Bufokinin: a substance P-related peptide from the gut of the toad, *Bufo marinus* with high binding affinity but low selectivity for mammalian tachykinin receptors

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A tachykinin peptide, termed bufokinin, was isolated in pure form from an extract of the intestine of the toad, *Bufo marinus*, and its primary structure was established as: Lys-Pro-Arg-Pro-Asp-Gln-Phe-Tyr-Gly-Leu-Met.NH₂. This sequence was confirmed by chemical synthesis and shows four amino acid substitutions (Arg¹ → Lys, Lys³ → Arg, Gln¹ → Asp and Phe¹ → Tyr) compared with substance P. Binding parameters for synthetic bufokinin and mammalian tachykinins were compared using receptor-selective radioligands and crude membranes from rat tissues enriched in the NK-1 (submandibular gland), NK-2 (stomach fundus) and NK-3 (brain) receptors. In terms of inhibiting the binding of the selective radioligands, bufokinin (Kᵢ = 0.3 nM) was 1.8-fold more potent than substance P at the rat NK-1 site, but it was only 2-fold less potent (Kᵢ = 2.8 nM) than neurokinin A at the NK-2 site and only 2-fold less potent (Kᵢ = 48 nM) than neurokinin B at the NK-3 site. Thus, bufokinin shows relatively high affinity but lack of selectivity for all three tachykinin binding sites in rat tissues. © Munksgaard 1998.

Key words: bufokinin; tachykinin; substance P; neurokinin A; neurokinin B; Amphibia

The tachykinins are a family of polypeptides that are defined structurally by the amino acid sequence -Phe-Xaa-Gly-Leu-Met.NH₂ (Xaa = Phe, Tyr, Val or Ile) at the COOH-termini of the molecules; they were first identified by their myotopic actions on vascular, gastrointestinal and other smooth muscle preparations. Immunohistochemical studies have shown that tachykinin-related peptides are present in nervous tissues from all classes of vertebrates studied up to this time (reviewed in ref. 1). Although recent work from our own (2) and other laboratories (3) has led to structural characterization of several tachykinins from non-mammalian vertebrates, our knowledge of the structural and functional evolution of the family is fragmentary.

The Amphibia, as extant representatives of the first terrestrial vertebrates, occupy a crucially important position in phylogeny. The skins of frogs have proved a rich source for the purification of novel tachykinin peptides (4), but relatively few tachykinin have been isolated from amphibian nervous and gastrointestinal tissues. A substance P (SP)-related peptide, termed ranakinin, was isolated together with neurokinin B (NKB) from the brain of the European green frog, *Rana ridibunda* (5) and a neurokinin A (NKA)-related peptide ([Leu',Ile']NKA) from the intestine of the same species (6). Three structurally related tachykinins, termed ranatachykinin A, B and C, have been isolated from the brain and/or intestine of the bullfrog, *Rana catesbeiana* (3), and two tachykinins with limited structural similarity to SP and NKA, from the intestine of a salamander, *Amphiuma tridactylum* (2).

Mammalian tissues contain three well characterized, high-affinity binding sites for the tachykinins, termed the NK-1, NK-2 and NK-3 receptors, which differ in their ability to bind endogenous tachykinin ligands (7). SP is the most receptor-selective of the ligands and binds with highest affinity to the NK-1 receptor; NKA binds preferentially to the NK-2 receptor and NKB to the NK-3 receptor. All three subtypes belong to the G-protein-coupled receptor family and their activation is associated with activation of the inositol polyphosphate-Ca²⁺ signaling pathway (8). The pharmacological properties of tachykinin receptors in amphibian tissues are not well characterized. In the frog *R. ridibunda*, ranakinin stimulates corticosteroid secretion from the adrenal gland by a mechanism that involves

Abbreviations: SP, substance P; SP-LI, substance P like immunoreactivity; NKA, neurokinin A; NKB, neurokinin B.
activation of phospholipase C via a pertussis toxin-sensitive G-protein and mobilization of Ca^{2+} ions from intracellular stores (9, 10). However, a study of the effect of tachykinin antagonists on the effects of ranakinin has concluded that the stimulatory action of the peptide on steroid secretion is mediated by a receptor which differs appreciably in properties from the mammalian NK1 receptor (11).

