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Novel bradykinins and their precursor cDNAs from European yellow-bellied toad (*Bombina variegata*) skin

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Two novel bradykinin-related peptides (Ala3,Thr6)-bradykinin and (Val1,Thr3,Thr6)-bradykinin, were identified by a systematic sequencing study of peptides in the defensive skin secretion of the yellow-bellied toad, *Bombina variegata*. These peptides are the first amphibian skin bradykinins to exhibit amino acid substitutions at the Pro3 position of the bradykinin nonapeptide. Previously reported bradykinins from other *Bombina* species were not detected. Respective precursor cDNAs, designated BVK-1 and BVK-2, respectively, were cloned from a skin library by 3′- and 5′-RACE reactions. BVK-1 contained an open-reading frame of 97 amino acids encoding a single copy of (Ala3,Thr6)-bradykinin and similarly, the open-reading frame of BVK-2 consisted of 96 amino acids encoding a single copy of (Val1,Thr3,Thr6)-bradykinin. Synthetic replicates of each novel bradykinin were found to be active on mammalian arterial and small intestinal smooth muscle preparations. The structural diversity of bradykinins in amphibian defensive skin secretions may be related to defence against specific predators.

Keywords: amphibian; bradykinin; skin; precursor; cloning.

The skins of anuran amphibians, in addition to mucous glands, contain highly specialized poison glands, which, in reaction to stress or attack, exude a complex noxious cocktail of biologically active molecules [1]. These secretions often contain a plethora of peptides among which bradykinin or structural variants have been identified [2–5].

The kallikrein-kininogen system appears to be a common target for certain venom components of many invertebrates and vertebrates. Among snakes, venoms from members of the Crotalinae (pit vipers) contain low molecular mass peptides that inhibit angiotensin-converting enzyme – a major bradykinin inactivating protease – thus potentiating the activity of endogenous bradykinin [6]. These bradykinin-potentiating peptides were the leads in developing angiotensin-converting enzyme inhibitor drugs that are frontline pharmaceuticals in the treatment of hypertension. Peptides with angiotensin-converting enzyme inhibitory activity have also been isolated from scorpion venom [7,8]. Wasp venoms contain bradykinin-related peptides that are responsible for the reddening, oedema and intense pain associated with envenomation by these insects [9,10]. These molecular strategies appear to be related to either potentiation of the effects of endogenous bradykinin by inhibition of catabolic proteases (snakes and scorpions) or by local delivery of supraphysiological quantities of bradykinins (wasps and amphibians).

Although canonical bradykinin (RPPGFSPFR) has been isolated from the European common frog (*Rana temporaria*), with a range of minor N- and C-terminally extended forms [11,12], other species of ranid frog have been found to contain structural variants. These include (Thr6)-bradykinin and (Thr6)-bradykinyl-IAPEIV in *R. rugosa* and (Val1, Thr6)-bradykinin and (Val1,Thr6)-bradykinyl-VAPAS in *R. nigromaculata* [13,14]. Additional structural variants such as phyllokinin (bradykinyl-IYsulfated) have been found in the skins of several South and Central American leaf frogs (*Phyllomedusa* sp.) and bombinakinin O (bradykinyl-GKFH) in *Bombina orientalis* [15,16].

Attempts to generate and subsequently isolate/characterize endogenous bradykinins in the anuran amphibian circulation, by activation of the kallikrein-kininogen system, have met with no success. The use of standard activation procedures in a range of species generated no detectable bradykinin activity [17,18]. The apparent absence of a circulatory kininogen in this group of tetrapods poses a fundamental question as to the nature of the protein(s) responsible for the generation of bradykinins in anuran skin.

In our laboratory, we have identified bradykinin and (Thr6)-bradykinin in the skin secretion of *B. orientalis* and a novel N-terminally extended bradykinin, maximakinin (DLPKINRKGP-bradykinin), in the skin secretion of *Bombina maxima* (SWISSPROT accession nos P83060,

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Abbreviations: Fmoc, 9 fluorenylmethoxycarbonyl; LC, liquid chromatography; MS, mass spectrometry; UPM, universal primer mix; EC_{50}, concentration required to elicit 50% release of the maximum effect.

Note: The nucleotide sequences of *Bombina variegata* skin kininogens 1 and 2 have been deposited in the EMBL Nucleotide Sequence Database under the accession codes AJ320269 and AJ318509, respectively. The sequences of the novel isolated bradykinin-related peptides can be accessed in SWISSPROT under accession numbers P83056 and P83057, respectively.

