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Identification of novel bradykinin-potentiating peptides (BPPs) in the venom gland of a rattlesnake allowed the evaluation of the structure–function relationship of BPPs

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ABSTRACT

Aiming to extend the knowledge about the diversity of bradykinin-potentiating peptides (BPPs) and their precursor proteins, a venom gland cDNA library from the South American rattlesnake (*Crotalus dursissus terrificus*, *Cdt*) was screened. Two novel homologous cDNAs encoding the BPPs precursor protein were cloned. Their sequence contain only one single longer BPP sequence with the typical IPP-tripeptide, and two short potential BPP-like molecules, revealing a unique structural organization. Several peptide sequences structurally similar to the BPPs identified in the precursor protein from *Cdt* and also from others snakes, were chemically synthesized and were bioassayed both *in vitro* and *in vivo*, by means of isolated smooth muscle preparations and by measurements of blood pressure in anaesthetized rats, respectively. We demonstrate here that a pyroglutamyl residue at the N-terminus with a high content of proline residues, even with the presence of a IPP moiety characteristic of typical BPPs, are not enough to determine a bradykinin-potentiating activity to these peptides. Taken together, our results indicate that the characterization of the BPPs precursor proteins and identification of characteristic glutamine residues followed by proline-rich peptide sequences are not enough to predict if these peptides, even with a pyroglutamyl residue at the N-terminus, will present the typical pharmacological activities described for the BPPs.

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Abbreviations: BPPs, Bradykinin-potentianting peptides; ACE, angiotensin I-converting enzyme; Bk, bradykinin; *Bj*, *Bothrops jararaca*; *Cdt*, *Crotalus durissus terrificus*; *Ahb*, *Gloydius blomhoffii* (former *Agkistrodon halys blomhoffii*); *Tg*, *Trimeresurus gramineus*; *Tf*, *Protobothrops flavoviridis* (former *Trimeresurus flavoviridis*); ORF, open reading frame; IPP-tripeptide, Ile-Pro-Pro tripeptide; ISP-tripeptide, Ile-Ser-Pro tripeptide; <E, pyroglutamic acid or pyroglutamate residue

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1. Introduction

Sergio Ferreira was the first to show that the venom of the Brazilian pit viper *Bothrops jararaca* (*Bj*) contained peptides that greatly enhanced the smooth-muscle contraction induced by bradykinin (Bk) [1]. These peptides, named bradykinin-potentiating peptides (BPPs), repress both the degradation of the hypotensive nonapeptide Bk and the generation of the hypertensive peptide angiotensin II, by inhibiting the activity of angiotensin I-converting enzyme (ACE, EC 3.4.15.1) [2,3]. As a whole, the action of the BPPs in the animal results in the decrease of the blood pressure [4,5]. The BPPs were the first natural inhibitors of the ACE described [1,6], and their discovery was essential for the development of the first active-site directed inhibitor of ACE [7,8]. Characteristically, the BPPs consist of 5–14 amino acid residues, with a pyroglutamyl residue at the N-terminus and a proline residue at the C-terminus [1,9]. Larger BPPs share similar features including a high content of proline residues and the tripeptide Ile-Pro-Pro (IPP-tripeptide) at the C-terminus of the peptide [10].

Since their discovery, a number of BPPs have been identified and isolated from the venom of snakes by using protein chemistry techniques [9–14]. Later, with the employment of molecular biology technology, our group was the first to clone a cDNA encoding the BPPs precursor protein [15]. The BPPs precursor protein of *Bj* consists of 265 amino acid residues, with seven BPPs arranged in tandem in its N-terminal segment, and a C-type natriuretic peptide at the C-terminus [15]. Interestingly, Northern blot analysis of distinct tissues of this snake identified a similar size BPPs precursor mRNA transcript in the brain and spleen [15]. More recently, we have cloned and sequenced the cDNA encoding the BPPs precursor from the *Bj* brain, which was found to be very similar, but not identical, to the venom gland transcript [16]. Both, biochemical and pharmacological properties of the BPPs, and their presence within snake brain regions correlated with the neuroendocrine functions, have suggested a physiological role for these peptides, most likely in the regulation of the vascular tonus [16,17].

Although several works describing a number of new cDNAs encoding precursor proteins containing BPP-like sequences have been published in the last few years [18–22], the processing mechanisms involved in the release of the bioactive peptides from these precursor proteins remain unknown. In addition, up to now it has not been possible to recognize any putative processing sites for known proteolytic enzymes in the isolated precursor proteins, which could contribute to the release of BPPs. So forth, the identification of new putative BPP sequences in any new cloned precursor protein has been solely based on the presence of a glutamine residue (‘Q’), which is converted to a pyroglutamic acid (‘<E’) residue by a pyroglutamyl cyclase, followed by a single proline residue up to 4–5 amino acids after (in short peptides) or a high content of proline residues with a typical IPP-tripeptide at the C-terminus (for the longer peptides). In fact, several BPPs have been identified in this way [18–22].

In the present work, we report the cloning and characterization of two cDNAs coding for the BPPs precursor protein from the venom gland of the South American rattlesnake *Crotalus durissus terrificus* (*Cdt*), which belongs to the Crotalinae subfamily. In order to evaluate whether the putative peptides identified in several known precursor proteins possess the structural requirements for the pharmacological activity described for the BPPs, the selected peptide sequences were chemically synthesized and their Bk-potentiating activity were bioassayed. By this strategy, we show here that the presence of a pyroglutamic acid and a proline residue in each extremity of the peptides are not enough to determine the characteristic biological activity of BPPs.

2. Materials and methods

2.1. Materials

Cdt crotamine-plus venom gland cDNA library was the same as reported elsewhere [23]. Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA, USA). Radioactive reagents and Hybond-N nylon filters were from Amersham Biosciences (Buckinghamshire, UK). All BPPs were chemically synthesized by the solid phase method and purified according to Hirata et al. [24], and their sequences were confirmed by mass spectrometry analysis. BK was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. cDNA library screening

The *Cdt* venom gland cDNA library was screened by hybridization using a [alpha-^{32}P] radioactive-labeled DNA probe. The radioactive probe was obtained by the random primer labeling method using the RediPrime^{TM} II Kit (Amersham Biosciences), and the complete cDNA sequence encoding the BPP-CNP precursor (clone NM 96), isolated from *Bj* venom gland [15], as template. Pre-hybridization of the filters was performed for 2 h, at 42 °C, in pre-hybridization solution (6× SSC, 50% formamide, 0.5% SDS, 5× Denhardt’s, and 0.1 mg/ml herring sperm DNA). The hybridization was performed by the addition of the radioactive probe to the pre-hybridization solution, and the incubation conditions were the same as above, except by the incubation time that was extended for 16 h. The filters were washed under high stringent conditions, as follow: three times in 2× SSC and 1% SDS, and three times in 0.1× SSC and 0.1% SDS, at 65 °C. The positive plaques were detected after exposition of the membranes to X-ray films, at −80 °C, for suitable time, in the presence of intensifier screens. Positive clones were collected in SM buffer (10 mM NaCl; 8 mM $MgSO_4$; 50 mM Tris–HCl pH 7.5; 0.01% gelatin), containing 0.3% chloroform. Latter, the recombinant phagemids containing the BPP cDNA precursors were rescued by *in vivo* excision.

