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Tachykinins (Substance P, Neurokinin A and Neuropeptide γ) and Neurotensin from the Intestine of the Burmese Python, *Python molurus*

J. MICHAEL CONLON,*[1] THOMAS E. ADRIAN* AND STEPHEN M. SECOR†

*Department of Biomedical Sciences, Creighton University School of Medicine, Omaha, NE 68178-0405
†Department of Physiology, University of California at Los Angeles School of Medicine, Los Angeles, CA 90095-1751

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CONLON, J. M., T. E. ADRIAN AND S. M. SECOR. *Tachykinins (substance P, neurokinin A and neuropeptide γ,) and neurotensin from the intestine of the burmese python,* Python molurus. PEPTIDES **18**(10) 1505–1510, 1997.—Peptides with substance P-like immunoreactivity, neurokinin A-like immunoreactivity and neurotensin-like immunoreactivity were isolated in pure form from an extract of the intestine of the Burmese python (*Python molurus*). The primary structure of python substance P (Arg-Pro-Arg-Pro-Gln-Gln-Phe-Tyr-Gly-Leu-Met-NH_2) shows one amino acid substitution ($Phe^8 \rightarrow Tyr$) compared with chicken/alligator substance P and an additional substitution ($Lys^3 \rightarrow Arg$) as compared with mammalian substance P. The neurokinin A-like immunoreactivity was separated into two components. Python neuropeptide γ (Asp-Ala-Gly-Tyr-Ser-Pro-Leu-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 shows three substitutions ($Gly^5 \rightarrow Ser$, $Gln^6 \rightarrow Pro$ and $Ile^7 \rightarrow Leu$) compared with alligator neuropeptide γ and an additional substitution ($His^4 \rightarrow Tyr$) compared with mammalian neuropeptide γ. Python neurokinin A (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met.NH_2) is identical to human/chicken/alligator neurokinin A. Python neurotensin (pGlu-Leu-Val-His-Asn-Lys-Ala-Arg-Pro-Tyr-Ile-Leu) is identical to chicken/alligator neurotensin. The data are indicative of differential evolutionary pressure to conserve the amino acid sequences of reptilian gastrointestinal peptides. © 1997 Elsevier Science Inc.

Python Tachykinin Neurotensin Substance P Neurokinin A Neuropeptide γ

THE REPTILES are the extant representatives of the stock from which birds and mammals have evolved and so occupy a crucially important position in phylogeny. The fossil record suggests that crocodilians and birds have developed from a common group of ancestors whereas present day chelonians (turtles and tortoises) and squamates (snakes and lizards) have arisen from more ancient divergences. The position of chelonians is controversial as the traditional view that these animals represent the only surviving clade of 'primitive' anapsid reptiles has been recently challenged (18). Immunohistochemical studies have shown that the gastrointestinal tract of all major classes of reptiles contain the complex distribution of most, if not all, of the regulatory peptides found in mammals (3,9–11,14,16,29). However, few regulatory peptides from reptilian gastrointestinal tissues have been characterized structurally and studies have been confined to the alligator, *Alligator mississipiensis.* Gastrin-releasing peptide, vasoactive intestinal polypeptide and somatostatin-14 have been isolated from an extract of alligator stomach (26) and neurotensin has been purified from alligator intestine (19). In addition, tachykinins (27) and neuropeptide Y (26), peptides with a dual location in the brain and gastrointestinal tract, have been purified from alligator brain.

The initial impetus for the current study was our interest in the Burmese python *Python molurus* (Squamata: Serpentes: Pythonidae) as a novel animal model with which to study the effects of neuroendocrine peptides upon intestinal adaptation (20–22). In its natural habitat or in captivity, the python may fast for over a year and then, in a single meal, consume prey that weight over 100% of its own body mass (24). Feeding results in profound and rapid adaptive changes in the gastrointestinal tract such as increases in mucosal mass, nutrient uptake and oxygen consumption rate (20–22) and a marked release of gastrointestinal and pancreatic hormones into the circulation (1). The role of neuroendocrine factors in mediating these changes is not understood and so our laboratories have embarked upon a program of studies to purify regulatory peptides from the python gastrointestinal tissues in order to investigate their possible involvement in the post-prandial responses. In the present study, we describe the isolation and characterization of three tachykinins, corresponding to mammalian substance P, neurokinin A and neuropeptide γ, and neurotensin from the intestine of the Burmese python.

