Primary structure and receptor-binding properties of a neurokinin A-related peptide from frog gut

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A tachykinin peptide was isolated from an extract of the intestine of the European green frog, *Rana ridibunda*, and its primary structure was established as: His-Lys-Leu-Asp-Ser-Phe-Ile-Gly-Leu-Met.CONH₂. This sequence was confirmed by chemical synthesis and shows two amino acid substitutions (leucine for threonine at position 3 and isoleucine for valine at position 7) compared with neurokinin A. Binding parameters for synthetic [Leu³,Ile⁷]neurokinin A and mammalian tachykinins were compared using receptor-selective radioligands and crude membranes from tissues enriched in the NK1, NK2 and NK3 receptors. [Leu³,Ile⁷]Neurokinin A was approx. 3-fold less potent than substance P in inhibiting the binding of [¹²⁵I]-labelled [Sar⁹,Met(O₂)¹¹]substance P (labelled with Bolton-Hunter reagent) to rat submandibular gland (NK1 receptor), 8-fold less potent than neurokinin A in inhibiting the binding of [2-¹²⁵I]iodohistidine¹]neurokinin A to rat stomach fundus (NK2 receptor) and 6-fold less potent than neurokinin B in inhibiting the binding of [¹²⁵I]-Bolton-Hunter-labelled scyliorhinin II to rat brain (NK3 receptor). Thus the frog neurokinin A-related peptide shows moderate affinity but lack of selectivity for all three tachykinin-binding sites in rat tissues. This non-selectivity is similar to that displayed by the molluscan tachykinin, elaidoisin, which also contains an isoleucine residue in the corresponding position in the molecule.

INTRODUCTION

Amphibian skin has proved a rich source for the isolation of numerous tachykinin peptides which share features of structural similarity (Erspamer, 1981). In particular, the peptides terminate in the common sequence -Phe-Xaa-Gly-Yaa-Met.CONH₂ where Xaa is an aromatic or branched-chain aliphatic amino acid and Yaa is leucine or methionine. Structure-activity relationships determined using synthetic tachykinin analogues have shown that this region of the tachykinin peptides is of particular importance in receptor binding and in mediating the actions of the tachykinins on mammalian vascular and other smooth muscles (Regoli *et al.*, 1987; Burcher *et al.*, 1991). Antisera raised against amphibian tachykinins have proved valuable in demonstrating that multiple tachykinin peptides are present in nervous tissues of higher vertebrates (Maggi, 1985). In addition to substance P, neurokinin A (Xaa = Val and Yaa = Leu) and several biosynthetically related *N*-terminally extended forms, and neurokinin B (Xaa = Val, Yaa = Leu) have been isolated from mammalian [reviewed in Takeda *et al.* (1990)] and avian (Conlon *et al.*, 1988) tissues. More recently, amphibian nervous tissue has been shown to contain tachykinin peptides. A substance P-related peptide, termed ranakinin (Xaa = Tyr, Yaa = Leu), was isolated together with neurokinin B from the brain of the European green frog, *Rana ridibunda* (O'Harte *et al.*, 1991), and four structurally related tachykinins, termed ranatachykinins A, B, C and D, have been isolated from the brain and/or intestine of the bullfrog, *Rana catesbeiana* (Kozawa *et al.*, 1991).

Mammalian tissues contain three well-characterized high-affinity binding sites for the tachykinins, termed the NK1, NK2 and NK3 receptors, which differ in their ability to bind endogenous tachykinin ligands [reviewed in Burcher *et al.* (1991)]. Substance P binds with highest affinity to the NK1 receptor,

neurokinin A to the NK2 receptor and neurokinin B to the NK3 receptor. In the present study, a neurokinin A-related peptide has been isolated from the intestine of *R. ridibunda* and its receptor-binding activity is compared with the mammalian tachykinins using membrane preparations from rat tissues (submandibular gland, stomach fundus and brain) that are enriched in NK1, NK2 and NK3 receptors respectively.

EXPERIMENTAL

Materials

Synthetic peptides were supplied by Peninsula Laboratories (Belmont, CA, U.S.A.) or by Auspep (Melbourne, Australia). The NK1 receptor-selective radioligand [¹²⁵I]Bolton-Hunter-labelled [Sar⁹,Met(O₂)¹¹]substance P (Lew *et al.*, 1990) and the NK3 receptor-selective radioligand [¹²⁵I]Bolton-Hunter-labelled scyliorhinin II (Mussap & Burcher, 1990) were prepared and purified as previously described. The NK2 receptor radioligand [2-¹²⁵I]iodohistidine¹]neurokinin A was purchased from Amersham International, U.K. The specific radioactivity of all radioligands was greater than 60 TBq/mmol. Reagents for peptide synthesis were supplied by Applied Biosystems (Foster City, CA, U.S.A.).