2.3. DNA sequencing

The nucleotide sequence was determined by the dideoxy chain-termination method using the BigDye^{TM} Terminator Cycle Sequence Kit and the ABI 3100 automatic system (Applied Biosystems, Foster City, CA, USA).

2.4. Sequence alignments

Alignment of the nucleotide and the deduced amino acid sequences of the *Cdt* BPP–CNP precursors and Asian snakes venom gland precursor proteins were performed using the Clustaw W program, available at http://www2.ebi.ac.uk/clustalw/. The Asian snakes precursor proteins sequences used for the alignment were obtained from Higuchi et al. [18].

2.5. Peptide 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 3–20% CH_3CN/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.

2.6. Mass spectrometric analysis

Molecular mass analyses of synthetic peptides were performed by ESI mass spectrometry on a Q-TOF Ultima API (Micromass, Manchester, UK) under positive ionization mode and/or by MALDI-TOF mass spectrometry on an Ettan MALDI-TOF/Pro system (Amersham Biosciences, Sweden), using alpha-cyano-4-hydroxycinnamic acid as matrix.

2.7. Animals

Experiments were performed using male guinea pigs (150–250 g body weight) and male Wistar rats (230–300 g body weight), which were bred in animal care facility of the Butantan Institute (Sao Paulo, SP, Brazil) and in the Biologic Sciences Institute (CEBIO, Federal University of Minas Gerais, Brazil), respectively. Before setting up the experiments, the animals were kept under 12-h light–12-h dark cycle, and were allowed to have water and food *ad libitum*. 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 [25].

2.8. Bk-potentiation on isolated guinea pig ileum

The Bk-potentiation assays on isolated guinea pig ileum were performed essentially as previously described [15]. Male guinea pigs (150–250 g body weight) were fasted for 24 h before experiments. 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 4.5 cm of the isolated ileum were mounted in a 5 ml chamber containing continuously aerated Tyrode 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 for Bk were obtained in the absence (control) or presence of 3×10^{-6} M of pure peptide. Dose–response curves were fitted through a non-linear regression and pD_2 values ($-\log EC_{50}$) were calculated using the curve-fitting program Graph-Pad PRISM 4.0 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± S.E.M.

2.9. Bk-potentiation on blood pressure in anesthetized rat

The assays of Bk-potentiation on blood pressure was performed essentially as follow [10]: before the experiment, male Wistar rats (250–300 g) were anesthetized with sodium urethane 12% (1.0 ml/100 g weight body intraperitoneal), and a polyethylene catheter (PE-10 connected to a PE-50) was inserted into the abdominal aorta through the femoral artery for blood pressure measurements. For intravenous (i.v.) bolus injections, a polyethylene catheter was implanted into the femoral vein. The cannulas were closed by a metallic pin and filled with isotonic saline solution. Pulsed arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) were continuously monitored by a solid-state strain gauge transducer connected to a computer using a data acquisition system (MP 100; BIOPAC systems, Inc., Santa Barbara, CA). The rats were kept anesthetized during the experiments. After blood pressure stabilization, the BK hypotensive response on blood pressure was standardized using doses of 0.5 and 1.0 μg. For testing and comparisons of the effect of injection of the synthetic peptides, 60 nmol of each peptide were injected in bolus, in each animal, followed by 0.5 μg of Bk injections in 5, 10, 15, 20, 25 and 30 min. The number of animals (n) used for each peptide was **Cdt1a** ($n = 5$), **Cdt1b** ($n = 5$), **Cdt2** ($n = 4$), **Cdt3** ($n = 4$) and **Ahb1** ($n = 5$). As positive control, **BPP-5a** (60 nmol, $n = 4$) was used. All peptides were dissolved in sterile isotonic saline (0.9% NaCl) immediately before use. Statistical comparisons were made using the effect of 0.5 μg of Bk before the drug injection as reference. Data are expressed as the MAP ± S.E.M. of the hypotensive effect (Figs. 4 and 6) and the percentage of Maximal Potentiation (%), which mean the increase of the hypotensive areas obtained by integrating the record drawn with a scanner for a single dose of Bk (0.5 μg), after synthetic BPP infusion, compared to the control areas elicited by single dose of Bk (0.5 μg) injected before the synthetic BPP infusion. In the described conditions, this single dose is in the middle of the linear portion of the log-dose–response curve of Bk and the observed hypotensive effect area was considered as 100%.

For evaluation of possible interference of these BPP-like peptides in the renin-angiotensin system due to inhibition of ACE, the activity of conversion of angiotensin I to angiotensin II by ACE was also evaluated in vivo. The vasopressor

