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Bradykinin-related peptides in the venom of the solitary wasp *Cyphononyx fulvognathus*

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ABSTRACT

Bradykinin (BK) and its related peptides are widely distributed in venomous animals, including wasps. In fact, we have previously purified a novel BK-related peptide (BRP) named *Cd*-146 and the threonine^6-bradykinin (Thr^6-BK) from the venom of the solitary wasp *Cyphononyx fulvognathus*. Further survey of this same wasp venom extract allowed the structural characterization of two other novel BRPs, named here as fulvonin and cyphokinin. Biochemical characterization performed here showed that although the high primary structure similarity observed with BK, these wasp peptides are not good substrates for angiotensin I-converting enzyme (ACE) acting more likely as inhibitors of this enzyme. In pharmacological assays, only those more structurally similar to BK, namely cyphokinin and Thr^6-BK, were able to promote the contraction of guinea-pig ileum smooth muscle preparations, which was completely blocked by the B_2 receptors antagonist HOE-140 in the same way as observed for BK. Only fulvonin was shown to potentiate BK-elicited smooth muscle contraction. Moreover, the 2 new wasp BRPs, namely fulvonin and cyphokinin, as well as *Cd*-146 and Thr^6-BK, showed hyperalgesic effect in the rat paw pressure test after intraplantar injection. This effect was shown here to be due to the action of these peptides on BK receptors, since the hyperalgesia induced by both *Cd*-146 and fulvonin was blocked by B_1 receptor antagonist, while the effect of both cyphokinin and Thr^6-BK was reversed by B_2 antagonist. This data give support to a better understanding of the function and targets of the kinin-related peptides widely found in several insect venoms.

1. Introduction

Bradykinin (BK) [RPPGFSPFR] and its related peptides are widely distributed in venomous animals such as frogs and wasps. In the case of wasp, the first component found in social wasp venom was a kinin-like or BK-related peptide (BRP) that was described as a pain-producing substance [1]. Since then, many peptides of this class have been found in social wasp venoms, which are collectively known as wasp kinins [2].

BRPs have also been found in solitary wasp venoms as neurotoxins. The venoms of two scoliid wasps, *Megascolia flavifrons* and *Colpa interrupta*, contain both threonine^6-bradykinin (Thr^6-BK) and megascoliakinin, which act presynaptically blocking the nicotinic acetylcholine receptors in the insect central nervous system [3–5].

Abbreviations: BK, bradykinin; ACE, angiotensin I-converting enzyme; Thr^6-BK, threonine^6-bradykinin; FRET, fluorescence resonance energy transfer; Fmoc, *N*-9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; CPY, carboxypeptidase Y; APM, aminopeptidase M; CPB, carboxypeptidase B; CID, collision-induced dissociation; PSD, post-source decay; HOE-140, icatibant.

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In particular, the Thr^6-BK is one of the most widely distributed kinin-related peptide [6]. It has been isolated and characterized from different species, including frogs, alligators, turtle, snake, and mainly from insects, as wasp and ant [3–5,7–15]. However, up to now, little is known about the Thr^6-BK pharmacological activity and its mechanism of action in mammals, with some few reports of paw oedema and nociceptive behavioural responses after local (intraplantar) injection in unanaesthetized rats [12], and a central anti-nociceptive effects after intracerebroventricular injection to rats [15].

Our survey of solitary wasp venoms allowed identifying Thr^6-BK peptide in four species of wasp inhabiting Japan [16]. Of these, the venom of *Cyphononyx fulvognathus* (formerly known as Cyphononyx *dorsalis* [17]) contained a novel type of BRP, e.g., *Cd*-146 [SETGNTVTVKGFSPLR], besides the well-known Thr^6-BK [RPPGFTPFR] [18]. In addition, three new proteins were further identified in this venom: an arginine kinase-like protein that was highly homologous to that of honeybee, an elastase-like protein that was homologous to that of fire ant, and an unknown protein that was not homologous to any protein in the database [19]. In the present work, we further surveyed the extract of this same wasp venom and found two other completely novel BRPs, named fulvonin [SIVLRGKAPFR] and cyphokinin [DTRPPGFTPFR]. Herein we report the chemical, biochemical and pharmacological characterization of these new peptides found in the *C. fulvognathus* venom besides the known *Cd*-146 and Thr^6-BK peptides.

Interestingly, we verified that, in fact, those peptides showing the highest structural similarity to BK, which were the case of Thr^6-BK and cyphokinin, were able to contract smooth muscle preparation, while the other two peptides studied here, namely fulvonin and *Cd*-146, could not. On the other hand, all these peptides were able to inhibit ACE as well as to induce the hyperalgesic effect in living rats after intraplantar injection. The use of specific BK-receptors antagonists also allowed the identification of BK-receptors as the target of these wasp peptides.

2. Materials and methods

2.1. Drugs

Rabbit lung angiotensin I-converting enzyme (ACE), carboxypeptidase B (CPB), captopril, bradykinin, BPP-5a and BK-receptor antagonists [Lys-(des-Arg^9, Leu^8)-BK and HOE-140] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). α-Cyano-4-hydroxycinnamic acid was purchased from Aldrich Chemical Company Inc. (Milwaukee, WIS, USA). Carboxypeptidase Y (CPY) was obtained from Applied Biosystems (Framingham, MA, USA), while Sequazyme kit and aminopeptidase M (APM) were from Boehringer-Mannheim (Indianapolis, IN, USA). HPLC grade acetonitrile (AcN), trifluoroacetic acid (TFA) and all other chemical reagents were from Nacalai Tesque (Kyoto, Japan). The internally quenched fluorescent substrate Abz-FRK(2,4-dinitrophenol)P-OH was kindly provided by Dr. Adriana Carmona (Department of Biophysics, Universidade Federal de São Paulo - UNIFESP, São Paulo, Brazil).

2.2. Animals

Male guinea pigs (150–250 g body weight) and Wistar rats (170–190 g body weight), which were bred in the animal care facility of the Butantan Institute (São Paulo, SP, Brazil) were used throughout this study. Animals were housed in a temperature-(21 ± 2 °C) and light-controlled (12/12 h light/dark cycle) room and were allowed to have water and food *ad libitum*. All behavioural tests were performed between 9:00 a.m. and 4:00 p.m. All animals were caged and handled under ethical conditions according to international rules of animal care, stated by the International Animal Welfare Recommendations, and in accordance with the Guidelines for the Use of Animals in Biochemical Research [20]. All *in vivo* experiments were in accordance with the Guidelines for the Ethical Use of Conscious Animals in Pain Research published by the International Association for the Study of Pain [21] and were approved by the Institutional Animal Care Committee of the Butantan Institute (CEUAIB, protocol number 532/2008).

