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ISOLATION OF FOUR NOVEL TACHYKININS FROM FROG (*Rana catesbeiana*) BRAIN AND INTESTINE

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SUMMARY: In a survey for unknown bioactive peptides in frog (*Rana catesbeiana*) brain and intestine, we isolated four novel peptides that exhibit potent stimulant effects on smooth muscle preparation of guinea pig ileum. By microsequencing and synthesis, these peptides were identified as Lys-Pro-Ser-Pro-Asp-Arg-Phe-Tyr-Gly-Leu-Met-NH_2 (ranatachykinin A), Tyr-Lys-Ser-Asp-Ser-Phe-Tyr-Gly-Leu-Met-NH_2 (ranatachykinin B), His-Asn-Pro-Ala-Ser-Phe-Ile-Gly-Leu-Met-NH_2 (ranatachykinin C) and Lys-Pro-Asn-Pro-Glu-Arg-Phe-Tyr-Ala-Pro-Met-NH_2 (ranatachykinin D). Ranatachykinin (RTK) A, B and C conserve the C-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, which is common to known members of the tachykinin family. On the other hand, RTK-D has a striking feature in its C-terminal sequence, Phe-Tyr-Ala-Pro-Met-NH_2, which has never been found in other known tachykinins, and may constitute a new subclass in the tachykinin family.

It is well known that amino acid sequences of several bioactive peptides are well conserved between mammalians and amphibians (1,2). In fact, we have identified in porcine spinal cord a series of neuromedins highly homologous to amphibian peptides (3,4,5). Therefore, we began a systematic search for unidentified bioactive peptides in frog brain and intestine using a bioassay system for a stimulant effect on guinea pig ileum. Here we report isolation and complete amino acid sequences of four novel bioactive peptides. By structural analyses, these peptides are found to have highly homologous sequences to other known tachykinins. Thus, we designated them ranatachykinin (RTK) A, B, C and D. Up to now, the mammalian tachykinin family has been shown to be composed of three distinct peptides, substance P, neuromedin K[@] and neuromedin L (3,4,6,7). Diversity of tachykinins in a single frog species may suggest the presence of a complex peptidergic regulation system in amphibians.

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@ Neuromedin K is also known as neurokinin B (or β) (3,6), and neuromedin L as substance K or neurokinin A (or α) (4,6,7).

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MATERIALS AND METHODS

Isolation: Frog (*Rana catesbeiana*) brain (47 g wet weight from 157 frogs) and intestine (98 g wet weight from 25 frogs) were collected immediately after decapitation and kept on ice before extraction. Diced brain and intestinal tissue were each boiled for 8 min in 10 volumes of water to inactivate intrinsic proteases. After cooling, glacial acetic acid was added (final concentration: 1.0M), and boiled tissue was homogenized with a Polytron mixer for 5 min, and stirred at 4℃ for 12 hr. The supernatants obtained after centrifugation at 14,500 × *g* for 35 min were diluted two-fold with water and loaded onto a reverse phase C-18 column (90 ml, Chemco LC-SORB SPW-C-ODS). After washing with 0.5M CH_3COOH, the adsorbed materials on the column were eluted with 60% CH_3CN in 0.1% trifluoroacetic acid (TFA). The eluates were evaporated in vacuum to dryness. The residual materials were dissolved in 1M CH_3COOH and loaded on an SP-Sephadex C-25 columun (H^+-form, 15 × 50 mm) pre-equilibrated with 1M CH_3COOH. Successive elutions with 1M CH_3COOH, 2M pyridine and 2M pyridine-CH_3COOH (pH 5.0) afforded three respective fractions SP-Ⅰ, SP-Ⅱ and SP-Ⅲ. Each dry concentrate of the SP-Ⅲ fraction prepared from brain and intestine was used as starting material for the present purification. Gel filtration of SP-Ⅲ fractions was performed on a Sephadex G-50 column (fine, 1.8 × 134 cm). An aliquot of each fraction was submitted to a bioassay for stimulant activity on guinea pig ileum. Fractions exhibiting major ileum contractile activity were pooled and lyophilized. The lyophilizates obtained from intestine were dissolved in 10mM $HCOONH_4$ (pH 6.5) containing 10% CH_3CN and subjected to CM ion exchange HPLC on a TSK gel CM-2SW column (7.6 × 300 mm, Tosoh) using a linear gradient elution of $HCOONH_4$ from 10mM to 0.5M (pH 6.5) in the presence of 10% CH_3CN at a flow rate of 2.0 ml/min. In the case of brain extracts, this step was omitted. The ileum contractile fractions of brain and intestine were then subjected to reverse phase HPLC on a Chemcosorb 3ODS-H column (8.0 × 75 mm, Chemco) with a linear gradient elution of CH_3CN from 0% to 60% in 0.1% TFA for 120 min at a flow rate 2.0 ml/min. Final purification was also performed by reverse phase HPLC on a diphenyl column (219TP5215, 2.1 × 150 mm, Vydac) or Chemcosorb 3ODS-H (2.1 × 75 mm, Chemco). Column effluents in HPLC were monitored by measuring absorbance at 210 nm and 280 nm, simultaneously.