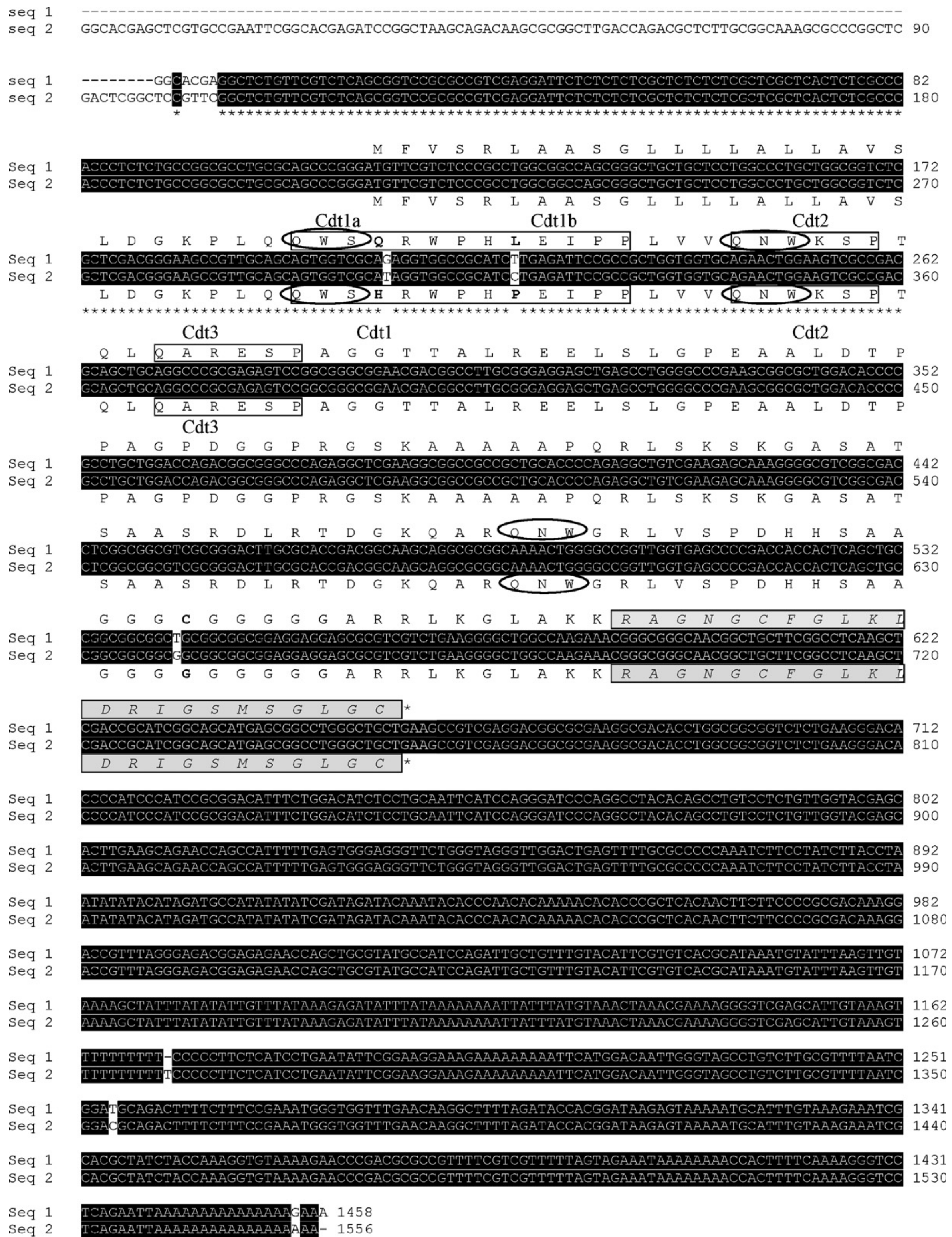

Fig. 1 – Sequence alignment of the BPP-CNP precursor cDNAs isolated from the venom gland of a single rattlesnake *Crotalus durissus terrificus*. Alignment of the nucleotide and the respective deduced amino acid sequences of a BPP-CNP precursor from a crotamine-plus rattlesnake venom gland were performed using the Clustaw W program, available at http://www2.ebi.ac.uk/clustalw/. The synthesized BPP-like sequences are indicated by boxes and the identified in Table 1 with the respective molecular weight. The circles indicate the tripeptide sequences. The CNP sequence is indicated by grey boxes. Insertions or deletions are represented by gaps (-), and boldface type letters indicate the amino acid substitutions. Numberings of base pairs (bp) of the cDNA sequence are indicated in columns at right.

responses to i.v. injections of angiotensin I (300 ng/kg) and/or angiotensin II (100 ng/kg) were obtained at 20 min intervals, before and after each BPP-like peptide (60 nmol) or captopril (0.1 mg/kg) infusion. For statistical comparison between angiotensin I and angiotensin II vasopressor response, before and after BPP infusion, the effects before BPP infusion were considered as 100% of activity.

2.10. Statistical analysis

One-way analysis of variance (ANOVA) followed by Newman–Keuls test were performed to determine significance of differences of the values EC_{50} on the Bk-potentiation assays on isolated guinea pig ileum. The significance level was considered as $p < 0.05$. Statistical analyses of Bk-potentiation and vasopressor response of angiotensin I or II, before and after BPP or captopril infusion, on anaesthetized rat blood pressure were performed with paired Student's *t*-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Sequence analysis of the BPP-CNP precursor cDNAs from Cdt venom gland

Screening of the *Cdt* venom gland cDNA library, allowed the isolation of two novel homologous cDNAs coding for BPPs precursor proteins [GenBank accession no. AF308593 and AF308594], preliminary named as 'seq 1' and 'seq 2' (Fig. 1). Both sequences, of about 1.5 kb in length, show a predicted open reading frame (ORF) of 540 bp, encoding proteins of 180 amino acid residues showing approximately 99% of similarity to each other (Fig. 1). A more detailed comparison of these sequences, allowed us to verify the presence of three point mutations determining a non-synonymous amino acid substitutions in the protein coding segment. In addition, four point mutations were seen in the 5′ and 3′ untranslated regions. A high similarity was also observed for the comparison with the nucleotide and amino acid