Note: a website is available at http://www.FrogPharm.com

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P83059, P83055, respectively), the latter was recently described by another group using the name bombinakinin M [19]. We found that none of these bradykinins were present in our archived LC/MS files of *Bombina variegata* skin secretion. Although both *B. orientalis* and *B. maxima* are from eastern Asia, we deemed it highly unlikely that their European congenerics were devoid of members of this peptide family. Here we describe the methodology employed in addressing this question which has led to the discovery of two novel, bradykinin-related peptides in the defensive skin secretion of the yellow-bellied toad (*B. variegata*) and the subsequent cloning of their precursor cDNAs from a skin-derived cDNA library. In addition, both peptides were chemically synthesized and tested for bioactivity using two different mammalian smooth muscle preparations.

MATERIALS AND METHODS

Identification and structural analysis of novel bradykinins

Specimens of *B. variegata* ($n = 12$) were obtained from a commercial source as captive-bred metamorphs and were raised to maturity in our vivarium for a period of 18 months. Skin secretions were obtained by mild transdermal electrical stimulation [20]. Secretions were washed from toads with distilled-deionized water, snap frozen in liquid nitrogen and lyophilized. 5 mg quantities of freeze-dried secretion were reconstituted in 0.5 mL of trifluoroacetic acid/water (0.1 : 99.9, v/v), clarified by centrifugation and subjected to LC/MS using an LCQ^{TM} electrospray ion-trap mass spectrometer interfaced with a gradient HPLC system (both supplied by ThermoFinnegan, San Jose, CA, USA). The gradient employed was formed from (trifluoroacetic acid)/water (0.1 : 99.9, v/v) to trifluoroacetic acid/water/acetonitrile (0.1 : 19.9 : 80.0, v/v/v) in 240 min at a flow rate of 1 mL·min^{-1}. The effluent from the chromatographic column was flow split with 10% of flow entering the mass spectrometer and 90% routed to a fraction collector. Fractions (900 μL) were collected automatically at minute intervals. Doubly charged ions were identified by isotopic resolution and subjected to MS/MS fragmentation. Sequence tags generated from fragment ions were database trawled on-line. Peptides displaying C-terminal fragment ion structural features of bradykinin (-PFR- y3-*m*/*z* 419, y2-*m*/*z* 322, y1-*m*/*z* 175) were further subjected to structural analysis by automated Edman degradation using an Applied Biosystems 491 Procise sequencer. Fifty microliters of the 900 μL fractions containing the putative bradykinin-related peptides were used in each case. The novel bradykinins identified were subsequently synthesized by solid-phase Fmoc chemistry using an Applied Biosystems 433 peptide synthesiser. Products were purified and structures confirmed by LC/MS/MS. For pharmacological experiments, standardization of each synthetic peptide was achieved by acid hydrolysis of a known gravimetric quantity of lyophilisate followed by amino acid analysis using an Applied Biosystems PTH-amino acid analyser.

cDNA cloning

Dorsal skin was excised from two dead adult toads, frozen in liquid nitrogen and polyadenylated mRNA was isolated using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK) and reverse transcribed. The cDNA was subjected to a 5′- and 3′- RACE procedure to obtain full-length preprobradykinin nucleic acid sequence data using a SMART-RACE kit essentially as described by the manufacturer (Clontech, UK). Briefly, the 3′-RACE reactions employed a UPM primer (supplied with the kit) and a sense primer (Brady-S1; 5′-AARGGICCIMG ICCICCIGGITTY-3′) that was complementary to the amino acid sequence -KGPRPPGF- of maximakinin (SWISSPROT accession no. P83055). The PCR products were gel-purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated capillary DNA sequencer. The sequence data obtained from these 3′-RACE products were used to design a specific antisense primer for the 5′-RACE reaction, BVAS-1 (5′-ATATCAGGGGACGCTACTTC-3′). Subsequent products were gel-purified, cloned and sequenced as described above. Following acquisition of these data, specific sense primers were designed to the N-terminal coding region of each respective peptide (Ala3,Thr6 BK-5′-ATCAGCCGCCTACAAAGACGTCCAGCG-3′, Val1, Thr3,Thr6-BK-5′-TCAGCCGCCTACAAAGAGTTCCA AC-3′) and employed in 3′-RACE reactions. Products were gel-purified, cloned and sequenced.

Smooth muscle pharmacology

Male albino Wistar rats (200–350 g) were killed by asphyxiation followed by cervical dislocation. The tail artery was prepared as previously described [21]. Incubation buffer was 95% O_2/5% CO_2 oxygenated Krebs' solution (NaCl 118 mM, KCl 4.7 mM, $NaHCO_3$ 25 mM, NaH_2PO_4 1.15 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 1.1 mM, glucose 5.6 mM). Constriction or dilation of the arterial smooth muscle preparation was detected by an increase or decrease in pressure generated by water column displacement using pressure transducers connected to a MacLab System (AD Instruments Pty Ltd. Australia). Data were displayed graphically on a Macintosh computer. Viability was determined using a range of bolus phenylephrine (5×10^{-6} M–1×10^{-3} M) 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 (5×10^{-5} M) after preconstriction with phenylephrine (1×10^{-5} M). After perfusion of arterial preparations with 1×10^{-5} M phenylephrine to obtain constriction plateaux, relative relaxation was recorded following applications of bradykinins in the range of 1×10^{-10} to 1×10^{-5} M. Peptides were added consecutively in increasing concentrations, each addition being followed by a wash out and equilibration phase.