Bioassay: Effects on the contractility of freshly isolated preparations of guinea pig ileum and rat duodenum were measured according to the described method (8). Ileum and duodenum were bathed in Tyrode's solution.

Sequence analysis: Amino acid analyses of these peptides were carried out with an amino acid analyzer (Hitachi 835), after acid hydrolysis of the peptide in 6M HCl containing 0.1% phenol and 0.02% 2-mercaptoethanol at 110℃ for 20 hr. After oxidation of methionine residues with 0.03% H_2O_2 in 1M HCOOH at room temperature for 30 min, amino acid sequence analyses of the peptides were performed by a gas-phase sequencer equipped with phenylthiohydantoin (PTH)-amino acid analyzing HPLC system (Model 470A/120A, Applied Biosystems). PTH-amino acids were detectable as low as 0.1 pmol. The C-terminal amide structure was verified by thermolysinization of the peptides, followed by dansylation, according to the method of Tatemoto and Mutt (9).

Synthesis: RTK-A, RTK-B and RTK-C were synthesized by solid phase techniques conducted on benzhydrylamine resin, and RTK-D on p-methyl-benzhydrylamine resin. Purification was performed by CM-52 ion exchange chromatography and reverse phase HPLC. Correct synthesis was confirmed by amino acid analysis and sequencing.

RESULTS AND DISCUSSION

Basic peptide fractions (SP-Ⅲ) obtained from acid extracts of frog brain and intestine showed the most potent guinea pig ileum stimulant activities among the three SP-Sephadex fractions. Thus, we started the present purification from the SP-Ⅲ fractions of frog brain and intestine.

First, we purified RTK-A and RTK-B from frog brain. The brain SP-Ⅲ fraction (dry weight: ca. 40 mg) was subjected to Sephadex G-50 gel filtration. Although guinea pig ileum

stimulant activities were observed in a wide range of fractions **a**~**e**, major activity emerged in fractions **d** and **e** (Fig. 1a). Fractions **d** and **e** were each submitted to reverse phase HPLC on a C-18 column. The first reverse phase HPLC of fraction **d** yielded one major peak of ileum contractile activity eluted at 52.5 min along with several minor peaks (Fig. 2a), and RTK-A was already purified to a homogeneous state by this step. In the case of fraction **e**, ileum contractile activity was separated into two peaks eluted at 52.5 min and 55 min (Fig. 2b). The second peak of ileum contractile activity was further purified by reverse phase HPLC on a diphenyl column to give a homogeneous RTK-B (Fig. 2c). The first peak in Fig. 2b was also identified as an N-terminally shortened form of RTK-A (data not shown). Homogeneity of RTK-A and RTK-B thus obtained was confirmed by another reverse phase HPLC.

Next, we purified RTK-C , RTK-D and RTK-A from frog intestine. The intestinal SP-Ⅲ fraction (dry weight: ca. 60 mg) was separated by Sephadex G-50 gel filtration. Ileum contractile activity was observed in a wide range of fractions A~E, but major activity emerged in fraction D (Fig. 1b). Fraction D was lyophilized and subjected to CM ion exchange HPLC. As shown in Fig. 1c, seven peaks of ileum contractile activity were observed, and RTK-C and RTK-D were isolated from peaks eluted at 30-33 min and 48-49.5 min, respectively. RTK-A was also isolated from the peak eluted at 52.5-54 min. The three peaks of ileum contractile activity were each purified by reverse phase HPLC on a C-18 column to homogeneous states, as shown in Figs. 3a~3c. Purity of the peptides was confirmed by another HPLC system. Purification of the other peaks with ileum contractile activity found in brain and intestinal extracts is now going on.