Preparation of frog gut extract

Adult frogs (*Rana ridibunda*) were obtained from a commercial source (Couetard, St-Hilaire de Riez, France). The small intestine was collected from 400 specimens and immediately frozen on dry ice. The tissue (267 g wet weight) was cut into small (approx. 1 cm) pieces and boiled for 15 min in 0.5 M-acetic acid (2.5 l). After centrifugation (1600 *g* for 30 min at 4 °C), the supernatant was pumped at a flow rate of 2 ml/min through 12 Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA, U.S.A.) connected

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in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:29:1, by vol.) and freeze-dried.

Radioimmunoassay

Neurokinin A-like immunoreactivity was measured by use of an antiserum (NKA-2) directed against the C-terminal region of neurokinin A. Full details of the specificity of the antiserum and the radioimmunoassay procedure have been provided previously (Conlon, 1991).

Purification of frog neurokinin A

The gut extract, after partial purification on Sep-Pak cartridges, was redissolved in 0.1% (v/v) trifluoroacetic acid/water (5 ml) and chromatographed on a Sephadex G-25 (fine) column (75 cm × 2 cm) equilibrated with 1 M-acetic acid at a flow rate of 36 ml/h. Fractions (3 ml) were collected and the presence of NKA-like immunoreactivity was determined by radioimmunoassay at appropriate dilution. The fractions denoted by the bar in Fig. 1 were pooled (total volume = 18 ml) and pumped at a flow rate of 2 ml/min on to a Vydac 218 TP reversed-phase C₁₈ h.p.l.c. column (1 cm × 25 cm) (Separations Group, Hesperia, CA, U.S.A.) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min, held at this concentration for 40 min and then raised to 49% (v/v) over 60 min with linear gradients. Absorbance was measured at 214 nm and 280 nm. Individual peaks were collected by hand. The fraction denoted by the bar (Fig. 2a) was rechromatographed on a Supelcosil LC-18-DB reversed-phase C₁₈ column (1 cm × 25 cm) (Supelco Inc., Bellefonte, PA, U.S.A.) equilibrated with acetonitrile/water/trifluoroacetic acid (140:859:1, by vol.) at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 38% (v/v) over 70 min. The fraction denoted by the bar (Fig. 2b) was rechromatographed on a Vydac 214 TP reversed-phase C₄ column (0.46 cm × 25 cm) equilibrated with acetonitrile/water/trifluoroacetic acid (140:859:1, by vol.) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 35% (v/v) over 50 min. Frog neurokinin A was purified to apparent homogeneity by rechromatography of the fraction denoted by the bar (Fig. 2c) on a Vydac 219 TP reversed-phase phenyl column (0.46 cm × 25 cm) under the same elution conditions used with the C₄ column.

Structural characterization

The primary structure of the frog neurokinin A-related peptide was determined by automated Edman degradation using approx. 130 pmol of peptide. Details of the instruments used have been provided previously (Conlon *et al.*, 1991). The detection limit for phenylthiohydantoin derivatives of amino acids was 0.5 pmol.

Peptide synthesis

[Leu⁸,Ile⁷]Neurokinin A was synthesized (0.5 mmol scale) on a *p*-methylbenzhydrylamine resin using an Applied Biosystems model 430A peptide synthesizer. *t*-Butyloxycarbonyl-protected amino acid derivatives were coupled as their hydroxybenzotriazole-active esters following the manufacturer's standard protocols. The peptide was cleaved from the resin using a mixture of trifluoromethanesulphonic acid/thioanisole/ethanedithiol/trifluoroacetic acid (2:2:1:20, by vol.) and was purified by gel-permeation chromatography [Sephadex G-25 in 50% (v/v) acetic acid] and reversed-phase h.p.l.c. The final yield of purified peptide was 25% of the theoretical amount. The identity of the peptide was confirmed by amino acid analysis (mol of residue/mol of peptide: Asx 1.0, Ser 0.7, Gly 1.0, His 1.0, Met 1.0, Ile 1.0, Leu 2.1, Phe 1.0, Lys 1.1), automated Edman degradation (Table 1) and low-resolution fast-atom bombard-

ment mass spectrometry (observed mass 1159; calculated mass for the protonated form 1159). The retention times of endogenous and synthetic [Leu⁸,Ile⁷]neurokinin A were compared by chromatography on a Vydac 218 TP h.p.l.c. column (0.46 cm × 25 cm) equilibrated with acetonitrile/water/trifluoroacetic acid (150:849:1, by vol.) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 35% (v/v) over 30 min. The oxidized form of frog neurokinin A ([Leu⁸,Ile⁷,Met(O)¹⁰]neurokinin A) was prepared as previously described (Conlon *et al.*, 1986b).