[1] To whom correspondence should be addressed.

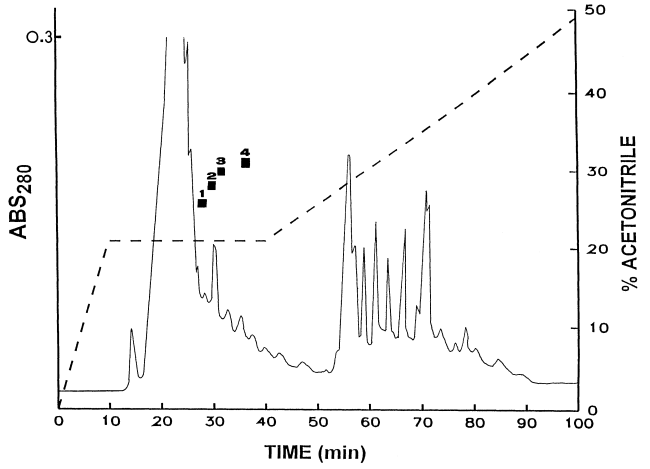

FIG. 1. Purification by reversed-phase HPLC on a semipreparative Vydac C-18 column of regulatory peptides in an extract of python intestine after partial purification by gel permeation chromatography. Fraction 1 contained neurotensin, fraction 2 contained substance P, fraction 3 contained neurokinin A and fraction 4 contained neuropeptide γ and these peptides were purified further. The concentration of acetonitrile in the eluting solvent is shown by a broken line.

METHOD

Tissue Extraction

Whole small intestine was removed from three subadult specimens (1 male; 1.6–2.2 m in total length) immediately after sacrifice. Tissue (125 g) was frozen in liquid nitrogen and stored at −70°. The animals had been fasting for 56, 227 and 355 days. The tissues were pooled and extracted with ethanol/0.7 M HCl (3:1 v/v; 1200 ml) at 0°C using a Waring Blender as previously described (19). After centrifugation (1600 *g* for 1 h at 4°C), ethanol was removed from the supernatant under reduced pressure. Peptide material was isolated from the extract using 8 Sep-Pak C-18 cartridges (Waters Associates). Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:29:1, v/v/v.) and freeze-dried.

Radioimmunoassay Procedure

Substance P-like immunoreactivity (SP-LI) was measured using antiserum P-4 that is directed against the COOH-terminal region of substance P. The antiserum shows <0.5% cross-reactivity with neurokinin A and with substance P that does not contain an α-amidated methionine residue or is extended from its COOH-terminus by a glycine residue. Neurokinin A-like immunoreactivity (NKA-LI) was measured with antiserum NKA-2 that is directed against the COOH-terminal region of neurokinin A and shows full cross-reactivity with neuropeptide K and neuropeptide γ and approximately 25% cross-reactivity with neurokinin B. The antiserum also requires an α-amidated COOH-terminal residue for reactivity. Full details of the radioimmunoassay procedures and specificities of the antisera have been provided in a review (6). Neurotensin-like immunoreactivity (NT-LI) was measured using antiserum 2/7 directed against a site in the *C*-terminal to central region of pig neurotensin as previously described (19). The antiserum cross-reacts with chicken neurotensin but shows negligible reactivity with xenopsin and neuromedin *N*.