Amino acid compositions of RTK-A, RTK-B, RTK-C and RTK-D were determined as follows: RTK-A; Asp 0.9 (1), Ser 1.1 (1), Pro 1.8 (2), Gly 1.1 (1), Met 0.9 (1), Leu 1.0 (1), Tyr 0.9 (1), Phe 1.0 (1), Lys 1.1 (1), Arg 1.1 (1). RTK-B; Asp 1.0 (1), Ser 1.9 (2), Gly 1.1 (1), Met 0.9 (1), Leu 1.1 (1), Tyr 1.9 (2), Phe 1.1 (1), Lys 1.0 (1). RTK-C; Asp 1.0 (1), Ser 1.1 (1), Pro 1.1 (1), Gly 1.2 (1), Ala 1.1 (1), Met 0.9 (1), Ile 1.0 (1), Leu 1.1 (1), Phe 1.0 (1), His 1.1 (1). RTK-D; Asp 1.0 (1), Glu 1.1 (1), Pro 2.8 (3), Ala 1.1 (1), Met 0.9 (1), Tyr 0.9 (1), Phe 1.0 (1), Lys 1.1 (1), Arg 1.1 (1). Based on amino acid analysis data, RTK-A, -B, -C and-D were found to comprise 11, 10, 10 and 11 amino acid residues. Their respective yields were estimated to be 6.00 nmol for RTK-A and 0.60 nmol for RTK-B starting from 47 g of frog brain, and 3.40 nmol for RTK-C, 4.87 nmol for RTK-D and 2.75 nmol for RTK-A starting from 98 g of frog intestine. The four peptides were submitted to sequence analysis with a gas-phase sequencer after oxidation of Met residues to Met sulfoxides in order to increase recovery yields in Edman degradation. Edman degradation proceeded in good yields and PTH- amino acids were clearly identified up to the C-terminal ends (Figs. 4a~4d). C-terminal Met amide structure of these four peptides was definitely identified by the method of Tatemoto and Mutt