In the present study, a novel tachykinin-related peptide has been isolated from the intestine of the cane toad, *Bufo marinus*, 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 NK-1, NK-2 and NK-3 receptors, respectively.

**EXPERIMENTAL PROCEDURES**

**Materials**

Synthetic peptides were supplied by Peninsula Laboratories (Belmont, CA) or by Auspep (Melbourne, Australia). The NK-1 receptor-selective radioligand 125I-Bolton-Hunter-labelled [Sar9,Met(O2)11]substance P (12), the NK-2 receptor-selective radioligand [125I]-labelled [Lys8,Tyr10],MeLeu6,Nle18]neurokinin A-(4–10) peptide (13) and the NK-3 receptor-selective radioligand [125I]-Bolton-Hunter-labelled scylorhinin II (14) were prepared and purified as described previously. The specific radioactivity of all radioligands was >60 TBg/mmol. Reagents for peptide synthesis were supplied by Applied Biosystems (Foster City, CA).

**Preparation of frog gut extract**

Adult toads (*B. marinus*) of both sexes were obtained from a commercial source. The small intestine was collected from 82 specimens and immediately frozen on dry ice. The tissue (99 g wet weight) was homogenized with ethanol/0.7 M HCl (3:1 vol/vol; 800 mL) using a Waring blender and stirred for 2 h at 0°C as described previously (6). After centrifugation (4000 g, 30 min, 4°C), ethanol was removed from the supernatant under reduced pressure. After a further centrifugation (4000 g, 30 min, 4°C), the extract was pumped at a flow rate of 48 mL/h. Fractions (8 mL) were collected and the presence of SP-LI was determined by radioimmunoassay at the appropriate dilution. The fractions containing maximum SP-LI were pooled (total volume, 32 mL) and injected onto a (25 × 1 cm) Vydac 218TP510 (C-18) column equilibrated with 0.1% (vol/vol) trifluoroacetic acid/water at a flow rate of 2 mL/min. The concentration of acetonitrile was raised to 21% during 10 min, held at this concentration for 30 min and raised to 49% during 60 min using a linear gradient. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. SP-LI in the fractions was measured by radioimmunoassay at the appropriate dilution.

The fraction denoted by the bar (Fig. 1A) 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% during 40 min using a linear gradient. Bufokinin was purified to apparent homogeneity by successive chromatographies on (25 × 0.46 cm) Vydac 219TP54 phenyl and (25 × 0.46 cm) Vydac 218TP54 (C-18) columns under the same elution conditions used with the C-4 column.

**Structural characterization**

The primary structure of bufokinin was determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for online detection of phenylthiohydantoin amino acids under gradient elution conditions. The detection limit was 1 pmol. The amino acid composition was determined after vapor phase hydrolysis (6 M HCl) by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer. Phenylthiocarbamoyl amino acid derivatives were identified by HPLC using an Applied Biosystems model 130A separation system, and the detection limit was 2 pmol. Full details of the methods used have been provided previously (6).

**Peptide synthesis**

Bufokinin was synthesized by solid-phase methodology on a 0.025 mmol scale using an Applied Biosystems model 432 synthesizer. Fluorenlymethoxycarbonyl (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/ethanedithiol/thioanisol (90:3:3:4, 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. The retention times of endogenous and synthetic bufokinin were compared by chromatography on a Vydac 218TP54 column using the conditions shown in Fig. 1D.

**Competitive binding studies**

Male Wistar rats (200–300 g) were killed by cervical dislocation. The submandibular gland, stomach fundus and brain minus cerebellum were quickly removed, frozen and stored at −70°C. On the day of the experiment, crude membrane preparations were prepared from these tissues as described previously [submandibular gland crude membrane preparations were prepared from these tissues suspended in an incubation buffer consisting of 50 mM Tris-HCl buffer, pH 7.4, containing bovine serum albumin (0.02% wt/vol), 3 mM MnCl₂ and the peptidase inhibitor, chymostatin (4 pg/mL) at 25°C. Incubations were terminated with 125I-Bolton-Hunter-labelled substance P (100 pmol/L) for 90 min (12); stomach fundus membranes (20 mg/mL) were incubated with 125I-labelled [Lys, Tyr, Met, Nle] neurokinin A-(4–10) peptide (100 pmol/L) for 60 min (13), and brain membranes (40 mg/mL) were incubated with 125I-Bolton-Hunter-labelled scyllo-inulin II (100 pmol/L) for 90 min (14). Incubations were terminated by rapid filtration and washing with ice-cold buffer (50 mM Tris-HCl, pH 7.4, containing 0.02% bovine serum albumin and 3 mM MnCl₂) using a Brandel cell harvester. The filter papers were soaked in 0.01% polyethyleneimine (submandibular gland) or 0.5% (brain) or 1.5% (fundus) bovine serum albumin solution.