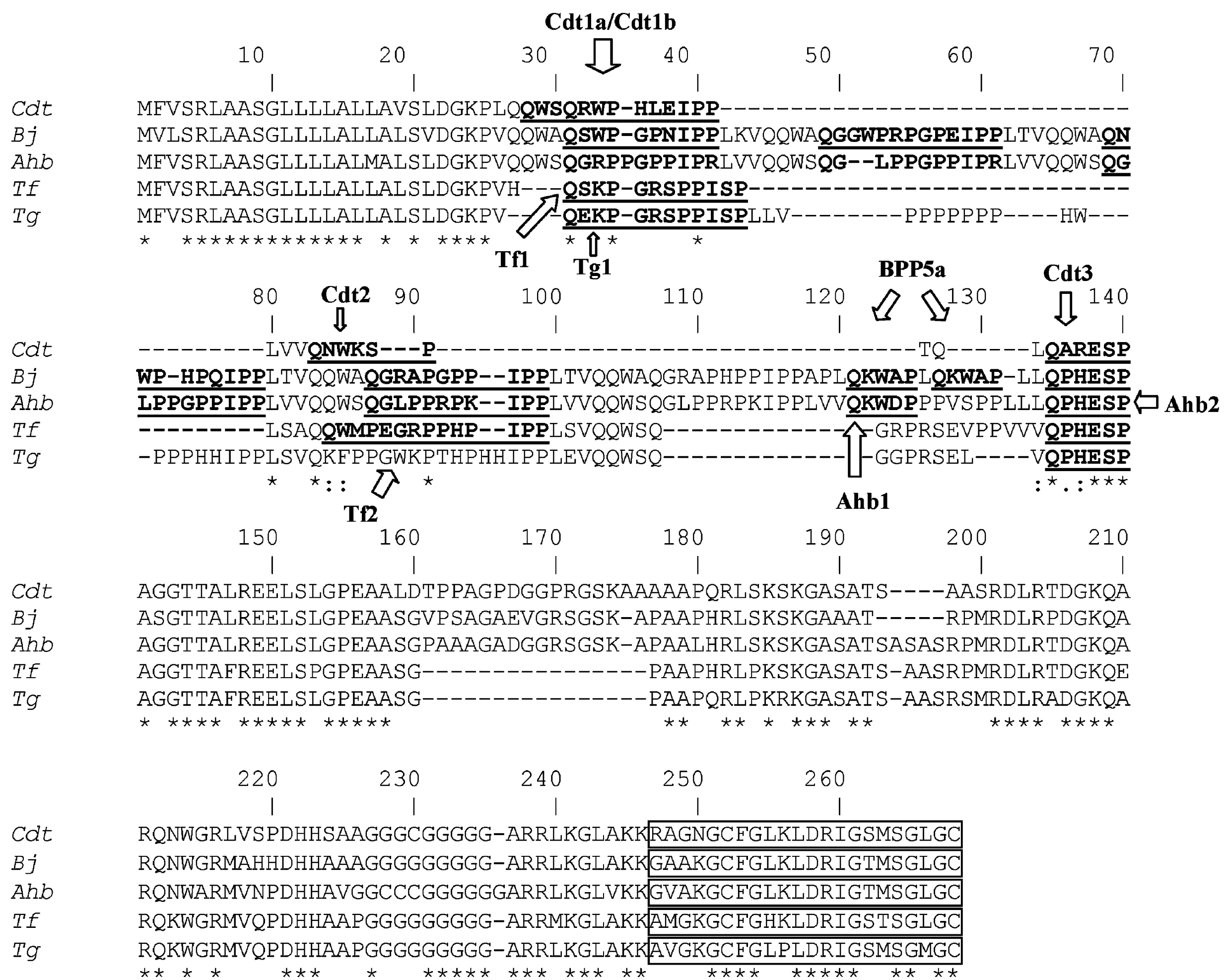

Fig. 2 – Alignment of the deduced amino acid sequences of BPP-CNP precursors from several snakes belonging to the *Crotalinae* subfamily. Peptide sequences displaying BPP-like structural features are indicated in boldface type letters and are underlined. The correspondent synthesized sequences are listed in Table 1. The blomhotin-like peptides are in boldface type letters and the CNP sequences are indicated by boxes. Insertions or deletions are represented by gaps (-), and the asterisks (*) indicate identical residues. *Cdt*: *Crotalus durissus terrificus*, *Bj*: *Bothrops jararaca*; *Ahb*: *Gloydius blomhoffii* (former *Agkistrodon halys blomhoffii*); *Tf*: *Protobothrops flavoviridis* (former *Trimeresurus flavoviridis*); *Tg*: *Trimeresurus gramineus*.

sequences of precursor protein from *Bj* venom gland (about 70 and 60%, respectively) [15]. As observed in other BPPs precursor proteins isolated to date, the rattlesnake BPPs precursor also presents a peptide sequence homologous to the human CNP at its C-terminus (Fig. 2). However, the identified region that corresponds to the BPP domain encompasses only a single copy of a peptide sequence showing the characteristic IPP-tripeptide at the C-terminus. Interestingly, two out of the three non-synonymous substitutions observed in the protein-coding region of the precursor protein were located within the sequence of this single long BPP-like sequence. Therefore, the isoform 'seq 1' contains one single putative BPP of 10 (**Cdt1b**) or 13 (**Cdt1a**) amino acid residues, while the isoform 'seq 2' contains only a single BPP isoform of **Cdt1a** (namely **Cdt1**), of 13 amino acid residues, with the IPP-tripeptide at the C-terminus (Fig. 1).

On the other hand, the alignment of this precursor protein with others precursors from snakes belonging to the subfamily *Crotalinae* [18] led to identify in the BPPs coding domain of these precursor proteins at least two novel small putative peptides, showing a glutamine residue followed by a proline residue interspaced by five amino acid residues, in the *Cdt* precursor protein (Figs. 1 and 2). These peptides, named as **Cdt2** and **Cdt3**, were also chemically synthesized, and their pharmacological activity was evaluated.

3.2. *BPPs precursor proteins alignment and identification of putative new peptides*

Molecular cloning and sequence analysis of cDNAs encoding BPPs precursor or their homologues from several species of Asian snakes belonging to the subfamily *Crotalinae* have been achieved by Higuchi et al. [18]. Sequence analysis has revealed that BPPs has eventually originated novel peptides like Leu3-blomhotin, with an arginine residue at the C-terminus of the bioactive peptides in place of a proline residue, which is usually found at the C-terminus of BPPs [19].

Another distinctive character contained in the BPPs domains of some of these BPPs precursors from Asian snakes is their length. They were found to be shorter, with only one or two BPP-like sequences in each precursor protein (Fig. 2). In the same way, the deduced primary structures of BPP precursor proteins from *Cdt*, characterized in this work, are distinct to the precursors from *Bothrops*, which generally contain several copies of BPPs. So forth, the BPP precursor proteins from *Cdt*, characterized herein, showed shorter BPP domains with only one single BPP sequence containing the typical IPP-tripeptide per precursor molecule (Figs. 1 and 2).

The alignment of this precursor protein with others precursors from snakes belonging to the subfamily *Crotalinae* also allowed identifying sequences of putative peptides showing the typical BPPs features in Asian snakes precursor