For intestinal smooth muscle preparations, 1-cm segments 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 segments 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. The intestinal

smooth muscle preparations were exposed to peptides as described previously and relative changes in tension were recorded.

RESULTS

Identification and structural analysis of novel bradykinins

Analysis of stimulated skin secretions by LC/MS/MS, with subsequent sequence calling based upon fragment ions, revealed the presence of two, novel bradykinin-related peptides (Fig. 1). As the more readily fragmented doubly charged ions from each peptide were used for MS/MS fragmentation, the resultant spectra contained both singly and doubly charged fragment ions. The theoretical and observed fragment ions from each peptide are shown in Table 1. The primary structures, RPAGFTPFR (Ala3,Thr6-bradykinin) (theoretical mass 1049.23 Da, observed mass 1049.31 Da) and VPTGFTPFR (Val1, Thr3,Thr6-bradykinin) (theoretical mass 1022.20 Da, observed mass 1022.5 Da), were confirmed by automated Edman degradation of respective peptides identified from RP-HPLC fractions (Fig. 2). Bradykinin (1060.22 Da) and bombinakinin O (1529.76 Da) were not detected in *B. variegata* skin secretion despite exhaustive interrogation of LC/MS archived data with deduced singly and doubly charged ion molecular masses of each peptide. The structures of amphibian skin bradykinin-related peptides are compared with those described in the present study in Table 2.

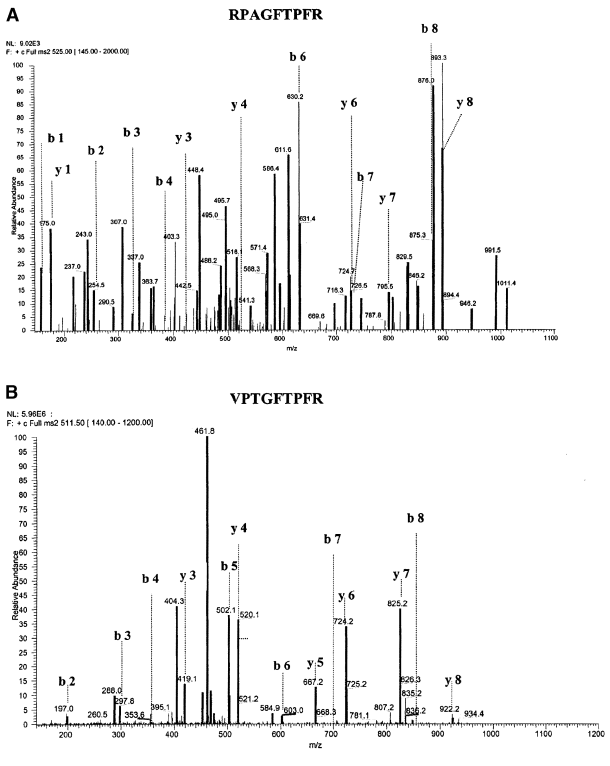

Fig. 1. MS/MS spectra of novel *B. variegata* skin bradykinin-related peptides. (A) (Ala3,Thr6)-bradykinin and (B) (Val1,Thr3,Thr6)-bradykinin.

cDNA cloning

Two different preprobradykinin cDNAs were consistently cloned from the skin library and each encoded a single copy of a novel bradykinin-related peptide (Ala3,Thr6)-bradykinin (BVK-1) or (Val1,Thr3,Thr6)-bradykinin (BVK-2) (Figs 3 and 4, respectively). Open-reading frames consisted of 97 and 96 amino acid residues, respectively. Alignments of both open-reading frame DNA (Fig. 5) and amino acid sequences (Fig. 6), using the ALIGNX programme of the VECTOR NTI bioinformatics suite (Informax), revealed that bradykinin-related peptide encoding regions, were located at identical positions within the precursors. Both preprobradykinins exhibited a high degree of both nucleotide and primary structural similarity with heterogeneity predominantly localized to the C-terminal regions. Mature peptides were flanked both N- and C-terminally by identical propeptide convertase processing sites probably involving a protease with Arg-X cleavage specificity. Both bradykinin-related peptides were flanked at the C-terminus by the tetrapeptide sequence, -GKFH-, a motif previously identified as the extension to bradykinin found in bombinakinin O from *B. orientalis*, a related species from Korea and Northern China [16].

