Rohdei-litorin: a new peptide from the skin of *Phyllomedusa rohdei*

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The bombesin-litorin family of peptides is characterized by the common amino acid sequence -Gly-His-X-Met-NH_2 at the C-terminus, where X is a hydrophobic or aromatic residue. A new member of this family, rohdei-litorin, has been isolated from amphibian skin and its structure shown to be: Glp-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2. This new peptide displayed a greater affinity than other members of the family for rat urinary bladder receptors. A litorin-like peptide, with high affinity for this kind of receptor, has already been described in mammalian spinal cord and named neuromedin B. Rohdei-litorin shares with neuromedin B the entire C-terminal octrapeptide and may be considered the amphibian counterpart of this mammalian neuropeptide.

Rohdei-litorin *Amino acid sequencing* *Amphibian skin* *Neuromedin*

1. INTRODUCTION

Extracts of the skin of the South American hilyd frogs belonging to the genus *Phyllomedusa* contain a number of active peptide families, including the litorin-ranatensin family with the C-terminal pentapeptide -Val-Gly-His-Phe-NH_2, and the new phyllolitorin family with the C-terminal sequence -Val-Gly-Ser-Phe(Leu)-Met-NH_2, in which a serine residue replaces the usual histidine residue [1,2].

This paper describes the isolation, structure elucidation and preliminary pharmacological studies of a new litorin from methanol extracts of *Phyllomedusa rohdei* skin, designated rohdei-litorin.

2. MATERIALS AND METHODS

2.1. *Amphibian material*

2397 specimens of *P. rohdei* were collected during the period January–April 1966 near Rio de Janeiro. The skins were removed immediately after killing, spread out and dried. Soon after their arrival in the laboratory by air mail, the dried skins were extracted twice with a volume of 80% methanol 15–20-times their weight. The combined extracts were filtered and kept at 3–4°C.

2.2 *Bioassay*

The following test preperations were used in the bioassay of litorin-like activity: (a) isolated smooth muscle preparations: rat uterus and guinea-pig large intestine; (b) in situ preparations: rat urinary bladder, guinea-pig gall bladder. Methods have been described in detail in [3].

2.3. *Isolation procedure*

The methanol extract corresponding to 30 g dried skin was concentrated to a small volume, washed with petroleum ether to remove fats, and then evaporated to dryness. The residue was dissolved in 10 ml distilled water plus 190 ml of 99% ethanol and the solution loaded on a column of 170 g alkaline alumina (Merck, Darmstadt). The column was eluted first with 3 portions (200 ml each) of 95% ethanol and then with ethanol-water mixtures of descending concentrations of ethanol. All the litorin-like activity emerged in the 95%

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ethanol eluate with a peak in the second fraction. Only penta- and tetra-tryptophyllins emerged together with the litorin-like peptide; the bulk of other peptides was eluted by lower concentrations of ethanol. Purification of the active alumina eluates (equivalent to 3 g tissue) was carried out by gel filtration on Sephadex G-25 superfine using 0.1 M acetic acid as eluent (flow rate 3 ml/15 min).

Final purification of the active fraction was achieved by reverse-phase high-performance liquid chromatography (HPLC) on an Aquapore RP-300 column (4.6×250 mm, Brownlee Labs), using a Beckman model 332 instrument, under the conditions detailed in the legend to fig.1A. Column effluent was monitored by measuring the absorbance both at 220 and 280 nm with a Beckman 165 Spectrophotometer.