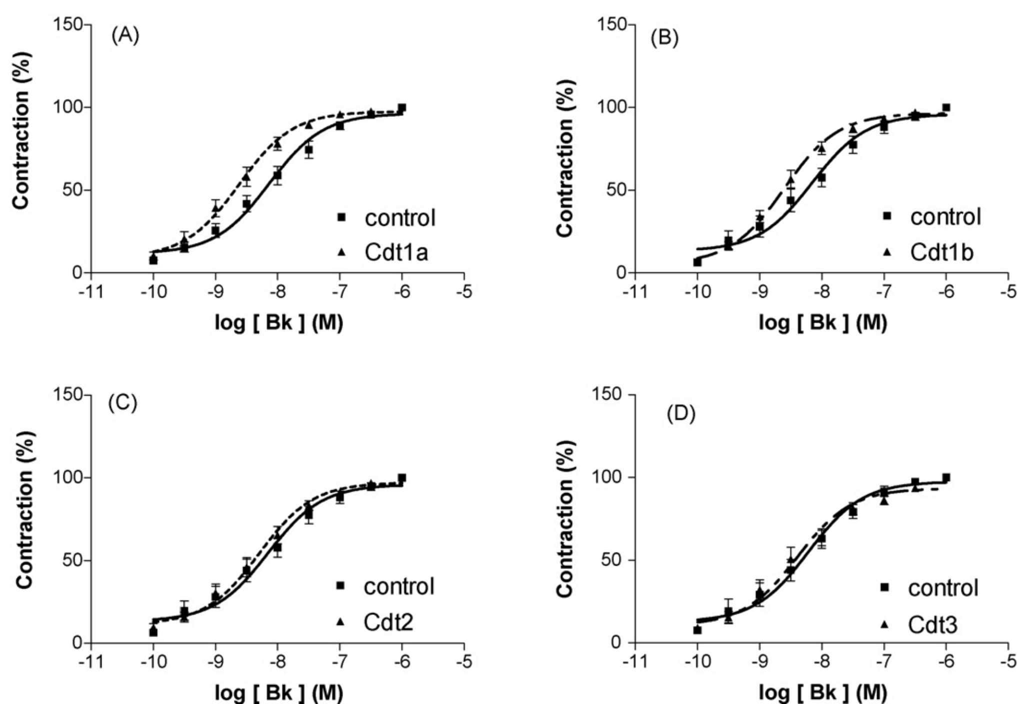

Fig. 3 – Bradykinin potetiation effect of rattlesnake putative BPPs on isolated guinea pig ileum. The putative BPPs identified in the BPP-CNP precursor protein from the rattlesnake *Cdt* were synthesized and were used to generate the potentiation curve (dotted line) that was compared to the control curve obtained using only Bk (continuous line). Panel A, *Cdt1a* [<EWSQRWPHLEIPP]; B, Cdt1b [<ERWPHLEIPP]; C, Cdt2 [<ENWKSP]; D, Cdt3 [<EARESP]. Each point represents a mean value of nine independent experiments, while the vertical lines correspond to the mean ± S.E.M. The responses are expressed as percentages of the maximum response obtained in the first curve for Bk.

Table 1 – Peptide sequences synthesized and evaluated by bioassays

Peptide	Sequence	MW	pD2 ± S.D. (control − Bk)	pD2 ± S.D. (sample)	N	
Cdt1a	<EWSQRWPHLEIPP	1656.87	8.134 ± 0.076	8.619 ± 0.072	9	*
Cdt1b	<ERWPHLEIPP	1255.45	8.153 ± 0.101	8.604 ± 0.058	9	*
Cdt2	<ENWKSP	741.80	8.153 ± 0.101	8.295 ± 0.074	9	–
Cdt3	<EARESP	669.69	8.213 ± 0.099	8.392 ± 0.089	9	–
Ahb1	<EKWDP	655.71	8.323 ± 0.126	8.785 ± 0.141	3	*
Ahb2	<EPHESP	676.69	8.239 ± 0.099	8.456 ± 0.096	4	–
Tf1	<ESKPGRSPPISP	1233.40	8.384 ± 0.167	8.611 ± 0.203	4	–
Tf2	<EWMPEGRPPHPIPP	1621.89	8.542 ± 0.097	8.634 ± 0.085	5	–
Tg1	<EEKPGRSPPISP	1275.43	8.344 ± 0.089	8.492 ± 0.096	5	–
P5	WAQKP	628.73	7.893 ± 0.076	8.021 ± 0.090	4	–

The identification, amino acid sequences, molecular weight and the potency (pD2 values), which is the negative logarithm of the mean value of the concentration of the agonist (Bk) producing a half-maximal response (EC_{50}) in the presence or absence of the sample synthetic peptide, of the synthesized peptides presenting BPP-like features are listed. The *N* is the number of independent assays, and the asterisks (*) indicate those peptides that were statistically able to potentiate the Bk effect on the isolated guinea pig ileum, considering that statistical significance was set at $p < 0.05$.

proteins, in the same position of **Cdt2** and **Cdt3**, namely **Tf2** and **Ahb2**, and also aligned with the *Bj*-**BPP-5a**, namely **Ahb1**, from the *Protobothrops flavoviridis* (former *Trimeresurus flavoviridis*, *Tf*) and *Gloydius blomhoffii* (former *Agkistrodon halys blomhoffii*, *Ahb*) precursor proteins, respectively (Fig. 2). Note that these peptides had not been predicted in the previous work published by Higuchi et al. [18].

Besides those peptides, two other peptides, **Tf1** and **Tg1**, from the *Tf* and *Trimeresurus gramineus* (*Tg*) precursor proteins, respectively, which appear aligned to the **Cdt1** and were suggested to be a BPP [18], despite showing a ISP-tripeptide in the C-terminus instead of the typical IPP-tripeptide, were also synthesized and evaluated in this work.

3.3. *Bradykinin-potentiating activity of synthetic putative BPPs from South American rattlesnake precursor protein*

Since the BPP precursor isoform ‘seq 1’ showed a single long peptide of 10 or 13 amino acid residues with the conserved IPP-tripeptide at the C-terminus, namely, **Cdt1a** [<EWSQRWPHLEIPP] and **Cdt1b** [<ERWPHLEIPP], respectively (Fig. 1), both peptides were synthesized and pharmacologically evaluated. Interestingly, both peptides were able to shift to the left the dose–response curve of Bk on isolated guinea pig ileum with similar potency (Fig. 3 and Table 1). On the other hand, the short peptides identified in the *Cdt* precursor, namely **Cdt2** [<ENWKSP] and **Cdt3** [<EARESP], did not cause any change in this assay (Fig. 3 and Table 1).

Surprisingly, when these peptides were evaluated *in vivo*, not only the two long putative peptides from BPP precursor isoform ‘seq 1’, namely **Cdt1a** [<EWSQRWPHLEIPP] and **Cdt1b** [<ERWPHLEIPP], but also the short hexapeptide, **Cdt3** [<EARESP] were able to potentiate the hypotensive effect mediated by Bk on the blood pressure of anesthetized rats (Fig. 4, Table 2). The maximal potentiation response occurred at 5 min after administration of **Cdt1a** (42.3 ± 5.8%, $n = 5$), **Cdt1b** (64.0 ± 18.0%, $n = 5$), and **Cdt3** (60.3 ± 10.2%, $n = 5$) peptides, but a significant effect was also observed after 10 min only for the **Cdt1a** (Fig. 4, Table 2). In contrast, the administration of **Cdt2** at same dose did not cause any significant change on Bk effect. All these peptides caused transient hypotension on rat blood pressure (**Cdt1a**, −4.6 ± 1.4 mmHg; **Cdt1b**, −6.2 ± 2.6 mmHg; **Cdt2**, −8.8 ± 2.0 mmHg; **Cdt3**, −2.2 ± 0.8 mmHg).