Smooth muscle pharmacology

Synthetic replicates of the novel bradykinin-related peptides produced dose-dependent relaxation of rat arterial smooth muscle and constriction of intestinal smooth muscle, though molar potencies were found to be different when compared to bradykinin (Figs 7 and 8). In arterial smooth muscle (Ala3,Thr6)-bradykinin was approximately 10 times more potent by ED_{50} but produced a lower maximal response than bradykinin (Fig. 7). In contrast (Val1,Thr3,Thr6)-bradykinin was approximately 100 times less potent than bradykinin in inducing arterial smooth muscle relaxation (Fig. 8). Both novel bradykinin-related peptides were less potent (by approximately two orders of magnitude) than bradykinin in constricting small intestinal smooth muscle.

DISCUSSION

(Ala3,Thr6)-bradykinin and (Val1,Thr3,Thr6)-bradykinin represent two novel bradykinin-related peptides isolated from the defensive skin secretion of the European yellow-bellied toad, *B. variegata*. Their presence in skin and their primary structures were confirmed by cloning of two different but very similar precursor cDNAs each encoding a single copy of the respective peptides.

The technique of LC/MS employed here is an extremely versatile technology when applied to amphibian skin peptide research. The archiving of complete molecular mass data for a defensive skin secretion from a given species, renders it possible to interrogate such data sets in a number of ways using manufacturer's software. Computed molecular masses of charged parent ion series can be used to determine the presence of known peptides from other species in a very rapid manner. The finding of coincident and identically charged parent ion series is highly indicative of structural identity. Subsequent MS/MS fragmentation and comparison of generated fragment ion profiles with those predicted from the query sequence can establish

Table 1. Predicted MS/MS singly and doubly charged fragment ions of (Ala3,Thr6)-bradykinin and (Val1,Thr3,Thr6)-bradykinin. Observed ions are in bold typeface.

Residue	b	y	(+1)	Sequence	#	b	y	(+2)
(Ala3,Thr6)-bradykinin								
R1	**157.1**	1048.6	9	R	1	79.1	524.8	9
P2	**254.2**	**892.4**	8	P	2	127.6	**446.7**	8
A3	**325.2**	**795.4**	7	A	3	163.1	398.2	7
G4	**382.2**	**724.4**	6	G	4	**191.6**	362.7	6
F5	529.3	**667.3**	5	F	5	265.1	334.2	5
T6	**630.3**	520.3	4	T	6	315.7	**260.6**	4
P7	**727.4**	**419.2**	3	P	7	**364.2**	210.1	3
F8	**874.4**	322.2	2	F	8	437.7	161.6	2
R9	1030.6	**175.1**	1	R	9	**515.8**	88.1	1
(Val1,Thr3,Thr6)-bradykinin								
V1	100.1	1021.5	9	V	1	50.5	511.3	9
P2	**197.1**	**922.5**	8	P	2	99.1	**461.7**	8
T3	**298.2**	**825.4**	7	T	3	149.6	413.2	7
G4	**355.2**	**724.4**	6	G	4	178.1	362.7	6
F5	**502.3**	**667.3**	5	F	5	251.6	**334.2**	5
T6	**603.3**	**520.3**	4	T	6	302.2	**260.6**	4
P7	700.4	**419.2**	3	P	7	350.7	210.1	3
F8	**847.4**	322.2	2	F	8	424.2	161.6	2
R9	1003.5	**175.1**	1	R	9	**502.3**	88.1	1

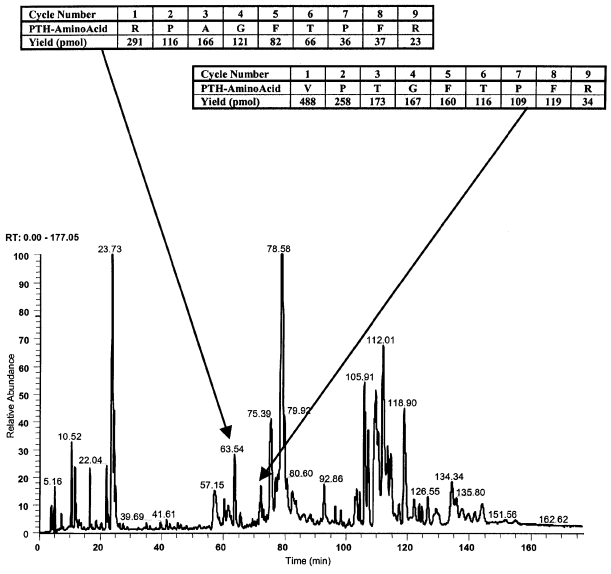

Fig. 2. RP-HPLC chromatogram (*y* axis = relative abundance at λ214nm in normalized arbitrary absorbance units) of *B. variegata* skin secretion. Retention times of novel bradykinin-related peptides are indicated below respective automated Edman degradation data.

identity beyond doubt. Likewise, failure to identify charged parent ion series is highly indicative of the absence of the query sequence. In this study, we have successfully used fragment ion identity from a conserved primary structural domain at the C-terminus of bradykinin ('sequence tags') as a tool for locating putative related peptides, in the absence of known variants in the skin secretion.

