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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™ 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™ Terminator Cycle Sequence Kit and the ABI 3100 automatic system (Applied Biosystems, Foster City, CA, USA).