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RESEARCH PAPER

# Inhibition of acute nociceptive responses in rats after i.c.v. injection of Thr$^6$-bradykinin, isolated from the venom of the social wasp, *Polybia occidentalis*

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**Background and purpose:** In this work, a neuroactive peptide from the venom of the neotropical wasp *Polybia occidentalis* was isolated and its anti-nociceptive effects were characterized in well-established pain induction models.
**Experimental approach:** Wasp venom was analysed by reverse-phase HPLC and fractions screened for anti-nociceptive activity. The structure of the most active fraction was identified by electron-spray mass spectrometry (ESI-MS/MS) and it was further assessed in two tests of anti-nociceptive activity in rats: the hot plate and tail flick tests.
**Key Results:** The most active fraction contained a peptide whose structure was Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-OH, which corresponds to that of Thr$^6$-BK, a bradykinin analogue. This peptide was given by i.c.v. injection to rats. In the tail flick test, Thr$^6$-BK induced anti-nociceptive effects, approximately twice as potent as either morphine or bradykinin also given i.c.v. The anti-nociceptive activity of Thr$^6$-BK peaked at 30 min after injection and persisted for 2 h, longer than bradykinin. The primary mode of action of Thr$^6$-BK involved the activation of $B_2$ bradykinin receptors, as anti-nociceptive effects of Thr$^6$-BK were antagonized by a selective $B_2$ receptor antagonist.
**Conclusions and implications:** Our data indicate that Thr$^6$-BK acts through $B_2$ bradykinin receptors in the mammalian CNS, evoking antinociceptive behaviour. This activity is remarkably different from that of bradykinin, despite the structural similarities between both peptides. In addition, due to the increased metabolic stability of Thr$^6$-BK, relative to that of bradykinin, this peptide could provide a novel tool in the investigation of kinin pathways involved with pain.
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**Keywords:** wasp venom; neuroactive peptides; Thr$^6$-bradykinin; bradykinin; anti-nociception; $B_2$ receptor

**Abbreviations:** ACN, acetonitrile; AUC, area under the curve of antinociception; BK, bradykinin; ESI-MS, electron-spray mass spectrometer; IA, index of antinociception; i.c.v., intracerebroventricular; i.p., intraperitoneal; RP-HPLC, reverse phase-high performance liquid chromatography; TFA, trifluoroacetic acid; Thr$^6$-BK, threonine$^6$-bradykinin

## Introduction

Since the characterization of bradykinin (BK) in 1949, kinins have been implicated in a wide range of physiological processes, including vascular permeability, control of blood pressure and pain (Rocha e Silva *et al*., 1949; Bhoola *et al*., 1992; Leeb-Lundberg *et al*., 2005). Moreover, BK and related peptides exert potent algesic effects by stimulation of the peripheral nervous system (Calixto *et al*., 2000; Marceau and Regoli, 2004).

In the central nervous system (CNS), BK given into the lateral ventricles or other brain sites, may induce a variety of effects, such as sedation, catatonia, noradrenaline depletion, hyperthermia, hypertension, antidiuretic hormone release and anti-aversive effects (Graeff *et al*., 1971; da Silva and Rocha e Silva, 1971; Pelá *et al*., 1996; Couto *et al*., 2006). BK exerts its action through two subtypes of G protein-coupled receptors; $B_1$ and $B_2$. Whereas BK activates receptors of the $B_2$

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type, $B_1$ receptors are stimulated by kinin metabolites, such as Lys-des-Arg$^9$-BK, obtained after enzymic cleavage by a neuronal kininase (Leeb-Lundberg *et al*., 2005). The stimulation of $B_2$ receptors by centrally injected BK results in potent and dose-dependent antinociceptive effects pointing to the involvement of BK in the control of painful sensations (Pelá *et al*., 1996; Couto *et al*., 2006).

Since 1954 many peptides with chemical structures and physiological activities similar to BK have been reported in the venoms of animals, such as solitary and social species of wasps (Jaques and Schachter, 1954; Griesbacher *et al*., 1998) The presence of BK analogues in the venoms are considered to have mainly defensive purposes, as wasp stings often provoke pain, oedema and local lesions even in higher vertebrates, such as man (Nakajima, 1986; Mortari *et al*., 2005). These compounds can still act as neurotoxins, as they cause paralysis in insects by the presynaptic blockade of cholinergic transmission, probably caused by a non-competitive inhibition of choline uptake (Piek *et al*., 1987, 1990; Hue and Piek, 1989). However, there are no previous reports on the activity of venom-derived kinins in the mammalian CNS (Piek, 1991).

Therefore, the aims of the present study were to isolate and fully sequence the low-molecular-weight (MW) antinociceptive neurokinin from the venom of the social wasp *Polybia occidentalis occidentalis*. Furthermore, the effects of the purified molecule in the CNS of freely moving Wistar rats were assayed in two models of pain induction.

## Materials and methods

*Venom extracts*

Females of *P. occidentalis* were collected at the University of São Paulo (Campus of Ribeirão Preto City, São Paulo, Brazil), and they were killed by freezing at −20°C. After species identification by Professor Sidney Matheus (Entomology Laboratory, USP), the venom reservoirs were manually removed, crushed in Milli-Q grade water/acetonitrile (ACN) (1:1) and centrifuged at 5000 *g* for 10 min, at 4°C. Supernatants were carefully collected and filtered using Microcon 3 (Millipore, Billerica, MA, USA). The extract with compounds of MW lower than 3 kDa was collected, lyophilized and weighed.

*Fractionation and purification*

The extract was resuspended in 1:1 ACN/water $H_2O$ and 0.07% trifluoroacetic acid (TFA). The solution was chromatographed on a reverse-phase high-performance liquid chromatography (RP-HPLC) column ($C_{18}$ ODS, Jupiter 15 $\mu$m, 20 × 250 mm, Phenomenex, Torrence, CA, USA) at a flow rate of 5.0 ml/min and eluted using an isocratic gradient from 2% ACN/$H_2O$ (v/v) (containing 0.07% TFA) for 20 min, followed by 2–60% for 40 min and 60% for 20 min. The active fraction was chromatographed on a RP-HPLC column ($C_{18}$ ODS, Jupiter 5 $\mu$m, 4.6 × 150 mm, Phenomenex, Torrence, CA, USA) at a flow rate of 1.0 ml min$^{-1}$ and it was eluted by an isocratic gradient from 2% ACN/$H_2O$ (v/v) (containing 0.07% TFA) for 10 min, followed by 2–60% for 30 min and 60% for 10 min. Effluents from both columns were monitored photometrically at 214 nm.

