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Bradykinin-related peptides (BRPs) from skin secretions of three genera of phyllomedusine leaf frogs and their comparative pharmacological effects on mammalian smooth muscles☆

Yingchun Jiang[a,b,1], Xinping Xi[a,1], Lilin Ge[a,1], Nan Yang[a], Xiaojuan Hou[a], Jie Ma[a], Chengbang Ma[a], Yuxin Wu[a,b,c], Xiaoxiao Guo[a], Renjie Li[a,*], Mei Zhou[a], Lei Wang[a,*], Tianbao Chen[a], Chris Shaw[a]

[a] Natural Drug Discovery Group, School of Pharmacy, Queen's University, Belfast BT9 7BL, Northern Ireland, UK
[b] School of Medicine, Shenzhen University, PR China
[c] Division of Molecular Cardiology, Department of Internal Medicine, College of Medicine, Texas A&M University Health Science Center, Central Texas Veterans Health Care System, Temple, TX 76504, USA

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ABSTRACT

While bradykinin has been identified in the skin secretions from several species of amphibian, bradykinin-related peptides (BRPs) are more common constituents. These peptides display a plethora of primary structural variations from the type peptide which include single or multiple amino acid substitutions, N- and/or C-terminal extensions and post-translational modifications such as proline hydroxylation and tyrosine sulfation. Such modified peptides have been reported in species from many families, including Bombinatoridae, Hylidae and Ranidae. The spectrum of these peptides in a given species is thought to be reflective of its predator profile from different vertebrate taxa. Here we report the isolation of BRPs and parallel molecular cloning of their respective biosynthetic precursor-encoding cDNAs from the skin secretions of the Mexican leaf frog (*Pachymedusa dacnicolor*), the Central American red-eyed leaf frog (*Agalychnis callidryas*) and the South American orange-legged leaf frog (*Phyllomedusa hypochondrialis*). Additionally, the eight different BRPs identified were chemically synthesized and screened for bioactivity using four different mammalian smooth muscle preparations and their effects and rank potencies were found to be radically different in these with some acting preferentially through bradykinin B_1-type receptors and others through B_2-type receptors.

1. Introduction

The discovery of bradykinin (BK) has its roots in venom research going back as far as 1949 when Rocha et al. incubated trypsin or whole snake venom with plasma proteins [18]. The resultant digests were found to stimulate certain types of smooth muscle preparations, including the uterus and ileum of guinea pigs and rats and the intestine of rabbits. Moreover, when the digest was injected into the veins of rabbits and cats, a fall in arterial blood pressure was observed. The active agent (BK) was thus considered to be a general mammalian hypotensive and myotropic agent that could be used in the treatment of hypertension [19].

BK is biosynthesized in the liver as a relatively small domain within the structures of higher molecular weight protein precursors (the kininogens). The levels of BK in plasma and tissue fluids under normal physiological conditions are low and these are increased through the actions of certain proteases (kallikreins) on kininogens at sites of trauma, inflammation or infection [1,17]. However, the biosynthesis of BK and BK-related peptides (BRPs) in the skins of various amphibians, follows a quite different pathway to that observed in the plasma and tissues of mammals (the kallikrein-kinin system) [1,9,17].

Bradykinin and BRPs are encoded, like the majority of other groups of amphibian skin peptides, by precursor proteins that have a highly ordered and highly conserved structure consisting of an N-terminal signal peptide domain, followed by an acidic

☆ The nucleotide sequences of clones encoding respective BRP precursors from the skin secretions of the Mexican leaf frog (*Pachymedusa dacnicolor*), the Central American red-eyed leaf frog (*Agalychnis callidryas*) and the South American orange-legged leaf frog (*Phyllomedusa hypochondrialis*), have been deposited in the EMBL Nucleotide Sequence Database under the accession codes HE967329 through HE967331.

* Corresponding authors at: Natural Drug Discovery Group, School of Pharmacy, Queen's University, Belfast BT9 7BL, Northern Ireland, UK. Tel.: +44 02890 972200; fax: +44 02890 247794.
E-mail addresses: r.li@qub.ac.uk (R. Li), l.wang@qub.ac.uk (L. Wang).

[1] These authors contributed equally to this work.

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amino acid residue-rich (spacer) domain and terminating in a hypervariable mature peptide (BRP)-encoding domain [3–8,20,22]. Many amphibian skin BRP-encoding precursors, while having the N-terminal signal peptide domain, have multiple tandem-repeats of acidic spacer peptide/BRP-encoding domains, the latter either encoding identical BRPs or a variety of such [3–8,20,22].

The BRPs of amphibian skin occur in an astonishing diversity of primary structures in addition to the original nonapeptide [3–8,20,22]. This structural diversity includes single or multiple amino acid substitutions within the core nonapeptide sequence, extensions of various lengths at the N- and/or C-terminals, and one or more post-translational modifications which include hydroxylation of prolyl residues and/or sulfation of tyrosyl residues [7,8]. BRPs are of widespread distribution in amphibian skin, occurring in representative species of the major families, Ranidae and Hylidae and the minor families, Bombinatoridae and Ascaphidae [3–10,20,22].

