

Characterization of Bradykinin-Related Peptides Generated in the Plasma of Six Sarcopterygian Species (African Lungfish, Amphiuma, Coachwhip, Bullsnake, Gila Monster, and Gray's Monitor)

Zhihong Li,* Stephen M. Secor,† Valentine A. Lance,‡ Maria A. Masini,§
Mauro Vallarino,§ and J. Michael Conlon*,¹

*Regulatory Peptide Center, Department of Biomedical Sciences, Creighton University School of Medicine, Omaha, Nebraska 68178; †Department of Physiology, University of California at Los Angeles School of Medicine, Los Angeles, California 90095; ‡Center for Reproduction of Endangered Species, Zoological Society of San Diego, San Diego, California 92112; and §Istituto di Anatomia Comparata, Università di Genova, 16132 Genoa, Italy

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Incubation of heat-denatured plasma from six species occupying different evolutionary positions within the Sarcopterygian lineage [the dipnoan, *Protopterus annectens* (African lungfish); the urodele, *Amphiuma tridactylum* (three-toed amphiuma); the colubrid snakes, *Pituophis melanoleucus sayi* (bullsnake) and *Masticophis flagellum* (coachwhip); and the lizards *Heloderma suspectum* (Gila monster) and *Varanus Grayi* (Gray's monitor)] with trypsin generated bradykinin-related peptides that were detected by radioimmunoassay using an antiserum raised against mammalian bradykinin (BK). The peptides were purified by HPLC and their primary structures were established as lungfish [Tyr¹,Gly²,Ala⁷,Pro⁸]BK, amphiuma [Phe¹,Ile²,Leu⁵]BK, bullsnake and coachwhip [Val¹,Thr⁶]BK, Gila monster [Leu²,Thr⁶]BK, and Gray's monitor [Thr⁶]BK. Monitor BK is identical to the peptide generated in turtle and alligator plasma and coachwhip/bullsnake BK shows one amino acid substitution (Ala¹ → Val) compared with the peptide generated in the plasma of the python. The data provide further evidence for the widespread occurrence of a kallikrein–kininogen system in nonmammalian vertebrates but indicate that the

primary structure of BK has been poorly conserved during evolution. © 1998 Academic Press

The kallikrein–kininogen system of mammals involves the sequential action of several well-characterized proteolytic enzymes. Activation of Factor XII (Hageman factor) by contact with a charged surface results in the activation of plasma prekallikrein and a subsequent generation of bradykinin (BK) by the cleavage of high molecular mass kininogen (reviewed in Damas, 1996). There is accumulating evidence for the existence of at least some components of the kallikrein–kininogen system in nonmammalian vertebrates (Conlon, 1998). Treatment of plasma from a chelonian, the turtle *Psuedemys scripta* (Conlon *et al.*, 1990), and from a crocodilian, the alligator *Alligator mississippiensis* (Comeau *et al.*, 1992), with glass beads in the presence of a kininase inhibitor generates [Thr⁶]BK. This suggests that the blood of these reptiles contained all the components of the kallikrein–kininogen found in mammals. In contrast, contact activation of plasma from the chicken (Kimura *et al.*, 1987) and python *Python reticulatus* (Conlon and Lance, 1994) did not generate a kinin, indicating the absence of a prekallikrein activator that is itself activated by glass beads,

¹ To whom correspondence and reprint requests should be addressed. Fax: (402) 280-2690. E-mail: jmconlon@creighton.edu.

but treatment of these plasmas with mammalian kallikrein or trypsin generated [Thr⁶,Leu⁸]BK and [Ala¹,Thr⁶]BK, respectively.

Evidence that at least some components of the kallikrein-kininogen system may predate the appearance of tetrapods was provided by the observations that incubation of heat-denatured plasma from the trout, *Oncorhynchus mykiss* (Conlon *et al.*, 1996), and cod, *Gadus morhua* (Platzack and Conlon, 1997), with porcine glandular kallikrein and/or trypsin generated [Arg⁰,Trp⁵,Leu⁸]BK. Similarly, treatment of heat-denatured plasma from the gar, *Lepisosteus osseus* (Ginglymodi), and bowfin, *Amia calva* (Halecomorphi), with trypsin generated [Trp⁵]BK (Conlon *et al.*, 1995). Incubation of plasma from the "lower" actinopterygian fish, the shovelnose sturgeon, *Scaphirhynchus platyrhynchus* (Acipenseriformes), with trypsin generated [Met¹,Met⁵]BK (Li *et al.*, 1998).