2.4. *Structural analysis*

Amino acid analyses were performed with an LKB 4400 amino acid analyzer, equipped with a Spectra-Physics System I Computing Integrator after hydrolysis of the peptide (0.5–1 nmol) in 6 N HCl at 110°C for 24 h in vacuo. N-terminal analyses were performed by the dansyl chloride method according to Gray [4]. Amino acid sequences were determined by the dansyl-Edman technique as described by Hartley [5]. DNS-Trp was identified after hydrolysis with 4 M methanesulphonic acid [6,7]. The C-terminal sequence was analyzed by carboxypeptidase Y (Boehringer) digestion in 0.1 M ammonium acetate at 37°C for 1 h (3–5 μg enzyme/nmol peptide). Digestion with pyroglutamate aminopeptidase (Boehringer) was performed according to Schwabe and McDonald [8]. Methanolysis of the pyrrolidone ring was performed by dissolving 20 nmol of the peptide in 100 μl of a freshly prepared solution of methanol/acetyl chloride (95:8, v/v) and incubating for 24 h at room temperature. 35 nmol peptide was subdigested with α-chymotrypsin (Worthington) at 38°C at an enzyme/substrate ratio = 1:35. After 40 min the digest was directly injected onto the HPLC reverse-phase column and chromatographed under the conditions detailed in fig.1B.

3. RESULTS AND DISCUSSION

The active fraction from Sephadex G-25 was injected onto the reverse-phase column and eluted with a linear gradient of aqueous acetonitrile containing 0.2% trifluoroacetic acid as indicated in fig.1A. The amino acid composition of the active fraction from the reverse-phase column was determined after acid hydrolysis to be: Thr (0.9) Glu (1.0), Gly (1.0), Ala (0.9), Met (1.1), Leu (1.0), Phe (1.0), His (0.9) (mol/mol peptide). The presence of tryptophan was determined by spectrophotometric analysis. The peptide was unresponsive to N-terminal analysis. After treatment with methanolic HCl it became amenable to dansyl-Edman degradation and the following partial sequence was established: Glx-Leu-Trp-Ala-Thr-Gly. Reaction with dansyl-chloride of an aliquot of peptide which had been preincubated with pyroglutamate aminopeptidase yielded leucine as the N-terminal residue, thus indicating the presence of a pyroglutamic acid at the N-terminus of the original peptide. Carboxypeptidase Y digestion released Met (1.0 mol/mol peptide), Phe (0.8), His (0.7). After chymotryptic digestion of an aliquot of the peptide, 4 fragments were purified by reverse-phase HPLC (fig.1B); their amino acid

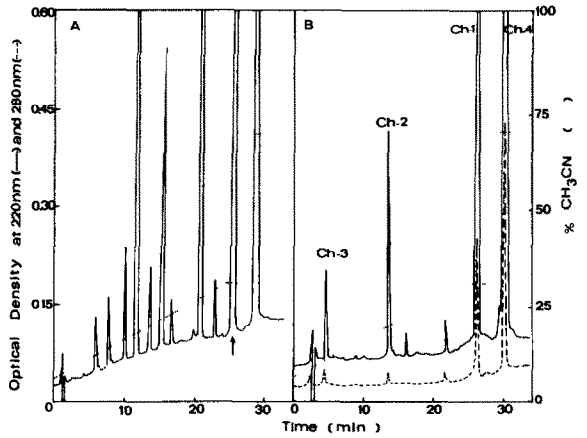

Fig.1. (A) Reverse-phase HPLC of the active fraction from Sephadex G-25. Flow rate: 1.2 ml/min. Column: Aquapore RP-300, 4.6×250 mm (Brownlee Labs). Solvent system: a 30 min linear gradient elution from 0.2% trifluoroacetic acid (TFA) in water to 0.2% TFA in 70% acetonitrile. (——) Absorbance at 220 nm; (···) % of solvent B in the gradient elution. The active fraction is indicated by the vertical arrow. (B) Reverse-phase HPLC of the chymotryptic digest of the active peptide. Conditions as in (– – –): Absorbance at 280 nm.