Neotropical hylid frogs of the sub-family, Phyllomedusinae, have long been considered to produce among the most chemically complex and biologically potent skin secretions of any amphibian taxon [12]. Thus, in this report, BRPs have been studied in the skins of three species of phyllomedusine frogs representative of three genera within the sub-family, namely, *Pachymedusa dacnicolor* (Mexico), *Agalychnis callidryas* (most of Central America) and *Phyllomedusa hypochondrialis* (most of Northern South America). A functional genomic approach was employed that consisted of "shotgun" molecular cloning of skin BRP precursor-encoding cDNAs, prediction of mature BRP sequences from these templates, localization of the predicted peptides in HPLC fractions of skin secretion and confirmation of their structures using mass spectrometric techniques, their solid-phase chemical synthesis and finally, their pharmacological evaluation using four different mammalian smooth muscle preparations.

Using this approach, eight different BRPs were identified resulting from differential processing of respective precursors and post-translational modification of products. Pharmacological evaluation of these BRPs in the mammalian smooth muscle preparations revealed substantial differences in their potencies and target receptors in a tissue-dependent manner.

2. Materials and methods

2.1. Specimen biodata and secretion harvesting

Specimens of *Pachymedusa dacnicolor*, *Agalychnis callidryas* and *Phyllomedusa hypochondrialis* ($n = 3$ in each case) were obtained from commercial sources and were kept for a period of 4 months prior to secretion harvesting. They were maintained in our purpose-designed amphibian facility at 18–25 °C under a 12/12-h light/dark cycle and fed multivitamin-loaded crickets three times per week. Defensive skin secretions were obtained from each specimen by either gently squeezing the paired parotoid and tibial glands or by massaging the dorsal skin with a latex-gloved finger. Secretions were then washed from the skin using deionized water, snap-frozen in liquid nitrogen and lyophilized. Lyophilizates were stored at −20 °C prior to analysis.

2.2. "Shotgun" cloning of BRP precursor-encoding cDNAs

Five mg of lyophilized skin secretion from each species were separately dissolved in 1 ml of cell lysis/mRNA stabilization solution (Dynal, UK). Polyadenylated mRNA was isolated from this stabilization solution using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK) and reverse-transcribed. Briefly, the 3′-RACE reactions employed an NUP primer (supplied with the kit) and a degenerate sense primer pool (3′-RACE; 5′-CCRVCNGGGTTYASSCCWTTY-3′) (R = A/G, V = A/C/G, N = A/C/T/G, Y = C/T, S = C/G, W = A/T) that was complementary to the nucleic acid sequence encoding the—PPGFSPF—and—PPGFTPF—internal amino acid sequences of skin bradykinin-like peptide (BRP) precursor transcripts from other phyllomedusine frog species [7,8]. The products of 3′-RACE reactions were gel-purified and cloned using a pGEM-T vector system (Promega Corp.) and sequenced using an ABI 3100 automated sequencer. Following acquisition of these data, a degenerate antisense primer pool (AS1, 5′-GCTCYTBAGATTAYSGCATSYTACWTT-3′) (B = T/C/G) was designed to a site in the 3′-untranslated region and was employed in 5′-RACE reactions. Products were likewise gel-purified, cloned and sequenced as described above.

2.3. Identification and structural analyses of deduced BRP homologs

Samples (5 mg) of lyophilized skin secretion from each species were separately dissolved in 1 ml of 0.05/99.95 (v/v) trifluoroacetic acid (TFA)/water and were clarified of microparticulates by centrifugation ($1500 \times g$) for 10 min. The clear supernatants were then each separately pumped directly onto a reverse phase HPLC column and peptides were eluted using a gradient formed from 0.05/99.95 (v/v) TFA/water to 0.05/19.95/80.00 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 1 ml/min. A Cecil CE4200 Adept (Cambridge, UK) gradient reverse phase HPLC system, fitted with an analytical column (Phenomenex C-5, 0.46 cm × 25 cm) was employed and fractions were collected automatically at 1 min intervals. Dead volume between column and fraction collector was minimal (20 μl). The molecular masses of peptides in each chromatographic fraction were determined by using matrix-assisted, laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using α-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as ±0.1%. The peptides with masses coincident with those of BRPs deduced from cloned precursor cDNAs, were each subjected to primary structural analyses by MS/MS fragmentation sequencing using an LCQ Fleet electrospray ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA).

2.4. Solid-phase peptide synthesis

Replicates of the eight different BRPs identified were chemically synthesized by solid-phase Fmoc chemistry using a PS3 automated solid-phase synthesizer (Protein Technologies, Inc., AZ, USA). Following cleavage from the resin and subsequent deprotection of side-chains, each synthetic peptide was analyzed by both reverse phase HPLC and MALDI-TOF mass spectrometry to establish both degree of purity and authenticity of structure. For pharmacological experiments, standardization of the synthetic peptide was achieved by acid hydrolysis of a known gravimetric quantity of lyophilizate followed by amino acid analysis using an Applied Biosystems PTH-amino acid analyzer.

2.5. Smooth muscle pharmacology

Adult male Wistar rats, weighing 200–250 g, were killed by asphyxiation with CO_2 followed by cervical dislocation in accordance with institutional animal experimentation ethics and UK animal research guidelines.