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A novel bradykinin-like peptide from skin secretions of the frog, *Rana nigrovittata*

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Abstract: A bradykinin-like peptide has been isolated from the skin secretions of the frog *Rana nigrovittata*. This peptide was named ranakinin-N. Its primary structure, RAEAVPPGFTPFR, was determined by Edman degradation and mass spectrometry. It is structurally related to bradykinin-like peptides identified from skin secretions of other amphibians. Ranakinin-N is composed of 13 amino acid residues and is related to the bradykinin identified from the skin secretions of *Odorrana schmackeri*, which is composed of 9 amino acid residues. Ranakinin-N was found to exert concentration-dependent contractile effects on isolated guinea pig ileum. cDNA sequence encoding the precursor of ranakinin-N was isolated from a skin cDNA library of *R. nigrovittata*. The amino acid sequences deduced from the cDNA sequences match well with the results from Edman degradation. Analysis of different amphibian bradykinin cDNA structures revealed that the deficiency of a 15-nucleotide fragment (agaatgatcagacgc in the cDNA encoding bradykinin from *O. schmackeri*) in the peptide-coding region resulted in the absence of a dibasic site for trypsin-like proteinases and an unusual -AEVA- insertion in the *N*-terminal part of ranakinin-N. The -AEAV- insertion resulted in neutral net charge at the *N*-terminus of ranakinin-N. Ranakinin-N is the first reported bradykinin-like peptide with a neutral net charge at the *N*-terminus. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: amphibian; bradykinin-like peptide; ranakinin-N; *Rana nigrovittata*; skin

INTRODUCTION

Over the past several decades, numerous studies have focused on the bioactive compounds present in amphibian skin secretions. These compounds include many peptides [1], which can generally be classified as having either (i) regulatory or hormonal functions or (ii) antimicrobial activity. The peptides in the first group are analogs of mammalian hormones and neurotransmitters [2]. The antimicrobial peptides are an important part of the amphibian innate immune system [3].

An important family of bioactive compounds having regulatory or hormonal functions from amphibian is the bradykinin-like peptide family, which is a counterpart of mammalian bradykinins. In the mammalian blood system, bradykinin is generated by the kallikrein–kinin system, which participates in a broad spectrum of biological activities and events in pathophysiological conditions [4,5]. To date, more than ten bradykinin-like peptides that are structurally and functionally related to mammalian bradykinin have been isolated from amphibian skin secretions [6–14].

Ranidae is the largest family of amphibians. Many bradykinin-like peptides and their biosynthetic precursor (preprobradykinin) have been identified from *Rana* and *Bombina* amphibians following our first report of the cDNA encoding an amphibian bradykinin peptide from *Bombina maxima*. No bradykinin-like peptide has been reported from *Rana nigrovittata*. In this study, we describe the isolation, characterization, cDNA cloning and biological activities of a novel bradykinin-like peptide derived from skin secretions of *R. nigrovittata*.

MATERIALS AND METHODS

Collection of Frog Skin Secretions

Adult specimens of *R. nigrovittata* of both sexes ($n = 30$; weight range 30–40 g) were collected in the Yunnan Province of China and skin secretions were collected as follows: Frogs were put into a cylindrical container, a piece of absorbent cotton immersed with anhydrous ether was put on the top of the container and the container was covered with a lid and permeated with volatilized anhydrous ether. Being stimulated by anhydrous ether for 1–2 min, the frog skin surface was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each frog with 0.1 M NaCl solution (containing 0.01 M EDTA). The collected solutions (500 ml total volume) were quickly centrifuged at 5000 *g* for 20 min and the supernatants were lyophilized.

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Peptide Purification

The lyophilized skin secretion sample of *R. nigrovittata* (1.2 g, total $OD_{280\ nm}$ of 300) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the elute was monitored at 280 nm. The contractile activity of the fractions on isolated guinea pig ileum was determined as indicated below under 'Bioassay'. The protein peak containing bradykinin-like activity was pooled (30 ml), lyophilized and resuspended in 2 ml 0.1 M phosphate buffer, pH 6.0, and purified further by C_{18} reverse-phase high-performance liquid chromatography (RP-HPLC, Hypersil BDS C_{18}, 30 × 0.46 cm) column as illustrated in Figure 1(B).