*Electrospray mass spectrometry*

Molecular mass spectral analyses of peptides were performed on a high-resolution q-TOF. Electron-spray mass spectrometer (ESI-MS) spectrum was acquired on an UltrOTOF apparatus (Bruker Daltonics, Billerica, MA, USA).

*Peptide sequencing by MS/MS*

A Quattro-LC instrument from Micromass (Manchester, UK) was used for peptide sequencing. Solutions were infused into the ESI source at 10 $\mu$l min$^{-1}$ using a Harvard Apparatus syringe pump model 1746 (Holliston, MA, USA). Deionized water (Milli-Q) was used throughout the study. The mass scan range was from *m/z*1000 to 2000. Experiments were performed with a cone voltage of 30 V, a capillary voltage of 3 kV and a dessolvation gas temperature of 80°C. A singly charged (protonated) molecule was submitted to collision-induced dissociation with argon gas at 10–50 eV collision energies. All MS/MS experiments were performed using continuous acquisition mode, scanning from *m/z*50 to 2000, with a scan time of 5 s.

Peptide sequencing was obtained using the ion products described in the mass spectra of each peptide, with the aid of AminoCalc software (Protana A/S).

*Animal care*

Male Wistar rats (220–250 g) from the animal house of the University of São Paulo were used in the assays. They were kept in wire-mesh cages in a room with a 12-h dark/light cycle (lights on at 0700 hour) with food and water *ad libitum*. Animals were maintained in accordance with the ethical statements of the Brazilian Society for Neuroscience and Behavior, which follow the guidelines for animal care prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA. Conditions of light and temperature (22°C) were kept constant in the housing and experimental rooms. Efforts were made to minimize the number and potential suffering of the animals used.

*Surgical preparation of rats*

All animals were anaesthetized by intraperitoneal (i.p.) administration of sodium thiopental (40 mg kg$^{-1}$; Cristalia, Brazil) for stereotaxic implantation of a stainless-steel guide cannula (10 mm) in the right lateral ventricle. The coordinates used were 0.9 mm posterior to bregma, 1.6 mm lateral from midline and 3.4 mm ventral from the surface of the skull according to the Atlas of Paxinos and Watson (1986). The cannula was fixed to the skull with acrylic resin and one stainless-steel screw. At the end of the surgery, each guide cannula was sealed with a stainless-steel wire to protect it from obstruction.

The animals were allowed to recover for 5–7 days after the surgery. After that, the rats were gently wrapped in a cloth,

hand-held and a thin dental needle was introduced through the guide cannula. The injection needle was linked to a polyethylene tube connected to a 10 $\mu$l Hamilton syringe, held by an infusion pump (Insight, Brazil) that injected a volume of 1 $\mu$l during 60 s.

*Antinociceptive assays*

Rats were tested for antinociceptive behaviour using the tail flick and hot-plate tests. In the tail flick test, the tail of each animal was laid across a nichrome wire coil, which was then heated by the passage of an electric current. The current raised the temperature of the coil at a rate of $9^{\circ}C\,s^{-1}$. The equipment was calibrated to obtain three consecutive baseline tail flick latencies between 2.5 and 3.5 s. If at any time the animal failed to flick its tail within 6 s (cutoff time), the current was automatically turned off and the tail of the animal was removed from the wire to avoid damage to the skin.

For the hot-plate test, animals were placed on a metal plate enclosed by Plexiglas walls, maintained at $55.5 \pm 0.5^{\circ}C$. The behavioural end point was the time measured in seconds at which the animal jumped off the plate or licked a hind paw. Rats were removed from the hot plate if they did not respond within 30 s (cutoff time) to prevent tissue damage.

Three baseline measurements of withdrawal latencies to noxious stimulus in the tail flick and hot-plate tests were taken at 5 min intervals before the testing session. For both tests, measurements of antinociception were made at 5, 10, 20, 30, 45, 60, 90 and 120 min, after receiving in separate groups ($n = 5$, per group), intracerebroventricular (i.c.v.) administration of physiological saline, BK (16, 8 and 4 nmol), morphine (24, 12 and 6 nmol) or peptide from the wasp venom (8, 4, 2 and 1 nmol).

Independent groups of Wistar rats were treated with concomitant i.c.v. administration of the $B_2$-receptor antagonist, D-Arg$^0$ (8 nmol) plus BK (8 nmol) or threonine$^6$-bradykinin (Thr$^6$-BK) (4 nmol).

As there were no significant statistical differences among the baseline latencies among the different experimental groups (unpaired Student's *t*-test; $P < 0.05$ in all cases), all the withdrawal latencies, that is, the time taken for the motor response (LA) were normalized by an index of antinociception (IA) using the formula:

$$\mathrm{IA} = \frac{(\text{test LA}) - (\text{average baseline LA})}{\text{cutoff} - (\text{average baseline LA})}$$

The results were expressed as means $\pm$ s.e.m. of IA values and plotted against time of the recordings of withdrawal reflexes. The area under the IA–time curves was calculated as area under the curve (AUC) – computer units.

*Histology*

At the end of the experiments, animals were killed by an overdose of sodium thiopental and perfused through the left ventricle first with saline (0.9%), and then with paraformaldehyde (4% phosphate-buffered saline (PBS), pH 7.4 at 4°C). After perfusion, animals were injected with toluidine blue dye in the same volume as injected in the experiments to mark the correct site of injection. The brains were immediately removed and placed into formaldehyde (4% PBS, pH 7.4) and kept at 4°C for 24 h. They were then rinsed in 10 and 20% sucrose (PBS, pH 7.3) at 4°C for 12 h each. Tissue pieces were embedded in Tissue Tek, frozen and cut (40 $\mu$m) on a cryostat (HM 505 E, Microm, Zeiss).

*Statistical analysis*

All normal data were submitted to repeated measures analysis of variance (ANOVA). In case of significant treatment-versus-time interaction, one-way ANOVAs followed by Duncan's test, at each time interval, were performed (SPSS for Windows, version 10.0; Chicago, IL, USA). The AUCs were analysed using one-way ANOVA, with $P < 0.05$ or $P < 0.001$ followed by Student–Newman–Keuls as a *post hoc* test. Probit methodology (Finney, 1971) was used to calculate $ED_{50}$ value (with 95% confidence interval). Moreover, curves described for all treatments, were compared by means of nonlinear regression with the F-test, considering differences significant when $P < 0.05$. The AUCs and dose–response curves of experiments were obtained with GraphPad Prism (version 4.0, GraphPad Software, San Diego, CA, USA).