The clade Sarcopterygii includes three extant groups—the Tetrapoda (amniotes and lissamphibians), the Actinista (the coelacanth, *Latimeria*), and the Dipnoi (lungfish) (Forey *et al.*, 1991). Our knowledge of the structures of BK-related peptides in nonmammalian sarcopterygians is fragmentary. In this study, BK-related peptides were isolated from trypsin-treated plasma from the African lungfish (Dipnoi), an amphibian, the amphiuma (Caudata), two North American snakes, the bullsnake and coachwhip (Colubridae), and two lizards, Gila monster (Helodermatidae) and Gray's monitor (Varanidae), and characterized structurally. The nomenclature used in this article to indicate amino acid substitutions is based upon the structure of mammalian BK with [Arg⁰]BK referring to BK extended from its N-terminus by an arginine residue.

MATERIALS AND METHODS

Plasma Collection

Blood was taken from African lungfish (19 adult and juvenile specimens) (Conlon *et al.*, 1997) and amphiuma (6 adult specimens) (Conlon and Yano, 1995) as previously described. Blood was drawn from the caudal vein of adult specimens of bullsnakes and coachwhips that originated in Texas and Arizona. The blood was collected into a plastic tube containing

heparin and immediately centrifuged. Blood was collected postmortem from a single specimen of Gray's monitor and a single specimen of Gila monster, both of which were housed in the San Diego Zoo. All plasma samples were stored at -20°C .

Generation of the Kinins

Activation with trypsin was carried out by a modification of the method of Diniz and Carvalho (1963). Plasma (lungfish, 7 ml; amphiuma, 50 ml; bullsnake, 20 ml; coachwhip, 10 ml; Gila monster, 23 ml; monitor lizard, 8 ml) was diluted 10-fold with 0.2% (v/v) acetic acid and heated on a boiling water bath for 30 min. After cooling to 25°C , the pH of the mixture was adjusted to 7.8 by addition of 0.5 M Tris-HCl. The denatured plasma was incubated with 1-tosylamide 2-phenylethyl chloromethyl ketone-treated trypsin (Sigma, St. Louis, MO) (1 mg/ml plasma) for 30 min at 37°C and centrifuged (1600g for 20 min). Peptide material was isolated from the supernatant using Sep Pak C-18 cartridges as previously described (Conlon *et al.*, 1990). Bound material was eluted with 70% (v/v) acetonitrile/water and lyophilized.

Contact activation of plasma from the amphiuma and bullsnake was carried out as previously described (Conlon *et al.*, 1990). Plasma (5 ml) was mixed with 0.9% (w/v) NaCl (5 ml) containing 4 mM 1,10-phenanthroline. After adjustment of the pH to 7.4 using 0.1 M Tris-HCl buffer, the mixture was shaken with glass beads (2.5 g) for 60 min at 25°C . The incubation mixture was filtered on a sintered glass funnel and peptide material was isolated using Sep Pak C-18 cartridges.

Radioimmunoassay

BK-like immunoreactivity (BK-LI) was measured as previously described (Balks *et al.*, 1988) except that antiserum BT4 was used. This antiserum was raised against mammalian BK in rabbits in 1987 as described (Balks *et al.*, 1988), but, because of the poor sensitivity of the antiserum in radioimmunoassay (approximate $\text{IC}_{50} = 5 \text{ ng/ml}$), it was never characterized until recently. The antiserum was used in this study because it showed the strongest cross-reactivity with trout BK ([Arg⁰,Trp⁵,Leu⁸]BK) (approximate $\text{IC}_{50} = 8 \text{ ng/ml}$),

but the immunoreactivity in serial dilutions of trout BK did not diminish in parallel with the mammalian BK standard curve in radioimmunoassay. A series of analogs of trout BK have been synthesized in which each residue in the peptide has been replaced by alanine in order to study the binding properties of the BK receptor in trout stomach (Jensen and Conlon, 1998). Analogs containing the substitutions Pro³ → Ala, Trp⁵ → Ala, Ser⁶ → Ala, and Arg⁹ → Ala showed greatly reduced reactivity toward antiserum BT4, whereas substitutions at other positions had little effect on binding. It is concluded, therefore, that the epitope in mammalian BK recognized by antiserum BT4 involves contributions from residues 3, 5, 6, and 9.