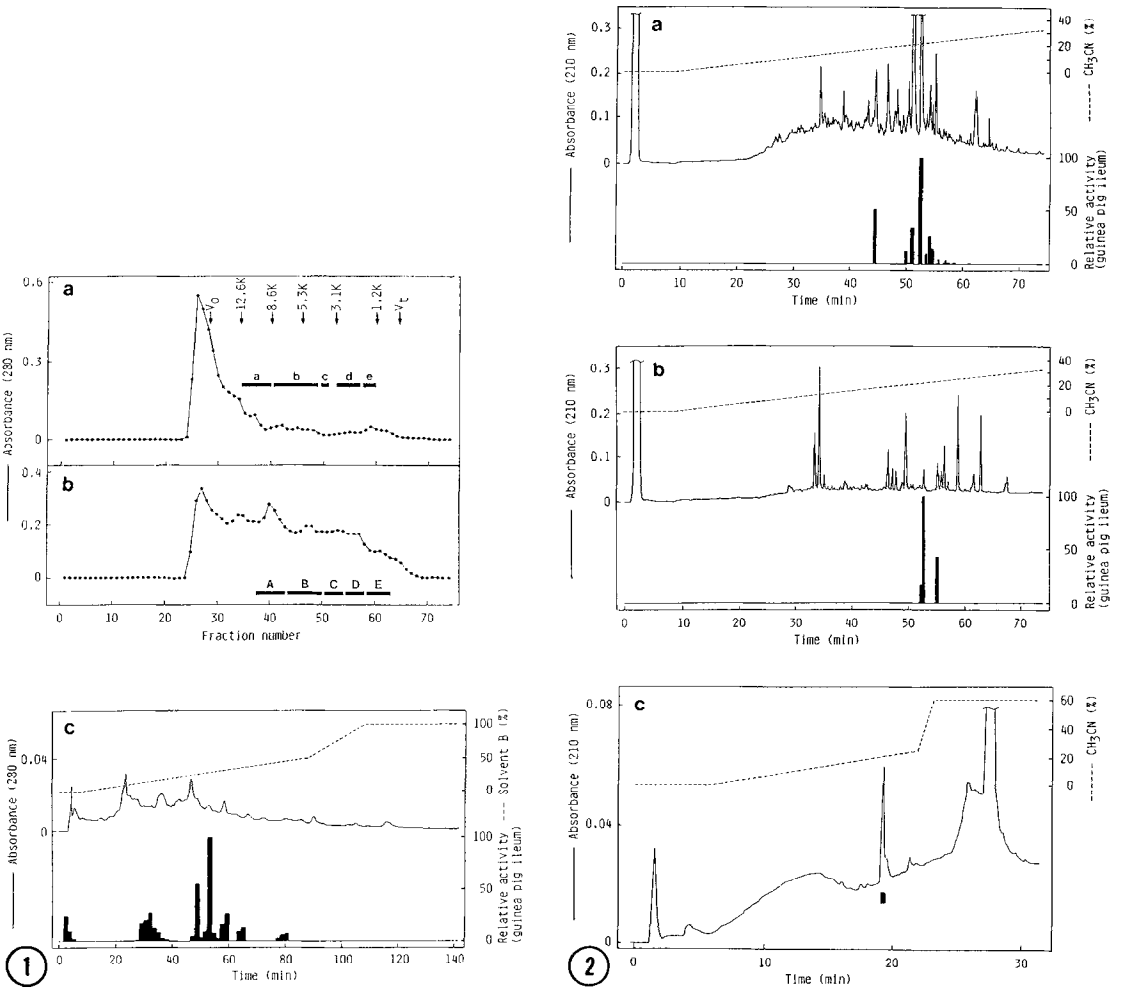
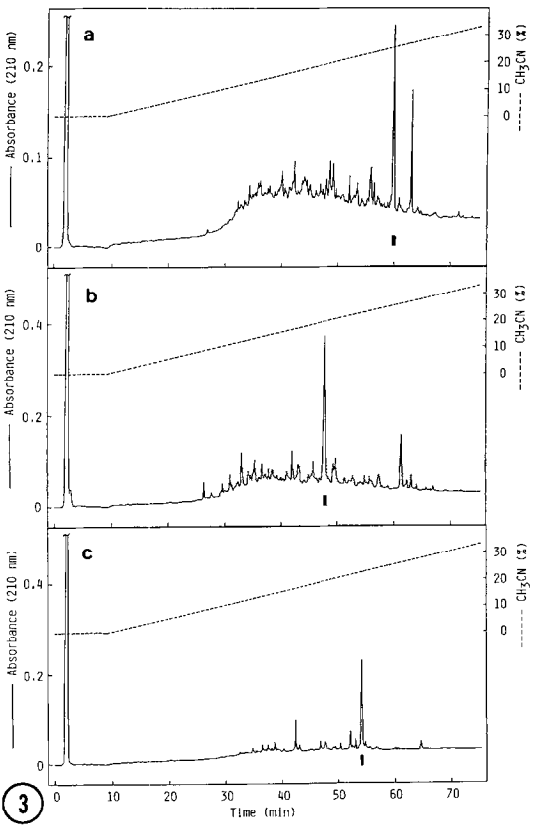


Figure 1. (a, b) Sephadex G-50 gel filtration of (a) frog brain and (b) frog intestinal extracts. Sample: SP-Ⅲ fraction of (a) frog brain and (b) frog intestine.
Column: Sephadex G-50 (fine), 1.8 × 134 cm.
Eluent: 1M CH_3COOH. Fraction size: 5 ml/tube.
Guinea pig ileum contractile activity was observed in black bar regions.
(c) Ion exchange HPLC of fraction D of frog intestine.
Sample: Ileum contractile fraction D in Fig. 1b.
Column: TSK gel CM-2SW, 7.6 × 300 mm (Tosoh). Flow rate: 2.0 ml/min.
Solvent system: Linear gradient elution from A to 50% B (80 min) followed by that from 50% B to 100% B (20 min).
(A) 10mM $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v),
(B) 1.0M $HCOONH_4$ (pH 6.5) : CH_3CN = 90 : 10 (v/v).