Competitive binding studies

Female Wistar rats (150–250 g) were killed by cervical dislocation and the submandibular glands, stomach fundus and brain minus cerebellum were quickly removed. Crude membrane preparations were prepared for these tissues as previously described (Lew *et al.*, 1990; Mussap & Burcher, 1990). Incubations were carried out at 25 °C in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.2% (w/v) BSA and chymostatin (4 µg/ml). Submandibular-gland membranes at a concentration equivalent to 10 mg of tissue/ml were incubated with [¹²⁵I]-labelled [Sar⁹,Met(O₂)¹¹]substance P (70 pmol/l) for 120 min, stomach fundus membranes (35 mg/ml) were incubated with [2-¹²⁵I]iodohistidine¹neurokinin A (50 pmol/l) for 90 min and brain membranes (40 mg/ml) were incubated with [¹²⁵I]-labelled scylorhinin II (80 pmol/l) for 90 min.

Under the conditions of incubation used, addition of chymostatin (4 µg/ml) resulted in an increase in the specific binding of [2-¹²⁵I]iodohistidine¹neurokinin A to stomach fundus membranes whereas leupeptin (4 µg/ml), bestatin (100 µM), phosphoramidon (1 µM) and enalapril (10 µM) has no significant effect on binding, and bacitracin (40 µg/ml) caused a decrease in specific binding. Consequently, only chymostatin was included in the incubation buffers. Kinetic analysis indicated that binding was relatively slow, reaching equilibrium after 90 min. At equilibrium, specific binding represented approximately 90% of the total radioactivity. Binding was reversible on addition of 1 µM-neurokinin A at equilibrium with complete dissociation of radioactivity after 240 min (C. J. Mussap & E. Burcher, unpublished work). Previous studies have shown that [¹²⁵I]-labelled [Sar⁹,Met(O₂)¹¹]substance P (Lew *et al.*, 1990) and [¹²⁵I]-labelled scylorhinin II (Mussap & Burcher, 1990) are stable under the incubation conditions employed and that inclusion of additional proteinase inhibitors in the incubation medium had no effect on specific binding.

Slope factors (pseudo-Hill coefficients) were calculated using the program EBDA (McPherson, 1985) and dissociation constants (*K_d*) were calculated using the iterative curve-fitting program LIGAND (Munson & Rodbard, 1980). Between three and five independent experiments were carried out with each radioligand, and data are expressed as means ± s.e.m. Comparisons were made using Student's *t* test for unpaired data and *P* < 0.05 was regarded as significant.

RESULTS

Purification of [Leu⁸,Ile⁷]neurokinin A

An extract of frog intestine, even at high dilution, contained material that inhibited the binding of [2-¹²⁵I]iodohistidine¹neurokinin A to an antibody raised against mammalian neurokinin A. The immunoreactivity in serial dilutions of the extract did not diminish in parallel with the neurokinin A standard curve and so it was concluded that radioimmunoassay could be used to detect but not quantify a frog neurokinin A-related peptide.

Fig. 1. Gel-permeation chromatography on Sephadex G-25 of an extract of frog intestine, after partial purification on Sep-Pak cartridges

The histogram shows the concentration of neurokinin A-like immunoreactivity measured with an antiserum directed against the C-terminal region of neurokinin A. The void volume of the column is shown by the arrow. The fractions denoted by the bar were pooled and subjected to further purification.

The neurokinin A-like immunoreactivity in the extract was eluted from a Sephadex G-25 column as a single peak (Fig. 1).