2.3. Purification

Two *C. fulvognathus* female wasps were collected in Kyoto (Japan), and immediately frozen in dry ice and stored at −75 °C. The venom sacs were dissected from the freshly thawed wasp abdomens, lyophilized and kept at −20 °C until use.

The lyophilized venom sacs were extracted (5×0.5 ml) with 1:1 AcN–water solution containing 0.1% TFA (AcN/H_2O/0.1% TFA) and the extracts were subjected to reverse-phase HPLC (Shimadzu Corp., Kyoto, Japan) using CAPCELL PAK C_{18}, 10 mm × 250 mm (Shiseido Co., Ltd., Tokyo, Japan) with a linear gradient from 5 to 65% of AcN/H_2O/0.1% TFA, at a flow rate of 2.5 ml/min, over 30 min. The observed peaks were manually collected and further submitted to the identification of the primary structure of the peptide.

2.4. Mass spectrometry

All mass spectra were acquired on a Voyager Elite MALDI-TOF MS spectrometer (Applied Biosystems, Framingham, MA, USA), equipped with a delayed extraction system and 337 nm pulsed nitrogen laser. The accelerating voltage was 20 kV. Argon gas was used as the collision gas for the CID/PSD experiment. A matrix, a-cyano-4-hydroxycinnamic acid was prepared at a concentration of 10 mg/ml in 1:1 AcN/0.1%TFA. The sample (0.5 μl) spotted onto the MALDI sample plate was mixed with the matrix (0.5 μl) and allowed to dry at room temperature.

2.5. Ladder sequencing

On-plate exopeptidase digestion was used for peptide ladder sequencing. The C- and N-terminals were sequenced using CPY (in 30 mM ammonium citrate buffer pH 6.0) and APM (in 10 mM Tris–HCl buffer pH 7.5, 2.5 μg/μl), respectively. The sample (0.5 μl) was mixed with the enzyme solution (0.5 μl) on the MALDI sample plate at room temperature. To compensate evaporation during digestion process, 0.5 μl of water was added to the solution. After 7 min, 0.5 μl of matrix was added and dried at room temperature.

2.6. Amino-acid sequencing

The amino-acid sequence of the peptides was analyzed by a gas-phase sequencer PPSQ-10 (Shimadzu Corp., Kyoto, Japan) based on automated Edman degradation.

2.7. Peptides synthesis

Peptides were synthesized on an automated PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan) by stepwise solid-phase method using *N*-9-fluorenylmethoxycarbonyl (Fmoc) chemistry. All the resins and Fmoc-L-amino acids were purchased from Nova Biochem (UK). Cleavage of the peptide from the resin was achieved by treatment with a mixture of TFA/1,2-ethanedithiol/ethyl methyl sulfide, at room temperature for 2 h. After removal of the resin by filtration and washing twice with TFA, the combined filtrate was added, dropwise, to diethyl ether at 0 °C, and then centrifuged at 3000 rpm for 10 min. Then, the crude synthetic

peptide was purified by preparative reverse-phase HPLC using YMC-Pack ODS, 20 mm × 150 mm (YMC Co. Ltd., Kyoto, Japan), with isocratic elution of 18–24% of AcN/H_2O/0.1% TFA, at a flow rate of 7 ml/min. Both the homogeneity and the sequence of each synthetic peptide were confirmed by analytical HPLC and MALDI-TOF MS.

2.8. Enzyme activity assay and determination of kinetic parameters

The ACE enzymatic activity was measured using the internally quenched fluorescent substrate Abz-FRK(2,4-dinitrophenol)P-OH [22], in 0.1 M Tris–HCl pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$ buffer, at 37 °C, in a spectrofluorometer RF-5301-PC (Shimadzu, Tokyo, Japan). The relative inhibition constants (K_i) were determined using the fluorogenic substrates described above in a concentration equal to the previously determined K_m, with increasing concentrations of the analyzed peptides as competitive inhibitors. To determine the K_i values, five solutions with nonfluorogenic synthetic peptides concentrations ranging from 0.1 to 100 μM were used to construct the graph (V_0/V_i) versus [I] (Fig. 2A, C, E, and G). In the (V_0/V_i) − 1 versus [I] plot, the slope represents $1/K_{i,app}$. The following equations were used to calculate the K_i values: $K_i = K_{i,app}/(1 + [S]/K_m)$, where [$S$] = molar concentration of the substrate, K_m = Michaelis–Menten constant, where V_0 = velocity of hydrolysis without the inhibitor, V_i = velocity of hydrolysis in the presence of the inhibitor, and [I] = molar inhibitor concentration.

The relative hydrolysis ratio was determined using peptides at a concentration of 50 μM, under zero-order kinetics, with less than 10% of the substrate consumed by the end of the incubation period, which varied from 10 min to 2 h. The enzyme concentration varied from 0.5 to 50 nM/assay. All assays were performed in triplicate. The enzymatic activities were normalized by protein concentration determined by the Bradford assay [23], using bovine serum albumin (BSA) as standard.

2.9. HPLC analysis and determination of the cleavage sites

Analytical HPLC was performed using a binary HPLC system Shimadzu 10A class-vp with a UV–Vis detector (220 and 365 nm) and a fluorescence detector (λ_{Ex} 280 nm and λ_{Ex} 350 nm), coupled to a C18 column (5 μm, 4.6 mm × 150 mm), which was eluted with the solvent systems A (TFA 0.1%) and B (water:AcN = 1:9; with TFA 0.1%), at a flow rate of 1 ml/min and a 10–80% gradient of B over 20 min.

