcholine-mediated abdominal constrictions in mice. On the other hand, the systemic kinin antagonistic action of NPC 18688 is quite selective for B_2 receptor subtypes, since at doses which caused significant inhibition of BK-mediated rat and mouse hindpaw oedema, NPC 18688 failed to affect the B_1-selective agonist des-Arg^9-BK-mediated oedema in paws that had been completely desensitized to BK. We have recently reported that BK induces rat paw oedema through stimulation of the constitutive B_2 receptor (Campos & Calixto, 1995; Campos *et al.*, 1995). However, following complete desensitization of the B_2 receptor with daily intraplantar injection of BK for seven consecutive days, des-Arg^9-BK (which is inactive in naive rats) produced marked paw oedema, an effect which may be prevented by dexamethasone treatment, suggesting the induction of B_1 receptors (Campos & Calixto, 1995; Campos *et al.*, 1995).

Of interest are the results indicating that systemic injection of NPC 18688 into mice produces significant inhibition of capsaicin-induced nociception, a recently-proposed model for neurogenic pain (Sakurada *et al.*, 1992; 1993). These results and the previously-reported data concerning the anti-hyperalgesic action of B_1 and B_2 antagonists against formalin-induced neurogenic pain (Haley *et al.*, 1989; Shibata *et al.*, 1989; Chapman & Dickenson, 1992; Corrêa & Calixto, 1993) support the view that kinins acting through B_1 and B_2 receptors participate in neurogenic pain. Interestingly, when injected locally, NPC 18688 did not produce a clear dose-related inhibition against either phase of formalin-induced algesic response. Additionally, at a higher concentration (20 nmol/paw) it completely lost its antinociceptive effect against the later phase of the formalin test as well as its antioedematogenic action. These results suggest that although NPC 18688 has no residual agonist activity when given topically at higher doses, it exhibits other unknown actions which abolish its anti-hyperalgesic and antioedema properties. The mechanism responsible for such effects was not examined further.

NPC 18688, even when administered at higher doses (up to 300 nmol kg^{-1}), was unable to produce an antinociceptive effect when analysed in the tail-flick and in hot-plate tests, two opioid-sensitive models, indicating that it has no analgesic action *per se*. Furthermore, we have also attempted to investigate whether the anti-hyperalgesic action of NPC 18688 might reflect some nonspecific central or peripheral depressant effect. As revealed in the rota-rod model, NPC 18688, even at higher systemic doses, did not display any motor dysfunction, as indicated by the lack of any detectable relaxant or sedative effects.

In summary, the current results have demonstrated that in contrast to those reported for some highly potent B_2 receptor antagonists, the pseudopeptide and selective B_2 receptor antagonist, NPC 18688, although less potent than some available second generation B_2 peptide receptor antagonists, did not display any residual agonist activity either when injected topically or when given systemically to mice. Furthermore, NPC 18688 produced topical and systemic long-lasting anti-hyperalgesic activity in several models of nociception in mice, suggesting that this newly-developed pseudopeptide and selective B_2 receptor antagonist may constitute a useful tool for investigating the participation of kinins in physiological and pathological processes. Thus, it may lead to the development of a third generation of orally-potent and highly selective long-lasting kinin B_2 receptor antagonists, useful for the management of clinical disorders involving BK and related kinins.

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