

Bradykinins and their precursor cDNAs from the skin of the fire-bellied toad (*Bombina orientalis*)[☆]

Tianbao Chen^a, David F. Orr^a, Anthony J. Bjourson^a, Stephen McClean^a,
Martin O'Rourke^a, David G. Hirst^a, Pingfan Rao^b, Chris Shaw^{a,*}

^a School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, Northern Ireland, UK

^b Institute of Biotechnology, Fuzhou University, Fuzhou 350002, Fujian Province, PR China

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Abstract

Bradykinin and (Thr⁶)-bradykinin have been identified in the defensive skin secretion of the fire-bellied toad, *Bombina orientalis*. The homologous cDNAs for both peptides were cloned from a skin library using a 3'- and 5'-RACE strategy. Kininogen-1 (BOK-1) contained an open-reading frame of 167 amino acid residues encoding four repeats of bradykinin, and kininogen-2 (BOK-2) contained an open-reading frame of 161 amino acid residues encoding two repeats of (Thr⁶)-bradykinin. Alignment of both precursor nucleotide and amino acid sequences revealed a high degree of structural similarity. These amphibian skin kininogens/preprobradykinins are not biologically analogous to mammalian kininogens.

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1. Introduction

The skins of anuran amphibians contain highly specialized exocrine glands, the granular or poison glands, which upon application of a stressor, secrete a complex cocktail of proteins, peptides, and biogenic amines [11]. Peptides often predominate and bradykinin or structural variants have been identified in species of frogs from most zoogeographical regions [7,8,15,16].

Authentic nonapeptide bradykinin has been isolated from the European common frog (*Rana temporaria*) in addition to a range of minor N- and C-terminally extended molecular forms [1,4]. However, other *Rana* species have been found to contain structural variants such as (Thr⁶)-bradykinin

and (Thr⁶)-bradykinyl-IAPEIV from *Rana rugosa* and (Val¹,Thr⁶)-bradykinin and (Val¹,Thr⁶)-bradykinyl-VAPAS from *Rana nigromaculata* [22,12]. Additional structural variants such as phyllokinin (bradykinyl-IYsulfate) have been found in the skins of several Neotropical leaf frogs (*Phyllomedusa* sp.) and bombinakinin O (bradykinyl-GKFH) in *Bombina orientalis* skin extracts [2,21].

Curiously, attempts to activate a kallikrein–kininogen system in the blood of anuran amphibians have met with no success. Glass bead treatment and trypsinization of blood plasma from the frogs, *Rana catesbeiana* and *Rana pipiens*, and the toads, *Bufo arenarum* and *Bufo marinus paracnemis*, generated no detectable bradykinin activity [13,18]. The apparent absence of a circulatory kininogen in this group of tetrapods poses a fundamental question as to the nature of the kininogens/preprobradykinins responsible for the generation of bradykinin and related peptides in anuran skin.

A systematic proteomic study of *B. orientalis* skin secretion in our laboratory failed to detect bombinakinin O, previously identified in skin extracts of this species [21]. However, peptides corresponding in molecular mass to authentic bradykinin and (Thr⁶)-bradykinin were detected in significant quantities.

Here, we describe the structural characterization of these peptides and subsequent cloning of their precursors from

Abbreviations: HPLC, high performance liquid chromatography; fmoc, 9-fluorenylmethoxycarbonyl; LC, liquid chromatography; MS, mass spectrometry; UPM, universal primer mix; EC₅₀, concentration required to elicit 50% release of the maximum effect

[☆] The nucleotide sequences of *Bombina orientalis* skin kininogen-1 and -2 have been deposited in the EMBL Nucleotide Sequence Database under the accession codes AJ316578 and AJ347942, respectively. The sequences of the isolated bradykinins can be accessed in the SwissProt database under the accession numbers P83060 and P83059, respectively.

* Corresponding author. Tel.: +44-2870-324611;

fax: +44-2870-324965.

E-mail address: c.shaw@ulst.ac.uk (C. Shaw).

a dorsal skin-derived cDNA library. Determination of the precursor structure of bradykinin was deemed particularly relevant and novel as this toad skin peptide is structurally identical to the mammalian homolog and the structure of its kininogen/preprobradykinin in amphibians was unreported. In addition, the biological activities of synthetic replicates of both peptides were compared using two different mammalian smooth muscle preparations.