2.10. BK-potentiation on isolated guinea-pig ileum

The BK-potentiation assays on isolated guinea-pig ileum were performed essentially as previously described [24]. Briefly, male guinea pigs, fasted for 24 h before experiments, were used. Segments of about 15 cm of the terminal ileum were removed, cleaned from surrounding tissues and the lumens were carefully washed with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.04 mM D-glucose), containing diphenyldramine (1 mg/l) and atropine (1 mg/l). After a resting period of 30 min, segments of 1–4.5 cm of the isolated ileum were mounted in a 3 or 5 ml chamber containing continuously aerated Tyrode's solution at 37 °C. Isometric contractions were recorded by means of isometric transducers coupled to a recording system (PowerLab/4SP, AD Instruments) under a load of 1.0 g. Concentration–response curves were obtained for BK and synthetic wasp peptides (Thr^6-BK and cyphokinin), in absence (control) or presence of captopril [15 and 50 nM] [25], and were fitted through a non-linear regression using the curve-fitting program GraphPad PRISM 4.0 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean percent values of maximum contraction achieved in control curve ± S.E.M.

To evaluate the influence of the wasp venom on BK response, single incubation of the BK EC_{50} concentration [3×10^{-8} M] was repeated several times until stabilization of the contractile response, when the wasp peptides or BPP-5a [0.4 μM each] were added and pre-incubated for 5 min. After this period, BK EC_{50} concentration was repeated and the contraction elicited by BK in the presence of each of these synthetic peptides was determined.

The contractile responses to the EC_{50} concentration of the wasp peptides cyphokinin and Thr^6-BK, or to BK, used as control, were individually recorded after single bath application. After observing the same contractile response to each of these wasp peptides or BK, the B_2 BK-receptor antagonist HOE-140 [1 μM] [26,27] was incubated for 10–15 min, before a new addition of the same concentration of the evaluated peptide, for instance, cyphokinin, Thr^6-BK or BK.

2.11. Nociceptive threshold evaluation

Nociceptive threshold was evaluated using an Ugo Basile® pressure apparatus, essentially as described by Randall and Selitto [28]. Briefly, a force (in g), with increasing magnitude (16 g/s), was applied to the paw. When the animals reacted by withdrawing the paw, the force needed to induce this response was considered the nociceptive threshold. To reduce stress, the rats were habituated to the apparatus one day before the experiments. The test was performed before and after the intraplantar injection of the peptides *Cd*-146, fulvonin, cyphokinin and Thr^6-BK or sterile saline (0.85% NaCl solution, 100 μl/paw), used as control. The nociceptive threshold after the intraplantar injections was evaluated at different times up to 6 h.

2.12. In vivo pharmacological treatments

Results obtained in preliminary studies determining a dose–response curve of the peptide *Cd*-146 (data not shown) demonstrated that the concentration of 10 μg/paw [5.9 pmol] of this peptide reduces the nociceptive threshold of the treated rats and, because of this, it was chosen to evaluate the hyperalgesic effect induced by all other wasp peptides. The peptides *Cd*-146, fulvonin, cyphokinin, and Thr^6-BK [5.9 pmol] were dissolved in sterile saline and injected by the subcutaneous route (s.c.) in the rat plantar region, in a total volume of 100 μl. The hyperalgesic activity was evaluated before and 30, 60, 120, 240 and 360 min after the treatments with the wasp peptides.

Aiming to verify the participation of BK receptors on the hyperalgesic effect induced by these wasp peptides, B_1 and B_2 receptors antagonists, namely Lys-(des-Arg^9, Leu^8)-BK (50 ng/50 μl) and icatibant (HOE-140, 5 ng/50 μl), respectively, were injected by intraplantar (s.c.) route, 20 min before the peptides administration. The nociceptive threshold of the animals was evaluated 60 min after *Cd*-146, fulvonin or cyphokinin [5.9 pmol] injection, or 10 min after Thr^6-BK [5.9 pmol] injection, that corresponds to the peak of hyperalgesic effect for each wasp peptides. It is worth to mention that the concentration of BK-receptors antagonists used here was enough to block the hyperalgesia induced by BK peptide (500 ng/50 μl, intraplantar route), employed here as control, in a dose known to cause hyperalgesia [29].

2.13. Presentation of data and statistical analysis

One-way analysis of variance (ANOVA) followed by Newman–Keuls test were performed to determine the significance of differences of the EC_{50} values obtained for BK-potentiation assays

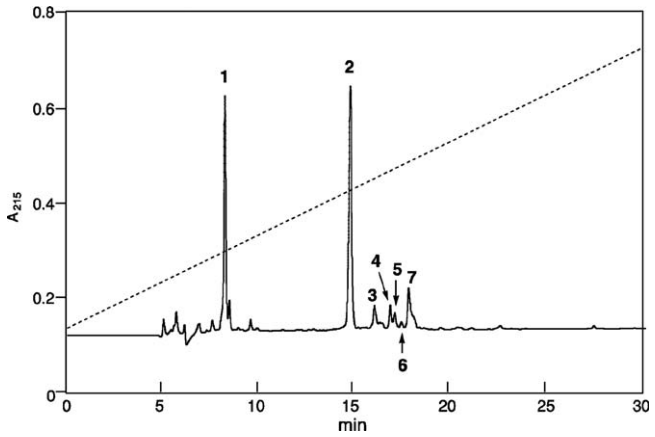

Fig. 1. HPLC profile of the crude extracts from the venom of *Cyphononyx fulvognathus*. The crude venom extracts were subjected to reverse-phase HPLC using CAPCELL PAK C_{18} (10 mm × 250 mm) with linear gradient of 5–65% CH_3CN/H_2O/0.1% TFA, over 30 min, at flow rate of 2.5 ml/min. UV absorption was monitored at 215 nm. (1) Adenosine; (2) *Cd*-125 [DTARLQWH]; (3) fulvonin; (4) *Cd*-146; (5) cyphokinin; (6) Thr^6-BK; (7) not determined yet.

on isolated guinea-pig ileum. The significance level was considered at $p < 0.05$. Results for pain threshold evaluation are presented as the mean ± S.E.M. Statistical evaluations of the data for hyperalgesia were carried out also using ANOVA, and sequential differences among mean values were evaluated according to Tukey's contrast analysis at $p < 0.05$ [30].