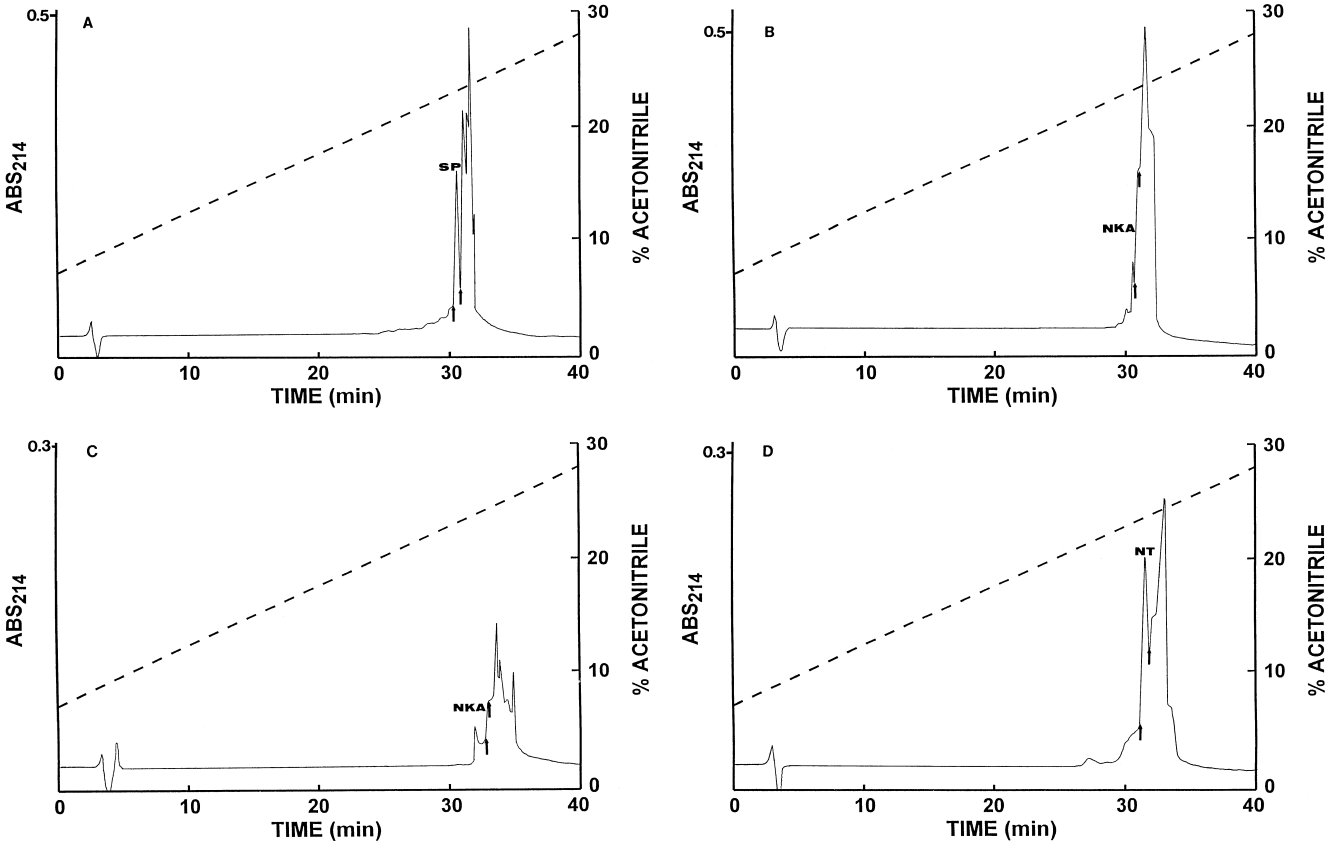

FIG. 2. Purification by reversed-phase HPLC on an analytical Vydac C-4 column of python (A) substance P, (B) neurokinin A, (C) neuropeptide γ and (D) neurotensin The arrows show where peak collection began and ended.