2. Materials and methods

2.1. Identification and structural analysis of bradykinins

Skin secretions were obtained from four adult specimens of *B. orientalis* by mild transdermal electrical stimulation [20]. Secretions were washed from the toads with distilled–deionized water, snap frozen in liquid nitrogen, and lyophilized. Five milligrams of freeze–dried secretion were subjected to LC/MS using an LCQ ion-trap electrospray mass spectrometer interfaced with a gradient HPLC system (ThermoQuest). Detected ions with molecular masses coincident with predicted masses of bradykinin and known variants were subjected to MS/MS fragmentation and sequenced. Primary structures were confirmed by automated Edman degradation using an Applied Biosystems 491 Precise sequencer. Each of the bradykinins was synthesized by solid-phase fmoc chemistry using standard protocols on an Applied Biosystems 433 peptide synthesizer. Products were purified and structures confirmed by LC/MS/MS.

2.2. Cloning of bradykinin cDNAs

Dorsal skin was excised from two euthanized adult *B. orientalis* toads, frozen in liquid nitrogen, and subsequently ground to a fine powder in this medium. Polyadenylated mRNA was isolated using magnetic oligo-dT beads as described by the manufacturer (DynaL Biotec, UK). The isolated mRNA was subjected to 5′- and 3′-rapid amplification of cDNA ends (RACE) procedures to obtain full-length kininogen/preprobradykinin nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed an UPM primer (supplied with the kit) and a sense primer (Brady-S1; 5′-AARGGICCMGICCCIGGITY-3′) that was complementary to the amino acid sequence, -KGP-RPPGF-, of maximakinin (SwissProt accession no. P83055). The four discrete bands of approximately 220, 330, 360, and 450 base pairs, that were produced in the 3′-RACE reaction, were gel purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer. The sequence data obtained from these 3′-RACE products were used to design a series of bradykinin-specific antisense primers. Two 5′-RACE primers were used in separate reactions, Brady-AS1 (5′-GCATTTTCAATGGATTTTGTGTGT-3′) and Brady-AS2 (5′-GTCAGTCAGAGCTGGTATTACAGAGG-3′), in conjunction with the UPM RACE primer. Generated PCR products were gel purified, cloned, and sequenced as described earlier.