*Drugs*

All drugs were dissolved in sterile physiological saline (NaCl, 150 mM) and injected i.c.v., as described above. The following drugs were used: BK (Sigma, St Louis, MO, USA), the competitive $B_2$ antagonist D-Arg$^0$-Hyp$^3$-Thi$^{5,8}$-D-Phe$^7$-BK (D-Arg$^0$) (Sigma) and the agonist of $\mu$-opioid receptors, morphine (Sigma).

## Results

*Purification and identification of active fraction from P. occidentalis venom*

The first step of the RP-HPLC fractionation of compounds with molecular masses lower than 3 kDa (30 mg) resulted in the chromatographic profile shown in Figure 1a. The active fraction is indicated with an arrow and this peak was re-chromatographed by HPLC (Figure 1b). In this second step, the chromatogram profile revealed the presence of the major fraction that was analysed and identified by ESI-MS.

ESI-MS spectrum of the active fraction showed the high purity of the isolated peptide with molecular ion peak at *m/z*1074.8 Da ($M + H^+$) (Figure 1c). The primary structure of the peptide present in the fraction was identified by MS/MS fragmentation. The complete series of b-ions starts with the ion *m/z*157, followed in sequence by *m/z*254, 351, 412, 555, 656, 753, 900 and 1075 (Figure 2). The MS/MS fragmentation profile was compared to that of peptides found in other animal venoms (Piek, 1991; Chen *et al*., 2002). From this analysis, the isolated peptide consisted of nine amino acids and its primary sequence was Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-OH, which corresponded to that of the BK analogue, Thr$^6$-BK.

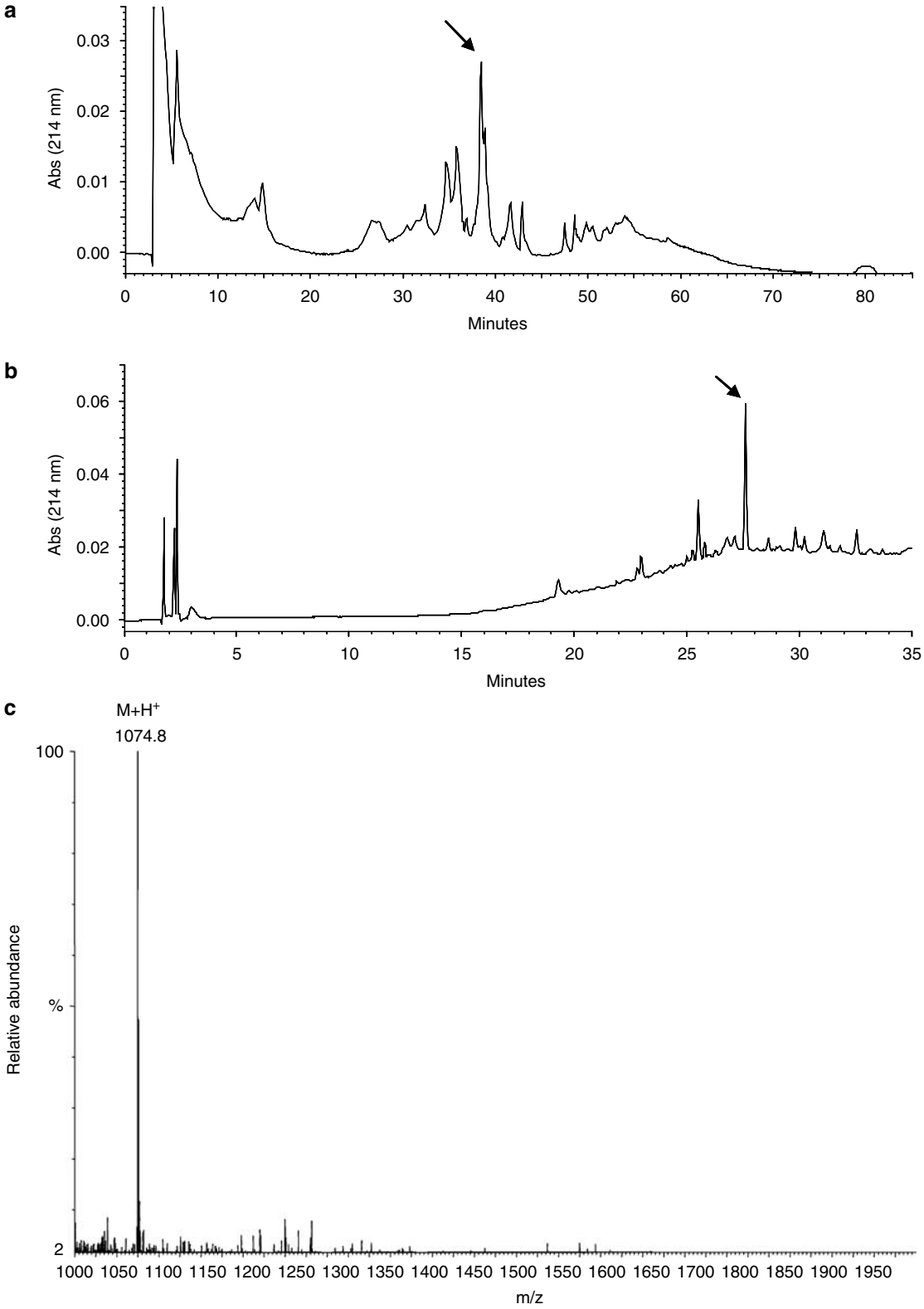


**Figure 1** Isolation and identification of the neurokinin Thr$^6$-BK extracted from the venom of the social wasp, *P. occidentalis*. (**a**) RP-HPLC profile of the low MW extract from the venom of *P. occidentalis*. Chromatography was carried out with a $C_{18}$ ODS column at a flow rate of 5.0 ml min$^{-1}$ and eluted by a linear gradient from 2% ACN/$H_2O$ (v/v) (containing 0.07% TFA) for 20 min, followed by 2–60% for 40 min and 60% for 20 min. (**b**) Chromatographic profile of purification of the fraction indicated by arrow in A. RP-HPLC using a $C_{18}$ column at a flow rate of 1.0 ml min$^{-1}$ and eluted by a linear gradient from 2% ACN/$H_2O$ (v/v) (containing 0.07% TFA) for 10 min, followed by 2–60% for 20 min and 60% for 10 min. (**c**) ESI mass spectrum of the Thr$^6$-BK, presenting a major peak at *m/z*1074.8.

