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The leech excitatory peptide, a member of the GGNG peptide family: isolation and comparison with the earthworm GGNG peptides

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Abstract A member of the GGNG peptide family was isolated from *Hirudo nipponia* (leech). GGNG peptides had only been isolated previously from earthworms. The C-terminus structure of the leech peptide, LEP (leech excitatory peptide), was –Gly–Gly–Asn–amide, while that of the earthworm peptides, EEP (earthworm excitatory peptide), was –Gly–Gly–Asn–Gly. LEP exerted 1000-fold more potent activities on leech gut than did EEP-2. On the other hand, EEP-2 was 1000-fold more potent than LEP on the crop-gizzard of the earthworm. Analog peptides of LEP and EEP-2 were synthesized, and the myoactive potency of each analog on the leech and earthworm tissues was compared.

Key words: Leech excitatory peptide; GGNG peptide family; Myoactivity; Structure–activity relationship; *Hirudo nipponia*

1. Introduction

We have been screening peptides of annelids that may be involved in regulation of physiological events, such as neurotransmission, osmoregulatory adaptation, gut motility, and reproductive events, etc. Addition to FMRFamide-related peptides [1–3], several peptides have been isolated and their biological properties characterized as follows: S-I amide peptides [4], myomodulin–CARP-related peptide as a putative excitatory neuromediator involved in the regulation of the gut motility [5], MATP-related peptides as gut motivative peptides [6] and *Eisenia* inhibitory pentapeptides [7] as gut inhibitory peptides. These peptides are members of the corresponding peptide family previously isolated from molluscan and arthropodan animals.

Recently, peptides that belong to the vertebrate peptide family have been isolated from earthworms and leeches. Lysine–conopressin, a member of the oxytocin vasopressin superfamily, was isolated from the pharyngobdellid leech *Erpobdella octoculata* by Salzet et al. [8]. Annetocin, another member of the super family, was isolated from the lumbricid earthworm *Eisenia foetida* using its nephridial movement as a measure of bioactivity [9]. This peptide has also been reported to potentiate spontaneous contractions of gut tissue [10]. Salzet et al. [11] isolated and structurally characterized methionine- and leucine-enkephalins from the brain of the rhynchobdellid leech *Theromyzon tessulatum* by means of ELISA and dot immunobinding assays. However, the biological activity of these peptides in leeches has not been reported. Angiotensin II amide was also structurally identified from the central nervous system of the leech *E. octoculata* using ELISA. Angiotensin II amide was demonstrated to exert a diuretic action in the leech [12].

The present study was designed to isolate bioactive substances involved in regulation of gut motility in the leech. A peptide from the leech *Hirudo nipponia* was isolated using contractility of the intestine of the leech as a bioassay system. The peptide was found to be structurally related to GGNG peptides, which were isolated from the earthworm, *E. foetida* and were potent exciters of spontaneous contraction of gut tissues [13]. The C-terminus residue of the leech peptide was an α-amidated aspargine, whereas that of the earthworm peptides was a glycine that followed an aspargine residue. The structure of the C-terminus of earthworm peptides appears to be a precursor form of a C-terminus α-amidation. The present study reports the isolation and chemical characterization of the leech GGNG peptide, and presents this structure–activity relationship between leech and earthworm GGNG peptides.