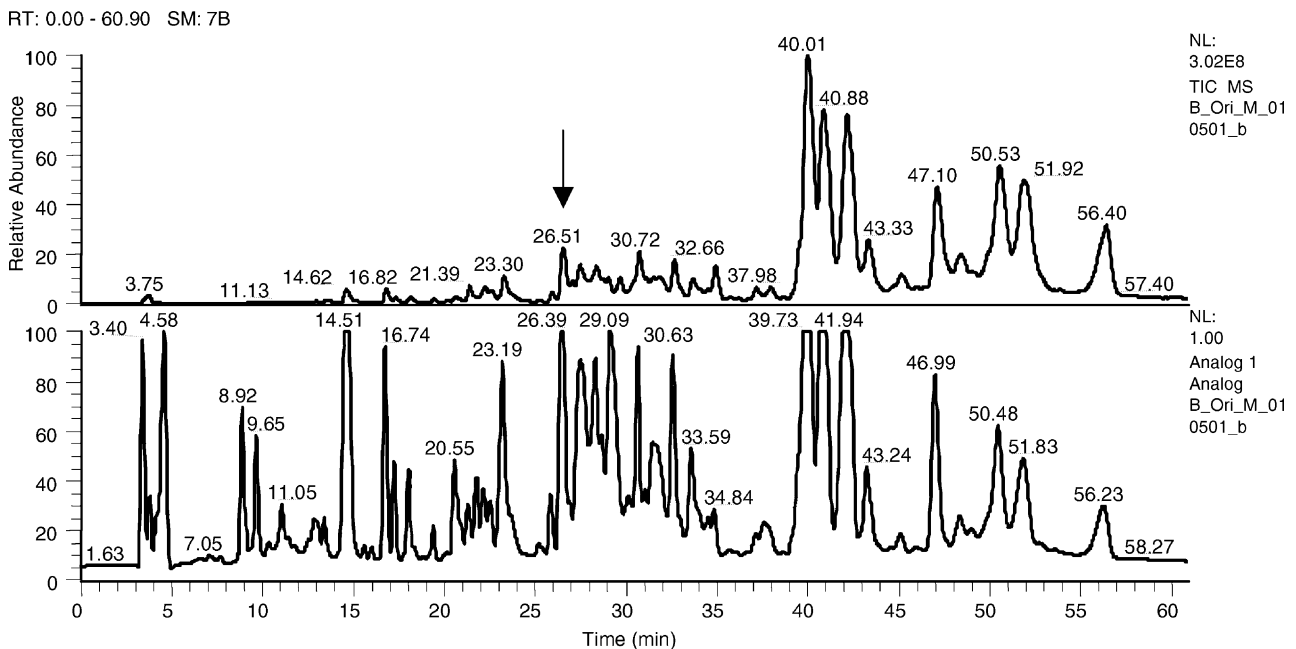


Fig. 1. LC/MS of *Bombina orientalis* venom showing total ion counts (upper panel) and UV absorbance at $\lambda = 214$ nm (lower panel). The retention time of the bradykinins is indicated by an arrow.

2.3. Smooth muscle pharmacology

Male albino Wistar rats (200–350 g) were euthanized by asphyxiation followed by cervical dislocation. The tail artery was prepared as described previously [9]. Incubation

buffer was 95% O₂/5% CO₂ oxygenated Krebs' solution (NaCl 118 mM, KCl 4.7 mM, NaHCO₃ 25 mM, NaH₂PO₄ 1.15 mM, CaCl₂ 2.5 mM, MgCl₂ 1.1 mM, and glucose 5.6 mM). Constriction or dilation of the arterial smooth muscle preparation was detected by an increase or decrease