3.4. *Bradykinin-potentiating activity of synthetic putative BPPs from Asian snakes BPPs precursor proteins*

Alignment with BPPs precursor from Asian snakes, belonging to the subfamily *Crotalinae*, allowed the identification of a number of characteristic BPP-like homologues peptide sequences, exactly in the BPP domain of the precursor proteins (Fig. 2). Herein, we chemically synthesized some of these putative peptides, mainly those not presenting the typical IPP-tripeptide at the C-terminus, to evaluate their pharmacological activity *in vitro* using isolated guinea pig ileum (Fig. 2 and Table 1).

Almost all synthetic peptides from Asian snakes venom gland, namely **Ahb2** [<EPHESP], **Tf1** [<ESKPGRSPPISP], **Tf2** [<EWMPEGRPPHPIPP] and **Tg1** [<EEKPGRSPPISP], assayed here did not show significant effect on the smooth muscle assay, with the only exception for the short peptide **Ahb1** [<EKWDP] (Fig. 5 and Table 1).

Interestingly, the pentapeptide **Ahb1** [<EKWDP], which in fact had not been predicted in the previous work published by Higuchi et al. [18], is very similar to the *Bj*-**BPP-5a** [<EKWAP], whose structure was the base for the design of the first active-site directed inhibitor of ACE, namely captopril [7,8].

Table 2 – Percentage of maximal potentiation of Bk hypotensive effect on rat blood pressure after intravenous bolus injection of 60 nmol of each synthetic peptides

Peptide	N	Maximal potentiation (%)	Time (min)
Cdt1a	5	42.3 ± 5.8	5
Cdt1b	5	64.0 ± 18.0	5
Cdt2	4	NS	–
Cdt3	4	60.3 ± 10.2	5
Ahb1	5	44.3 ± 5.4	5
BPP-5a	4	48.3 ± 4.7	10

NS: non significant; *N* is the number of animals used for each peptide.

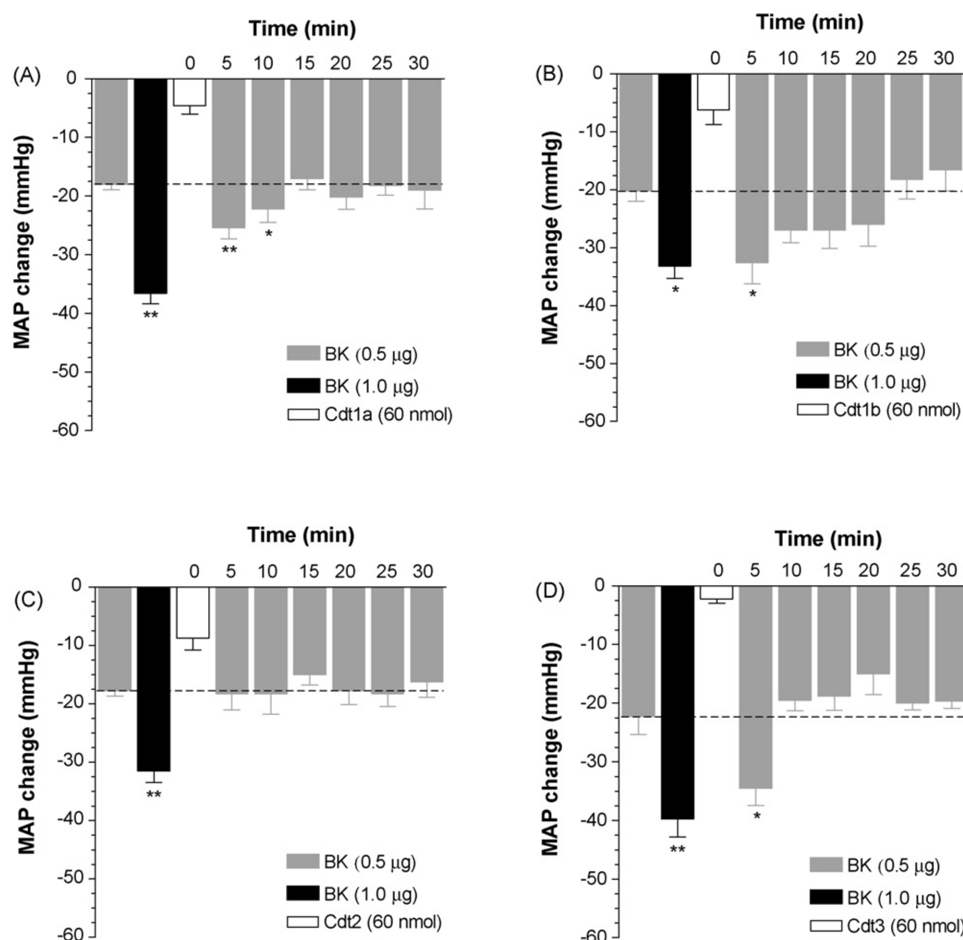

Fig. 4 – Hypotensive effect of intravenous bolus injections of Bk before and after *Cdts* peptides injection in the blood pressure of anesthetized Wistar rats. Panels A, *Cdt1a* ($n = 5$) B, Cdt1b ($n = 5$); C, Cdt2 ($n = 4$) and D, Cdt3 ($n = 5$). Data are expressed as mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison to the values obtained with 0.5 μg of Bk before peptide injection. MAP stands for Mean Arterial Pressure.

Moreover, *in vivo*, both structurally similar pentapeptides, **BPP-5a** [<EKWAP] and **Ahb1** [<EKWDP], were able to potentiate de Bk standard dose response (Fig. 6). The maximal potentiation response on blood pressure of anesthetized rat occurred at 5 min after administration of **Ahb1** (44.3 ± 5.8%, $n = 5$), similarly to that observed for **Cdt1a**, **Cdt1b** and **Cdt3** peptides (Figs. 4 and 6). However, the maximal potentiation effect of **BPP-5a** occurred after 10 min (48.3 ± 4.7%, $n = 4$) and remained until 15 min (Fig. 6 and Table 2).

Noteworthy, it was not possible to observe any potentiation of the Bk effect on the contraction of isolated guinea pig ileum, at the employed concentration, e.g. 3×10^{-6} M, for both peptides: the **P5** [WAQKP], which is a scrambled sequence of the pentapeptide *Bj*-**BPP-5a**, and the **Tf2**, which is a 14 amino acid residues long peptide showing the typical IPP-tripeptide at the C-terminus (Fig. 5 and Table 1).