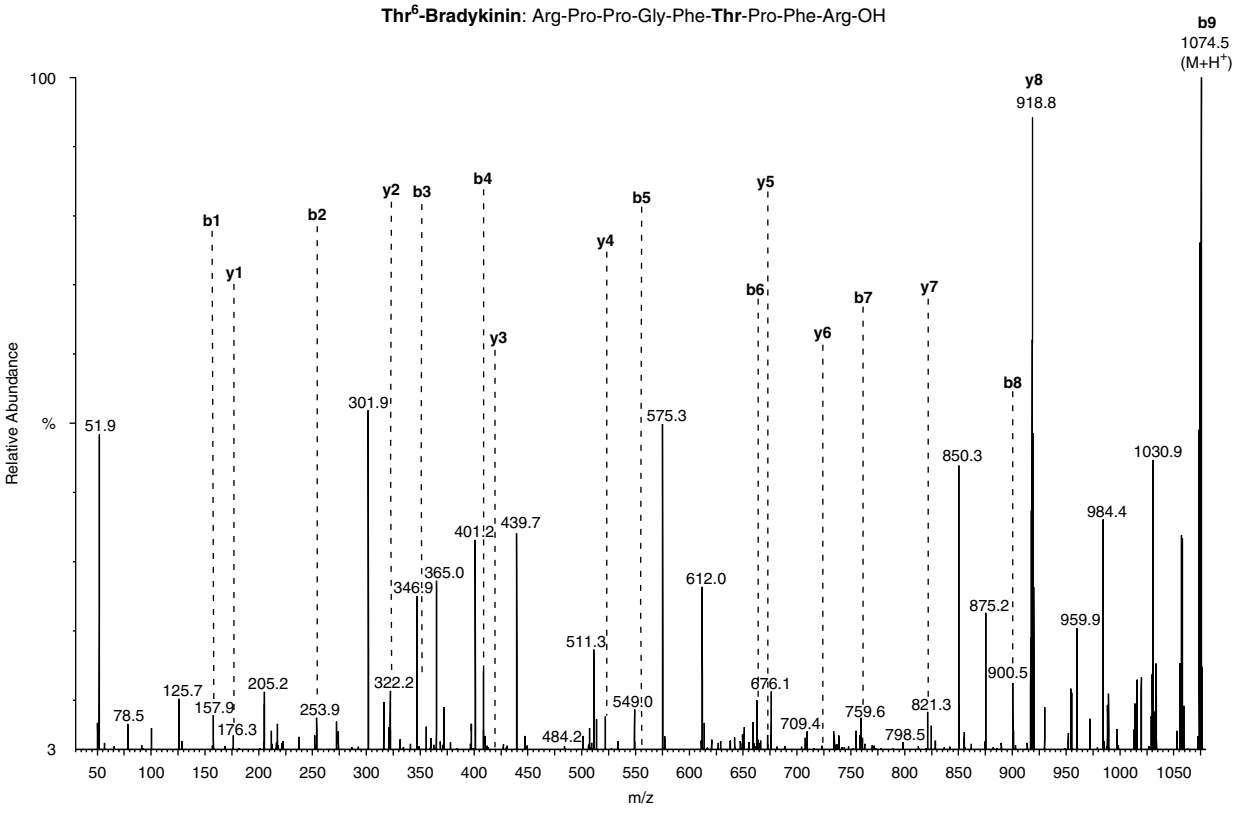


**Figure 2** ESI MS/MS spectrum of the active peptide, indicating the amino-acid sequence on the top of the figure.

*Antinociceptive assays*

The peptide purified from the venom of *P. occidentalis* induced a dose-dependent antinociceptive effect in the tail flick test after i.c.v. administration. Significant differences in the effect of treatment ($F(4,21) = 13.36$; $P < 0.0001$), time ($F(9,21) = 7.27$; $P < 0.0001$), and treatment-versus-time interaction ($F(36,79) = 1.612$; $P < 0.02$), were observed. One-way ANOVAs showed a significant effect for the treatments in all periods ($F(4,21)$ varying from 4.35 to 8.55; $P < 0.05$). Data from the tail flick test submitted to the *post hoc* analysis indicated that all doses of Thr$^6$-BK (8, 4, 2 and 1 nmol) produced significant antinociceptive effects when compared to control animals. The highest dose of Thr$^6$-BK (8 nmol) induced antinociceptive effects that were expressed over the whole experimental period (2 h). Moreover, at 30 and 45 min after i.c.v. injection, a significant difference between the highest and the lowest dose was observed (Figure 3a).

A dose-dependent antinociceptive effect was also observed in the hot-plate test. There were significant effects of treatment ($F(4,22) = 7.79$; $P < 0.0001$), time ($F(9,12) = 12.12$; $P < 0.0001$), and treatment-versus-time interaction ($F(36,198) = 3.33$; $P < 0.001$). One-way ANOVAs showed a significant treatment effect from 30 to 120 min ($F(4,22)$ varying from 3.00 to 7.68; $P < 0.05$). *Post hoc* analysis indicated that doses of Thr$^6$-BK 8 and 4 nmol produced significant increased hot-plate latencies of the rats when compared to the control group. In addition, Thr$^6$-BK at the dose of (8 nmol) was significantly different from the doses of 2 and 1 nmol (Figure 3c).

In both tests, the greatest antinociceptive effect was observed 30 min after microinjection of Thr$^6$-BK by i.c.v. route and the duration of the effect was also related to the dose (Figure 3a and c). The values of $ED_{50}$ and confidence intervals of the antinociceptive effect of Thr$^6$-BK in the tail flick and hot-plate tests were 2.94 nmol (1.54–3.49) and 6.82 nmol (3.17–7.57), respectively.

Similar antinociceptive effects were also observed in the AUC for both tail flick ($F(4,20) = 11.58$; $P < 0.001$) and hot-plate ($F(4,22) = 11.13$; $P < 0.0001$) tests. *Post hoc* analysis of data from the tail flick test showed significant antinociceptive effect for Thr$^6$-BK 8 nmol in comparison to the lower doses and the two higher doses (8 and 4 nmol) in comparison to the control (Figure 3b). The AUCs from the hot-plate test revealed significant differences for Thr$^6$-BK at the dose of 8 nmol in comparison to its lowest dose as well as the control animals (Figure 3d).