Figure 2. Purification of RTK-A and RTK-B by reverse phase HPLC.
Sample: (a) Ileum contractile fraction **d** in Fig. 1a.
(b) Ileum contractile fraction **e** in Fig. 1a.
(c) Ileum contractile fraction eluted at 55 min in Fig. 2b.
Column: (a, b) Chemcosorb 3ODS-H, 8.0 × 75 mm (Chemco).
(c) 219TP5215 diphenyl, 2.1 × 150 mm (Vydac).
Flow rate: (a, b) 2.0 ml/min. c) 0.3 ml/min.
Solvent system: (a, b) Linear gradient elution from A to B for 120 min.
(c) Linear gradient elution from 5% B to 100% B for 40 min.
H_2O : CH_3CN : 10% TFA = (A) 100 : 0 : 1, (B) 40 : 60 : 1 .
Ileum contractile activity in (c) was observed in black bar region.



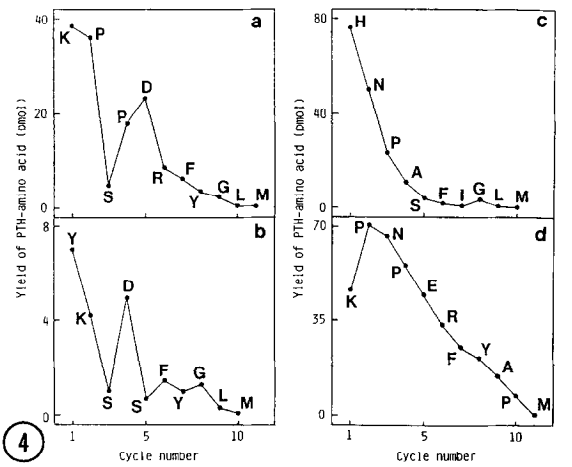


Figure 3. Final purification of (a) RTK-C, (b) RTK-D and (c) RTK-A by reverse phase HPLC.
Sample: (a) Ileum contractile fraction eluted at 30-33 min in Fig. 1c.
(b) Ileum contractile fraction eluted at 48-49.5 min in Fig. 1c.
(c) Ileum contractile fraction eluted at 52.5-54 min in Fig. 1c.
Chromatographic conditions are identical to those used in Figs. 2a and 2b.
Ileum contractile activiy was observed in black bar regions.

Figure 4. Yield of PTH- amino acid at each cycle of Edman degradation of (a) RTK-A, (b) RTK-B, (c) RTK-C and (d) RTK-D. One letter amino acid notation is used.

(9). Positive confirmation of the structures determined above was provided by co-chromatography of the native and synthetic peptides. Thus, the complete amino acid sequences of the four newly isolated peptides were unambiguously established (Fig. 5).

Amino acid sequences of RTK- A, - B, -C and -D are shown in Fig. 5, along with those of other known mammalian and non- mammalian tachykinins (10). RTK- A, -B and -C share the consensus sequence, Phe-X-Gly-Leu-Met-NH_2, of presently known members of the tachykinin family. As for the X residue, RTK- A and RTK- B have a Tyr, which is also found in physalaemin and uperolein. On the other hand, RTK- C has an Ile for the X- residue, as does eledoisin. None of these peptides has a Phe or a Val for the X residue, as mammalian tachykinins do. In the N- terminal regions, RTK-A has Pro residues at positions 2 and 4, as does substance P. Furthermore, RTK-A has a basic residue at the 6th position from the C-

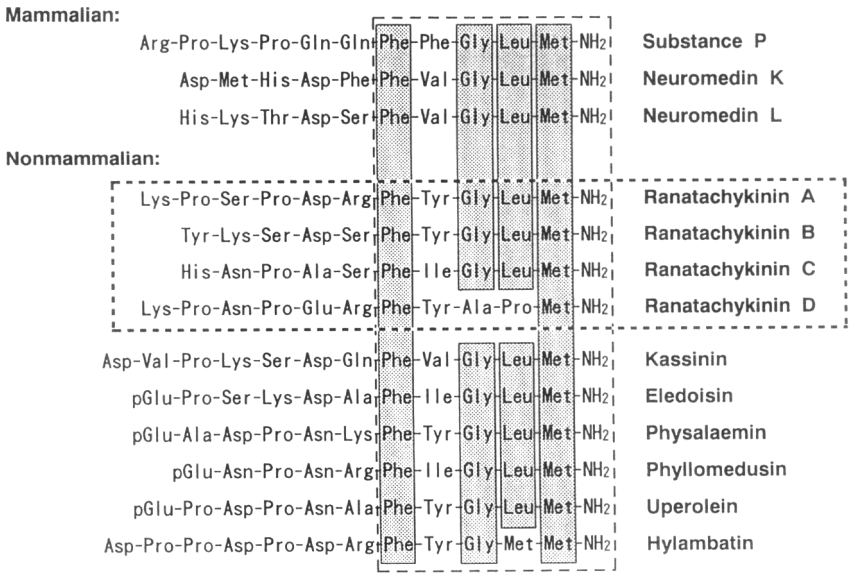


Figure 5. Amino acid sequences of the tachykinin family.