2. Materials and methods

2.1. Animals and purification

Leeches (*H. nipponia*) were obtained from a commercial source and kept without food in dechlorinated tap water that was constantly aerated at room temperature (20–25°C). Approximately 1000 leeches (200 g) were boiled for 10 min in 5 vol. of 4% acetic acid. After cooling, the same volume of ethanol was added, and then the mixture was homogenized in a Waring blender. The homogenate was centrifuged at 10000×*g* for 30 min (4°C). The pellet was extracted again with 50% ethanol/2% acetic acid. The combined supernatant was condensed to approximately 300 ml, acidified with TFA to a final concentration of 0.1%, and then centrifuged at 15000×*g* for 30 min (4°C). The supernatant was passed through four Sep-Pak Vac C_{18} syringes (12 ml, 2 g, Waters Associates) connected in series. The syringes were washed with 10% methanol/0.1% TFA, and the retained materials were eluted with 50% methanol/0.1% TFA. The eluate was condensed in vacuo to 2 ml and applied to a Sephadex G-25 (fine) column (1.5×100 cm) equilibrated with 10 mM acetic acid. The applied materials were eluted with 10 mM acetic acid at a flow rate of 0.25 ml/min and collected in 2 ml fractions. Each fraction was assayed on the intestine of the leech for contraction-eliciting activity (see below). Active fractions (No. 31–36) were pooled, condensed and subjected to reversed-phase HPLC (Asahipak, ODP-50, 6×250 mm) with

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Abbreviations: CARP, catch-relaxing peptide; MATP, molluscan myoactive tetradecapeptide; ELISA, enzyme-linked immunosorbent assay(s); TFA, trifluoroacetic acid; FAB-MS, fast atom bombardment mass spectrometry; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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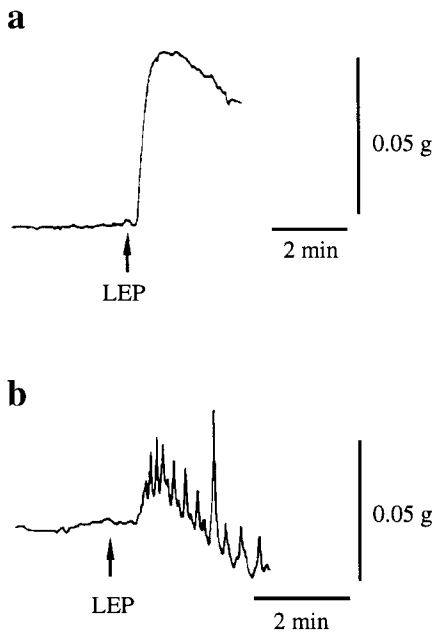

Fig. 1. Effect of LEP on spontaneous contractions of isolated intestine (a) and vagina (b) of leech *H. nipponia*. Peptides were applied at time indicated by arrow heads.

a 200 min linear gradient of 0–100% CH_3CN/0.1% TFA at a flow rate of 1 ml/min. Active fractions eluted at around 17–26% CH_3CN were collected and applied to a strong cation-exchange column (SP-5PW, Tosoh, 7.5×75 mm). The column was eluted with a 70 min linear gradient of 0–0.7 M NaCl/10 mM phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min. The bioactivity on the leech intestine was observed in the fractions eluted with 0.11–0.22 M NaCl. The fractions were subjected to separation by reversed-phase HPLC (Inertsil ODS-80A, GL Science, 10×250 mm) with a 100 min gradient of 10–30% CH_3CN/0.1% TFA at a flow rate of 0.5 ml/min. The fractions eluted at 22–28% CH_3CN were further separated by another reversed-phase column (ODS-80T_M, Tosoh, 4.6×150 mm) with a 50 min gradient of 20–30% CH_3CN/0.1% TFA (0.5 ml/ min). HPLC was repeated four more steps alternately using a cation-exchange (SP-5PW) and a reversed-phase (ODS-80T_M) column. The bioactive substance, which elicited contraction of the leech intestine, was finally purified as a single peak.

Table 1
Amino acid sequence analysis of the leech GGNG peptide

Cycle	Amino acid	Intact (pmol)	RPE[a] (pmol)
1	Ala	86	83
2	Lys	75	75
3	Cys	–	56[b]
4	Glu	63	65
5	Gly	45	42
6	Glu	51	53
7	Trp	31	26
8	Ala	44	42
9	Ile	39	36
10	His	31	26
11	Ala	38	37
12	Cys	–	28[b]
13	Leu	27	31
14	Gly	25	25
15	Gly	29	26
16	Asn	27	30

[a]Reductive *S*-pyridylethylation.
[b]Detected as *S*-pyridylethylcysteine.