In a recent review of bradykinins in nonmammalian vertebrates, it was stated that bradykinin was undetectable in the skins of several species of European amphibians of the closely related genera, *Bombina*, *Alytes* and *Discoglossus* [22]. While it may be true that canonical bradykinin is not present, the data presented here unequivocally demonstrate the presence of two, novel bradykinin-related peptides in a European bombinid toad. In addition, these data may provide an insight into the apparent absence of a circulating kallikrein-kinin system in anuran amphibians [17,18,23]. The high degree of primary structural variability of bradykinins in amphibian skin secretions, their differing spectra of bioactivity and the lack of information on bradykinin receptor subtypes and pharmacology in amphibians, may mean that, in the past, inappropriate bio- and/or immuno-assays have been employed to detect the endogenous circulatory kinins.

However, it is most probable that anuran amphibians expressing bradykinins in skin granular glands employ these for a quite different biological purpose. Each cloned amphibian skin preprobradykinin displays little structural similarity with cloned mammalian prekininogens [24] apart from the actual kinin coding sequence. Circulating kininogens represent a large pool of inactive peptide precursor and relatively small quantities are cleaved, often locally in tissues, to generate active kinin [25]. In contrast, amphibian skin preprobradykinins exhibit molecular dynamics generally associated with peptide hormone precursors in that the intact high molecular mass precursor is not detectable in secretions but rather the fully processed active peptides. Indeed, the amphibian's active kinin products are destined for export as products of a defensive skin secretion rather than for action as a local hormone within endogenous tissues. For all of these reasons, it is most probable that mammalian kininogens and amphibian skin probradykinins are not in the biological sense, homologous proteins.

Analysis of the cloned precursor primary structures, however, indicates generation of the bradykinins by

Table 2. Amino acid sequence alignments of amphibian skin bradykinins. Substituted and extension residues in bold. Fully conserved residues indicated by asterisks.

	* ** * *	
	RP**A**GF**T**PFR	*Bombina variegata*
	VP**T**GF**T**PFR	*Bombina variegata*
	RPPGFSPFR	*Rana temporaria* [11], *R. palustris* [28], *Bombina orientalis*
	RPPGF**T**PFR	*Rana rugosa* [13], *Bombina orientalis*
	VPPGF**T**PFR	*Rana nigromaculata* [14]
	RPPGFSPFR**GKFH**	*Bombina orientalis* [16]
DLPKINRKGP	RPPGFSPFR	*Bombina maxima* [19]
	RPPGFSPFR**IY(SO$_3$H)**	*Phyllomedusa* sp. [15]
	RPPGFSPFR**IA**	*Rana temporaria* [12]
	RPPGFSPFR**IAPASTL**	*Rana temporaria* [12]
	RPPGFSPFR**IAPASIL**	*Rana temporaria* [12]
	RPPGFSPFR**VAPAS**	*Rana nigromaculata* [14]
	IRRPPGFSP**L**R	*Rana palustris* [28]
	IRRPPGFSP**L**RIA	*Rana palustris* [28]
	AGIRRPPGFSP**L**R	*Rana palustris* [28]
	AGIRRPPGFSP**L**R**IA**	*Rana palustris* [28]