3.5. *Effects of BPP-like peptides on angiotensin I vasopressor response on anesthetized rat blood pressure.*

The same doses of angiotensin I and II, previously described by Rubin et al. [26], in which a marked and transitory vasopressor effect (almost 50 and 45 mmHg, respectively) could be observed in conscious rats was used. In this condition, an effective inhibition of the vasopressor response to angiotensin I, with no interference in the angiotensin II response, was observed after intravenous bolus injection of captopril (0.1 mg/kg). An inhibition of 78 ± 8.2% of vasopressor effect on blood pressure of anesthetized rat elicited by angiotensin I was observed for this well-known ACE inhibitor. On the other hand, no effect was verified for either angiotensin I or II, after intravenous bolus injection of the BPP-like peptides assayed here, suggesting that angiotensin is not involved in the observed effects.

4. Discussion

Recent works have suggested that the mechanism responsible for the enhancement of Bk activity by the BPPs cannot be entirely explained by the inhibition of ACE catalytic activity [17,27,28]. Thus, indicating that each BPP might act through distinct mechanism of action, probably involving different target proteins [16,17]. Based on this, a systematic and detailed

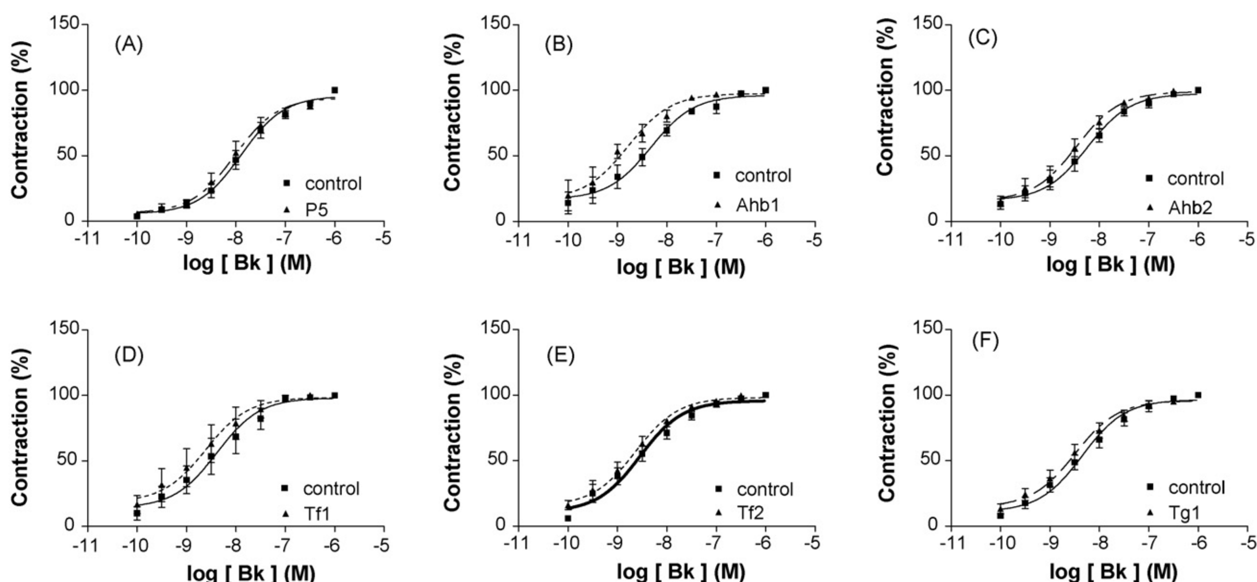

Fig. 5 – Bradykinin potetiation effect of Asian snakes putative BPPs on isolated guinea pig ileum. The putative BPPs identified in the BPP-CNP precursor protein were synthesized and were used to generate the potentiation curve (dotted line) that was compared to the control curve obtained using only Bk (continuous line). Panel A, P5; B, Ahb1; C, Ahb2; D, Tf1; E, Tf2; F, Tg1. Each point represents a mean value of independent experiments, while the vertical lines correspond to the mean ± S.E.M. The responses are expressed as percentages of the maximum response obtained in the first curve for Bk.

study of the structural diversity of members of BPP family became of utmost importance to delineate the minimal structural requirements for Bk-potentiating activity.

The molecular cloning and analysis of the deduced sequence of BPPs precursor proteins from the venom gland of the South American rattlesnake, *Cdt*, brings valuable information about the structural variation of the Bk-potentiating peptides genes and their products. While the *Cdt* cDNAs encoding BPPs precursor protein encompass sequences of 1.5 kb with ORFs of 540 bp, sharing about 99% of identity with each other (Fig. 1), the homologous cDNAs from *Bothrops* species are much longer and contain a higher number of BPPs sequences in each precursor protein [15]. Moreover, molecular analysis through Northern blot of total RNAs from the venom gland of this South American rattlesnake, revealed a single size precursor transcript (of about 1.5 kb) (data not shown), while in the venom gland of the viper *Bj*, at least two other longer related transcripts, of about 3.5 and 5.7-kb, were also observed [15]. Since the transcripts of *Cdt* and *Bj*-BPPs precursor are the product of highly homologous genes, they should be arised by independent evolution of an ancestral gene that duplicated before the divergence of these two *Crotalinae* species. Noteworthy, hybridization signals of BPPs transcripts were detected in several *Bj* tissues, including the brain, indicating a functional diversity and a physiological role for these peptides [15,16]. However, this was not observed for the *Cdt*, in which the expression seems to be limited to the venom gland of this snake (data not shown).

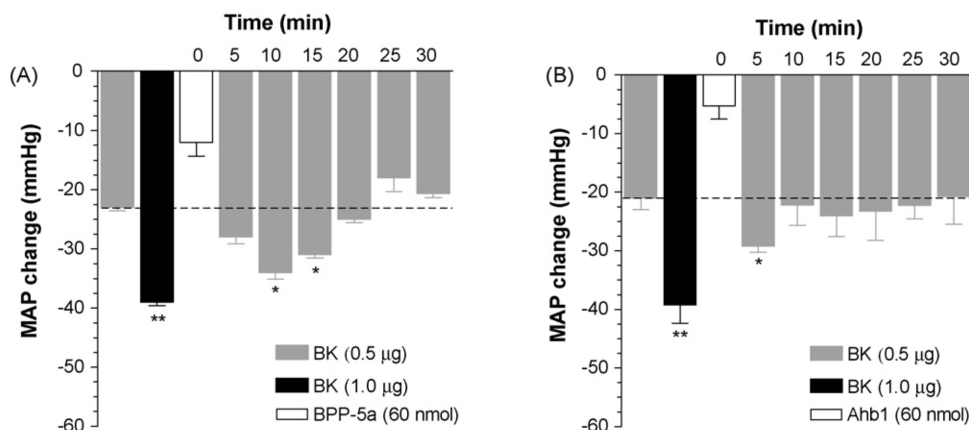

Fig. 6 – Hypotensive effect of intravenous bolus injections of Bk before and after BPP-5a and Ahb1 injections in the blood pressure of anesthetized Wistar rats. Panel A, *BPP-5a* ($n = 4$) and Panel B, Ahb1 ($n = 5$). Data are expressed as mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison to the values obtained with 0.5 μg of Bk before peptide injection.