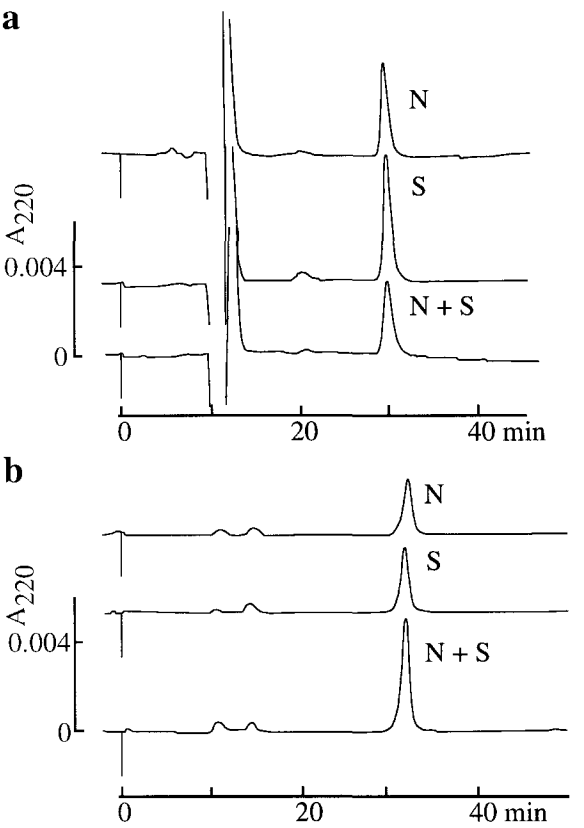

Fig. 2. Elution pattern of native peptide (N), synthetic peptide (S) and their mixture (N+S) on reversed-phase and cation-exchange HPLC. a: Elution with reversed-phase column (ODS-80T_M) was isocratically performed with 22% CH_3CN/0.1% TFA at 0.3 ml/min, where native peptide (1/63 of the total purified), synthetic peptide (100 pmol), and mixture (1/126 native and 50 pmol synthetic peptide) were injected, respectively. b: Cation-exchange column (SP-5PW) was eluted isocratically with 10 mM phosphate buffer excluding NaCl (pH 6.0), where native peptide (1/63), synthetic peptide (100 pmol), and mixture (1/63 plus 100 pmol) were injected, respectively.

2.2. Structure determination and synthesis

The purified substance was subjected to amino acid sequence analysis using an automated gas-phase sequencer (Shimadzu PSQ-1). The cysteine residues were detected as an *S*-pyridylethylated derivative [14] after reduction of the intramolecular disulfide bond. The molecular mass was determined by FAB-MS (JMS HX-110/110A, JEOL). According to the anticipated structure, the peptide was synthesized by a conventional solid-phase method using FastMoc™ (Perkin Elmer 431A Peptide Synthesizer), and then purified by reversed-phase HPLC. To confirm the inferred structure, the synthesized peptide was compared with its native counterpart with respect to biological activity and chromatographic behavior on reversed-phase and cation-exchange HPLC. In order to compare the structure–activity relationship between the leech and earthworm GGNG peptides, analog peptides were also synthesized in the same manner.

2.3. Biological activity

Muscle contraction in the intestine excised from *H. nipponia* was used as a measure of bioactivity [15]. The tension produced by intestinal contraction was recorded using force-displacement transducer (NEC San-ei, 45196A) and strain amplifier (NEC San-ei, AS1202). The methods for recording tension have been described elsewhere [9,10]. The biological activity of the isolated native peptides and synthetic peptides were also examined on the vagina of the leech *H. nipponia* and the crop-gizzard of the earthworm *E. foetida*. The tension produced by these muscle tissues was recorded in the same manner as that of the leech intestine. Bioactivity of the peptides were expressed as a percent change in the tension of the muscle contraction.