3. Results

3.1. Purification and chemical characterization of wasp venom peptides

The venom extracts of *C. fulvognathus* wasp were subjected to reverse-phase HPLC (Fig. 1), and the purity and complexity of each fraction was examined by MALDI-TOF MS. We previously purified two BK-related peptides (BRPs), named *Cd*-146 [SETGNTVTVKGFSPLR] and threonine^6-bradykinin (Thr^6-BK, [RPPGFTPFR]), from the fractions eluted at 17 and 17.7 min, respectively ([18] and also indicated in Fig. 1). *Cd*-146 seemed to be a new type of BRP, since only the 6 residues of the peptide C-terminal showed sequence similarity to BK ([18] and Table 1). However its biological evaluation remained to be investigated. On the other hand, Thr^6-BK has been found in many other wasp venoms [2–5].

Further survey of these HPLC fractions led to the characterization of two other new BRPs, named fulvonin and cyphokinin. The compound fulvonin was obtained from the fraction eluted at 16 min, showing high purity by MALDI-TOF MS with a monoisotopic protonated molecular ion peak at m/z 1243.8 [$(M + H)^+$]. By ladder sequencing [31], a partial amino-acid sequence of the C-terminal region of this peptide was revealed as being [-P-F-R] by carboxypeptidase Y (CPY)-digestion, while aminopeptidase M (APM)-digestion gave the sequence [S-I/L-V-I/L-R] for the N-terminal region. Edman degradation yielded a 10 amino-acids sequence as [SIVLRGKGPF]. Therefore, the whole sequence of fulvonin was concluded to be [SIVLRGKGPFR] with the theoretical $[M + H]^+$ = 1243.8, which was consistent with the observed value. Moreover, this sequence was also finally confirmed by the solid-phase synthesis of a peptide with the same sequence as deduced, followed by its analysis by MALDI-TOF MS, which gave a final molecular ion peak data that corroborated to the proposed sequence. This peptide is clearly a new type of BRP, since only the C-terminal half of this peptide is homologous to BK, and no description of similar sequence could be found in the accessible databanks.

Another novel peptide with a MS peak at m/z 1290.6 $(M + H)^+$, named cyphokinin, was obtained from the fraction eluted at 17.5 min. By ladder sequencing, the C-terminal amino acid was determined as [-R] by CPY-digestion, and the sequence [D-T-], at the N-terminal region, was obtained by APM digestion. To sequence the unidentified portion, the collision-induced dissociation/post-source decay (CID/PSD) spectrum of the APM digested peptide ($[M + H]^+$ = 1074.5) was measured. It was found that this spectrum was consistent with that of intact Thr^6-BK. These results indicated that the sequence of cyphokinin was [DTRPPGFTPFR] (theoretical $[M + H]^+$ = 1290.7), which was unambiguously confirmed by solid-phase synthesis of identical sequence peptide followed by MALDI-TOF MS analysis. Up to our knowledge, this peptide has never been described previously, and only two other wasp kinins including the whole sequence of Thr^6-BK inside its sequence have so far been reported ([2,14] and Table 1). Even showing that similarity to already known kinins (Table 1), to our point of view, it was not that obvious simply to expect the same biochemical and pharmacological activities, as described for BK, for these newly identified wasp peptides. Therefore, the biological activities of these four wasp BRPs described by the group were investigated here by using their synthetic analogs.

Table 1
Primary sequence and molecular weight of the studied wasp peptides, and hydrolysis of the peptides by ACE.

Peptides	MW	Sequence	Percentage of Hydrolysis (%) ACE
Bradykinin[a]	1060.2	RPPGFSP↓FR	100
Cd-146	1692.9	SETGNTVTVKGFSP↓LR	6.7
Fulvonin	1243.5	SIVLRGKAP↓FR	7.5
Cyphokinin	1290.5	DTRPPGFTP↓FR	27.3
Thr^6-BK	1074.3	RPPGFTP↓FR	22.7

[a] BK was totally cleaved by ACE in 1 h; (↓) cleavage point by ACE.

3.2. Effect of synthetic BK-like wasp peptides on the activity of angiotensin I-converting enzyme

Considering the observed high structural similarity of wasp peptides characterized in this work with BK (Table 1), we have enzymatically assayed them with somatic ACE to verify if they are substrate and/or inhibitors of this enzyme.

After incubation of 50 μM of each wasp peptide with somatic ACE (50 nM/assay) in 0.1 M Tris–HCl pH 7.0, containing 0.05 M NaCl and 10 μM $ZnCl_2$ (0.35–2.0 ml final volume) buffer, at 37 °C, overnight, the integrity of these peptides as well as their putative metabolites were monitored by analytical HPLC and MS analysis of each observed peak, for determination of cleavage site(s). After 18 h of incubation with ACE, all peptides were very slowly hydrolyzed, if compared to BK hydrolysis rate after 1 h incubation (Table 1). The wasp peptides were thus evaluated as competitive inhibitors of ACE using the FRET-based fluorescent substrate Abz-FRK(2,4-dinitrophenol)P-OH. Peptides fulvonin, cyphokinin and Thr^6-BK presented high affinities for ACE with inhibition constants (K_i) around 1–2 μM. The only exception was the *Cd*-146 peptide, which presented a lower affinity with a K_i about 60-fold higher than that observed for other wasp peptides (Fig. 2 and Table 2).

3.3. Activity of synthetic BK-like wasp peptides on isolated smooth muscle preparation

The inhibitory activity of these peptides on ACE enzymatic activity stimulated us to evaluate their action on the contraction of isolated smooth muscle preparations. In this assay, it was possible