Bioassay

Bradykinin activity was tested by assaying the contractile activity on isolated guinea pig ileum, mainly as described [10]. About 10 cm of the distal ileum of guinea pig of either sex (150–250 g body weight) was removed immediately after death and washed with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.04 mM D-glucose). Cut segments of 2 cm of the isolated ileum were mounted isotonically, under 1 *g* load, in a 5 ml muscle bath containing Tyrode solution maintained at 37 °C and bubbled with air. PcLab software package was used for the collection and analysis of the biological signal (Beijing Microsignalstar Technology Development Co.Ltd.). The peptides were quantified by UV absorbance at 215 and 225 nm using the formula: concentration (mg/ml) = $(A_{215} - A_{225}) \times 0.144$.

Structural Analysis

Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Fast atom bombardment (FAB) mass spectrometry was carried out on an Autospec-3000 spectrometer, equipped with a high-field magnet, using glycerol : 3-nitrobenzyl alcohol : dimethyl sulphoxide (1 : 1 : 1, v : v : v) as the mixed matrix. The ion gun was operated at 25 kV with a current of 1 μA, using Cs^+ as the bombarding gas.

Construction and Screening of a cDNA Library

Standard recombinant DNA techniques were used as described [15]. mRNAs were prepared from the total RNA of *R. nigrovittata* skin by oligo(dT) cellulose chromatography. A directional cDNA library was constructed with a plasmid cloning kit (SuperScript Plasmid System, GIBCO/BRL) following the instructions of the manufacturer, producing a library of about 2.3×10^5 independent colonies.

A PCR-based method [15] for high-stringency screening of DNA libraries was used for screening and isolating the clones with some modifications. Two oligonucleotide primers, S_1 (5′-AAGATGTTCACCTTGAAGGAATC-3′ according to the signal sequence of the cDNA encoding bradykinin from *Odorrana schmackeri*, in the sense direction) and a vector SP_6 promoter primer (5′-CATACGATTTAG GTGACACTATAG-3′, in the antisense direction) located in the 3′ part of the cloned insert were used in PCR reactions. All the oligonucleotide primers for PCR were prepared with a DNA synthesizer (Model 381A, Applied Biosystems). The PCR conditions were, 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C and 40 s at 72 °C. DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Microorganism Strains and Susceptibility Testing

Microorganisms including Gram-positive bacterium *Staphylococcus aureus* (ATCC2592), Gram-negative bacteria *Escherichia coli* (ATCC25922), *Bacillus dysenteriae* and fungus *Candida albicans* (ATCC2002) were obtained from Kunming Medical College and were first grown in Luria–Bertani (LB) broth or yeast extract–peptone–dextrose broth as in our previous report [3]. The minimal inhibitory concentrations (MICs) of the antimicrobial peptide against tested microorganisms were determined as in previous reports. The MIC was defined as the lowest concentration of test peptides inhibiting microorganism growth.

RESULTS

Purification of Bradykinin-like Peptide

The supernatant of *R. nigrovittata* skin secretions was fractionated into several peaks by Sephadex G-50 as illustrated in Figure 1(A), and the contractile activity of fractions on isolated guinea pig ileum was measured and shown to occur in the peak marked by a horizontal bar. The fractions with contractile activity on isolated guinea pig ileum were combined and applied to an RP-HPLC column, and more than 10 peaks were obtained from this separation as indicated in Figure 1(B). The peak with contractile activity on isolated guinea pig ileum (marked by an arrow) was collected.

Structural Characterization

The purified bradykinin-like peptide (indicated by an arrow, in Figure 1(B)), named 'ranakinin-N', was subjected to amino acid sequence analysis by automated Edman degradation. The amino acid sequence of ranakinin-N was RAEAVPPGFTPFR, composed of 13 amino acid residues. Its molecular weight was 1442.5 by FAB mass spectrometry analysis. This molecular weight matched well with the theoretical molecular weight (1442.8). There is a conserved PPGF motif as found in other bradykinin-like peptides. Compared with bradykinin composed of 9 amino acid residues (RPPGFSPFR), ranakinin-N has an insert of -AEAV- in its *N*-terminus. Analysis using the ExPASy MW/pI tool (http://www.expasy.ch/tools/pi_tool.html) showed that it has a predicted pI of 9.6. Ranakinin-N showed no antimicrobial activity in our experiments, although it is basic peptide with a positive charge.