Purification of the Kinins

The same procedure was used to purify BK-related peptides from all species studied and so only the purification of lungfish BK will be described in detail. The trypsin-treated plasma extract, after partial purification on Sep Pak cartridges, was redissolved in 1% (v/v) trifluoroacetic acid/water (3 ml) and chromatographed on a 90 × 1.6-cm column of Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 1 M acetic acid. The column was eluted at a flow rate of 24 ml/h and fractions (2 ml) were collected. Absorbance was measured at 280 nm. Aliquots of the fractions (100 µl) were lyophilized and reconstituted in the same volume of assay buffer (0.05 M sodium phosphate, pH 7.4, containing 0.4% (w/v) bovine serum albumin). The concentration of BK-LI in the fractions was determined by radioimmunoassay. Fractions containing BK-LI were pooled and injected onto a 25 × 1-cm Vydac 218TP510 C-18 reversed-phase-HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 14% over 10 min and to 35% over 50 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. The fraction containing BK-LI was rechromatographed on a 25 × 0.46-cm Vydac 214TP54 (C-4) column equilibrated with acetonitrile/water/trifluoroacetic acid (7.0/92.9/0.1) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 28% over 40 min using a linear gradient. Lungfish BK was purified to near

homogeneity by successive chromatographies on 25 × 0.46-cm Vydac 219TP54 (phenyl) and Vydac 218TP54 (C-18) columns. The concentration of acetonitrile in the eluting solvent was raised from 7 to 28% over 40 min and the flow rate was 1.5 ml/min.

Structural Analysis

Amino acid composition was determined by pre-column derivatization with phenyl isothiocyanate using an Applied Biosystems model 420A derivatizer, and separation of the phenylthiocarbamyl amino acid derivatives was determined by reversed-phase-HPLC. Hydrolysis in 5.7 M HCl (24 h at 110°C) of 1 nmol of peptide was carried out. The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin amino acids under gradient elution conditions as previously described (Conlon *et al.*, 1990). Mass spectrometry was performed on a Voyager RP MALDI-TOF instrument (Perspective Biosystems Inc., Framingham, MA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction, and the accelerating voltage in the ion source was 25kV. The accuracy of the mass determinations was within 0.05%.

RESULTS

Generation of BK-Related Peptides

The BK-related peptides generated in heat-denatured plasma from the six species by the action of trypsin were identified by their ability to inhibit the binding of [¹²⁵I]-labeled [Tyr⁰]BK to antiserum BT4. The antiserum was used to facilitate detection of the BK-related peptides during the chromatographic procedures. However, the immunoreactivity in serial dilutions of plasma from the lungfish, amphiuma, and both species of snake did not dilute in parallel with the mammalian BK standard, and so the radioimmunoassay could not be used for quantitation of the peptides.

Treatment of amphiuma and bullsnake plasma with glass beads under conditions previously shown to produce [Thr⁶]BK in turtle (Conlon *et al.*, 1990) and alligator plasma (Comeau *et al.*, 1992) did not generate BK-LI.