The deduced BPP precursor proteins from *Cdt* venom gland are unique in their structural organization. This distinction is evidenced by the comparison of these BPP precursor proteins with others from the Brazilian tropical arrowhead viper (*Bj*) and from Asian snakes, namely *Gloydius blomhoffi* (*Ahb*), *Trimeresurus gramineus* (*Tg*) and *Protobothrops flavoviridis* (*Tf*) (Fig. 2). Thus, only one typical BPP peptide with the IPP-tripeptide in the C-terminus is arranged per molecule of *Cdt* BPP percursor protein (Fig. 1), while several BPPs sequences are observed in the *Bothrops* precursor protein (Fig. 2). The BPP precursor from *Cdt* reported here is the shortest precursor homologue described up to date. Even so, at least two distinct short BPP-like sequences could be identified in each molecule of the deduced BPPs precursor proteins, beyond the long peptide with the IPP-tripeptide at the C-terminus (Fig. 1).

As stated above, the identification of putative BPPs has been basically based on either the characterization of the respective bioactive peptides in the venom of these animals or by simple and direct analysis of the primary structure of precursor proteins. The latter is frequently based uniquely on the presence of the peptide sequence stretches showing the typical features previously described for the BPPs [15], that is, the presence of a glutamine residue followed by a proline residue at the C-terminus. Thus, in the present study, we first cloned the cDNAs and analyzed their sequences, as well as the respective deduced protein sequences, for the characterization of *Cdt* BPPs precursor protein (Fig. 1). Once distinguished, all putative peptides that might correspond to *Cdt* BPP molecules were chemically synthesized and pharmacologically assayed to confirm their Bk-potentiating activity. Synthetic peptides corresponding to potential BPP molecules identified in precursor proteins from Asian snakes were also prepared, and the scrambled sequence of **BPP-5a** from *Bj*, namely **P5** [WAQKP], was concomitantly assayed as a control (Fig. 6 and Table 1).

Two types of classical pharmacological methods were employed in this work to evaluate the BPPs activities: *in vitro* assay using isolated guinea pig ileum contraction and *in vivo* hypotensive assay using anesthetized rats. By using these two methodologies, we observed that the *Cdt* synthetic peptides displaying the typical IPP-tripeptide were not only effective in eliciting smooth muscle contraction mediated by Bk *in vitro*, but also were able to potentiate the hypotensive effect of Bk *in vivo* (Figs. 3 and 4, and Tables 1 and 2). On the other hand, the short BPP-like peptides, **Cdt2** [<ENWKSP] and **Cdt3** [<EARESP], lacking the canonical IPP-tripeptide, were not able to potentiate the Bk effect on smooth muscle contraction (Fig. 3). Nevertheless, the hexapeptide **Cdt3** [<EARESP] was capable to potentiate the hypotensive effect of Bk on the blood pressure of anesthetized rats, while the **Cdt2** [<ENWKSP] was not (Fig. 4).

In this way, these data reinforce the indication that the mechanisms involved in the Bk-potentiation by BPPs on each pharmacological assay, e.g., contraction of isolated guinea pig ileum and on vasodilation in rats, are not the same, as it has also been shown by others [16,17]. It is of note that the pressor response of angiotensin I (300 ng/kg) and/or angiotensin II (100 ng/kg) before and after BPP's infusion was the same, showing that the renin-angiotensin system is not interfering in our assay. Moreover, in our assay, inhibition of angiotensin I vasopressor response of about 78% was observed only for captopril, which was shown to be an effective inhibitor of the circulating ACE [26]. On the other hand, no interference in the angiotensin II response was observed for this inhibitor.

On the other hand, *in vitro* pharmacological assays for almost all synthetic BPP-like peptides derived from Asian snakes precursor prepared have no Bk-potentiating activity. The only exception was the pentapeptide **Ahb1** [<EKWDP], which is structurally highly similar to the *Bj*-**BPP5a** [<EKWAP] and showed Bk-potentiating activity on both pharmacological assays employed here (Figs. 5 and 6, Tables 1 and 2).

Interestingly, the peptide **Ahb1** was found aligned to the **BPP5a**, when the precursor proteins from *Ahb* and *Bj* were compared (Fig. 2). On the other hand, the sequence of **Ahb2**, despite not showing a Bk-potentiation effect in the bioassays employed here (Fig. 5), was not observed only in the *Ahb* BPPs precursor proteins, but also in others precursor proteins from the Asian snakes studied herein. Surprisingly, the same sequence was also present in the Brazilian viper *Bj* precursor protein (Fig. 2). Unexpectedly, even longer peptides with the typical IPP-tripeptide at the C-terminus, which was the case of the **Tf2** [<EWMPEGRPPHPIPP], did not show Bk-potentiating effect in the evaluated doses (Fig. 5 and Table 1). Longer peptides with the ISP-tripeptide at the C-terminus, namely **Tg1** and **Tf1**, also did not show Bk-potentiating activity (Fig. 5 and Table 1), besides being previously described as a BPP [18]. Thus far, our data concerning to evaluation of the pharmacological effect of synthetic peptide fragments, derived from BPP precursor proteins from the South American rattlesnake and from Asian snakes, confirm that the structural analysis of the deduced sequence alone are not enough to identify novel BPPs and to determine if they are able or not to potentiate the Bk activity *in vitro* or *in vivo*.

Taken together, the data present in this work show that the presence of a pyroglutamyl and a proline residue at the N- and C-extremities of the peptide, respectively, is not enough to determine the Bk-potentiation activity neither *in vitro* nor *in vivo*, suggesting the necessity of sets of specific and distinct biological assays, affording strong criteria for the identification of new BPPs.

At the present, the mechanisms involved in the BPPs molecular processing leading to the release of these bioactive peptides are still unknown. From this point of view, it seems imperative to clarify the molecular process and enzymes involved in the proteolytic release of the BPPs, in order to correctly identify putative new sequences in precursor protein sequences as far characterized.

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