Figure 4a and c shows the comparison between the effects of i.c.v. administration of either BK (4, 8 and 16 nmol) or Thr$^6$-BK (8 nmol) in the tail flick and hot-plate tests. In the tail flick test, significant effects of treatment ($F(4,20) = 18.53$; $P < 0.01$), time ($F(7,14) = 2.26$; $P < 0.05$), and treatment-versus-time interaction ($F(28,78) = 1.67$; $P < 0.02$), were observed. One-way ANOVAs showed significant treatment effects from 20 to 120 min ($F(4,20)$ varying from 2.52 to 10.53; $P < 0.05$). *Post hoc* analysis indicated that Thr$^6$-BK produced significant increases in the tail flick latencies when compared to BK-treated groups (4 and 8 nmol) in the periods from 30 to 120 min. Moreover, the maximum effect for BK

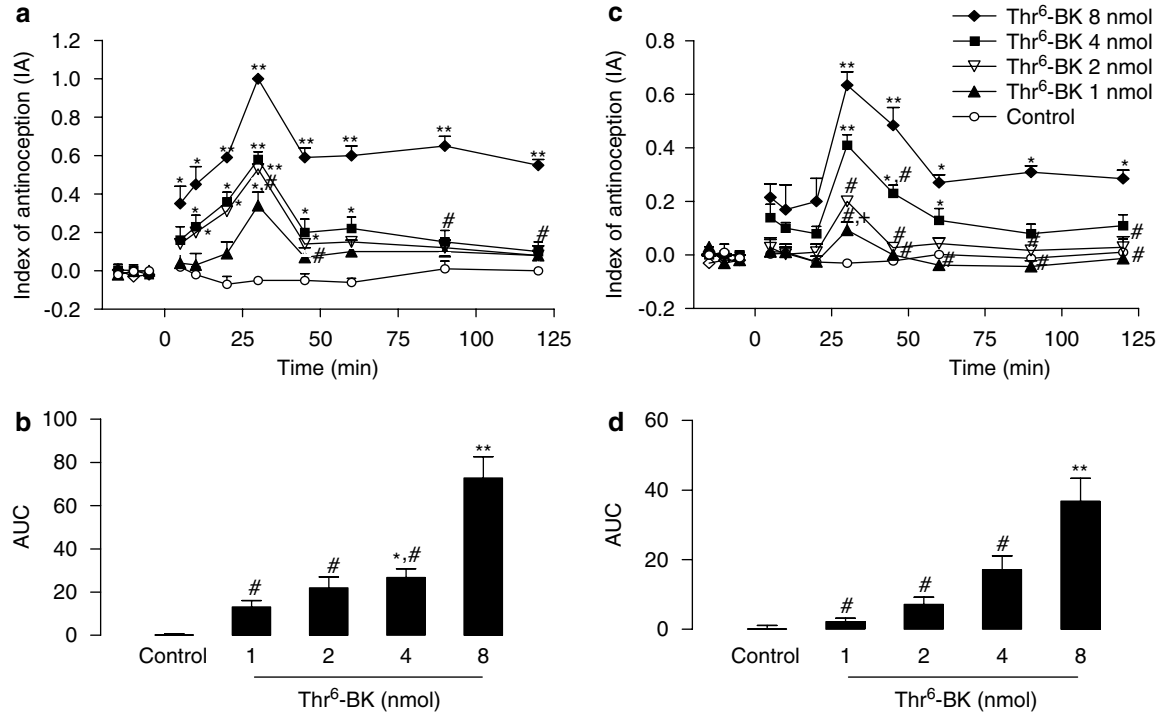


**Figure 3** Antinociceptive dose-dependent effect of Thr$^6$-BK injected i.c.v. in rats. (**a**) Time course of the dose-dependent increase in latencies in the tail flick test induced by i.c.v. injection of Thr$^6$-BK. (**b**) AUC of the groups shown in (**a**). (**c**) Time course of the dose-dependent increase in latencies in the hot-plate test induced by i.c.v. injection of Thr$^6$-BK. (**d**) AUC of the groups shown in (**c**). *Significantly different from control (*$P<0.05$ **$P<0.001$); $^{\#}$significantly different from the 8 nmol dose of Thr$^6$-BK, and + significantly different from the 4 nmol dose of Thr$^6$-BK. AUC, area under the curve of antinociception; IA, index of antinociception; Thr$^6$-BK, threonine$^6$-bradykinin.