Table 2
Structures of GGNG peptides isolated from earthworms *Eisenia foetida* and *Pheretima vittata* (EEP) and leech *Hirudo nipponia* (LEP)

Peptides	Structure	Source
EEP-1	A-P-K-C-S-G-R-W-A-I-H-S-C-G-G-G-N-G	*Eisenia* gut
EEP-2	G-K-C-A-G-Q-W-A-I-H-A-C-A-G-G-N-G	*Eisenia* whole body
EEP-3	R-P-K-C-A-G-R-W-A-I-H-S-C-G-G-G-N-G	*Pheretima* whole body
LEP	A-K-C-E-G-E-W-A-I-H-S-C-L-G-G-N-NH_2	*Hirudo* whole body
[Ala^1, Leu^{13}]EEP-2 [Ala^4, Gln^6]preLEP	A-K-C-A-G-Q-W-A-I-H-A-C-L-G-G-N-G	
[Glu^{4,6}]EEP-2 [Gly^1, Ala^{13}]preLEP	G-K-C-E-G-E-W-A-I-H-A-C-A-G-G-N-G	
[Ala^1, Glu^{4,6}, Leu^{13}]EEP-2 preLEP	A-K-C-E-G-E-W-A-I-H-A-C-L-G-G-N-G	
[Gly^1, Ala^{4,13}, Gln^6]LEP des-Gly^{17}-EEP-2 amide	G-K-C-A-G-Q-W-A-I-H-A-C-A-G-G-N-NH_2	
[Ala^4, Gln^6]LEP [Ala^1, Leu^{13}]des-Gly^{17}-EEP-2 amide	A-K-C-A-G-Q-W-A-I-H-A-C-L-G-G-N-NH_2	
[Gly^1, Ala^{13}]LEP [Glu^{4,6}]des-Gly^{17}-EEP-2 amide	G-K-C-E-G-E-W-A-I-H-S-C-A-G-G-N-NH_2	

EEP, earthworm excitatory peptide; LEP, leech excitatory peptide.

Physiological saline used for *H. nipponia* was prepared according to Norris and Calabrese [16] with minor modifications: 115 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES–NaOH (pH 7.2). Composition of saline for the earthworm *E. foetida* was as follows: 102 mM NaCl, 1.6 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES–NaOH (pH 7.2).

3. Results

The purified substance showed a single symmetrical peak during the final purification step of reversed-phase HPLC with an isocratic elution of 22% CH_3CN. A 1/200 aliquot of this substance elicited spontaneous contractions of the isolated leech intestine (Fig. 1a). The results of amino acid sequence analysis are shown in Table 1. The molecular mass of the peptide by FAB-MS was m/z $(M+H)^+$ of 1655.56. This value corresponds with the monoisotopic mass (1655.74) calculated from the amino acid sequence (Table 1) that assumes that two cysteine residues formed intramolecular disulfide bond and that the carboxyl terminus was amidated. The structure inferred by this analysis was as follows:

The structure inferred by this analysis was as follows:

Ala-Lys-Cys-Glu-Gly-Glu-Trp-Ala-Ile-His-Ala-Cys-Leu-Gly-Gly-Asn-amide.

The synthetic peptide was compared to its native counterpart by HPLC (Fig. 2). Both reversed-phase (Fig. 2a) and cation-exchange HPLC (Fig. 2b) demonstrated that the native peptide had the same retention time as the synthetic species. Furthermore, each mixture was eluted as a single peak. Thus, the above structure was confirmed. The leech peptide elicited rhythmic spontaneous contractions of the isolated leech vagina at 10^{-9} M or higher (Fig. 1b). This peptide was homologous with the three GGNG peptides, which were isolated from two species of earthworms, *E. foetida* and *Pheretima vittata* (Table 2) as potent excitatory peptides of spontaneous contractions of gut tissue in earthworms. The leech peptide