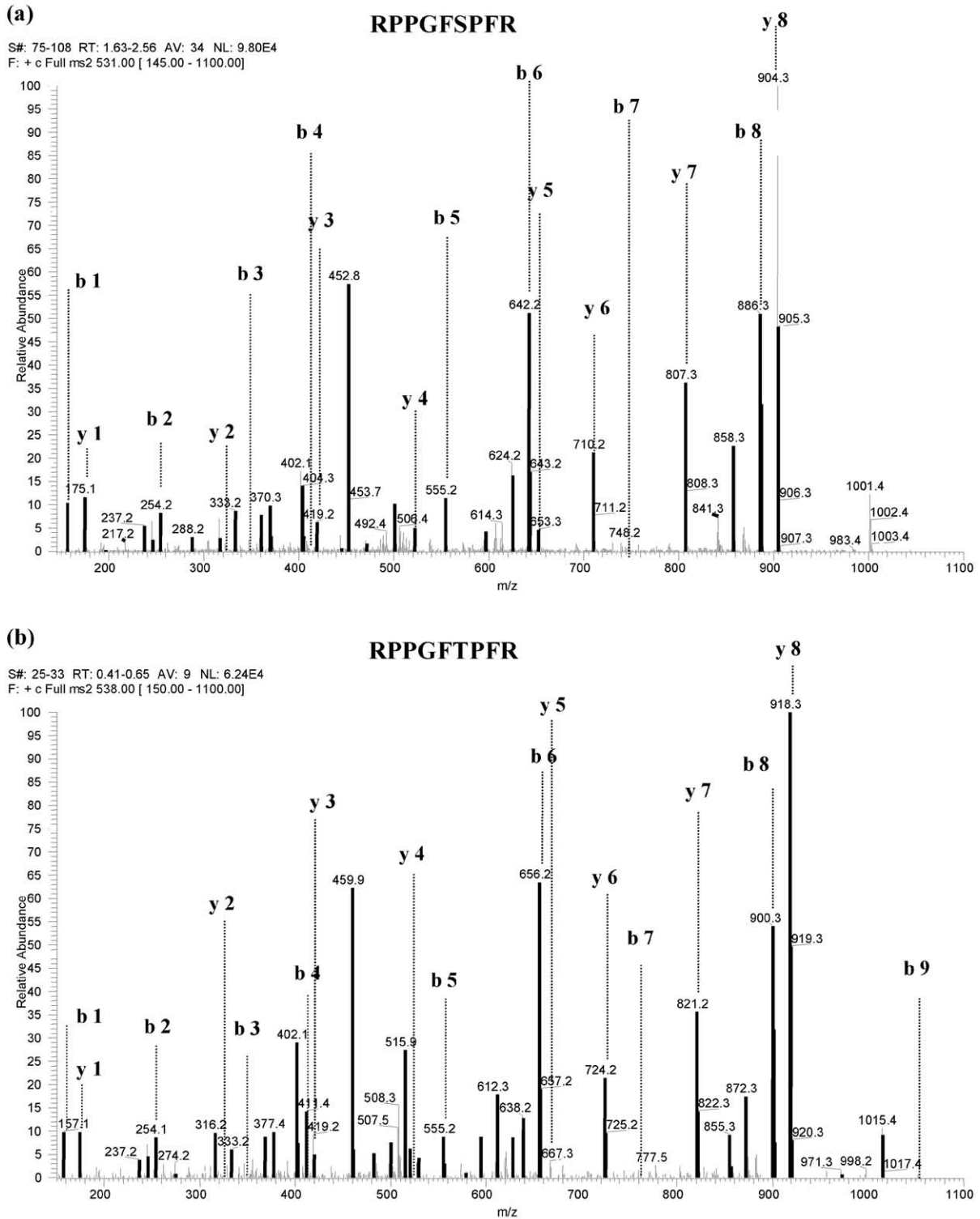


Fig. 2. MS/MS spectra of doubly charged ions of bradykinin ($m/z = 531$) (a) and (Thr⁶)-bradykinin ($m/z = 538$) (b). Identified b-ion and y-ion fragment series used in sequence calls are indicated in each case.

in pressure generated by water column displacement using pressure transducers connected to a MacLab System (AD Instruments Pvt. Ltd., Australia). Data were displayed graphically on a Macintosh computer. Viability was determined using a range of bolus phenylephrine (5 M–1 mM) exposures and the endothelial layer of the artery was removed by bubbling with oxygen for 10 s. Absence of the endothelial layer was confirmed by the lack of relaxation in response to a 30 min perfusion of acetylcholine (50 μ M) after precontraction with phenylephrine (10 μ M). For intestinal smooth muscle preparations, 1-cm thick rings of ileum were carefully placed onto the pins of a MacLab force transducer, one pin acting as a stationary fixed point

while the second pin was free, permitting application of tension to the smooth muscle. The muscle rings were gradually exposed to 0.1 g increments in resting tension until the spontaneous contractions originated from a resting tension of 0.5 g. The contracting muscle preparations were allowed to stabilize for 25 min before the application of bradykinins. After perfusion of arterial preparations with 10 μ M phenylephrine to obtain constriction plateaux, relative relaxation was recorded following applications of bradykinins in the range from 1×10^{-5} to 1×10^{-10} M. The intestinal smooth muscle ring preparations were exposed to peptide doses described as earlier and relative changes in tension were recorded.

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1   ACGCGGGGA GTGTCTGGTT GCTGAGCTCA CAGTTCTGTA ACAAGTGGCC
                                     M R L W F C L S F F I I
51  CTTCCAGCTC TGAAAATGAG ACTGTGGTTC TGTCTAAGTT TCTTCATCAT
                                     L C L E H F P G T L A D E R N V
101 CCTGTGCCTC GAGCATTTTC CAGGAACCCT GGCAGATGAA AGAAATGTTT
                                     P E S E E K T E Q F L R D L S E I
151 CAGAGAGTGA AGAAAAAATC GAGCAGTTCC TGCGGGATTT GTCTGAGATC
                                     S R L Q R R P P G F S P F R G K F
201 AGCCGCCTAC AAAGACGTCC ACCGGGGTTC AGCCCTTTCC GAGGAAAATT
                                     H S Q S L R D L S E I S R L Q R
251 CCATAGCCAG TCCCTGCGAG ATTTGTCTGA GATCAGCCGC CTACAAAGAC
                                     R P P G F S P F R G K F H S Q S M
301 GTCCACCGGG GTTCAGCCCT TTCCGAGGAA AATTCATAG CCAGTCCATG
                                     R D L S E I S R L Q R R P P G F S
351 CGGGATTTGT CTGAGATCAG CCGCCTACAA AGACGTCCAC CGGGGTTTCC
                                     P F R G K F H S Q S M R D L S E
401 CCCTTTCCGA GAAAATTC ATAGCCAGTC CATGCGGGAT TTGTCTGAGA
                                     I S R L Q R R P P G F S P F R G K
451 TCAGCCGCCT ACAAAGACGT CCACCGGGGT TCAGCCCTTT CCGAGAAAAA
                                     F H S Q S L R G L S E I K R L K T
501 TTCCATAGCC AGTCCCTGCG GGGTTTGTCT GAGATCAAGA GATTGAAAAC
                                     T H K I H *
551 TACACACAAA ATCCATTGAA AATGCACTCC GGGTTTGTCTG TGTTAAAAAT
601 AGATTTGAAA GTAGCGTCCC CTGGTATAAA TAAGCATTGT TATGTCACAT
651 GGTGTCACCT CTGTAATACC AGCTCTGACT GACATGGTTT ATTAACAGC
701 AGATTTGTGC TCTCATATTT CGAAAAAAAA AAAAAAAAAA AAA