Dissociation constants (Kₐ) were calculated using the iterative curve-fitting program LIGAND (16). Four independent experiments were carried out using each radioligand and data are expressed as means ± SEM.

**RESULTS**

**Purification of bufokinin**

An extract of toad intestine, even at high dilution, contained material that inhibited the binding of 125I-Bolton Hunter-labelled substance P to an antibody raised against mammalian SP. The immunoreactivity in serial dilutions of the extract did not diminish in parallel with the synthetic SP standard curve, so it was concluded that radioimmunoassay could be used to detect but not to quantitate the toad tachykinin.

The SP-LI in the extract was eluted from a Sephadex G-25 column as a single peak with maximum immunoreactivity at a slightly greater elution volume than mammalian SP (Kₐ between 0.75 and 0.90). The fractions containing SP-LI were pooled and chromatographed on a semi-preparative Vydac C-18 reversed-phase HPLC column (Fig. 1A). The SP-LI was eluted from the column under isocratic conditions as in a single fraction, denoted by the bar. No SP-LI was detected in the fraction corresponding to the retention time of mammalian SP. Rechromatography of this fraction on an analytical Vydac C-4 column (Fig. 1B) revealed that the material was heterogeneous, but the SP-LI was associated with the most prominent peak in the chromatogram. Bufokinin was purified to apparent homogeneity, as assessed by symmetrical peak shape, by successive chromatographies on an analytical Vydac phenyl (Fig. 1C) and Vydac C-18 (Fig. 1D) columns. The yield of purified peptide, determined by amino acid composition analysis, was 3.5 nmol.

**Characterization of bufokinin**

It was possible to assign without ambiguity phenylthiohydantoin derivatives of amino acids for 11 cycles of operation during sequence analysis of the peptide. The primary structure of bufokinin was established as: Lys(368)-Pro(417)-Arg(196)-Pro(437)-Asp(282)-Gln(334)-Phe(398)-Tyr(311)-Gly(358)-Leu(186)-Met(109). The values in parentheses show the yields of amino acid phenylthiohydantoin in picomoles. Bufokinin shows four amino acid substitutions (Arg→Lys, Lys→Arg, Asp→Asp, Phe→Tyr) compared with mammalian SP. The results of amino acid analysis demonstrated that bufokinin had the following composition: Asx 1.0 (1), Glx 1.0 (1), Gly 1.0 (1), Arg 1.3 (1), Pro 1.7 (2), Tyr 0.8 (1), Met 0.4 (1), Leu 1.0 (1), Phe 0.8 (1), Lys 0.7 (1) (mol of residue/mol of peptide). The values in parentheses show the number of residues predicted from the proposed structure. Agreement between the results of Edman degradation and amino acid composition analysis was good, which shows that the full sequence of the peptide had been obtained. The data indicated that the bufokinin sample was >95% pure. The strong reactivity of bufokinin with an antibody directed against the COOH-terminal region of mammalian SP suggested that the peptide terminated in an α-amidated methionine residue. The antisera used in the radioimmunoassay shows negligible reactivity with the COOH-terminal free acid form of substance P or with substance P extended from its COOH-terminus by a glycine residue (15). The primary structure of bufokinin was confirmed by chemical synthesis. A mixture of purified endogenous bufokinin (1 nmol) and COOH-terminally α-amidated form of synthetic [Lys, Arg, Asp, Tyr] substance P (1 nmol) eluted from a C-18 reversed-phase HPLC column as a single sharp and symmetrical peak.