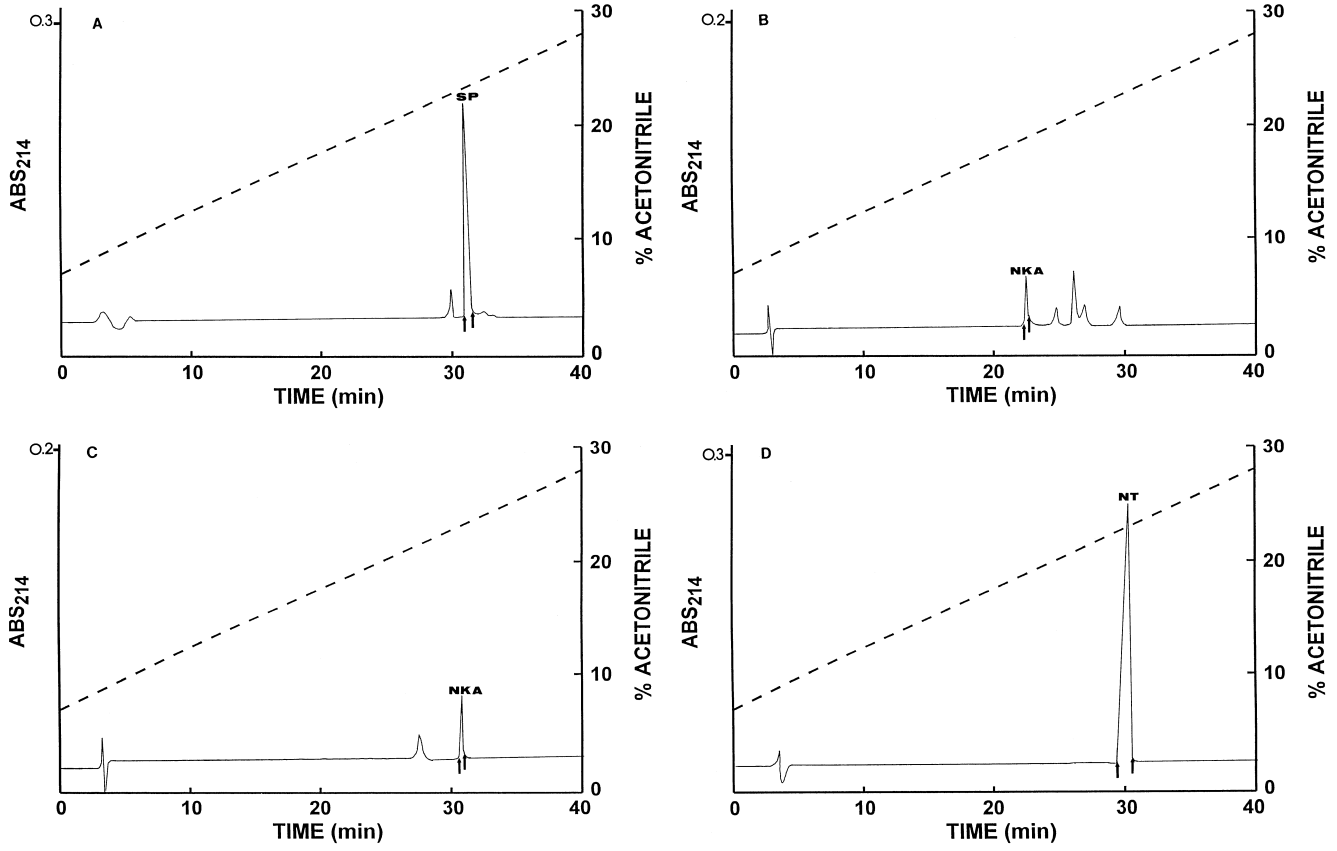

FIG. 3. Purification by reversed-phase HPLC on an analytical Vydac phenyl column of python (A) substance P, (B) neurokinin A, (C) neuropeptide γ and (D) neurotensin The arrows show where peak collection began and ended.

Gel Permeation Chromatography

The extract, after partial purification on Sep-pak cartridges, was redissolved in 1 M acetic acid (5 ml) and chromatographed on a 100 × 2.5 cm column of Sephadex G-25 (Pharmacia) equilibrated with 1 M acetic acid at a flow rate of 48 ml/h. Absorbance was measured at 280 nm and fractions (8 ml) were collected. SP-LI, NKA-LI and NT-LI in the fractions were measured at appropriate dilution.

Purification of the Python Peptides by HPLC

The fractions containing SP-LI from gel permeation chromatography were pooled and pumped at a flow rate of 2 ml/min onto a 25 × 1 cm Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The concentration of acetonitrile in the eluting solvent was raised to 21% over 10 min, maintained at this concentration for 30 min and raised to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. The fraction containing SP-LI (denoted by the bar 2 in Fig. 1) was rechromatographed on a 25 × 0.46 cm Vydac 214TP54 C-4 column equilibrated with acetonitrile/water/trifluoroacetic acid (7.0/92.9/0.1) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 28% over 40 min using a linear gradient. Python substance P was purified to apparent homogeneity by chromatography on a 25 × 0.46 cm Vydac 219TP54 phenyl column using the same elution conditions for the C-4 column.

Fractions from the semi-preparative C-18 column (Fig. 1) containing NKA-LI (denoted by bar 3 containing neurokinin A and by bar 4 containing neuropeptide γ) and NT-LI (denoted by bar 1) were separately chromatographed on 25 × 0.46 cm Vydac 214TP54 C-4 and 25 × 0.46 cm Vydac 219TP54 phenyl columns under the same conditions used for the purification of substance P.