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Fig. 3. Nucleotide sequence of *Bombina orientalis* skin kininogen/preprobradykinin-1 (BOK-1) cDNA encoding four copies of bradykinin (single underlined). The putative signal peptide (double underlined) and stop codon (asterisk) of the open-reading frame are indicated.

3. Results

3.1. Identification and structural analysis of bradykinins

Analysis of stimulated skin secretion by LC/MS/MS identified both bradykinin and its structural variant, (Thr⁶)-bradykinin, in approximately equal abundance (20–30 µg/mg freeze-dried secretion). The peptides were incompletely resolved by HPLC (Fig. 1), but each was structurally characterized by both MS/MS fragmentation (Fig. 2a and b) and automated Edman degradation (data not shown). Bombinakinin O (bradykinyl-GKFH), previously identified in a large skin extract from *B. orientalis* [21], was

not detected in several LC/MS experiments using different batches of skin secretion from different groups of toads.

3.2. Cloning of bradykinin cDNAs

From the *B. orientalis* skin cDNA library, two different preprobradykinin cDNAs were consistently cloned. One encoded four-spaced repeats of bradykinin (BOK-1) (Fig. 3) and the other, two-spaced repeats of (Thr⁶)-bradykinin (BOK-2) (Fig. 4). These had open-reading frames of 167 and 161 amino acid residues, respectively. BOK-2 contained a 36 amino acid residue insert not present in BOK-1 (or the latter exhibited a deletion of this sequence) that

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1   ACGCGGGGAG TGTCTGGTTG CTGAGCTCAC AGTTCTGTAA CAAGGATGAA
51  TTTTTTCTTC AGCTCCAAGG GACTGCCATG GGTTCACACG TCGCCCCTAA
101 CTACGCCAAT ATTTTTATGA ATTTATATGA AGAAAGTTCT GTGTCACTTC
            M R L W F C L S F F V V L      
151 CAGCTCTGAT AATGAGACTG TGGTTCTGTC TAAGTTTCTT CGTCGTCCTG
            C L E H F P G T L A D E R N N R D      
201 TGCCTGGAGC ATTTTCCAGG AACCCCTGGCA GATGAAAGGA ATAATCGTGA
      Y P I R T H L H G P H I P R N N
251 CTACCCATA AGAACCCACT TACATGGCCC TCATATACCA AGGAATAATC
      R D Y P I R T H L H G H H I P R N
301 GTGACTACCC CATAAGAACC CACTTACATG GCCATCATAT ACCAAGAAAT
      V P E S E E K T E Q F L R D L S E
351 GTTCCAGAGA GTGAAGAAAA AACTGAGCAG TTCCTGCGGG ATTTGTCTGA
      I S R L Q R R P P G F T P F R G
401 GATCAGCCGC CTACAAAGAC GTCCACCGGG GTTCACCCCA TTTCGAGGAA
      K F H S Q S L R D L S E I S R L Q
451 AATTCCATAG CCAGTCCCTG CGGGATTTGT CTGAGATCAG CCGCCTACAA
      R R P P G F T P F R G K F H S Q S
501 AGACGTCCAC CGGGGTTTAC CCCATTTCTGA GGAAAATTCC ATAGCCAGTC
      L R D M Y E I K G F K S A H G R
551 CCTGCGGGAT ATGTATGAAA TCAAGGGGTT CAAAAGTGCA CACGGACGCC
      P R V C P P G E Q C P I W V G *
601 CACGAGTCTG CCCTCCGGGT GAGCAGTGTC CGATATGGGT TGGGTAGTAG
651 CGTCCCCTGG TATAAATAAG CATTGTTATG TCACATGGTG TCACCTCTGT
701 AATACCAGCT CTGACTGACT AATCGATGGT TTATTAAACA GCAGATTTGT
751 GCTCTCAAAA AAAAAAAAAA AAAAAAAAAA