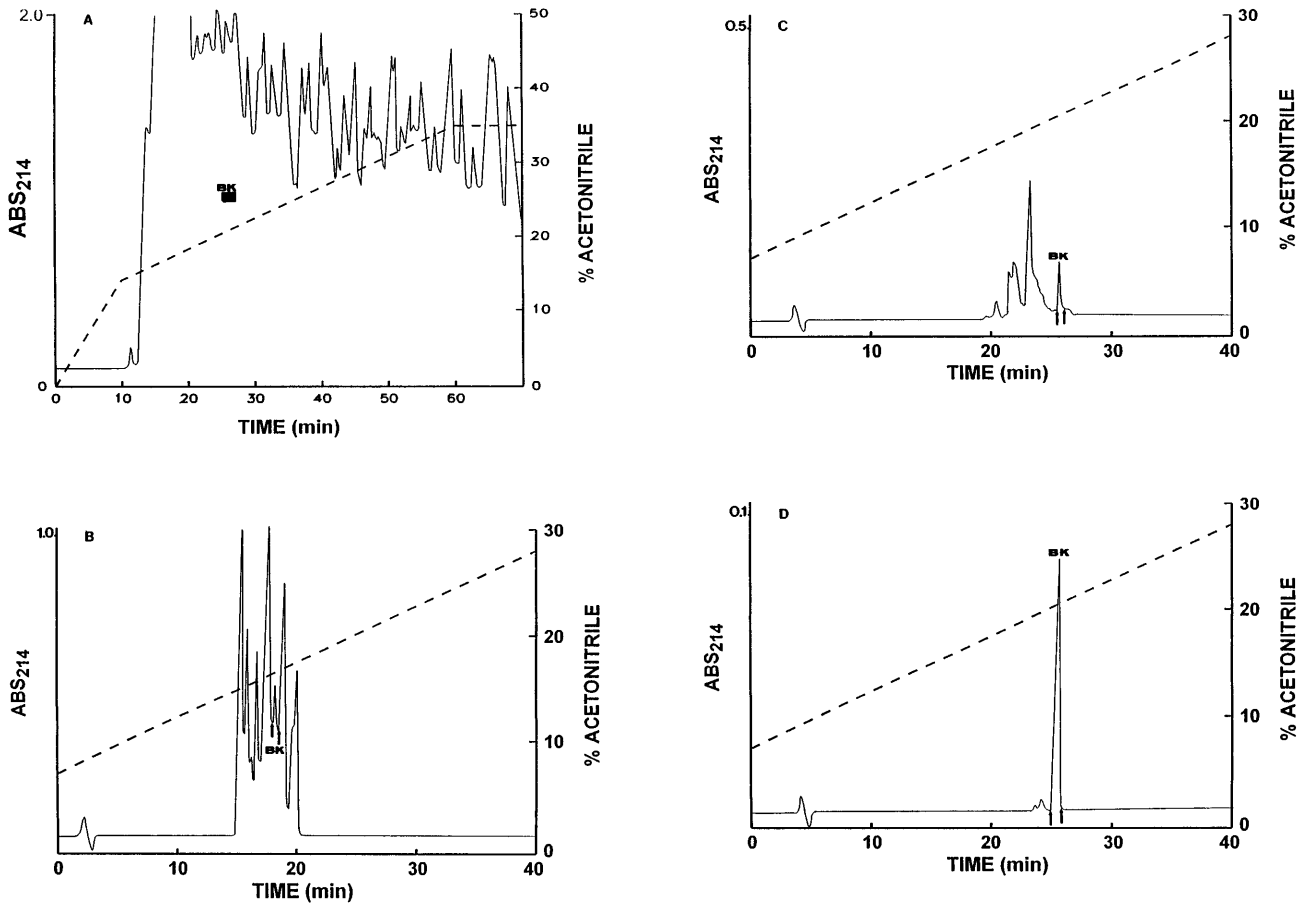


FIG. 1. Purification by reversed-phase-HPLC of lungfish bradykinin on (A) semipreparative Vydac C-18, (B) analytical Vydac C-4, (C) analytical Vydac phenyl, and (D) analytical Vydac C-18 columns. BK denotes the peaks containing bradykinin-like immunoreactivity. The arrows show where peak collection began and ended and (---) shows the concentration of acetonitrile in the eluting solvent.

Purification of the Bradykinin-Related Peptides

The BK-LI in the trypsin-treated lungfish plasma, after partial purification on Sep Pak cartridges, was eluted from a Sephadex G-25 gel permeation column as a broad, single peak with maximum immunoreactivity at the elution volume of mammalian BK. The immunoreactive fractions were pooled and injected onto a semipreparative Vydac C-18 HPLC column (Fig. 1A). BK-LI was eluted in the single fraction shown. Chromatography of this fraction on an analytical Vydac C-4 column (Fig. 1B) showed that the material was heterogeneous and the BK-LI was associated with the minor peak denoted by the bar. Further purification was accomplished on an analytical Vydac phenyl column (Fig. 1C), and the BK-LI was associated with the well-resolved late-eluting peak denoted by the

arrows. Lungfish BK was purified to near homogeneity by a final chromatography on an analytical Vydac C-18 column (Fig. 1D). The final yield of purified peptide was approximately 800 pmol.