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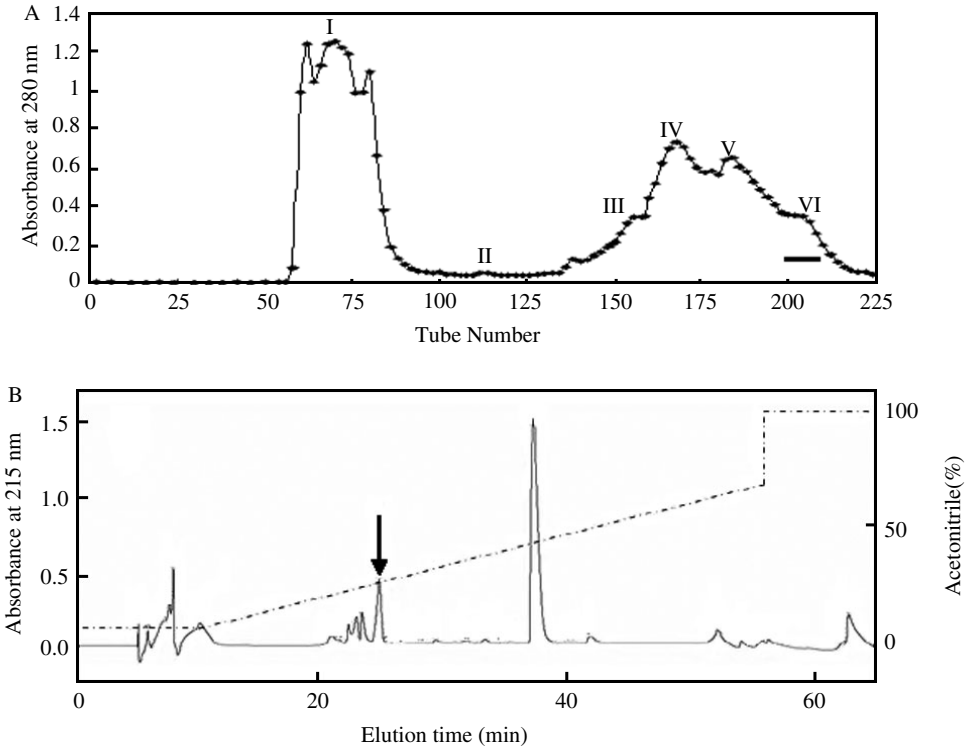

Figure 1 Fractionation of *R. nigrovittata* skin secretion. (A) Sephadex G-50 gel filtration of *R. nigrovittata* skin secretion. *R. nigrovittata* skin secretion was applied on a Sephadex G-50. (B) The fractions (indicated by a horizontal bar) with bradykinin-like activity from Sephadex G-50 were further purified on a Hypersil BDS C_{18} RP-HPLC column. The purified bradykinin-like peptide is indicated by an arrow.

cDNA Cloning

Upon screening of a skin cDNA library as indicated, several clones containing inserts of around 300 base pairs were identified and isolated. Both strands of these clones were sequenced (Figure 2). The complete nucleotide sequence of ranakinin-N and the deduced amino acid sequence are shown in Figure 2. The overall structure of ranakinin-N cDNA is similar to that of the cDNAs encoding bradykinin from other ranid amphibians. It was found to contain a coding region of 156 nucleotides. The encoded amino acid sequence corresponds to a polypeptide of 51 amino acids. Sequence identity between the cDNA from *R. nigrovittata* and the cDNA from *O. schmackeri* [16] is over 70%. However, some distinct differences were also observed. In the middle segment ranging from nucleotides 145–159, a deletion of the 15-nucleotide fragment (agaatgatcagacgc in the cDNA encoding bradykinin from *O. schmackeri*) in the peptide-coding region results in the absence of a dibasic site for trypsin-like proteinases. Furthermore, there is an unusual -AEAV- insertion in the *N*-terminal part of ranakinin-N. Ranakinin-N precursor possesses the same processing sites at its *C*-terminus as those of bradykinin-like peptides from other ranid species but they have different extensions in their *C*-terminus, suggesting that they do not share the same processing pathways. Ranakinin-N is cleaved from its precursor at its amino terminus following Lys-Arg residues (Figure 2).