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Fig. 4. Nucleotide sequence of *Bombina orientalis* skin kininogen/preprobradykinin-2 (BOK-2) cDNA encoding two copies of (Thr⁶)-bradykinin (single underlined). The putative signal peptide (double underlined) and stop codon (asterisk) of the open-reading frame are indicated.

consisted of a tandem repeat of an 18-mer with one amino acid substitution. Both preprobradykinins, however, exhibited a high degree of primary structural similarity with heterogeneity predominantly localized to the C-terminal regions. Alignment of open-reading frame sequences as both DNA (Fig. 5) and amino acid residues (Fig. 6), revealed that bradykinin and (Thr⁶)-bradykinin encoding regions (compensating for the insert in BOK-2) were located at identical positions within the precursors. Also the DNA and amino acid conserved sites were coincident. Mature peptides were

flanked both N- and C-terminally by identical propeptide convertase processing sites probably involving a protease with Arg–X cleavage specificity, except apparently where X = Pro.

3.3. Smooth muscle pharmacology

Synthetic replicates of bradykinin and (Thr⁶)-bradykinin produced dose-dependent relaxation of rat arterial smooth muscle and constriction of intestinal smooth muscle,

		1		50
BOK1-orf	(1)	ATGAGACTGTGGTTCTGTCTAAGTTTCTTCATCATCCTGTGCCTCGAGCA		
BOK2-orf	(1)	ATGAGACTGTGGTTCTGTCTAAGTTTCTTCGTCGTCCTGTGCCTGGAGCA		
		51		100
BOK1-orf	(51)	TTTTCCAGGAACCCCTGGCAGATGAAAGAAATGTTCCAGAGAGTG---AA		
BOK2-orf	(51)	TTTTCCAGGAACCCCTGGCAGATGAAAGGAATAATCGTGACTACCCCATAA		
		101		150
BOK1-orf	(97)	GAAAAAACTGAGCAGTTCCCTGCG-----GGATTGTCTGAGATCAGCC		
BOK2-orf	(101)	GAACCCACTTACATGGCCCTCATATACCAAGGAATAATC-GTGA-CTACC		
		151		200
BOK1-orf	(140)	GCCTACAAAGACGTCCACCGGGGTTCAGCCCTTTCCGAGGAAAAATTCCAT		
BOK2-orf	(149)	CCATAAGAAACCACCTTACATGG--CCATCATATACCAAGAAATGTTCCAG		
		201		250
BOK1-orf	(190)	AGC-----CAGTCCCTGCGAGATTTGTCTGAGATCAGC		
BOK2-orf	(197)	AGAGTGAAGAAAAACTGAGCAGTTCCTGCGGGATTTGTCTGAGATCAGC		
		251		300
BOK1-orf	(223)	CGCCTACAAAGACGTCCACCGGGGTTCCAGCCCTTTCCGAGGAAAAATTCCA		
BOK2-orf	(247)	CGCCTACAAAGACGTCCACCGGGGTTCCAGCCCAATTCGAGGAAAAATTCCA		
		301		350
BOK1-orf	(273)	TAGCCAGTCCATGCGGGATTTGTCTGAGATCAGCCGCTACAAAGACGTC		
BOK2-orf	(297)	TAGCCAGTCCCTGCGGGATTTGTCTGAGATCAGCCGCTACAAAGACGTC		
		351		400
BOK1-orf	(323)	CACCGGGGTTCCAGCCCTTTCCGAGGAAAAATTCATAGCCAGTCCATGCGG		
BOK2-orf	(347)	CACCGGGGTTCCAGCCCAATTCGAGGAAAAATTCATAGCCAGTCCCTGCGG		
		401		450
BOK1-orf	(373)	GATTTGTCTGAGATCAGCCGCTTACAAAGACGTTCCACCGGGGTTCCAGCCC		
BOK2-orf	(397)	GATATGTATGAAATCAAGGGGTTCAAAAG---TGCACACGG---ACGCCC		
		451		500
BOK1-orf	(423)	TTTCCGAGGAAAAATTCATAGCCAGTCCCTGCGGGGTTTGTCTGAGATCA		
BOK2-orf	(441)	-----ACGAGTCTGCCCT-----CCGGGTGAGCAGTGTCCGATAT--		
		501		532
BOK1-orf	(473)	AGAGATTGAAAACTACACACAAAATCCATTGA		
BOK2-orf	(476)	-GGGTTGGGTAG-----		