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  1 TCGATTAAGC AGGTGGTAAC AACGCAGAGT ACGCGGGGGA GTGTCTGATT

                                                      M  R
 51 GCTGAGCTCA CAGTTCTGTA CCAAGTTTCA CTTCCAGCTC TGATAATGAG

    .  L  W  F  C  L  S  F  L  I  I  L  C  V  E  H  F  P
101 ACTGTGGTTC TGTCTAAGTT TCCTCATCAT CCTGTGCGTG GAGCATTTTC

    .. G  T  L  A  V  E  R  N  V  P  E  S  E  E  K  T
151 CAGGAACCCT GGCAGTTGAA AGAAATGTTC CAGAGAGTGA AGAAAAAACT

     E  Q  F  L  R  D  L  F  E  I  S  R  L  Q  R  R  P
201 GAGCAGTTCC TGCGGGATTT GTTTGAGATC AGCCGCCTAC AAAGACGTCC

     A  G  F  T  P  F  R  G  K  F  H  S  Q  S  L  R  G
251 AGCGGGGTTC ACCCCTTTCC GAGGAAAATT CCATAGCCAG TCCCTGCGGG

    .. L  S  E  T  K  R  I  Y  N  A  I  W  P  C  K  H
301 GTTTGTCTGA GACCAAGAGG ATATATAATG CAATCTGGCC CTGCAAGCAC

     C  N  K  C  K  P  G  L  L  C  K  K  *
351 TGCAATAAAT GCAAACCGGG TTTGCTGTGT AAAAAATAGA TTTGAAAGTA

401 GCGTCCCCTG GTATAAATAA GCATTGTTAT GTCACATGGT GTCACCTCTG

451 TAATACCAGC TCTGATATGG TTTATTAAAC AGCAGATTTG TGCTCACAAA

501 AAAAAAAAAA AAAAAAAAAA AAA
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Fig. 3. Nucleotide sequence of *B. variegata* skin preprobradykinin-1 (BVK-1) cDNA encoding a single copy of (Ala3,Thr6)-bradykinin (double underlined). The putative signal peptide (single underlined) and stop codon (asterisk) of the open-reading frame are indicated.

```
  1 ACGGGGGAGT GTCTGGTTGC TGAGCTCACA GTTCTGTAAC AAGTTTCACT

                 M  I  L  W  F  C  L  N  F  L  I  V  L
 51 TCCAGCTCTG ATAATGATAC TGTGGTTCTG TCTAAATTTC CTCATCGTCC

    C  L  E  H  F  P  G  T  L  A  A  E  R  N  V  P
101 TGTGCCTGGA GCATTTTCCA GGAACCCTGG CAGCTGAAAG AAATGTTCCA

    Q  S  E  E  K  T  E  Q  F  L  R  D  L  S  E  I  S
151 CAGAGTGAAG AAAAAACTGA GCAGTTCCTG CGGGATTTGT CTGAGATCAG

    .  R  L  Q  R  V  P  T  G  F  T  P  F  R  G  K  F  H
201 CCGCCTACAA AGAGTTCCAA CGGGGTTCAC CCCTTTCCGA GGAAAATTCC

    .. S  Q  S  L  R  G  L  S  E  T  K  K  F  K  A  P
251 ATAGCCAGTC CCTGCGGGGT TTGTCTGAGA CCAAGAAGTT TAAAGCTCCA

     Y  N  I  H  W  H  C  K  P  G  L  L  C  K  N  F  N
301 TACAATATCC ATTGGCACTG CAAACCGGGT TTGCTGTGTA AAAATTTCAA

      *
351 TTGAAAGTAG CGTCCCCTGG TATAAATAAG CATTGTTATG TCACATGGTG

401 TCACCTCTGT AATACCAGCT CTGACTGACA TGGTTTATTA AACAGCAGAT

451 TTGTCCTCAC TAAAAAAAAA AAAAAAAAAA AAAAAAAA
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Fig. 4. Nucleotide sequence of *B. variegata* skin preprobradykinin-2 (BVK-2) cDNA encoding a single copy of (Val1,Thr3,Thr6)-bradykinin (double underlined). The putative signal peptide (single underlined) and stop codon (asterisk) of the open-reading frame are indicated.

proteolysis involving Arg-X cleavages (except apparently where X = Pro) consistent with the known cleavage site specificity of a kallikrein-type endoproteinase [26] but also with known vertebrate prohormone convertases [27]. The occurrence of additional molecular variants of bradykinins, especially phyllokinin (bradykinyl-IYsulfate) and C-terminally extended forms in *Rana* frogs [12–15], would imply that the preprobradykinin organization, described here for *B. variegata*, either differs from that present in other anuran amphibians or that cleavage specificities of skin peptide precursor convertases are different. Only one amphibian skin bradykinin-related peptide precursor cDNA has been cloned to date [19]. Bombinakinin M (*syn.