Peptide Synthesis

Python neuropeptide γ was synthesized by solid-phase methodology on a 0.025 mmol scale using an Applied Biosystems model 432 synthesizer. Fluorenylmethoxycarbonyl (Fmoc)-labelled amino acids were coupled as their hydroxybenzotriazole active esters following the manufacturer's standard protocols. The peptide was cleaved from the resin using trifluoroacetic acid/water/ethanedithol/thioanisol (900/30/30/40, by vol) and purified to near homogeneity by reversed-phase HPLC. Identity of the peptide was confirmed by amino acid analysis and automated Edman degradation. Synthetic neurokinin A was supplied by Peninsula Laboratories.

Structural Analysis

Amino acid compositions were determined by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer and model 130A separation system. The detection limit for phenylthiocarbamyl amino acids was 1 pmol. Hydrolysis (24 h at 110°C in 5.7 M HCl) of approximately 500 pmol of sample was performed. Automated Edman degrada-

TABLE 1

AUTOMATED EDMAN DEGRADATION OF TACHYKININS AND NEUROTENSIN FROM PYTHON INTESTINE

Cycle no.	Substance P	Neuropeptide γ	Neurokinin A	Neurotensin*
1	Arg (97)	Asp (19)	His (32)	Leu (616)
2	Pro (321)	Ala (23)	Lys (68)	His (227)
3	Arg (85)	Gly (17)	Thr (17)	Val (597)
4	Pro (284)	Tyr (12)	Asp (29)	Asn (213)
5	Gln (183)	Ser (3)	Ser (9)	Lys (229)
6	Gln (235)	Pro (11)	Phe (35)	Ala (463)
7	Phe (299)	Leu (12)	Val (25)	Arg (107)
8	Tyr (208)	Ser (3)	Gly (27)	Arg (148)
9	Gly (140)	His (5)	Leu (14)	Pro (278)
10	Leu (152)	Lys (9)	Met (5)	Tyr (261)
11	Met (97)	Arg (4)		Ile (89)
12		His (5)		Leu (26)
13		Lys (7)		
14		Thr (2)		
15		Asp (4)		
16		Ser (trace)		
17		Phe (5)		
18		Val (2)		
19		Gly (3)		
20		Leu (2)		
21		Met (trace)		

The values in parentheses show the yields of phenylthiohydantoin derivatives (pmol).

* Sequence analysis was carried out on the [des-pGlu[1]] derivative of neurotensin.

tion was performed using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin (PTH) amino acids under gradient elution conditions. The detection limit for the PTH derivatives was 1 pmol. Mass spectrometry was performed at Novo Nordisk A/S, Bagsvaerd, Denmark using a Voyager RP MALDI-TOF instrument (Perspective Biosystems) equipped with a nitrogen laser (337 nm). The accuracy of the mass determinations was within 0.02%.

The purified python neurotensin (approximately 1 nmol) was incubated for 18 h at 4°C with 1 μg pyroglutamyl aminopeptidase (Calbiochem) in 0.1 M sodium phosphate buffer, pH 8.0 containing 5% (v/v) glycerol, 10 mM EDTA and 5 mM dithiothreitol (total volume 100 μl). The reaction mixture was chromatographed on a 25 × 0.46 cm Vydac 218TP54 C-18 using the same elution conditions used for the C-4 column.

RESULTS

Purification of the Python Peptides

The SP-LI, NKA-LI and NT-LI in the extract of python small intestine, after partial purification on Sep-Pak cartridges, were eluted from a Sephadex G-25 column in the same fractions as a broad peak with K_{AV} between 0.65 and 0.85. The fractions containing immunoreactive material were pooled and chromatographed on a semi-preparative C-18 HPLC column as shown in Fig. 1. The resolving power of the column was sufficient to separate the different components into four distinct fractions. SP-LI was eluted in the fraction denoted by bar 2, NKA-LI in two non-overlapping fractions denoted by bars 3 and 4 and NT-LI in the fraction denoted by bar 1.