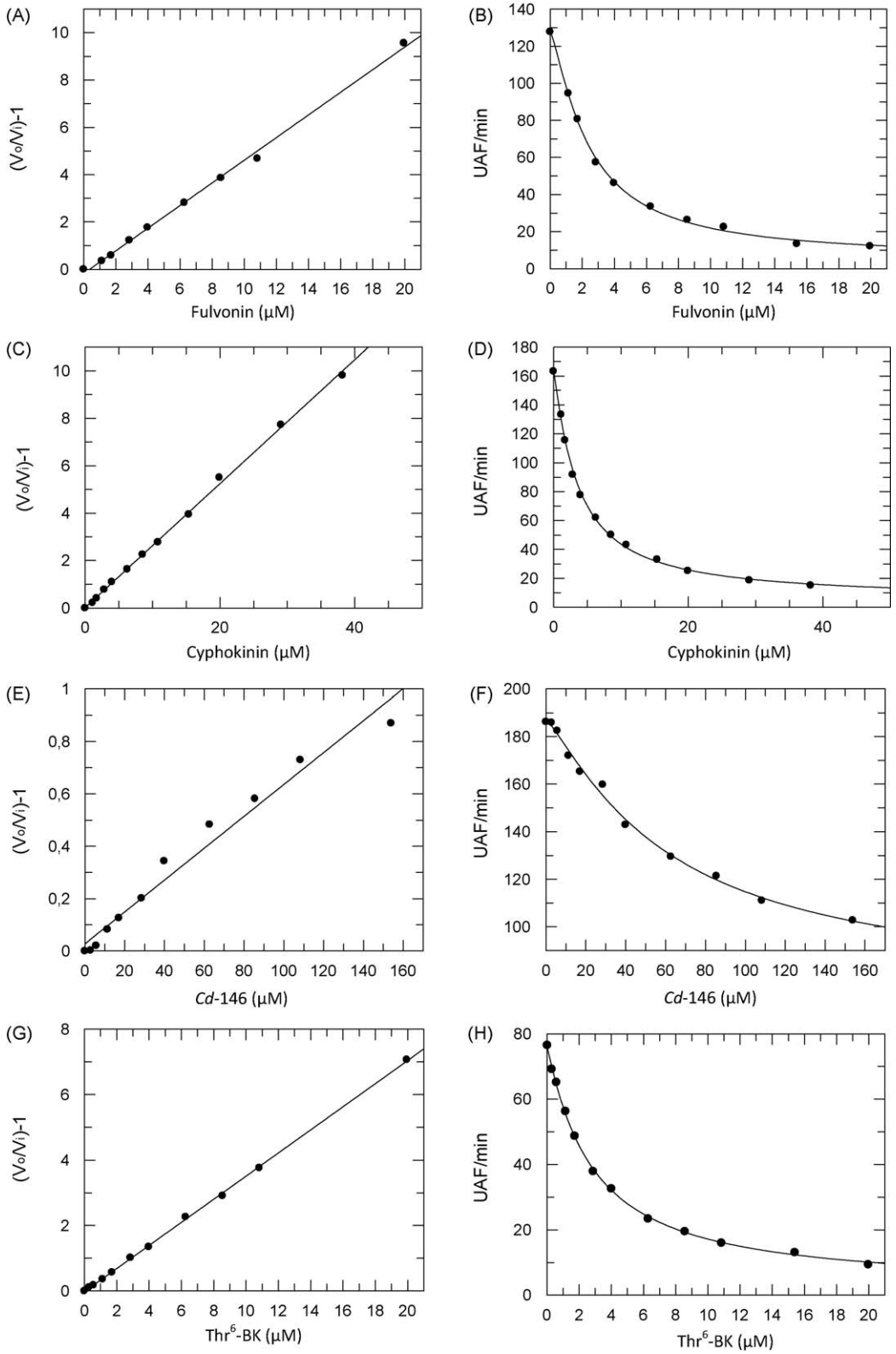

Fig. 2. Residual activity of somatic ACE in the presence of wasp peptides. The panels on left side show the linear fit of the log of the residual activity versus incubation time, and the panels on the right side show the points corresponding to a single exponential decay. Panels A and B, fulvonin [SIVLRGKAPFR]; C and D, cyphokinin [DTRPPGFTPFR]; E and F, *Cd*-146 [SETGNTVTVKGFSPLR]; G and H, Thr^6-BK [RPPGRTPFR]. The wasp peptides were evaluated as inhibitors of ACE using the FRET-based fluorescent substrate Abz-FRK(2,4-dinitrophenol)P-OH in 50 mM HEPES buffer pH 6.8, containing 200 mM NaCl and 10 mM $ZnCl_2$, followed by monitoring the fluorescence at λ_{Em} = 420 nm and λ_{Ex} = 320 nm in a Hitachi F-2000 spectrofluorometer, as describe in Section 2.

Table 2
Kinetic parameters for inhibition of somatic ACE.

Peptides	K_i (μM)
Cd-146	61.8
Fulvonin	1.2
Cyphokinin	2.2
Thr^6-BK	1.6

to observe that only the wasp peptides cyphokinin and Thr^6-BK induced concentration-dependent contractions of the guinea-pig ileum, mimicking the BK elicited concentration–response curve (Fig. 3), typically observed for BK in this preparation. The other two peptides, namely fulvonin and *Cd*-146, did not induce any direct contractile activity on this assay, even with concentrations up to 10^{-5} M (data not shown). The EC_{50} for the cyphokinin and Thr^6-BK peptides were 352.5 ± 64.7 and 26.7 ± 7.7 nM, respectively, while the EC_{50} for BK was shown to be 24.9 ± 4.6 nM (Fig. 3). As expected, only the BK curve was shifted to the left by the addition of captopril (15 and 50 nM) (supplemental material, S1A). The EC_{50} for the wasp peptides, namely cyphokinin and Thr^6-BK, on smooth muscle contraction was not affected by the presence of the same concentrations of captopril, although a decrease of the maximum contractions was observed for both wasp peptides (supplemental material, S1B and S1C). As cyphokinin and Thr^6-BK showed agonistic activity, they were also assayed in the presence of a competitive antagonist of B_2 BK-receptor (HOE-140). At the concentration used here [1 μM], pre-incubation with HOE-140 totally blocked the contraction elicited by cyphokinin and Thr^6-BK, and also by BK, used here as control (data not shown).