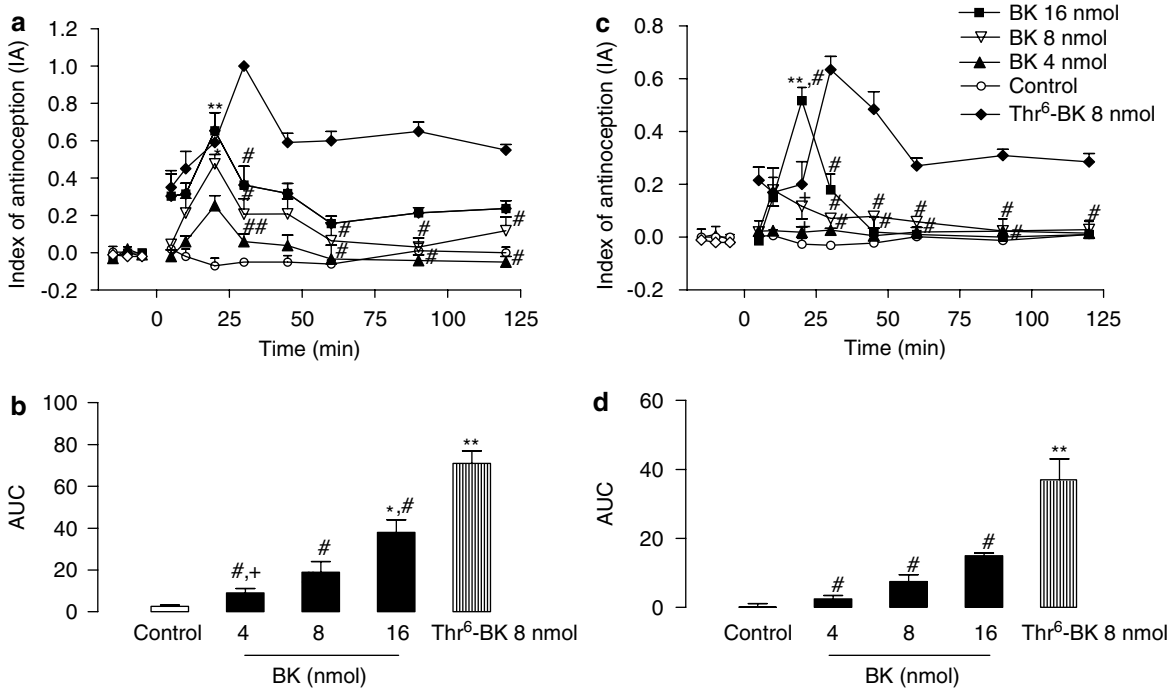


**Figure 4** Antinociceptive effect of i.c.v. injections of Thr$^6$-BK or BK. (**a**) Time course of the increase in latencies in the tail flick test induced by i.c.v. injection of Thr$^6$-BK (8 nmol) and BK (4, 8 and 16 nmol). (**b**) AUC of the groups shown in (**a**). (**c**) Time course of the increase in latencies in the hot-plate test induced by i.c.v. injection of Thr$^6$-BK (8 nmol) and BK (4, 8 and 16 nmol). (**d**) AUC of the groups shown in C. *Significantly different from control (*$P<0.05$ **$P<0.001$); $^{\#}$significantly different from 8 nmol Thr$^6$-BK, and + significantly different from 16 nmol BK. AUC, area under the curve of antinociception; IA, index of antinociception; Thr$^6$-BK, threonine$^6$-bradykinin.

occurred at 20 min, when no differences were observed between BK and Thr$^6$-BK treatments (Figure 4a).

Similar results were obtained in the hot-plate test. Significant effects of treatment ($F(4,23) = 8.78$; $P < 0.001$), time ($F(7,28) = 5.95$; $P < 0.0001$), and treatment-versus-time interaction ($F(28,73) = 7.74$; $P < 0.001$) were observed. One-way ANOVA indicated significant treatment effect from 20 to 120 min ($F(4,23)$ varying from 3.27 to 6.19; $P < 0.05$). BK presented a peak of activity at 20 min when this treatment was significantly different from Thr$^6$-BK ($P < 0.001$) (Figure 4c). The values of $ED_{50}$ and confidence intervals of the antinociceptive effect of BK in tail flick and hot-plate tests were 7.25 nmol (1.86–12.35) and 15.90 nmol (13.7–30.50), respectively.

When comparing the AUCs calculated for BK and Thr$^6$-BK, a significant antinociceptive effect was observed for Thr$^6$-BK (8 nmol) in relation to all doses of BK (4, 8 and 16 nmol) in the tail flick test ($F(4,22) = 16.39$; $P < 0.0001$) (Figure 4b), as well as the hot-plate test ($F(4,23) = 21.39$; $P < 0.0001$) (Figure 4d).

The potency of Thr$^6$-BK was also measured in relation to morphine, both administered i.c.v. In the tail flick test, significant effects of treatment ($F(4,18) = 31.56$; $P < 0.0001$), time ($F(9,12) = 10,18$; $P < 0.0001$), and treatment-versus-time interaction ($F(28,126) = 3.86$; $P < 0.0001$) were observed. One-way ANOVA showed a significant effect for treatments from 5 to 120 min ($F(4,18)$ varying from 2.01 to 8.11; $P < 0.05$). *Post hoc* analysis showed that the withdrawal latencies induced by Thr$^6$-BK at the dose of 8 nmol were significantly greater than morphine (12 nmol) at 30 min ($P < 0.05$) and greater than morphine (6 nmol) at 5–45 min and at 90 and 120 min after treatment (Figure 5a).

Analysis of the AUC results from the tail flick data showed statistical differences between the effects of Thr$^6$-BK and the lowest dose of morphine. The antinociceptive effects induced by morphine measured in this way were statistically greater than those of control for all highest doses given ($F(4,17) = 28.41$; $P < 0.001$; Figure 5b).

Analysis of the hot-plate latencies induced by Thr$^6$-BK and morphine showed significant effects of treatment ($F(4,22) = 9.94$; $P < 0.0001$), time ($F(7,19) = 11.79$; $P < 0.001$), and treatment-versus-time interaction ($F(28,73) = 4,27$; $P < 0.0001$). One-way ANOVAs showed a significant treatment effect from 20 to 60 min ($F(4,22)$ varying from 2.84 to 5.9; $P < 0.05$). *Post hoc* analysis revealed that the administration of the lowest dose of morphine did not increase latencies, compared to the saline-injected control. Moreover, there were significant differences between the latencies of animals treated with Thr$^6$-BK and the animals treated with morphine (6 nmol) at 30, 45 and 60 min after injection. The peak of morphine activity was at 45 min after treatment and this (24 nmol) drug was more effective than Thr$^6$-BK at 60 min (Figure 5c).

Figure 5d shows the AUC values for the hot-plate test results. In this set of experiments, Thr$^6$-BK induced a significantly greater increase in latencies than did morphine