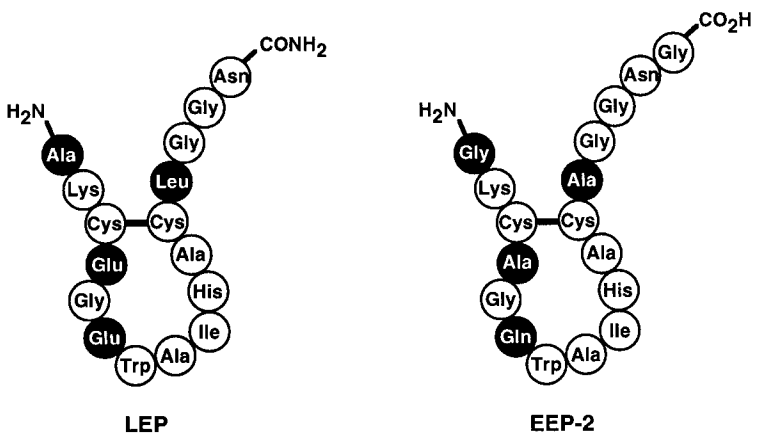

Fig. 3. Comparison of structures of LEP and EEP-2. Shaded circles indicate different amino acids. Each ring structures is stabilized by an intramolecular disulfide bond.

was tentatively named leech excitatory peptide (LEP), while the earthworm peptides were designated as earthworm excitatory peptides (EEP). Among the EEPs, EEP-2 showed 75% homology with LEP. Structural differences between LEP and EEP-2 were noted in the C-terminus and the amino acid residues inside and outside the ring structure formed by a disulfide bond (Fig. 3). The activity of the peptides on the crop-gizzard of the earthworm and on the intestine of the leech were compared. EEP-2 produced approximately 1000-fold greater excitation of the crop-gizzard motility than did LEP (Fig. 4a). In contrast, LEP exerted 1000 times more potent contractile effects on the intestine than did EEP-2 (Fig. 4b). Thus, the biological activity of these peptides appears to be species specific. Analog peptides of LEP and EEP-2, that contained changes at the C-terminus and/or at the amino acid residues inside and/or outside the ring structure were synthesized (Table 2), and their biological activity on the earthworm crop-gizzard or leech intestine was examined. Dose–response curves for each analog were obtained (Fig. 5a,b). Equiactive molar ratios (EMR) were estimated from the curves as the concentration at which the analog peptide exerted tension equal to EEP-2 or LEP at 10^{-8} M on each tissue (Table 3). The results can be summarized as follows. (1) On the earthworm crop-gizzard, [Ala^1, Leu^{13}]EEP-2 and [Glu^{4,6}]EEP-2 showed 1/3 and 1/5 of activity of EEP-2, respectively. [Ala^1, Glu^{4,6}, Leu^{13}]EEP-2 evoked activity 200 times less potent than that of EEP-2. The order of potency of EEP-2 and its analogs determined by EMR was EEP-2 > [Ala^1, Leu^{13}]EEP-2 ≥ [Glu^{4,6}]EEP-2 > [Ala^1, Glu^{4,6}, Leu^{13}]EEP-2. An EEP-2 analog with a leech-type C-terminus, des-Gly^{17}-