* maximakinin) is a N-terminal decapeptide-extended bradykinin from the skin secretion of the Chinese large-webbed bell toad, *Bombina maxima*. This precursor cDNA encodes six tandem repeat copies of the mature nonadecapeptide and interestingly, the convertase cleavage sites, both N- and C-terminally, are Arg-X, in common with those observed for the novel bradykinins in the present study. However, while the C-terminal cleavage site is identical to that observed in both precursors cloned in the present study, the N-terminal arginyl residue cleavage site has been substituted with a prolyl residue. The propeptide convertases in this species, appear to disregard the -Arg-Lys- doublet two positions proximal to this substitution and rather cleave at the C-terminal side of the single arginyl residue, seven positions proximal to this site (Table 2). This results in incorporation of the decapeptide extension on the bradykinin nonapeptide with an N-terminal aspartyl residue (Table 2). Strategically located single site amino acid substitutions can thus drastically affect the primary structure of

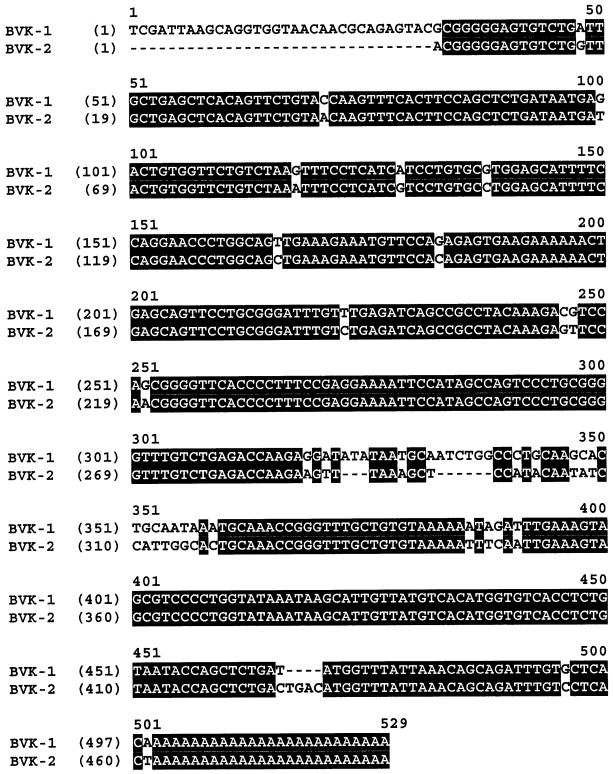

Fig. 5. Alignment of the nucleotide sequences of full-length clones of BVK-1 and BVK-2. Identical bases are shaded. The ALIGNX program of VECTOR NTI suite (Informax) was employed.

the mature peptide by altering propeptide convertase cleavage sites in the precursor as discussed. Each cloned precursor described in the present study, in contrast, generates a mature nonapeptide with internal amino acid substitutions that do not affect the appropriate propeptide convertase processing patterns. Additionally, although *B. maxima* is a congeneric of *B. variegata*, the latter species possesses skin bradykinin precursors that encode but single copies of mature peptides.

As previously stated, neither bombinakinin O (bradykinyl-GKFH) [16], a peptide isolated from *B. orientalis* skin extracts, nor canonical bradykinin, were detected in stimulated skin secretion from *B. variegata*. However, examination of cloned skin cDNA open-reading frames encoding both novel bradykinin-related peptides described here, indicates that in both cases, the coding sequences of mature peptides are flanked C-terminally in their respective precursors by the tetrapeptide sequence, -GKFH. The possibility was thus presented that cleavage at and removal of the lysyl residue in this sequence could expose

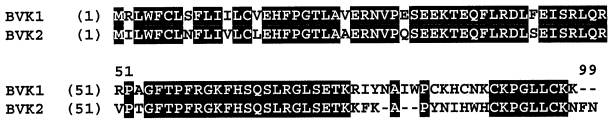

Fig. 6. Alignment of open-reading frame amino acid sequences of BVK-1 and BVK-2. Identical amino acid residues are shaded. ALIGNX programme of VECTOR NTI suite (Informax) employed.

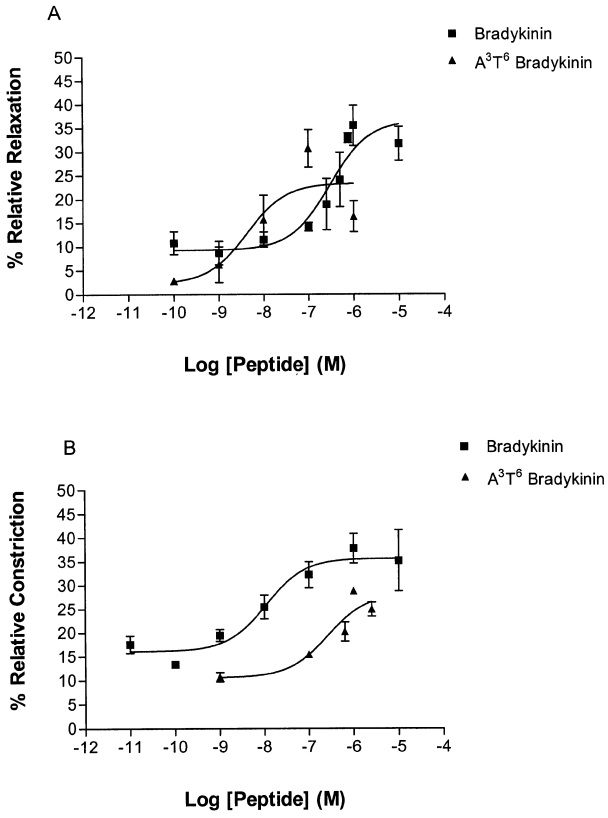

Fig. 7. Dose–response curves of bradykinin and (Ala3,Thr6)-bradykinin on rat arterial smooth muscle (A) and small intestinal smooth muscle (B) preparations. Each data point represents the mean ± SEM of six replicates.