Fig. 5. Alignment of open-reading frame DNA sequences of BOK-1 and BOK-2. Identical bases are shaded. AlignX program of Vector NTI suite (Informax) was employed.

		1		50
BOK1	(1)	MRLWFCLSF	IIILCLEHFPGTLADERN	-----
BOK2	(1)	MRLWFCLSF	VVLLCLEHFPGTLADERN	NNRDYPIRTHLHGPHIPRNNRDYP
		51		100
BOK1	(28)	-----	VPESSEKTEQFLRDLSEISRLQRRPPGFS	SPFRGKFHS
BOK2	(51)	IRTHLHGHHIPRNV	VPESSEKTEQFLRDLSEISRLQRRPPGFS	TPFRGKFHS
		101		150
BOK1	(65)	QSLRDLSEISRLQRRPPGFS	SPFRGKFHSQSMRDLSEISRLQRRPPGFS	SPF
BOK2	(101)	QSLRDLSEISRLQRRPPGFS	TPFRGKFHSQSLRDMYEIKGFKSAHG	----
		151		200
BOK1	(115)	RGKFHSQSMRDLSEISRLQRRPPG	-FSPFRGKFHSQSLRGLSEIKRLKTT	
BOK2	(146)	-----	RPRVCPPEQCPWVG	-----
		201		
BOK1	(164)	HKIH		
BOK2	(162)	----		

Fig. 6. Alignment of open-reading frame amino acid sequences of BOK-1 and BOK-2. Identical amino acid residues are shaded. AlignX program of Vector NTI suite (Informax) employed.

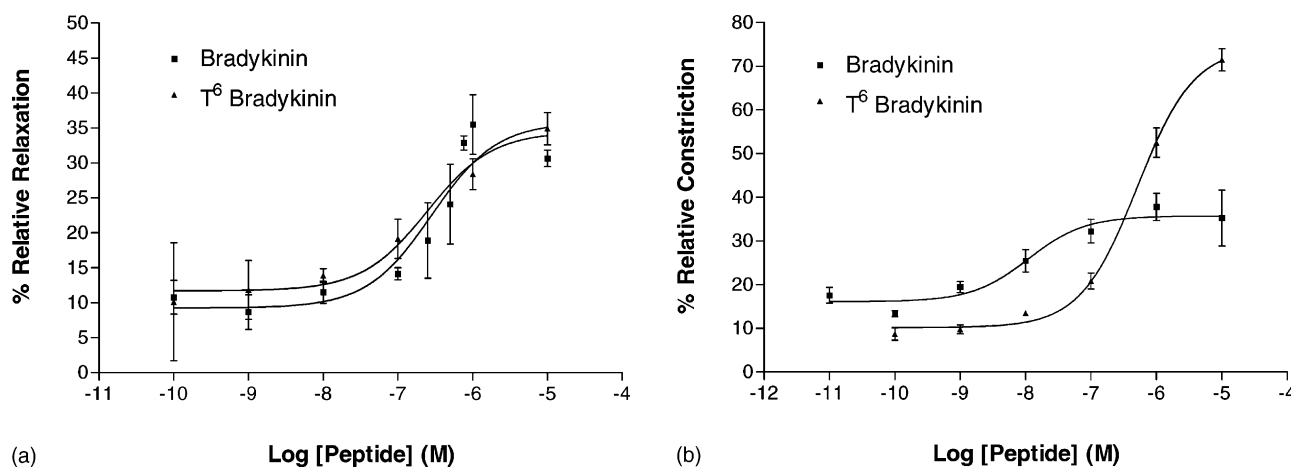


Fig. 7. Dose–response curves of bradykinin (■) and (Thr⁶)-bradykinin (▲) on rat arterial smooth muscle (a) and small intestinal smooth muscle (b) preparations. Each data point represents the mean \pm S.E.M. of six replicates.