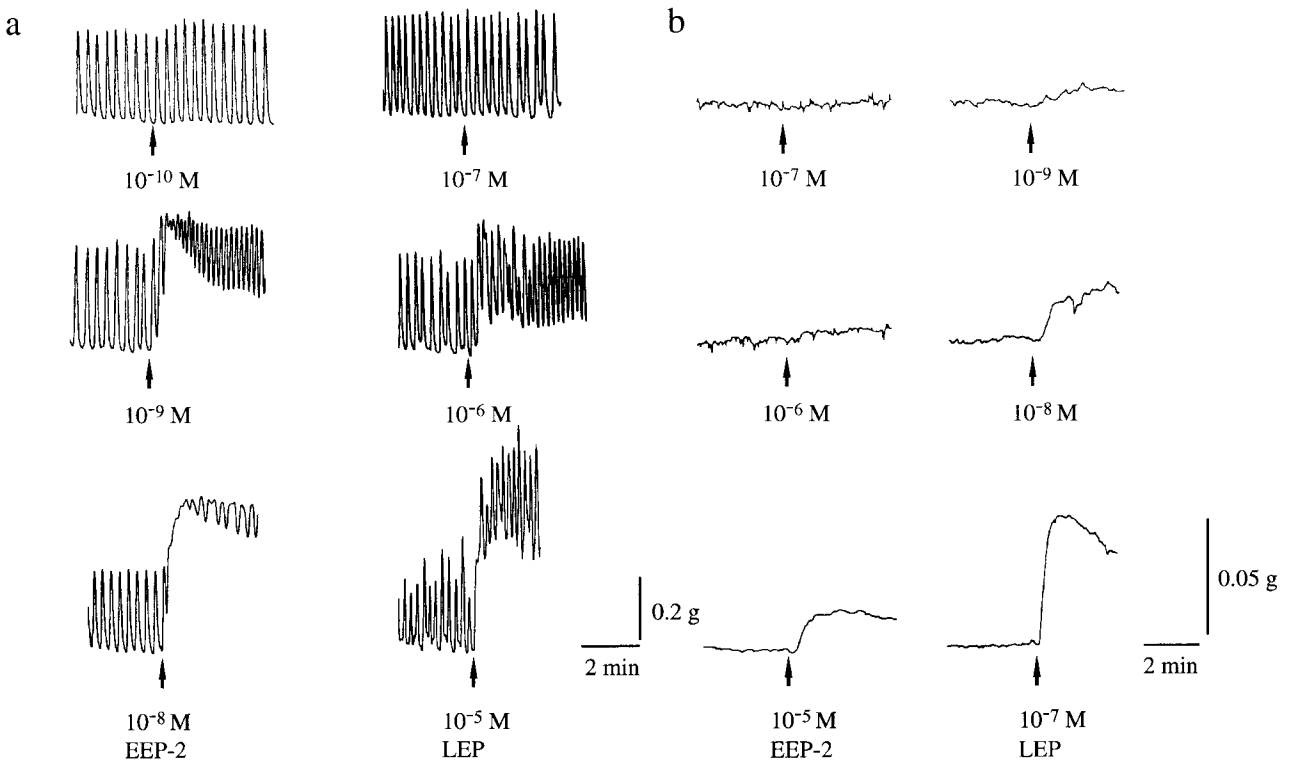

Fig. 4. a: Effects of EEP-2 and LEP on spontaneous contractions of isolated crop-gizzard preparations of earthworm *E. foetida*. b: Effects of LEP and EEP-2 on isolated intestinal preparations of leech *H. nipponia*. Peptides were applied at time indicated by arrow heads.

EEP-2 amide, showed 5-fold less activity than native EEP-2. The order of potency of the analogs was des-Gly^{17}-EEP-2 amide > [Glu^{4,6}]des-Gly^{17}-EEP-2 amide ≥ [Ala^{1}, Leu^{13}]des-Gly^{17}-EEP-2 amide ≫ [Ala^{1}, Glu^{4,6}, Leu^{13}]des-Gly^{17}-EEP-2 amide (LEP). Des-17-glycyl and C-terminus amidated analogs were 5- to 10-fold less active than the corresponding EEP-2 analogs. (2) On the leech intestine, LEP and preLEP, a precusor form of the C-terminus α-amidation of LEP,

A-K-C-E-G-E-W-A-I-H-A-C-L-G-G-N-G,

exhibited excitatory activity on the spontaneous contractions at thresholds of 10^{-9} M and 10^{-8} M, respectively. The order of potency determined by EMR of LEP and its analogs was LEP > [Gly^{1}, Ala^{13}]LEP = [Ala^{4}, Gln^{6}]LEP > [Gly^{1}, Ala^{4,13}, Gln^{6}]LEP and preLEP > [Ala^{4}, Gln^{6}]preLEP ≫ [Gly^{1}, Ala^{4,13}, Gln^{6}]preLEP (EEP-2) ≥ [Gly^{1}, Ala^{13}]preLEP. α-Deamidation and addition of Gly residue at the C-termini of [Gly^{1}, Ala^{13}]LEP and [Gly^{1},Ala^{4,13}, Gln^{6}]LEP reduced the activity to approximately 1/800 and 1/600, respectively.