The fractions denoted by the bar were pooled and chromatographed on a semipreparative Vydac C₁₈ reversed-phase h.p.l.c. column (Fig. 2a). The neurokinin A-like immunoreactivity was eluted from the column under isocratic conditions as a broad peak, denoted by the bar. This peak was rechromatographed on a semipreparative Supelcosil C₁₈ column (Fig. 2b) and the neurokinin A-like immunoreactivity was associated with the sharp peak denoted by the bar. Chromatography of this peak on an analytical Vydac C₄ column (Fig. 2c) revealed that neurokinin A-like immunoreactivity was associated with a minor peak, denoted by the bar, that was eluted before the most abundant peptides in the fraction. Rechromatography of this peak on a Vydac phenyl column (Fig. 2d) again showed that the neurokinin A-like immunoreactivity was associated with a minor peak that was eluted before the major peak in the chromatogram. The yield of peptide, estimated from the absorbance at 214 nm was approx. 150 pmol and 90 % of this material was subjected to automated Edman degradation.

Characterization of [Leu³,Ile⁷]neurokinin A

It was possible to assign without ambiguity phenylthiohydantoin derivatives of amino acid for nine cycles of operation during sequence analysis of frog neurokinin A (Table 1). The derivative of methionine was detected only in trace amount during cycle 10. The data indicated that the peptide was more than 95 % pure. The strong reactivity of frog neurokinin A with an antibody directed against the C-terminal region of neurokinin A suggested that the peptide terminated in an α -amidated methionine residue. The antiserum used in radioimmunoassay shows negligible reactivity with the C-terminal free acid form of neurokinin A or with neurokinin A extended from its C-terminus

Fig. 2. Purification of [Leu³,Ile⁷]neurokinin A by successive reversed-phase h.p.l.c. on (a) Vydac C₁₈, (b) Supelcosil LC-18-DB, (c) Vydac C₄ and (d) Vydac phenyl columns

In each chromatogram, the broken lines show the concentration of acetonitrile in the eluting solvent and the bars show the peaks containing neurokinin A-like immunoreactivity. The arrows show when peak collection began and ended.

Table 1. Determination of the primary structure of frog neurokinin A-related peptide by automated Edman degradation

Cycle no.	Endogenous		Synthetic	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	His	34	His	105
2	Lys	53	Lys	198
3	Leu	78	Leu	212
4	Asp	29	Asp	101
5	Ser	14	Ser	110
6	Phe	49	Phe	176
7	Ile	34	Ile	183
8	Gly	37	Gly	139
9	Leu	16	Leu	41
10	Met	Trace	Met	9

by a glycine residue (Conlon, 1991). Frog neurokinin A shows two amino acid substitutions (Leu for Thr-3 and Ile for Val-7) compared with neurokinin A previously isolated from mammalian tissues. The primary structure of frog neurokinin A was confirmed by chemical synthesis. Purified endogenous [Leu³,Ile⁷]-neurokinin A was eluted from a C₁₈ reversed-phase h.p.l.c. column as a single peak with a retention time between 17 and 18 min (Fig. 3). The C-terminally α -amidated form of synthetic [Leu³,Ile⁷]neurokinin was eluted under the same conditions of chromatography with a retention time of 24.5 min. However, the oxidized form of frog neurokinin A, prepared by treatment of the peptide with hydrogen peroxide (Conlon *et al.*, 1986b), was eluted from the column with a retention time of 17.6 min. Similarly, a mixture of endogenous frog neurokinin A (approx. 5 pmol) and the oxidized form of synthetic [Leu³,Ile⁷]neurokinin A (5 pmol) were co-eluted from the column as a single peak as determined by radioimmunoassay. It is concluded therefore that frog neurokinin had been converted into the oxidized form during the extraction and/or purification procedure. The tachykinin, carassin, isolated from goldfish brain (Conlon *et al.*, 1991), was also obtained in the oxidized form.

Competitive binding studies

The abilities of synthetic [Leu³,Ile⁷]neurokinin A and mammalian tachykinins to displace the binding of selective radio-labelled tachykinin ligands to rat tissues that are enriched in NK1 receptors (submandibular gland), NK2 receptors (stomach fundus) and NK3 receptors (brain) are compared in Table 2.

Table 2. Binding parameters for [Leu³,Ile⁷]neurokinin A and mammalian tachykinins in competing against ¹²⁵I-labelled [Sar⁹,Met(O₂)¹¹]substance P in rat submandibular gland, [2-¹²⁵I]iodohistidine¹neurokinin A in rat stomach fundus and ¹²⁵I-labelled scyliorhinin II in rat brain

Slope factors (psuedo-Hill coefficients) were calculated using the program EBDA and are shown as means $\pm$ S.E.M. Dissociation constants (K_d) were calculated using the iterative curve-fitting program LIGAND and are shown as means $\pm$ S.E.M. of three to five independent experiments analysed simultaneously.