terminus, as physalaemin and phyllomedusin do. RTK-B has a sequence (Tyr-Lys-Ser-Asp-Ser-) homologous to that of neuromedin L (His-Lys-Thr-Asp-Ser-) in its N-terminal half. Thus, these three *Rana catesbeiana* tachykinins are found to completely conserve the consensus sequence of the tachykinin family in the C-terminal half but have large variations in the amino acid sequence of the N-terminal half.

It should be emphasized that RTK-D has a unique structure in the C-terminal half, i.e., Phe-Tyr-Ala-Pro-Met-NH_2, in which the Ala-Pro sequence has never before been found in the tachykinin family. As an exception to the consensus sequence, hylambatin has been reported to have a Phe-Tyr-Gly-Met-Met-NH_2 sequence (11), but this is only one homologous amino acid substitution (Leu→Met). Even though it has a unique structure, RTK-D shows typical contraction patterns in guinea pig ileum and rat duodenum assays, and induces depressor effects, when injected into anesthetized rats, in a manner similar to that of other tachykinins. Thus, we conclude that this peptide is a member of the tackykinin family. RTK-D is homologous to RTK-A and hylambatin in the six N-terminal residues. The unique structure of RTK-D suggests a possibility that the tachykinin family is divided into several subclasses and that mammals may have a counterpart of this amphibian tachykinin.

In order to characterize pharmacological properties of these four tachykinins, relative potencies of the peptides on the contractility of guinea pig ileum and rat duodenum were measured and compared with the mammalian tachykinins, physalaemin and eledoisin (Table 1). RTK-A elicited strong effects in both assays in a manner similar to eledoisin. RTK-B exhibited relatively strong effects on rat duodenum, while RTK-C and RTK-D elicited

Table 1. Relative potency of tachykinins on guinea pig ileum and rat duodenum

Peptide	Relative potency	
	Guinea pig ileum*	Rat duodenum**
Substance P	100	2
Ranatachykinin A	68	40
Ranatachykinin B	20	10
Ranatachykinin C	44	3.3
Ranatachykinin D	26	0.5
Physalaemin	77	0
Eledoisin	83	29
Neuromedin K	39	69
Neuromedin L	15	100

Relative potency was calculated on molar basis by taking substance P as 100 in guinea pig ileum assay* (n=5), and neuromedin L as 100 in rat duodenum assay** (n=4).

relatively strong effects on guinea pig ileum and weak effects on rat duodenum. Mammalian tachykinin receptors have been pharmacologically classified into at least three groups; SP-P, SP-N and SP-E or NK-1, NK-2 and NK-3 (12,13,14,15). By these classifications, RTK-A may be an agonist for SP-E type receptor, while RTK-C and RTK-D for SP-P type receptor. Recently, Nakanishi et al. have directly demonstrated the presence of three types of tachykinin receptors in mammals by cDNA cloning (16,17,18,19). Although so far nothing is known about amphibian tachykinin receptors, the presence of four tachykinins exhibiting different potencies in the two bioassays suggests diversity in the amphibian tachykinin receptor system.

Most amphibian tachykinins have been isolated from skin, which is known to contain bioactive peptides in extremely high concentrations, where they are assumed to assist in protection against enemies. As an exception, entero-hylambatin and entero-kassinin have been identified in frog intestine (20). In the present study, we were able to isolate four different peptides eliciting different phamacological activities in *Rana catesbeiana* brain and gut. Identification of four different tachykinins in brain and intestine of the same frog species suggests that each frog tachykinin participates in regulating frog brain and gut function in a different manner. These results indicate that frog has already acquired a complex peptidergic regulation system both in ligands and receptors comparable to the mammalian system. Furthermore, identification in RTK-D of a unique C-terminal structure in frog may raise the possibility of further diversity in the mammalian tachykinin system.

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