4. Discussion

Three novel myoactive peptides that are highly homologous with each other have previously been isolated from two species of Oligochaeta, *E. foetida* and *P. vittata*. The structure of the peptides was not related to that of any known peptides, and the peptides were termed GGNG peptides based on their unique structure in the C-terminus [13]. The present study is the first report of isolation and chemical characterization of GGNG peptide from the Hirudinea (Annelida). We have isolated the same peptide from another species of leech, *Whitmania pigra*. The leech GGNG peptide identified in the present study differed specifically from the earthworm GGNG peptides in the C-terminus, that is, –Gly–Gly–Asn–amide in the leech peptide and –Gly–Gly–Asn–Gly in the earthworm peptides. α-Amidation of the carboxyl terminus is a ubiquitous modification of bioactive peptides, which is usually mandatory for exertion of bioactivity [17]. Such α-amidation of peptides is catalyzed by an α-amidating enzyme, which utilizes the amino group of a C-terminus glycine residue present in the intermediate precursor [18–20]. Based on this relationship, we cloned the cDNA that encodes LEP and EEP-2 in order to compare their precursor structures. The deduced precursor structure showed that both peptides are flanked by a dibasic

Table 3
Equiactive molar ratios (ENR) of LEP and EEP-2 analogs on the earthworm crop-gizzard and leech intestine assays

Peptides	EMR
Earthworm crop-gizzard assay	
-GGNG type	
EEP-2	1
[Ala^{1}, Leu^{13}]EEP-2	3
[Glu^{4,6}]EEP-2	5
[Ala^{1}, Glu^{4,6}, Leu^{13}]EEP-2	220
-GGN-amide type	
des-Gly^{17}-EEP-2 amide	5
[Ala^{1}, Leu^{13}]des-Gly^{17}-EEP-2 amide	30
[Glu^{4,6}]des-Gly^{17}-EEP-2 amide	20
LEP	1000
Leech intestine assay	
-GGN-amide type	
LEP	1
[Gly^{1}, Ala^{13}]LEP	2
[Ala^{4}, Gln^{6}]LEP	1
[Gly^{1}, Ala^{4,13}, Gln^{6}]LEP	6
-GGNG type	
preLEP	3
[Gly^{1}, Ala^{13}]preLEP	800
[Ala^{4}, Gln^{6}]preLEP	8
EEP-2	600