a terminal glycyl residue that could function as an amide donor. That is, a possibility existed that both bradykinin-related peptides described here, could be C-terminally amidated. To exclude this possibility, each natural peptide was subjected to MS/MS fragmentation either alone or mixed with its appropriate synthetic C-terminal free acid replicate. The natural and synthetic peptides produced identical b and y fragment ion series indicating unequivocally that the natural forms were not C-terminally amidated. Carboxy-terminal amidation would have produced a y fragment ion series exhibiting a 1 Da decrement in m/z ratios. This sensitivity is well within the capability of the ES-MS system employed.

The primary structural diversity displayed within amphibian skin bradykinins may not simply be due to phylogenetic differences but may reflect a degree of specific predator-mediated natural selection. Other major bioactive peptides found in amphibian 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 [1]. Until quite recently, the primary structures of endogenous bradykinins in nonmammalian vertebrates were unknown. Activation of the circulatory kallikrein-kinin system in a small but representative sample of species and isolation of the generated kinin has established that canonical bradykinin

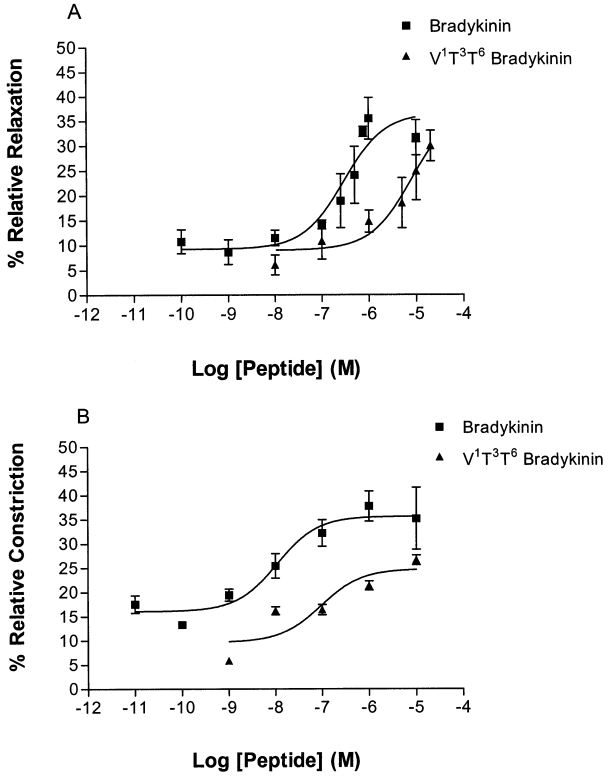

Fig. 8. Dose–response curves of bradykinin and (Val1,Thr3,Thr6)-bradykinin on rat arterial smooth muscle (A) and small intestinal smooth muscle (B) preparations. Each data point represents the mean ± SEM of six replicates.

is probably restricted to mammals [22]. Structure–activity studies using bradykinin analogues and alanine scan series, in a range of preparations of mammalian smooth muscle types, have indicated an almost absolute requirement for Arg1, Pro2, Gly4, Phe5, Pro7, Phe8 and Arg9 [25]. Interestingly, most of these residues are fully conserved in amphibian skin bradykinins with the exception of the (Leu8)-bradykinins from *R. palustris* [28], that in common with avian bradykinin, share this site substitution [22] (Table 2). However, these structure–activity data would explain the relatively low activity of (Val1,Thr3,Thr6)-bradykinin in both of the smooth muscle preparations employed in this study, in contrast to the activity observed for the Ala3,Thr6-substituted variant (Figs 7 and 8). Crocodilians, chelonians and varanid lizards possess (Thr6)-bradykinin whilst colubrid and crotalid snakes possess (Val1,Thr6)-bradykinin [22]. Indeed, a threonyl residue at position 6 appears to be present in virtually all bradykinins from nonmammalian, tetrapod vertebrates studied to date [22].

The freshwater habitats of amphibians possessing bradykinins in their defensive skin secretions would be rich in an array of predatory species predominantly from these taxa. We contend, on this basis, that the spectrum of bradykinins possessed by each species is reflective and perhaps, by natural selection of peptide libraries generated in dermal venom glands, dictated by predominant predators cohabiting the amphibian biotopes. *B. variegata*, from Europe, on this basis, would possess specific defence against chelonian and colubrid snake predators, both of which occur in the same habitats as the toad.

The focused study of bioactive peptides produced by the amphibian integument can thus provide much valuable information into structure–activity relationships and natural molecular evolutionary strategies that can be relevant to a broad range of disciplines within the biosciences.

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