	Submandibular gland		Stomach fundus		Brain	
	Slope	K_d (nM)	Slope	K_d (nM)	Slope	K_d (nM)
[Leu ³ ,Ile ⁷]-Neurokinin A	0.87 $\pm$ 0.03	2.9 $\pm$ 0.7	0.80 $\pm$ 0.03	27 $\pm$ 5	0.76 $\pm$ 0.03	95 $\pm$ 40
Substance P	1.42 $\pm$ 0.11	0.95 $\pm$ 0.13	0.56 $\pm$ 0.10	45 $\pm$ 33	0.57 $\pm$ 0.08	120 $\pm$ 50
Neurokinin A	1.01 $\pm$ 0.09	5.3 $\pm$ 1.0	0.76 $\pm$ 0.08	3.6 $\pm$ 0.8	0.61 $\pm$ 0.13	50 $\pm$ 25
Neurokinin B	1.01 $\pm$ 0.09	63 $\pm$ 14	0.70 $\pm$ 0.30	80 $\pm$ 40	0.74 $\pm$ 0.16	15 $\pm$ 5

Fig. 3. Comparison of the retention times on reversed-phase h.p.l.c. of (a) endogenous frog neurokinin A, (b) synthetic [Leu³,Ile⁷]neurokinin A and (c) synthetic [Leu³,Ile⁷]neurokinin A after treatment with hydrogen peroxide

Details of the elution conditions are given in the text. The peak of endogenous [Leu³,Ile⁷]neurokinin A was detected by radioimmunoassay.

[Leu³,Ile⁷]Neurokinin A was slightly but not significantly more potent (1.8-fold) than neurokinin A in competing for binding sites in the submandibular gland, slightly but not significantly less potent (1.9-fold) in competing for binding sites in the brain but appreciably less potent (8-fold) for competing for binding sites in the stomach fundus. For each of the three tissues studied, analysis of the binding data obtained with [Leu³,Ile⁷]neurokinin A demonstrated that the slope factors (psuedo-Hill coefficients) were not significantly different from unity, consistent with the presence of a single class of binding site for the peptide. In this series of experiments, the rank order of potency and the

dissociation constants of substance P, neurokinin A and neurokinin B were consistent with previously published data.

DISCUSSION

This study has contributed to our understanding of the molecular evolution of the tachykinin family of polypeptides and complements previous work that involved the isolation of a substance of P-related peptide (ranakinin) and neurokinin B from the brain of *Rana ridibunda* (O'Harte *et al.*, 1991). Frog neurokinin A contains two amino acid substitutions (Leu for Thr-3 and Ile for Val-7) compared with neurokinin A (Fig. 4). The primary structure of [Leu³,Ile⁷]neurokinin A is also compared with the structures of other tachykinins that contain an isoleucine residue in the C-terminal region of the molecule (Xaa = Ile). Such peptides have been classified as belonging to the eledoisin subfamily of tachykinins (Schoofs *et al.*, 1990), the members of which comprise, in addition to eledoisin, which is isolated from salivary gland of a mollusc, the Mediterranean octopus, *Eledone moschata* (Erspamer & Anastasi, 1962), phyllomedusin from the skin of the hylid frog, *Phyllomedusa sawagei* (Melchiorri & Negri, 1984), entero-kassinin from the gut of the African rhacophorid frog, *Kassina senegalensis* (Melchiorri & Negri, 1984), and ranatatchykinin C isolated from the gut of the bullfrog, *Rana catesbeiana* (Kozawa *et al.*, 1991). A tachykinin of the eledoisin subfamily has never been isolated from mammalian tissues but the presence of peptides with eledoisin-like immunoreactivity in rat brain has been reported (Theodorsson-Norheim *et al.*, 1986).

The evolutionary relationship between the tachykinin peptides identified in frog skin and the neuropeptides identified in mammalian and amphibian nervous tissues is unclear and it is not known whether such peptides are homologous. Structural analysis of mRNAs directing the synthesis of substance P in bovine (Nawa *et al.*, 1983) and rat (Kawaguchi *et al.*, 1986) brains has established that substance P and neurokinin A are derived from the post-translational processing of the same biosynthetic precursor (either β - or γ -preprotachykinin). Elucidation of the primary structures of ranakinin and frog neurokinin A will facilitate the screening of a *Rana ridibunda* cDNA library (Hilaro *et al.*, 1990) in order to characterize frog preprotachykinin(s) and to determine the biosynthetic relationship between the two peptides.