though molar potencies were found to vary with preparation (Fig. 7a and b). Both peptides were essentially equipotent in relaxing arterial smooth muscle, whereas in contrast, (Thr⁶)-bradykinin was found to be less potent (by approximately two orders of magnitude) than bradykinin in constricting small intestinal smooth muscle. Although less potent than bradykinin in this smooth muscle preparation, (Thr⁶)-bradykinin was found to produce a much greater maximal response than bradykinin. These data indicate, however, that (Thr⁶)-bradykinin possesses the ability to activate bradykinin receptors in both smooth muscle preparations from the rat and, hence, contains the minimal ligand structure–activity requirement for this purpose.

4. Discussion

The dermal granular glands in the dorsal skin of *B. orientalis* synthesize and secrete a complex defensive secretion which includes bradykinin and a structural variant, (Thr⁶)-bradykinin, in approximately equal quantities. Cloning of skin cDNAs revealed that both peptides are encoded on different kininogens and that these are expressed in dorsal skin.

The apparent absence of a circulating kallikrein–kinin system in anuran amphibians [6,13,18] may be explained in a number of ways. The variability of bradykinin structures in frog skin secretions, the different spectra of homologous bradykinin bioactivity and the lack of information

on bradykinin receptor subtypes and pharmacology in non-mammalian vertebrates, may mean that inappropriate bio and/or immunoassays have been employed to detect the generated kinin [6,14]. More likely, however, the anuran amphibians expressing kininogens in skin granular glands are employing their product for a quite different biological purpose, that is as a component of a chemically and biologically complex exocrine defensive skin secretion. Each cloned frog skin kininogen displays little structural similarity with cloned mammalian kininogens [19] apart from the actual kinin coding sequence, indicating that they are not in the biological sense, homologous proteins.

Circulating kininogens represent a large pool of inactive peptide precursor and relatively small quantities are cleaved, often locally in tissues, to generate active kinin [3]. In contrast, frog skin kininogens exhibit molecular dynamics generally associated with peptide hormone precursors in that the intact high molecular weight precursor is not detectable in secretions but rather the fully processed active peptides. The structural features of frog skin kininogens, especially the presence of a putative hydrophobic signal peptide, is likewise consistent with secretion via a regulated secretory pathway for cell export and, hence, these proteins may be regarded in systematic terms as prepro-bradykinins. Analysis of the cloned precursor primary structures, however, indicates generation of active kinins by proteolysis at single arginyl residues consistent with the known cleavage site specificity of a kallikrein-type endoproteinase [3] but also with known vertebrate prohormone convertases [17]. The absence of published data on skin bradykinin precursor organization in other frog taxa and the presence of additional molecular variants, especially phyllokinin (bradykinyl-IYsulfate) and C-terminally extended bradykinins in *Rana* frogs [2,4,12,22], would suggest that the preprobradykinin organization described here is different to that in other anuran amphibians. Alternatively, in these other taxa, cleavage specificities or differential expression of skin peptide precursor convertases may be different.

The primary structural diversity found in frog skin bradykinins may reflect a degree of specific predator-mediated selection. Other major bioactive peptides found in frog skin secretions, including bombesins, tachykinins, and caeruleins, have active sites that are highly conserved among vertebrate taxa and, hence, these would activate homologous receptors in a wide spectrum of predatory species [11]. Bradykinin in *B. orientalis* skin secretion is identical in structure with that found in mammals, whereas (Thr⁶)-bradykinin is the bradykinin homolog of crocodylians, chelonians, and varanid lizards [5]. The freshwater habitats of frogs possessing bradykinins in their defensive skin secretions would be rich in an array of predatory species, including representative species of these taxa. We, thus, suggest that the spectrum of bradykinins present in the defensive skin secretions of frogs may be reflective of, and perhaps, by natural selection of peptide libraries generated in dermal venom glands, dictated by predominant predators cohabit-

ing the amphibian biotopes. *B. orientalis*, from north-east Asia, on this basis, would possess specific defense against mammalian and chelonian predators.

The systematic study of bioactive peptides in anuran skin provides much insight into natural molecular evolutionary strategies and demonstrates the need for focused research efforts on a group of tetrapod vertebrates whose global diversity and abundance continues in freefall [10].

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