Considering that these wasp peptides were shown to be more likely competitive inhibitors of ACE than substrates of this enzyme, this same smooth muscle preparation was used to evaluate the ability of the wasp peptides to potentiate the contraction elicited by BK, as described for a number of BK-potentiating peptides (BPPs) [32,33]. After pre-incubation of the guinea-pig ileum with 0.4 μM of each synthetic wasp peptide with no agonist activity, namely *Cd*-146 and fulvonin, the contraction elicited by BK was significantly potentiated only in the presence of fulvonin or in the presence of the pit viper peptide BPP-5a, used as a positive control

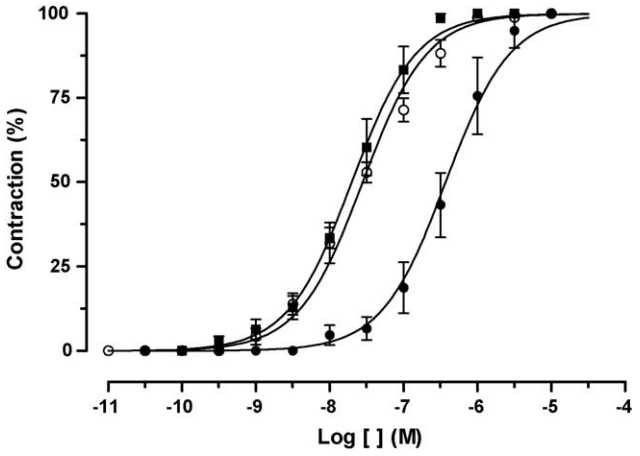

Fig. 3. Effect of wasp peptides on isolated guinea-pig ileum. Concentration–response curves to BK and synthetic wasp peptides. The calculated EC_{50} values were 24.9 ± 4.6 nM for BK [RPPGFSPFR] = ○; 352.5 ± 64.7 nM for cyphokinin [DTRPPGFTPFR] = ●; and 26.7 ± 7.7 nM for Thr^6-BK [RPPGRTPFR] = ■. Each point represents a mean value of 9 independent experiments, while the vertical lines correspond to the standard error of the mean (mean ± S.E.M., n = 9). The responses are expressed as percentages of the maximum response obtained in the first curve for BK or for the wasp peptides.

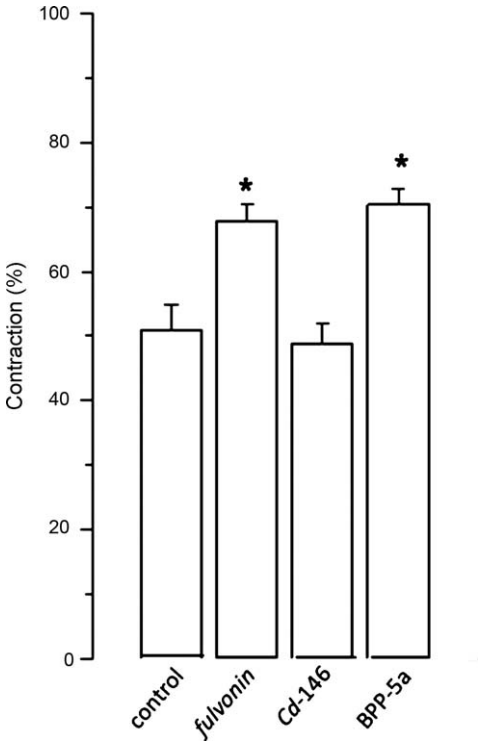

Fig. 4. BK-potentiating effects of wasp peptides on smooth muscle contraction. Graphical representation of the contraction values in percentage (mean ± S.E.M.) induced by the EC_{50} concentration of BK (3×10^{-8} M), in the presence of the wasp (fulvonin and *Cd*-146) and snake (BPP-5a) peptides. The control represents the contraction induced for BK EC_{50} concentration. The (*) indicates significant difference from the control ($p < 0.05$).

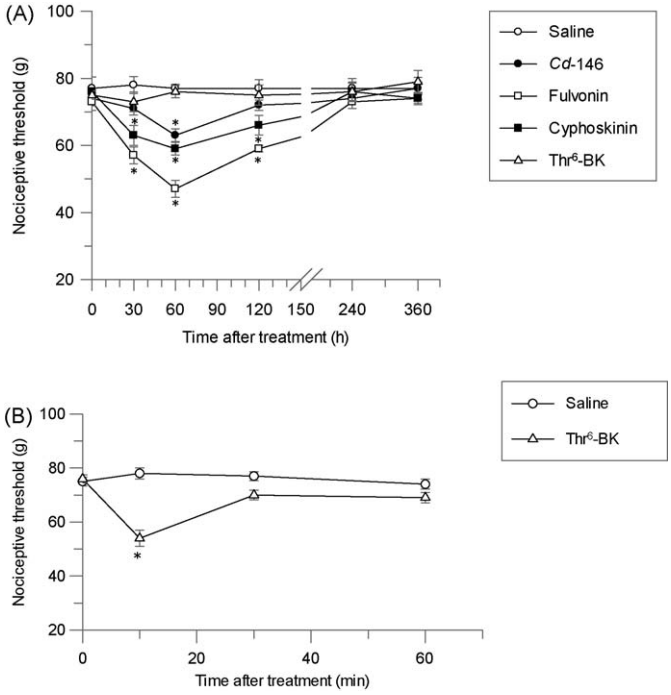

Fig. 5. Evaluation of hyperalgesia induced by *Cd*-146, fulvonin, cyphokinin and Thr^6-BK peptides. Nociceptive threshold, expressed in grams (g), was estimated in the rat paw pressure test applied before (time 0) and after (30, 60, 120, 240 and 360 min) peptides injection (Panel A) or before (time 0) and after (10, 30 and 60 min) Thr^6-BK injection (Panel B). Peptides *Cd*-146 (●), fulvonin (□), cyphokinin (■) and Thr^6-BK (△) [5.9 pmol] or saline (control) were injected by intraplantar (s.c.) route. Data represent mean values ± S.E.M. for 5 rats per group. *Significantly different from mean values obtained before treatment (time 0).

(Fig. 4). The wasp peptide *Cd*-146 did not cause any significant interference on BK contraction effect.

3.4. Characterization of the effect of BK-like wasp peptides on the nociceptive threshold

To evaluate the time-course of the wasp peptides effect on the nociceptive threshold of rats, the animals were submitted to the nociception evaluation before and after intraplantar injection of 5.9 pmol of *Cd*-146, fulvonin, cyphokinin and Thr^6-BK peptides. The obtained data demonstrated that the peptides *Cd*-146, fulvonin and cyphokinin decrease the nociceptive threshold of the experimental animals, characterizing the phenomena of hyperalgesia, with the maximum effect observed 60 min after each peptide administration (Fig. 5A). The highest and longest lasting hyperalgesic effect was observed for the fulvonin wasp peptide (Fig. 5A). At this condition, no significant effect was observed for the peptide Thr^6-BK (Fig. 5A).