Table 1

Amino acid composition of the chymotryptic fragments

Amino acid	Ch-1	Ch-2	Ch-3	Ch-4
Thr		0.9		0.9
Glu	1.0			1.0
Gly		1.0		1.1
Ala		1.0		0.9
Met			1.0	
Leu	0.9			0.9
Phe		0.9		1.0
His		1.0		0.8
Trp	0.8[a]			+[b]
N-terminal residue	−[c]	Ala	Met	−[c]

[a]Determined after hydrolysis with 4 M methanesulphonic acid [7]
[b]The presence of tryptophan was indicated by the absorbance at 280 nm
[c]Unreactive with DNS-Cl

compositions and results of N-terminal analysis are reported in table 1. An aliquot of fragment Ch-3 loaded on the amino acid analyzer without previous acid hydrolysis chromatographed in the position of authentic methionine amide. Fragment Ch-2, submitted to dansyl-Edman degradation, gave the sequence: Ala-Thr-Gly-His-Phe.

From the results described above, the complete amino acid sequence of the peptide was determined to be: Glp-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2, as shown in fig.2.

The relative potencies of litorin, phyllolitorin and rohdei-litorin are shown in table 2. Rohdei-litorin presents sharp divergence of activity in comparison to litorin. It is twice as potent as litorin on the rat urinary bladder and 4–5- and 10–12-times less potent on the guinea-pig large intestine and

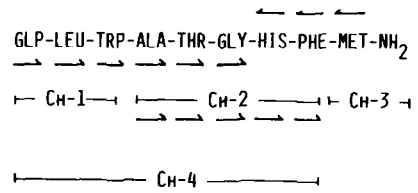

Fig.2. Complete amino acid sequence of the active peptide. (→). By dansyl-Edman degradation. (←) By carboxypeptidase Y digestion. CH, chymotryptic peptides.

guinea-pig gall bladder, respectively. On the other hand, in all bioassays used, rohdei-litorin was more potent than phyllolitorin.

The structure of this new nonapeptide is compared with that of other litorin-like peptides in fig.3. Rohdei-litorin shows in its sequence amino acid substitutions recalling on the one hand ranatensins C and R from Japanese frogs, and on the other the phyllolitorins from the Brazilian frog *P. sauvagei*. Thus, leucine at position 8 from the C-terminus is peculiar to all the litorin-like peptides so far isolated from *Phyllomedusa* skin, being also present in neuromedin B whereas threonine at position 5 from the C-terminus is common to rohdein-litorin, to ranatensins C and R and again to neuromedin B. Thus neuromedin B may be properly considered as the mammalian counterpart of the amphibian litorin-ranatensin peptide family. In fact, it shares with ranatensins C and R the entire C-terminal heptapeptide and with rohdei-litorin the entire C-terminal octapeptide. On the basis of results obtained in parallel bioassays it seems highly probable that the spectrum of biological activity of rohdei-litorin is very similar to that of neuromedin B. Synthetic analogues of rohdei-litorin will help to clarify the relative importance of the leucine and threonine

Table 2

Parallel bioassays of litorin-like peptides

	Litorin	Rohdei-litorin	Phyllolitorin
Rat uterus	100	40–120	3–15
Guinea-pig large intestine	100	20–25	1.5–3
Guinea-pig gall bladder (in situ)	100	8–10	2–4
Rat urinary blader (in situ)	100	200–230	100–120

The potency of litorin has been considered to be arbitrarily 100. Relative potencies are calculated on the basis of threshold doses

AMPHIBIAN:

HYLA	Glp-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2	LITORIN
PHYLLOMEDUSA	Glp-Leu-Trp-Ala-Val-Gly-Ser-Phe-Met-NH_2	PHYLLOLITORIN
	Glp-Leu-Trp-Ala-Val-Gly-Ser-Leu-Met-NH_2	LEU^8-PHYLLOLITORIN
	Glp-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2	ROHDEI-LITORIN
RANA	-Pro-Gln-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2	C-TERMINAL NONAPEPTIDE OF RANATENSIN C

MAMMALS:

	Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2	NEUROMEDIN B

Fig.3. Amino acid sequences of litorin-like peptides.

residues in determining the sharp quantitative differences in peripheral activity observed between rohdei-litorin and litorin.

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