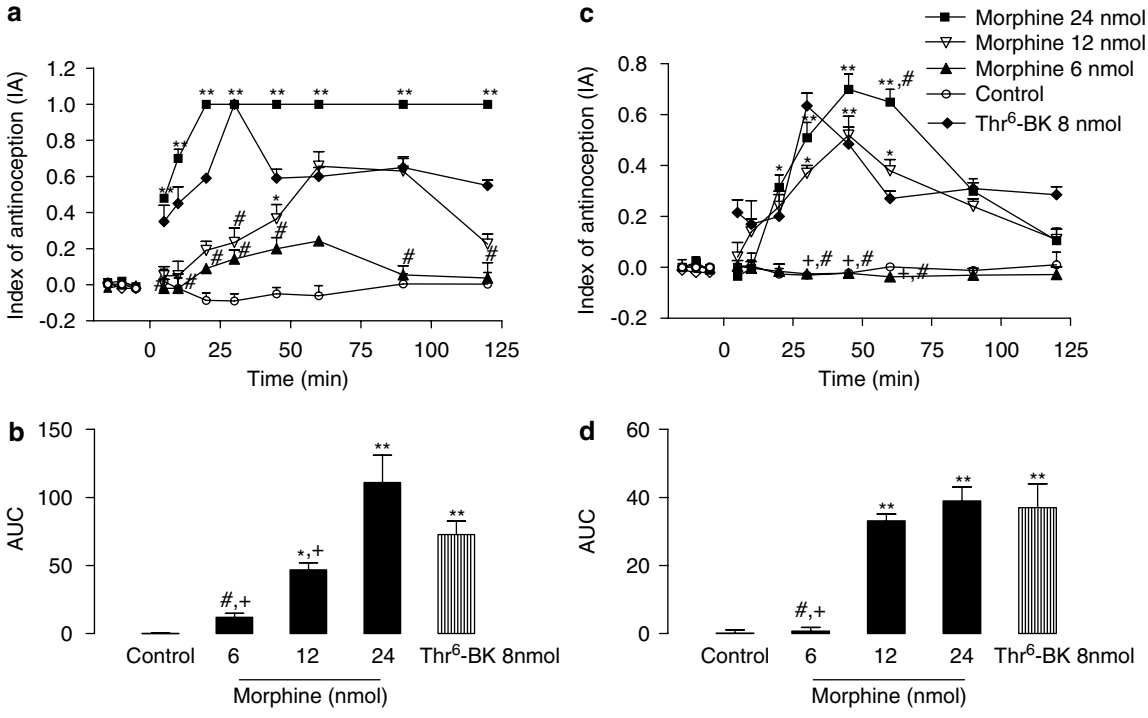


**Figure 5** Antinociceptive effect of i.c.v. injections of Thr$^6$-BK or morphine. (**a**) Time course of the increase in latencies in the tail flick test induced by i.c.v. injection of Thr$^6$-BK (8 nmol) and morphine (6, 12 and 24 nmol). (**b**) AUC of the groups shown in A. (**c**) Time course of the increase in latencies in the hot-plate test induced by i.c.v. injection of Thr$^6$-BK (8 nmol) and morphine (6, 12 and 24 nmol). (**d**) AUC of the groups shown in (**c**). *Significantly different from control (*$P < 0.05$ **$P < 0.001$); $^{\#}$significantly different from 8 nmol Thr$^6$-BK; and + significantly different from 24 nmol morphine. AUC, area under the curve of antinociception; IA, index of antinociception; Thr$^6$-BK, threonine$^6$-bradykinin.

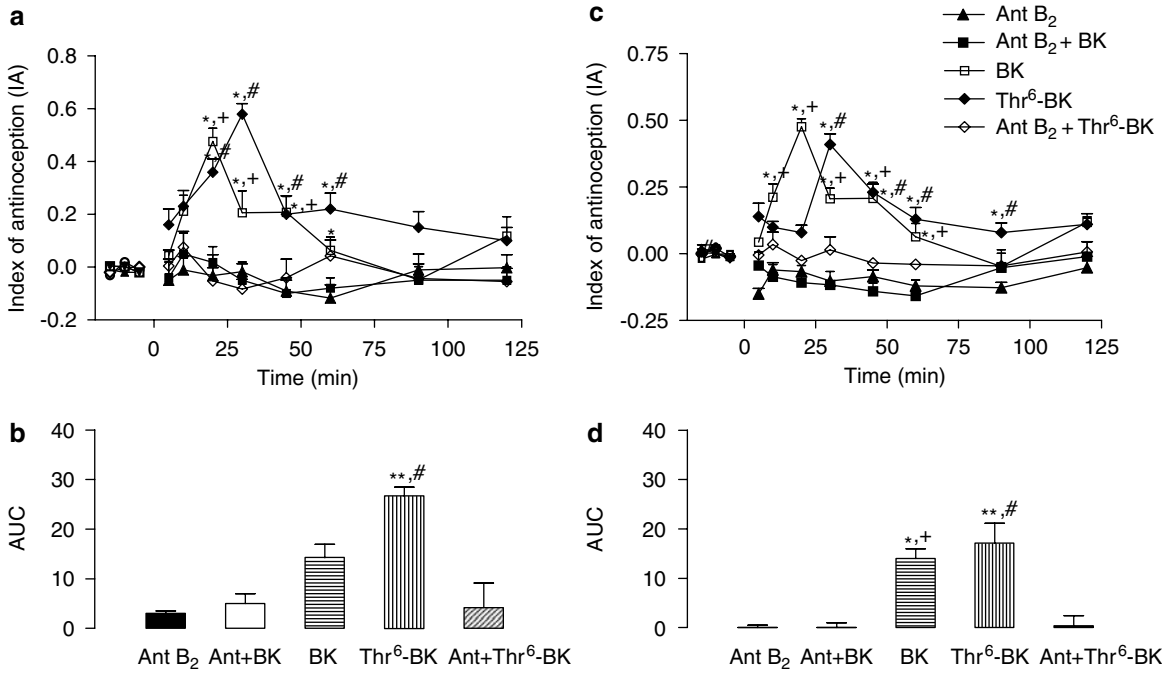


**Figure 6** Effect of concomitant i.c.v. injection of a selective antagonist of $B_2$ receptors and Thr$^6$-BK or BK on antinociceptive actions of the kinins. (**a**) Time course of the latencies in the tail flick test induced by concomitant i.c.v. injection of $B_2$-receptor antagonist (Ant $B_2$) and Thr$^6$-BK (4 nmol) or $B_2$-receptor antagonist and BK (8 nmol). (**b**) AUC of the groups shown in A. (**c**) Time course of the increase in latencies in the hot-plate test induced by concomitant i.c.v. injection of $B_2$-receptor antagonist and Thr$^6$-BK (4 nmol) or $B_2$-receptor antagonist and BK (8 nmol). (**d**) AUC of the groups shown in C. *Significantly different from $B_2$-receptor antagonist alone (*$P<0.05$ **$P<0.001$); $^{\#}$significantly different from the $B_2$-receptor antagonist and Thr$^6$-BK (4 nmol); and + significantly different from the $B_2$-receptor antagonist and BK (8 nmol). AUC, area under the curve of antinociception; IA, index of antinociception; Thr$^6$-BK, threonine$^6$-bradykinin.

only when compared to the lowest dose of morphine (6 nmol; $P<0.05$). In addition, morphine treatment at all highest doses produced AUC values significantly different from those for the control group ($F(4,21)=12.70$; $P<0.0001$). The $ED_{50}$ values and confidence intervals for the antinociceptive effect induced by morphine in tail flick and hot-plate tests were 7.8 nmol (5.1–12.03) and 13.35 nmol (7.05–24.5), respectively.

Finally, the curves as well as the $ED_{50}$ calculated for all highest treatments were compared. In this case, significant differences were reported for Thr$^6$-BK in comparison to both, morphine and BK (tail flick – $F(2,56)=21.61$, $P<0.00001$; hot plate – $F(2,64)=5.44$, $P<0.0066$). However, no differences were observed between dose-effect curves described for morphine and BK. Therefore, the absolute values of $ED_{50}$ indicate that Thr$^6$-BK was 2.65- and 2.1-fold more potent than morphine and 2.5- and 3.36-fold more potent than BK in the tail flick and in the hot-plate tests, respectively.