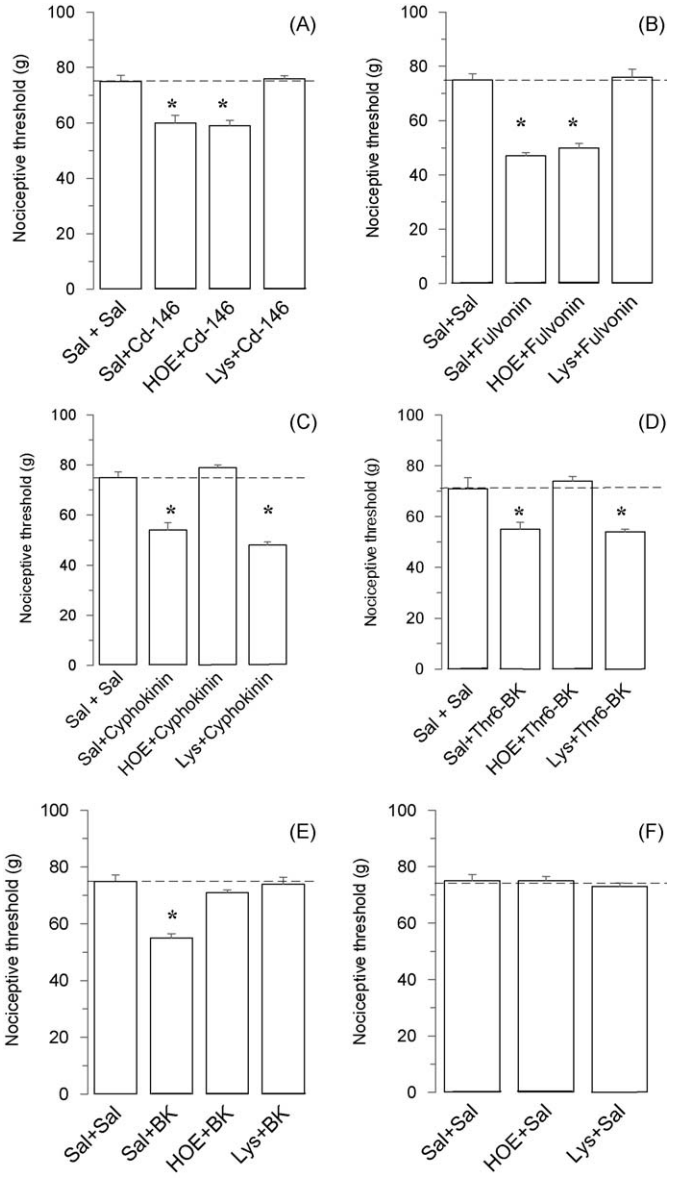

Fig. 6. Effect of BK antagonists on *Cd*-146, fulvonin, cyphokinin and Thr^6-BK peptides-induced hyperalgesia. Nociceptive threshold was estimated in the rat paw pressure test applied before (dash line) and 60 min after *Cd*-146 (5.9 pmol, Panel A), fulvonin (5.9 pmol, Panel B) and cyphokinin (5.9 pmol, Panel C) peptides or BK (500 ng, Panel E), used as a positive control, or before (Initial Measure) and 10 min after (Final Measure) Thr^6-BK (5.9 pmol, Panel D). Lys-(Des-Arg^9,Leu^8)-BK (Lys, 50 ng/50 μl) and icatibant (HOE-140, 5 μg/50 μl), antagonists of B_1 and B_2 receptors, respectively, were injected by intraplantar route 20 min before peptides administration. The effect of antagonists, *per se*, is shown on Panel F. Data represent mean values ± S.E.M. for 5 rats per group. *Significantly different from mean values obtained in the control group (Sal + Sal).

However, it had been demonstrated in the literature that Thr^6-BK peptide induces a painful behaviour after peripheral injection, but this effect was observed much earlier than the period of time employed in our first evaluation [12]. Then, the peripheral hyperalgesic effect induced by Thr^6-BK was re-evaluated as early as 10 min after its administration. At this new condition, it was observed that Thr^6-BK peptide induces a hyperalgesic effect that was no longer observed after 10 min, which could explain the absence of effect observed previously after a 60 min interval (Fig. 5B).

3.5. Evaluation of the effects of BK-receptors antagonists on hyperalgesia induced by the wasp peptides

The hyperalgesic effect induced by the wasp peptides *Cd*-146 and fulvonin was blocked by a B_1 BK-receptors antagonist (Fig. 6A and B), while the hyperalgesic effect of cyphokinin and Thr^6-BK was blocked by the B_2 receptor antagonist (Fig. 6C and D). It is important to point out that the antagonist concentrations used here were able to block the hyperalgesia induced by BK (Fig. 6E), which was used as a control, and at the same time the antagonist did not cause any effect *per se* on the nociceptive threshold of the rats (Fig. 6F).

4. Discussion

In this study, we have purified and chemically characterized two novel BK-related peptides (BRPs) from the venom of the solitary wasp *C. fulvognathus* collected in Japan. The primary structure of these peptides were determined by MALDI-TOF MS and ladder sequencing, followed by further confirmation by chemical synthesis of peptides with identical sequences, which were also analyzed by HPLC and by MALDI-TOF MS. These synthetically obtained wasp peptides were then biochemically and pharmacologically characterized, as well as the two other BRPs previously isolated from this same wasp venom [18].

BK and its related peptides are widely distributed in venomous animals, for example, frogs and insects. In most cases, they include a wide variation of the primary sequence or only a single amino-acid substitution compared to the sequence of BK. In fact, these types of sequence modifications have been widely reported for the wasp kinins, e.g., the BRPs found in wasp venoms [2–5]. Accordingly, cyphokinin is a typical wasp kinin; that is, it is highly homologous to Thr^6-BK with only 2 extra amino-acid residues elongating this peptide at its N-terminus. On the other hand, *Cd*-146 and fulvonin show a lesser similarity to BK sequence. *Cd*-146 has a kinin-like sequence at the C-terminus (6 residues), while fulvonin has only 4 residues identical to BK (Table 1). As far as we know, no other peptide showing these features have been found or reported before, and therefore they are structurally a new type of wasp kinin. This stimulated us to study the biochemical and pharmacological features of these novel wasp peptides.