BK-related peptides from the other species were purified to near homogeneity, as assessed by a symmetrical peak shape, using the same procedure, and the final yields of purified peptides were amphiuma, 8 nmol; bullsnake, 12 nmol; coachwhip, 4 nmol; Gila monster, 2 nmol; and Gray's monitor, 500 pmol. The low yield of BK from Gray's monitor plasma may be a consequence of the fact that it had been stored for 6 years at -20°C . A second peptide, identified by amino acid sequence analysis as $[\text{Leu}^2, \text{Thr}^6]\text{des-Arg}^1\text{-BK}$, was isolated from Gila monster plasma in yield of approximately 1 nmol.

	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg									Reference
Mammals										
Chicken	-	-	-	-	-	Thr	-	Leu	-	Kimura et al.,1987
Alligator	-	-	-	-	-	Thr	-	-	-	Comeau et al.,1992
Turtle	-	-	-	-	-	Thr	-	-	-	Conlon et al.,1990
Monitor	-	-	-	-	-	Thr	-	-	-	This study
Gila monster	-	Leu	-	-	-	Thr	-	-	-	This study
Python	Ala	-	-	-	-	Thr	-	-	-	Conlon and Lance,1994
Coachwhip	Val	-	-	-	-	Thr	-	-	-	This study
Bullsnake	Val	-	-	-	-	Thr	-	-	-	This study
Amphiuma	Phe	Ile	-	-	Leu	-	-	-	-	This study
Lungfish	Tyr	Gly	-	-	-	-	Ala	Pro	-	This study
Trout	-	-	-	-	Trp	-	-	Leu	-	Conlon et al.,1996
Cod	-	-	-	-	Trp	-	-	Leu	-	Platzack and Conlon,1997
Bowfin	-	-	-	-	Trp	-	-	-	-	Conlon et al.,1995
Gar	-	-	-	-	Trp	-	-	-	-	Conlon et al.,1995
Sturgeon	Met	-	-	-	Met	-	-	-	-	Li et al., 1998

FIG. 2. A comparison of the primary structures of bradykinin-related peptides generated in the plasma of species from different vertebrate taxa. (—) denotes residue identity.

Structural Characterization

The primary structures of the BK-related peptides were established without ambiguity by automated Edman degradation. In each case, it was possible to identify phenylthiohydantoin amino acids during nine cycles of operation of the sequencer, and the amino acid sequences are shown in Fig. 2. The amino acid composition of bullsnake BK [found: Gly 1.1 (1), Arg 1.1 (1), Thr 1.0 (1), Pro 3.0 (3), Val 0.9 (1), Phe 1.8 (2) residues/mol peptide]; coachwhip BK [found: Gly 1.2 (1), Arg 1.1 (1), Thr 1.2 (1), Pro 2.6 (3), Val 1.0 (1), Phe 1.8 (2) residues/mol peptide] and amphiuma BK [found: Ser 0.9 (1), Gly 1.1 (1), Arg 1.1 (1), Pro 1.8 (2), Ile 1.2 (1.0), Phe 2.7 (3) residues/mol peptide] are consistent with the proposed structures. The values in parenthe-

ses show the number of residues predicted from the sequence analysis data. The proposed structure of lungfish BK was confirmed by mass spectrometry (observed molecular mass, 951.1 ± 0.3 amu; calculated molecular mass, 951.3 amu).

DISCUSSION

This study extends our knowledge of the primary structures of the BK-related peptides generated in the plasma of nonmammalian species by the action of exogenous proteases. The amino acid sequences of these peptides are compared in Fig. 2. The BK-related peptide generated in the plasma of a varanid lizard,

Gray's monitor, is identical to that generated in the plasma of a chelonian (Conlon *et al.*, 1990) and crocodylian (Comeau *et al.*, 1992), whereas the peptide generated in the plasma of the Gila monster (family Helodermatidae) contains the substitution (Pro² → Leu). It is generally accepted that snakes evolved from lizards and the recent identification of the mid-Cretaceous fossil *Pachyrhachis problematicus* as a snake with legs suggests that mosasauroids (a group of extinct marine lizards) and snakes are sister taxa (Caldwell and Lee, 1997). The data demonstrate that plasmas from the more highly derived bullsnake and coachwhip (family Colubridae), like that from the relatively more primitive python (family Boidea), contain a kininogen that is cleaved by trypsin to generate a BK that is distinct from mammalian BK. Like the python, but unlike the turtle and alligator, plasma from a colubrid snake does not contain a prekallikrein activator, such as Factor XII, that is itself activated by a glass surface. The mechanism by which prekallikrein in snake plasma is activated remains to be elucidated. The common primary structure of bullsnake and coachwhip BK differs from python BK by the single substitution (Ala¹ → Val) and from mammalian BK by the additional substitution (Ser⁶ → Thr). The peptide [Val¹, Thr⁶]BK has been isolated from the skin of the Japanese frog, *Rana nigromaculata* (Nakajima, 1968), but a recent study (Conlon and Aronsson, 1997) has suggested the biosynthesis of BK-related peptides in frog skin does not involve the kallikrein-kininogen system. A threonine residue at position 6 is not present in peptides isolated from the plasma of the lungfish, amphiuma, and the actinopterygian species, suggesting that the substitution (Ser⁶ → Thr) occurred in an ancestor of the amniotes.