Myotropic Effects on Isolated Guinea Pig Ileum

The contractile activities of ranakinin-N and amolopkinin were evaluated in isolated guinea pig ileum in aerated Tyrode's solution at 37 °C. To a 5 ml organ bath, 0.075, 0.15, 0.3, 0.6 and 1.2 nM ranakinin-N or amolopkinin was added at 10 min intervals serially. As shown in Figure 3, those two peptides were found to elicit concentration-dependent contractile effects on isolated guinea pig ileum. At the same concentrations, amolopkinin had stronger contractile ability on isolated guinea pig ileum than ranakinin-N.

DISCUSSION

Like bradykinin, ranakinin-N was found to possess contractile activity as tested by using smooth muscle preparation isolated from guinea pig ileum. However, the physiological role played by ranakinin-N is not entirely clear. It is interesting to note the four-residue segment -AEAV- insertion in the *N*-terminal part of bradykinin. The -AEAV- insertion resulted in neutral net charge at the *N*-terminus of ranakinin-N. All other known bradykinin-like peptides have positive net

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B1 atgttcaccatgaagaaatccctgttactccttttctttcttgggaccatctccatgtct  60
B2 *********t*****g**********************************aa*tt*tct
B1  M  F  T  M  K  K  S  L  L  L  L  F  F  L  G  T  I  S  M  S   20
B2  *  *  *  L  *  E  *  *  *  *  *  *  *  *  *  *  *  N  L  *
B1 ctctgtgaagaaaagagagatgccgatgaagaagaaactgaaggggaagctaaaatggaa 120
B2 ******a*gc**g**********t********c****a****c**********gt****
B1  L  C  E  E  K  R  D  A  D  E  E  E  T  E  G  E  A  K  M  E   40
B2  *  *  K  Q  E  *  *  *  *  *  D  *  N  *  R  *  *  *  V  *
B1 gacataaaaagagcggaagccgtc----------------ccacctggatttactccattt 180
B2 **tg*t**********g*tactcaagaatgatcagacgc********g****gc******