In our *in vitro* biochemical assays, these peptides were significantly less susceptible to hydrolysis by ACE than BK (Table 1), specially the peptides *Cd*-146 and fulvonin that show lesser similarity to BK sequence than the cyphokinin and Thr^6-BK. But, as fulvonin presented high affinity for this peptidase, it might suggest that this peptide act more likely as a good inhibitor of this enzyme (Table 2 and Fig. 2A and B). The subtle amino-acid changes near the hydrolyzed peptide bond by ACE (P_3, P_2 and P_1') at the wasp peptides, as shown in Table 1, is not enough to explain such

differences observed in the hydrolyses rates and in the affinities for ACE compared to BK [34–36]. These differences observed in the kinetic assays with ACE may result probably from the distinct nature of ACE-peptide substrate interactions more distant from the hydrolyzed peptide bond. In pharmacological assays, it was shown that only cyphokinin and Thr^6-BK exhibited a concentration-dependent contraction of smooth muscle in guinea-pig ileum preparation. The potency of cyphokinin was lower (around 10 times) than that observed for BK and Thr^6-BK, with a EC_{50} of 352.5 ± 64.7 nM for cyphokinin, and 26.7 ± 7.7 and 24.9 ± 4.6 nM for Thr^6-BK and BK, respectively (Fig. 3). Moreover, as expected, only the BK curve was shifted to the left by the addition of captopril, while the EC_{50} for the wasp peptides, namely cyphokinin and Thr^6-BK, was not affected by the presence of the same concentrations of captopril (supplemental material S1). This is in good agreement to the fact that only BK is a good substrate of ACE, while the wasp peptides were shown to be more likely inhibitors of ACE than substrate. However, remarkably, a significative dose-dependent decrease of the maximum contraction was observed for both wasp peptides at these used concentrations of captopril (supplemental material S1), suggesting a possible non-competitive effect. It is also of note that the smooth muscle contraction elicited by these two wasp peptides was completely blocked by the B_2 receptor antagonist HOE-140 (data not shown), suggesting the involvement of this BK-receptor subtype on this mechanism of action of these wasp peptides.

Taking into account the inhibitory effect of some of the wasp peptides on somatic ACE activity, the effect of the wasp peptides on the BK-potentiation assay in the guinea-pig ileum preparations was evaluated. At least under the concentrations and conditions employed here, only the wasp peptide fulvonin potentiated the contraction elicited by the BK EC_{50} concentration (Fig. 4) in the isolated smooth muscle preparation, indicating a clear difference in their pharmacological features regardless of their structural similarities. This data also suggest that these wasp BRPs deserve a better characterization of their structure–activity relationship in the future.

The most prominent acute symptoms caused by wasp venoms are the formation of a localized cutaneous oedema and pain, and these symptoms can be observed even in higher vertebrates, such as man [12,15]. Several different types of compounds have been described in the venom of solitary and social wasps. Among them, peptides with chemical structures and physiological activities similar to BK [16,37,38]. For many years it has been known that BK is an inflammatory mediator involved in the nociceptive process [39], and that BK as well as bradykinin-like peptides produce pain and hyperalgesia due to their ability to excite and/or sensitize nociceptors [40–42]. Then, based on these facts and also considering the high primary structural similarity of these wasp peptides (*Cd*-146, fulvonin, cyphokinin and Thr^6-BK) with BK, we investigated if these four peptides could cause hyperalgesia in mammals contributing to pain observed in the wasp stings.

Our data demonstrated that the 2 new wasp peptides fulvonin and cyphokinin, as well as *Cd*-146 and Thr^6-BK, induced hyperalgesic effect in the rat paw after intraplantar injection. This hyperalgesic effect was due to the action of these peptides on distinct BK receptors, since the hyperalgesia induced by fulvonin and *Cd*-146 was blocked by the B_1 receptor antagonist [Lys-(des-Arg^9, Leu^8)-BK while the hyperalgesia induced by both cyphokinin and Thr^6-BK was blocked by the B_2 receptor antagonist HOE-140.

The B_2 receptors are the best characterized kinin receptors, being largely expressed in a constitutive manner and widely expressed throughout the central and peripheral nervous system, mediating most of physiological effects of kinins [43,44]. Concerning B_1 receptors, although with only a few exceptions they are present under normal conditions (for review see Calixto et al. [45]), their expression occurs rapidly in certain pathological conditions or by the action of proinflammatory agents [44,46–48]. In fact, in most situations, as tissue injury, noxious stimuli or stressful conditions, their synthesis may be upregulated occurring rapid induction of the expression of this receptor [44,45,49,50].

In agreement with our data, a number of works has provided evidences of the early presence of B_1 receptors in many inflammatory conditions. In fact, the involvement of these receptors were observed in the nociception [51], oedema [52] and plasma extravasations [53] at 15, 30 and 120 min, respectively. In addition, the increase of B_1 receptor mRNA expression in rat subcutaneous paw tissue after intraplantar injection of LPS could be evident as early as at 1 h following its injection [54,55].

Interestingly, in our data, the highest and longest lasting hyperalgesic effect was detected for fulvonin, which has the lowest structural similarity to BK, with only 4 amino-acid residues of the hendedecapeptide fulvonin in common to the nonapeptide BK. It is of note to mention that fulvonin was the single wasp peptide assayed here to show the ability to potentiate the effect of BK on isolated smooth muscle preparation.

Our *in vivo* data confirms the results obtained in the pharmacological assays performed in the guinea-pig ileum preparation, where the smooth muscle contraction elicited by cyphokinin and Thr^6-BK peptides was completely blocked by the B_2 receptor antagonist HOE-140 (data not shown). Moreover, our data corroborates a report from Griesbacher et al. [12] demonstrating that the hyperalgesic effect induced by Thr^6-BK is due to a peripheral action, since when centrally injected, this peptide induces anti-nociception [15], and this effect involves the activation of B_2 receptors.

In conclusion, including our previous work [18], our group has described four sequences of wasp BRPs from solitary wasp venom. The pharmacological evaluations revealed that they show distinct biological activities, which always involve the activation of BK-receptors, although not necessarily acting on the same BK-receptors subtypes. The data presented here, indicate clear subtle differences in the pharmacological features of each wasp peptide, regardless of their high structural similarities to BK, and also contribute to a better understanding of the structure–activity relationship of kinins and related peptides, which clearly still deserves a better characterization in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.08.020.

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