The present study has led to the first structural characterization of a BK-related peptide that has been generated in the plasma of an amphibian. Although the skins of numerous species of frogs contain high concentrations of BK-related peptides (Pisano, 1979), evidence that amphibian blood may be associated with a kallikrein-kininogen system has been lacking. For example, incubation of heat-denatured amphiuma plasma with pig pancreatic kallikrein generated a very high concentration of an oxytocic agent (equivalent to 2.9 µg of BK/ml of plasma) that contracted the isolated rat uterus (Dunn and Perks, 1975), but our earlier attempts to characterize this substance led to the

isolation of angiotensin II not BK (Conlon and Yano, 1995). The amphiuma peptide contains a leucine residue at position 5 compared with phenylalanine in all BK-related peptides isolated from sarcopterygians and tryptophan in all peptides isolated from actinopterygians, except the sturgeon which contains methionine. It is noteworthy that the amino acid substitutions (Trp → Phe) and (Met → Phe) cannot be accomplished by a single base change in the nucleotide sequence of the codon specifying the amino acids but the substitutions (Leu → Trp), (Leu → Phe), and (Leu → Met) can be accomplished by single-base transformations. This observation invites speculation that the "ancestral" BK may have contained a leucine at position 5.

Phylogenetic relationships among the Sarcopterygii, particularly the evolutionary relationships between the coelacanth, lungfishes, and tetrapods, have been a matter of controversy, but paleontological (Panchen and Smithson, 1987; Forey *et al.*, 1991), morphological (Cloutier and Ahlberg, 1996; Vallarino *et al.*, 1996), and molecular (Meyer and Wilson, 1990; Hedges *et al.*, 1993) analyses generally favor the hypothesis that lungfish and tetrapods are sister groups. This study has shown that trypsin treatment of lungfish plasma generates a BK-related peptide, which resembles the peptide from the amphiuma in that it contains an aromatic amino acid at position 1 and a substitution of the strongly conserved proline residue at position 2. The presence of glandular kallikrein-like activity (kininogenase) and kininase in the kidney of *P. annectens* has been demonstrated previously (Masini *et al.*, 1996).

The biological activity of the BK-related peptides in the species from which they were isolated is unknown. Previous work has demonstrated that the substitution (Ser⁶ → Thr) has little or no effect on the cardiovascular actions of BK in the turtle or rat (Conlon *et al.*, 1990). In contrast, [Ala¹]BK was only one-tenth as potent as BK in lowering blood pressure in the rat following intraarterial injection of the peptides (Regoli and Barabe, 1980). This suggests that the changes in the N-terminal region of the BK-related peptides from lungfish, amphiuma, and colubrid snakes may appreciably attenuate their cardiovascular actions in mammals. Consistent with this prediction, we have shown that bolus intraarterial injections of a synthetic replicate lungfish BK in doses up to 100 nmol into an anesthetized dog produce no change in arterial pres-

sure or blood flow in the bronchial artery (T. Pissari and J. M. Conlon, unpublished data). However, there is increasing evidence that the pharmacological properties of kinin receptors in nonmammalian vertebrates are appreciably different from the corresponding receptors in mammals. For example, mammalian BK has no effect on blood pressure in the cod, whereas cod BK is potently hypertensive in this species (Platzack and Conlon). Similarly, trout BK contracts isolated smooth muscle from the trout stomach and small intestine, whereas mammalian BK is without effect (Jensen and Conlon, 1997). Thus, it is clearly worthwhile to study the biological activity of lungfish BK in the lungfish.

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