**Competitive binding studies**

The abilities of synthetic bufokinin and mammalian tachykinins (SP, NKA and NKB) to displace the binding of selective radiolabelled tachykinin ligands to rat tissues that are enriched in NK1 receptors (subman-
Purification and binding properties of bufokinin

FIGURE 1
Purification by reversed-phase HPLC of bufokinin on (A) semi-preparative Vydac C-18, (B) analytical Vydac C-4, (C) analytical Vydac phenyl and (D) analytical Vydac C-18 columns. The peaks designated SPLI contained substance P-like immunoreactivity. The concentration of acetonitrile in the eluting solvent is shown by a broken line, and the arrows show where peak collection began and ended. The arrow designated SP shows the retention time of mammalian substance P.

dibular gland), NK2 receptors (stomach fundus) and NK3 receptors (brain) are compared in Table 1. For each of the three tissues studied, analysis of the binding data obtained with the tachykinins using the LIGAND program indicated the presence of a single class of binding site for the peptides. In this series of experiments, the dissociation constants of SP, NKA and NKB were consistent with previously published data (12–14).

DISCUSSION
The amino acid sequence of bufokinin is compared with that of SP and with the primary structures of SP-related peptides from other non-mammalian vertebrates in Fig. 2. The data confirm previous conclusions that evolutionary pressure to conserve the amino acid sequence of the tachykinins has been selective, acting primarily on the functionally important C-terminal region (Phe-Xaa-Gly-Leu-Met.NH$_2$) (2). The structural criteria for designating a non-mammalian tachykinin, such as bufokinin, as “SP-related” rather than “NKA-related” are somewhat arbitrary. SP-related tachykinins generally contain at least one basic residue and one proline residue in the N-terminal region of the peptide, and bufokinin shares the structural motif Arg/Lys-Pro-Xaa-Pro with SP and several other tachykinins in the series. Similarly, SP-related peptides, including bufokinin, generally contain an aromatic residue (Phe or Tyr) in the C-terminal region (corresponding to position 8 in mammalian SP), although the tachykinins from the cod and the lamprey contain an aliphatic residue at this position. The cod and lamprey peptides, however, do contain the Arg/Lys-Pro-Xaa-Pro sequence, and additional tachykinins with closer structural similarity to NKA have been isolated from these species (17, 18).

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In contrast, functional criteria clearly designate bufokinin as “SP-related” because the peptide binds with high affinity to the rat NK-1 receptor. Compared with SP, bufokinin is 1.8-fold more potent in inhibiting the binding of the NK-1 receptor-selective radioligand [125I]-Bolton-Hunter-labelled [Sar⁹, Met(O²)⁷] substance P in rat submandibular gland (NK-1 receptor), [125I]-labelled [Lys⁵, Tyr(13), MeLeu⁶, Nle¹⁰] neurokinin A-(4-10) peptide in rat stomach fundus (NK-2 receptor) and [125I]-Bolton-Hunter-labelled scyliorhinin II in rat brain (NK-3 receptor) (Table 1). At the NK-2 receptor, bufokinin was approximately 2-fold less potent than the endogenous ligand NKA but was 12-fold more potent than NKB. Structure-activity studies have shown that an Asp residue in the position analogous to residue 5 of SP promotes high-affinity binding of the ligand to NK-2 receptors (20) so that the relatively high affinity of bufokinin for the NK-2 receptor may be a consequence of the presence of the Asp residue at position 5 in the peptide. In contrast to ranakinin, which was a relatively poor ligand at the NK-3 receptor (Kᵣ = 160 nm) (21), bufokinin was only 2-fold less potent than NKB at the NK-3 receptor (Table 1). This result was unexpected because bufokinin contains an aromatic residue at position 8 instead of valine in the equivalent position in NKB suggesting that this residue may be relatively unimportant in determining high-affinity binding to the NK-3 receptor. The non-selective receptor binding profile of bufokinin is similar to that of the molluscan tachykinin, eledoisin. Eledoisin binds with comparable affinity to bufokinin at NK-3 receptors (Kᵣ = 20 nm) (14) and at NK-2 receptors (Kᵣ = 5.9 nm) (21), but eledoisin is appreciably less potent than bufokinin at the NK-1 receptor (Kᵣ = 5.8 nm) (12). It may be significant that eledoisin (pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met.NH₂) contains an Asp residue in the position corresponding to residue 5 of bufokinin.

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Purification and binding properties of bufokinin


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