After rechromatography on an analytical Vydac C-4 column (Fig. 2A), the SP-LI was eluted as the sharp peak delineated by the arrows. Python substance P was purified to apparent homogeneity, as assessed by symmetrical peak shape, by chromatography on an analytical Vydac phenyl column (Fig. 3A). The final yield of pure peptide was 2 nmol. The peak of NKA-LI designated by bar 3 (subsequently shown to contain neurokinin A) was eluted from an analytical C-4 column as a shoulder to the major peak in the chromatogram shown in Fig. 2B. Python neurokinin A was separated from non-immunoreactive components by chromatography on a Vydac phenyl column (Fig. 3B) and the peptide was eluted as a sharp symmetrical peak (final yield 100 pmol). The peak of NKA-LI designated by bar 4 (subsequently shown to contain neuropeptide γ) was eluted from an analytical C-4 column as a poorly-resolved shoulder to the major peak in the chromatogram shown in Fig. 2C. Python neuropeptide γ was purified to near homogeneity by chromatography on a Vydac phenyl column (Fig. 3C) and final yield of pure material was 100 pmol. After rechromatography on an analytical Vydac C-4 column (Fig. 2D), the peak containing NT-LI was eluted as the sharp peak delineated by the arrows. Python neurotensin was purified to apparent homogeneity by chromatography on a Vydac phenyl column (Fig. 3D) and final yield of pure material was 3 nmol.

Structural Characterization

The primary structures of the python peptides were established by automated Edman degradation (Table 1). The amino acid sequences of substance P and neurokinin A were determined without ambiguity and the data indicated that python substance P contained two amino acid substitutions ($Lys^3 \rightarrow Arg$ and $Phe^8 \rightarrow Tyr$) compared with mammalian substance P whereas python neurokinin A is identical to mammalian neurokinin A. Attempts to determine the amino acid sequence of neurotensin were unsuccessful indicating the peptide did not contain a free α-amino group. However, sequence analysis was accomplished after removal of the N-terminal pyroglutamyl residue by incubation with pyroglutamyl aminopeptidase. Python neurotensin is identical to chicken neurotensin (4). The proposed sequence of python substance P and neurotensin were confirmed by determination of their amino acid compositions [substance P: Glx 1.7 (2), Gly 1.1 (1), Arg 1.9 (2), Pro 1.7 (2), Tyr 0.7 (1), Met 0.6 (1), Leu 1.1 (1), Phe 1.0 (1) mol residue/mol peptide; neurotensin: Asx 1.2 (1), Glx 1.1 (1), His 0.9 (1), Arg 2.2 (2), Ala 1.1 (1), Pro 1.0 (1), Tyr 0.9 (1), Val 0.9 (1), Ile 1.0 (1), Leu 2.0 (2), Lys 1.0 (1) mol residue/mol peptide]. The values in parentheses show the number of residues predicted from the proposed structure. There was insufficient pure material to determine the amino acid composition of python neuropeptide γ but the proposed structure was confirmed by mass spectrometry and chemical synthesis. The observed molecular mass of the peptide was 2344.6 compared with a calculated mass of 2344.7 for the proposed structure. An additional component with molecular mass 2360.6 was observed in the spectrum which corresponds to the methionine-sulfoxide form of the peptide. The retention time of synthetic python neuropeptide γ on an analytical Vydac phenyl column, under the conditions of chromatography shown in Fig. 3C, was 30.5 ± 0.3 min compared with a retention time of 30.7 min for the endogenous peptide. The retention of synthetic neurokinin A under these conditions was 22.4 ± 0.2 min compared with a retention time of 22.5 min for python neurokinin A.

Substance P

Human	R	P	K	P	Q	Q	F	F	G	L	M
Chicken	-	-	R	-	-	-	-	-	-	-	-
Alligator	-	-	R	-	-	-	-	-	-	-	-
Python	-	-	R	-	-	-	-	Y	-	-	-
Green Frog	K	-	N	-	E	R	-	Y	-	-	-
Bullfrog	K	-	S	-	D	R	-	Y	-	-	-
Salamander	D	N	-	S	V	G	-	-	Y	-	-

Neurokinin A

Human	H	K	T	D	S	F	V	G	L	M
Chicken	-	-	-	-	-	-	-	-	-	-
Alligator	-	-	-	-	-	-	-	-	-	-
Python	-	-	-	-	-	-	-	-	-	-
Frog	-	-	L	-	-	-	I	-	-	-
Salamander	-	-	*	-	A	-	I	-	-	-

Neuropeptide γ

Human	D	A	G	H	G	Q	I	S	H	K	R	H	K	T	D	S	F	V	G	L	M
Alligator	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Python	-	-	-	Y	S	P	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Neurotensin