In competitive binding studies carried out in rat tissues enriched in NK1, NK2 and NK3 receptors, [Leu³,Ile⁷]neurokinin A showed moderate affinity but lack of selectivity for each of these sites. The peptide was approximately 3-fold less potent than

substance P in inhibiting the binding of ¹²⁵I-labelled [Sar⁹,Met(O₂)¹¹]substance P to NK1 receptors in submandibular gland, 8-fold less potent than neurokinin A in inhibiting the binding of [2-¹²⁵I]iodohistidine¹neurokinin A to NK2 sites in rat stomach fundus and 6-fold less potent than neurokinin B in inhibiting the binding of ¹²⁵I-labelled scyliorhinin II to NK3 sites in rat brain. This lack of selectivity may be a consequence of the presence of an isoleucine residue in the receptor-binding C-terminal region of the molecule. Eledoisin also shows moderate affinity for all three tachykinin receptors in rat tissues (Burcher & Buck, 1986; Lee *et al.*, 1986). The relative lack of affinity of [Leu³,Ile⁷]neurokinin A compared with mammalian neurokinin A for the NK2 receptor is surprising. Previous structure-activity studies have shown that neurokinin A-(4-10)-peptide is equipotent with neurokinin A, both in competitive binding studies and in contracting isolated smooth muscle (Regoli *et al.*, 1987; Chassaing *et al.*, 1991). In consequence, an amino acid substitution at position 3 in the molecule would be expected to have only a minor effect on receptor-binding activity. The observed 8-fold fall in binding affinity suggests that the presence of the hydrophobic leucine residue at position 3, together with the bulky isoleucine residue at position 7, may induce a change in the conformation of the C-terminal region of the molecule or may hinder sterically the interaction of the ligand with the NK2 receptor. Eledoisin and the other members of the eledoisin subfamily of tachykinins do not contain a hydrophobic amino acid residue in the N-terminal region of the molecule.

The lack of selectivity of [Leu³,Ile⁷]neurokinin A towards the mammalian NK2 receptor contrasts with the high affinity of the frog substance P analogue, ranakinin, for the mammalian NK1 receptor (O'Harte *et al.*, 1991). Ranakinin is equipotent with substance P in inhibiting the binding of ¹²⁵I-labelled [Sar⁹,Met(O₂)¹¹]substance P to NK1-binding sites in rat submandibular gland membranes. [Leu³,Ile⁷]Neurokinin A also shows highest affinity for the NK1 receptor and, in this respect, resembles the tachykinin, scyliorhinin I (Ala-Lys-Phe-Asp-Lys-Phe-Tyr-Gly-Leu-Met.CONH₂) isolated from the intestine of the dogfish, *Scyliorhinus canicula* (Conlon *et al.*, 1986a). Scyliorhinin I bound to NK1 sites in the rat submandibular gland with $K_d = 0.9$ nM, compared with $K_d = 2.0$ nM for binding to NK2 sites in hamster bladder and $K_d = 95$ nM for binding to NK3 sites in rat brain (Buck & Krstenansky, 1987). It is of interest to note that a phylogenetic study has reported that amphibian nervous tissue contains a receptor with ligand-binding properties similar to that of the mammalian NK1-binding site but no mammalian NK2-type binding site (Dietl & Palacios, 1991).

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Frog neurokinin A	His-Lys-Leu-Asp-Ser-Phe-Ile-Gly-Leu-Met.NH ₂
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met.NH ₂
Enterokassinin	Asp-Glu-Pro-Asn-Ser-Asp-Gln-Phe-Ile-Gly-Leu-Met.NH ₂
Phyllomedusin	pGlu-Asn-Pro-Asn-Arg-Phe-Ile-Gly-Leu-Met.NH ₂
Ranatatchykinin C	His-Asn-Pro-Ala-Ser-Phe-Ile-Gly-Leu-Met.NH ₂
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met.NH ₂

Fig. 4. Comparison of the primary structures of neurokinin A-related peptides from amphibia, mammals and a mollusc

[Leu³,Ile⁷]Neurokinin A is the peptide from *Rana ridibunda* isolated in this study, enterokassinin, phyllomedusin and ranatatchykinin C are peptides isolated from other amphibian species and eledoisin was isolated from a mollusc.

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