B1  D  I  K  R  A  E  A  V  _  _  _  _  _  P  P  G  F  T  P  F   48

B2  *  V  *  *  *  G  Y  S  R  M  I  R  R  *  *  *  *  S  *  *
B1 cgtaaaccgtaacaaagtttaaacttgaattggaaatcatctgatgtggaacatttagct  240
B2 ***gttg*acctgcgtcttctctc
B1  R  K  P  •
B2  *  V  A  P  A  S  S  L
B1 aaatgcacaacagatgtctagaaaaaaaattaaataaaaaggtcacacaaaaaaaaaaaaa 300
B1 aaaaaaaaaaaaaaaaa                                             303
```

Figure 2 The nucleotide sequences encoding ranakinin-N and bradykinin from *O. schmackeri* (see Ref. 16) and the deduced amino acid sequences of the precursor polypeptides. The sequence of mature ranakinin-N is boxed. B1: nucleotide sequence encoding ranakinin-N; B2: The nucleotide sequence encoding bradykinin from *O. schmackeri* (see Ref. 12). Gaps (–) have been introduced to optimize the sequence homology. The asterisk (*) indicates the same amino acids or nucleotides. ● indicates the stop codon.

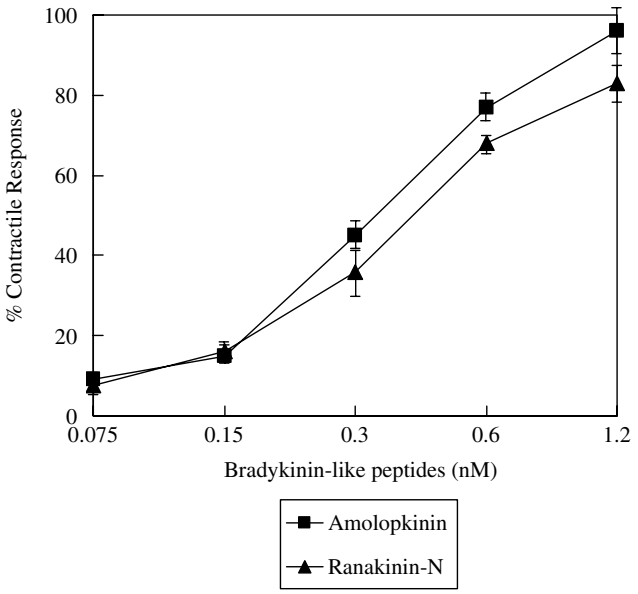

Figure 3 Concentration–response curves of ranakinin-N and amolopkinin (see Ref. 13) on isolated rat ileum. The ileum was stimulated with increasing concentrations of ranakinin-N previously incubated for 10 min, at 30 °C, with 1 Tyrode solution. Each point represents the mean ±SEM of five different experiments. The 100% contractile activity was the contractile ability of the ileum induced by 2 nM ranakinin-N.

charges. Ranakinin-N is the first reported bradykinin-like peptide with a neutral net charge at the *N*-terminus. Most of the known bradykinin-like peptides comprise bradykinin extended from its *N*-terminus or its *C*-terminus by an amino acid residue segment except amolopkinin found in *Amolops loloensis* [17]. Ranakinin-N reported here comprises a bradykinin with an insertion in its *N*-terminal part.

The generation of bradykinin in mammalian blood system by the action of kallikrein–kinin system has been well documented [4,5]. Bradykinin is a hydrolysis product produced by limited proteolysis of kallikrein on kininogens. There are three types of kininogens in mammals: high-molecular-weight and low-molecular-weight kininogens and T-kininogens [12,18,19]. They are single-chain glycoproteins consisting of three domains: a bradykinin moiety, an *N*-terminal heavy chain and a *C*-terminal light chain, bridged by a disulfide linkage. The light chain of the high-molecular-weight kininogen is involved in an initial step of the endogenous clotting cascade. Additionally, the heavy chain of kininogen may act as a cysteine proteinase inhibitor. All bradykinin-like peptides and their precursors from mammals are highly homologous. It suggests that mammals share similar or the same mode of biosynthesis of bradykinin. Different from mammalian bradykinin and its precursor, amphibian bradykinin-like peptides and their precursors are extremely variable (Figure 4). Amphibian bradykinin-like peptide may result from *N*- or *C*-terminal extension or internal insertion of bradykinin as reported in this study, although their precursors may have the same processing sites. It suggests that amphibians may not share a similar mode of biosynthesis of bradykinin.

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Bradykinin                              RPPGFSPFR (Ref. 12)
Ranakinin-N                          RAEAVPPGFTPFR (IN)(This report)
Amolopkinin                           RAPVPPGFTPFR (IN)(Ref.13)
Ranakinin R                             RPPGFTPFRIAPEIV (CE)(Ref. 20)
Des-Arg9-bradykinin                     RPPGFSPF (CE)(Ref. 12)
Leu8 Bradykinin-related Peptides      IRRPPGFSPLR (NE)(Ref. 5)
Bombinakinin M              DLPKINRKGPRPPGFSPFR (NE)(Ref. 10)
Bombinakinin O                       GPRPPGFSPFRGKFH (NE & CE)(Ref. 19)
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Figure 4 Sequence comparison of amphibian bradykinin-like peptides. The amino acids identical to the first line are highlighted. IN: insertion; CE: *C*-terminal extension; NE: *N*-terminal extension; CE&NE: *C*-terminal extension and *N*-terminal extension.

A large number of bradykinin-like peptides with diverse structures have been identified from amphibian skins, but their biological roles are still not clear. Amphibians, being the first group of organisms forming a connecting link between land and water, are forced to adopt and survive in a variety of conditions laden with pathogens and predators. Therefore, they are endowed with an excellent chemical defense system composed of pharmacological and antimicrobial peptides [20]. Bradykinin-like peptides distributed in amphibian skins may perform defensive roles alone or synergetically with other compounds. The diversity of bradykinin-like peptides and their precursors may extend their spectrum of biological activities.

Our current study on ranakinin-N increases the diversity of bradykin-like peptides and their processing modes, and may also inspire more consideration about their actual biological roles.

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