Human	<E	L	Y	E	N	K	P	R	R	P	Y	I	L
Chicken	-	-	H	V	-	-	A	-	-	-	-	-	-
Alligator	-	-	H	V	-	-	A	-	-	-	-	-	-
Python	-	-	H	V	-	-	A	-	-	-	-	-	-
Frog	-	S	H	I	S	-	A	-	-	-	-	-	-

FIG. 4. Comparison of the amino acid sequences of the tachykinins and neurotensin isolated from the intestine of the Burmese python compared with the sequences of the corresponding peptides from other tetrapods. Sequence identity is denoted by (−) and deletion of a residue by (*).

DISCUSSION

The amino acid sequences of the python tachykinins and neurotensin are compared with the corresponding peptides from the human and other non-mammalian tetrapods in Fig. 4. The data indicate that evolutionary pressure to conserve the amino acid sequences of these peptides has not been uniform. Sequence analysis of cloned cDNAs from various mammalian tissues has identified mRNAs directing the synthesis of five biosynthetic precursors of the tachykinins (α-, β- γ- and δ-preprotachykinin A and preprotachykinin B) [reviewed in (5)]. The α- and δ-preprotachykinin A contain the sequence of substance P only, β-preprotachykinin A contains substance P, neurokinin A and its 36 amino acid residue NH_2-terminally extended form, neuropeptide K and γ-preprotachykinin A contains the sequence of substance P, neurokinin A and its 21 amino acid residue NH_2-terminally extended form, neuropeptide γ. Preprotachykinin B is encoded by a separate gene and contains the sequence of neurokinin B only. Python substance P, like the corresponding peptide from the chicken (7) and alligator (27) contains a single conservative substitution (Arg → Lys) at position 3 compared with human substance P but, in common with the peptides from the green frog *Rana ridibunda* (15), bullfrog *Rana catesbeiana* (13) and the salamander *Amphiuma tridactylum* (28) contains a tyrosine residue at position 8. The biological activity of python substance P was not investigated in this study but previous work has demonstrated that synthetic [Arg^3] substance P is equipotent with substance P in contracting the guinea pig ileum (7) and ranakinin, the substance P-related peptide from *R. ridibunda*, is equipotent with substance P in binding to the mammalian NK-1 receptor in rat submandibular gland membranes (2,15). It is probable, therefore, that the substitutions at positions 3 and 8 in python substance P will not have a marked effect on biological potency. As shown in Fig. 4, the primary structure of neurokinin A has been fully conserved among those amniotes yet studied whereas the amino-terminal region of neuropeptide γ is more variable. Python neuropeptide γ contains three amino acid substitions compared with alligator peptide (27) and an additional substitution compared with human neuropeptide γ. A structure-activity study employing N-terminally truncated analogs of neuropeptide γ has shown that the N-terminal region of

the peptide is not important in binding of the ligand to NK-2 receptors in rat fundus (2). The isolation of neuropeptide γ from python intestine provides evidence that in snakes, as well as in mammals and crocodilians, the primary transcript of the preprotachykinin A gene can generate several mRNAs by an alternative splicing mechanism. Neuropeptide K (8) and neurokinin B (25) have been identified in low concentration in extracts of the intestines of mammalian species but these peptides were not detected in the extract of python intestine.

Like neurokinin A, the amino acid sequence of neurotensin has been more strongly conserved among the reptiles than among amphibia (Fig. 4). Python neurotensin is identical to chicken (4) and alligator (19) neurotensins although this amino acid sequence differs from human neurotensin at 3 sites. In contrast, neurotensin from the European common frog *Rana temporaria* (23) differs from the peptide isolated from *R. ridibunda* by the substitution ($Ser^2 \rightarrow Ala$) and from the peptide isolated from the cane toad *Bufo marinus* by the additional substitution ($Ile^4 \rightarrow Val$) (J. M. Conlon, unpublished data). The effects of neurotensin on gastrointestinal motility in mammals are complex, varying with both species and tissue. The effects of the peptide in non-mammalian vertebrates has not been studied extensively but it has been shown that low dose infusions of chicken neurotensin into the chicken result in a relaxation of the ileum (17) whereas mammalian neurotensin had an excitatory action on gastric smooth muscle from the amphibian *Necturus maculosus* (mud puppy) (12).

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