Analysis of the experiments using the selective $B_2$-receptor antagonist in the antinociceptive effect of Thr$^6$-BK in the tail flick test showed significant effects of treatment ($F(4,18)=25.89$; $P<0.0001$), time ($F(9,10)=5.76$; $P<0.006$), and treatment-versus-time interaction ($F(36,38)=2.74$; $P<0.003$). One-way ANOVA showed a significant treatment effect from 20 to 60 min ($F(4,18)$ varying from 5.10 to 15.10; $P<0.02$). *Post hoc* analysis showed there was significant antagonism of the antinociceptive effects of the injection of Thr$^6$-BK alone when the $B_2$-receptor antagonist was added to Thr$^6$-BK, up to 45 min. At 60 min, some loss of antagonism was seen. The $B_2$-receptor antagonist also abolished the antinociceptive effects of BK over the same period (Figure 6a).

The simultaneous injection of the $B_2$-selective antagonist and Thr$^6$-BK or BK completely inhibited the antinociceptive effects of both drugs at all times of the hot-plate test. Significant effects of treatment ($F(4,22)=11.13$; $P<0.0001$), time ($F(9,14)=2.41$; $P<0.05$), and treatment-versus-time interaction ($F(36,50)=2.01$; $P<0.01$). One-way ANOVAs showed a significant treatment effect from 10 to 90 min ($F(4,22)$ varying from 4.91 to 12.00; $P<0.007$). *Post hoc* analysis showed significant differences between the treatments of $B_2$-receptor antagonist plus Thr$^6$-BK and Thr$^6$-BK; and $B_2$-receptor antagonist plus BK and BK alone (Figure 6c).

Figure 6b and d show the AUCs after concomitant microinjections of $B_2$-receptor antagonist and Thr$^6$-BK or BK for both tests. In this case, i.c.v. injection of $B_2$-receptor antagonist reduced the AUC induced by BK and Thr$^6$-BK in hot-plate test ($F(4,22)=5.11$; $P<0.05$) and tail flick test ($F(4,18)=6.21$; $P<0.01$).

## Discussion

In this study, we isolated and fully sequenced a BK analogue extracted from the venom of the social wasp *P. occidentalis occidentalis,* Thr$^6$-BK. This kinin is the most active kinin isolated from the venoms of other social wasps (Watanabe *et al*., 1976; Nakajima *et al*., 1984). Our data demonstrated

that Thr$^6$-BK exerts remarkable antinociceptive effects when injected directly in the rat CNS, in both tail-flick and hot-plate tests. This is the first time that neurokinins isolated from arthropod venoms have been assayed in the mammalian CNS. However, the oedematogenic effects of Thr$^6$-BK injected into the rat paw have been reported by Griesbacher *et al* (1998). These authors compared the inflammatory responses to Thr$^6$-BK with those to BK and observed a difference in potency for the peptides (Thr$^6$-BK > BK). Both pain and inflammation are provoked by BK injected peripherally due to the stimulation of afferent nerve terminals (Armstrong *et al*., 1952 in Öztürk, 2001).

The neurokinin Thr$^6$-BK is a small linear peptide with nine amino-acid residues; Arg-Pro-Pro-Gly-Phe-**Thr**-Pro-Phe-Arg-OH, highly homologous to the endogenous mammalian peptide, BK, except for its secondary structure (Pellegrini *et al*., 1997). This structural difference is a result of one amino-acid residue substitution in position 6, where there is serine for BK and threonine for Thr$^6$-BK. Indeed, this substitution offers a more stable molecular conformation, as it occurs in a region that defines the $\beta$-turn at the C terminus. Moreover, this modification in the region of the $\beta$-turn might also determine a high receptor affinity and thus, a greater potency for Thr$^6$-BK than that observed for BK in *in vitro* investigations (Pellegrini and Mierke, 1997).

Our results demonstrated that this difference in potency between Thr$^6$-BK and BK was also observed in these two models of thermally induced nociception. Indeed Thr$^6$-BK was even more potent than morphine, which is widely used in the clinical treatment of pain. Furthermore, the effects of this molecule on antinociception were dose- and time-dependent, although it differed in both onset and duration of activity, compared to BK and morphine.

In our assays, Thr$^6$-BK remained active longer than BK, a finding that could be a result of a more stable conformation of its secondary structure. This in turn, probably protects Thr$^6$-BK from hydrolysis by neuronal kininases, preserving the effects of the peptide on $B_2$ receptors (Pellegrini and Mierke, 1997) and thus increasing its effectiveness.

Investigations focusing the neural effects of BK and analogues are particularly important in pain research, since BK is thought to play a role in central pain transmission (Millan, 1999). The antinociceptive effects of BK have been reported elsewhere (Laneuville and Couture, 1987; Laneuville *et al*., 1989; Pelá *et al*., 1996; Couto *et al*., 1998) and demonstrated to be independent of other physiological variables, including blood pressure (Couto *et al*., 1998).

BK exerts its action through two subtypes of G protein-coupled receptors. Intact BK is the characteristic agonist for the $B_2$ receptor, whereas kinin metabolites, such as Lys-des-Arg$^9$-BK or des-Arg$^9$-BK, produced by neuronal endopeptidase action, activate the $B_1$ receptor, which is far less expressed in normal tissues (Calixto *et al*., 2000; Ongali *et al*., 2003; Leeb-Lundberg *et al*., 2005). In this regard, Pelá *et al*. (1996) demonstrated that $B_2$-receptor agonists exerted antinociceptive effects even after the administration of $B_1$-receptor antagonists. These authors attributed the observed antinociceptive effect to the activation of descending adrenergic pathways, which are stimulated by presynaptic $B_2$ receptors (Laneuville *et al*., 1989; Couto *et al*., 1998).

In summary, our findings indicate that i.c.v. injections of Thr$^6$-BK exert a potent antinociceptive effect with potency approximately two-fold higher than BK and morphine. Moreover, this effect was fully inhibited by the $B_2$-receptor selective antagonist D-Arg$^0$, indicating that Thr$^6$-BK acts on $B_2$ receptors. The actions of BK, as well as its analogues, such as Thr$^6$-BK on the CNS deserve to be better investigated to provide novel alternatives in the study and treatment of pain-related disorders.

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## Conflict of interest

The authors state no conflict of interest.

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