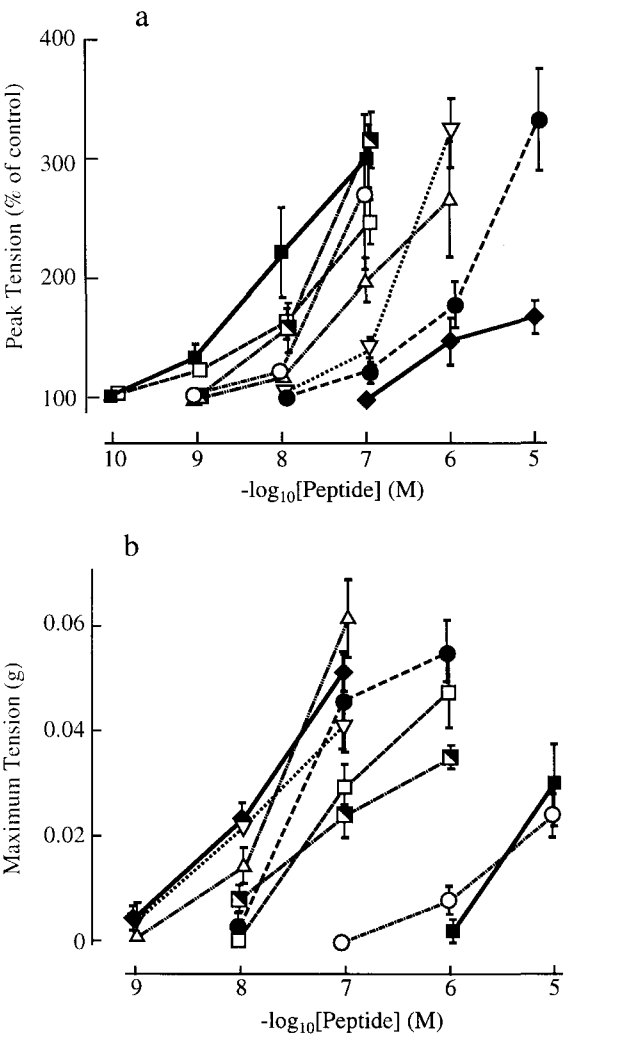

Fig. 5. a: Dose–response relationship between effects of native (EEP-2 and LEP) and analog peptides on tension of spontaneous contractions of isolated crop-gizzard preparations of earthworm *E. foetida*. Parameter is expressed as percent change in tension over 2 min recording just before and after application of peptides. Values are expressed as mean ± SE (n = 5–7). b: Dose–response relationship between effects of native (LEP and EEP-2) and analog peptides on tension produced by intestinal contraction of leech *H. nipponia*. Tension is expressed as maximum tension of contraction produced during 2 min just after application of peptides (n = 3–4). – ■ –, EEP-2([Gly^{1}, Ala^{4,13}, Gln^{6}]preLEP). – ◥ –, [Ala^{1}, Leu^{13}]EEP-2([Ala^{4}, Gln^{6}]preLEP). – ○ –, [Glu^{4,6}]EEP-2([Gly^{1}, Ala^{13}]preLEP). – ● –, [Ala^{1}, Glu^{4,6}, Leu^{13}]EEP-2(preLEP). – □ –, des-Gly^{17}-EEP-2 amide ([Gly^{1}, Ala^{4,13}, Gln^{6}]LEP). – ▽ –, [Ala^{1}, Leu^{13}]des-Gly^{17}-EEP-2 amide ([Ala^{4}, Gln^{6}]LEP). – △ –, [Glu^{4,6}]des-Gly^{17}-EEP-2 amide ([Gly^{1}, Ala^{13}]LEP). – ◆ –, [Ala^{1}, Glu^{4,6}, Leu^{13}]des-Gly^{17}-EEP-2 amide (LEP).

processing signal, –Lys–Arg–, at the N-termini and by a dibasic site preceded by a glycine residue, –Gly–Lys–Arg–, at the C-termini [21]. Therefore, there may be some significant difference between the leech and earthworm in the α-amidating-enzyme system; the α-amidating activity may not be localized to the subcellular system that produces EEP-2. Despite the wide-spread acknowledgment of the essentiality of C-terminal α-amidation for bioactivity in most peptides, des-Gly^{17}-EEP-2 amide exhibited 5-fold less bioactivity on the earthworm tissue than did EEP-2. However, EEP-2 showed 1000-fold more potent activity on the earthworm tissue than did LEP. The peptides with –Gly–Gly–Asn–Gly structure at their C-terminus were 5- to 10-fold more potent on the earthworm crop-gizzard than those with –Gly–Gly–Asn–amide. Changes in the amino acid residues at positions 1, 13 or 4, 6 of EEP-2 to LEP-type slightly decreased the activity. Four changes at positions of 1, 4, 6, and 13 of EEP-2 resulted in a decrease to 1/200 activity of EEP-2. These results suggest that the –Gly–Gly–Asn–Gly structure is preferable to the –Gly–Gly–Asn–amide structure on the earthworm tissue and that the recognition sites of the peptides may be located both in the C-terminus and inside and outside residues of the ring structure (Fig. 3). On the leech tissues, changes in the positions inside and/or outside the ring to EEP-2-type slightly decreased the activity. Changes at the C-terminus and the outside positions of the ring reduced the activity remarkably. These results suggest that both the –Gly–Gly–Asn–amide structure and the residues outside the ring structure may play important roles for recognition by receptors on the leech intestine. Although LEP and EEP-2 are highly homologous with each other, the recognition sites in the target tissues of the leech and earthworm may differ. Experiments are now in progress to characterize the receptors in the crop-gizzard of the earthworm and in the intestine of the leech.

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