Running Head: Three isoforms of NK̂1-like receptor in the toad

Molecular Identification and Characterization of Three Isoforms of Tachykinin NK̂1-like Receptors in the Cane Toad, *Bufo Marinus*

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ABSTRACT

The tachykinin peptide, bufokinin, isolated from the cane toad intestine, is important in intestinal and cardiovascular regulation in the toad. In this study, three tachykinin NK₁-like receptor isoforms, bNK₁-A, bNK₁-B, bNK₁-C, encoding proteins of 309, 390 and 371 amino acids, respectively, were cloned from the toad brain and intestine. These isoforms differ only at the intracellular C-terminus. The bNK₁-A and bNK₁-B isoforms are similar to the truncated and full-length forms of the mammalian NK₁ receptor, whereas bNK₁-C is unique and does not correspond to any previously described receptor. RT-PCR studies demonstrated that three isoform transcripts are widely distributed in the toad with high expression in gut, spinal cord, brain, lung and skeletal muscle. When expressed in COS-7 cells, bufokinin showed similar high affinity (IC₅₀ 0.6-0.8 nM) in competing for [¹²⁵I]Bolton-Hunter bufokinin binding at all receptors, but the binding affinities of substance P (SP) and neurokinin A (NKA) were very different at each isoform. When expressed in Xenopus oocytes, the truncated isoform, bNK₁-A, was inactive, whereas bNK₁-B and bNK₁-C produced changes in chloride current when stimulated by tachykinins (minimum concentrations: bufokinin 0.1 nM, SP 1 nM and NKA 10 nM). A marked desensitization of the response was seen to subsequent applications of tachykinins, as experienced by the mammalian NK₁ receptor. In summary, our study describing three isoforms of NK₁-like receptor from the toad suggests that the alternative splicing of NK₁ receptor is a physiologically conserved mechanism, and raises a fundamental question as to the physiological role of each isoform.

KEY WORDS

Bufokinin, tachykinin receptors, sequencing and cloning, Xenopus oocyte expression, amphibian
INTRODUCTION

Tachykinins are multifunctional brain, gut and skin peptides that possess a variety of biological roles in the central and peripheral nervous systems as well as in the cardiovascular and immune systems. Their actions include vasodilatation, plasma extravasation, smooth muscle contraction, secretion, neuronal excitation, and processing of sensory information; in addition, they have extensive pro-inflammatory properties (13). In mammals, substance P (SP), neurokinin A (NKA) and neurokinin B are the best known members of this family and act preferentially through tachykinin NK1, NK2 and NK3 receptors, respectively (27). Most recently, several new mammalian tachykinins (hemokinin and the endokinins) have been isolated from non-neural tissues (refer 27). The tachykinin family is phylogenetically ancient and has been well conserved throughout evolution, with numerous structurally-related tachykinins isolated from a wide range of vertebrates and invertebrates (15). The amino acid sequences of tachykinins from mammals, birds and reptiles are similar whereas those from amphibians and fish are quite divergent, possibly reflecting their evolutionary position (15, 31).

The three mammalian tachykinin receptors have been cloned and intensively studied, mainly in mammalian species (2). They belong to a G-protein-coupled receptor (GPCR) superfamily with the seven membrane-spanning domain structure typical for receptors of this family. After activation by agonist, the intracellular loops of the receptors interact with a Gq/G11-protein, leading to phospholipase C (PLC) activation, inositol 1,4,5-triphosphate (IP3) formation and an increase in intracellular calcium. Stimulation of cyclic AMP (cAMP) production via Gαs-protein may be an alternate signalling transduction pathway in certain systems (25).

Since mammalian and non-mammalian tachykinins have broadly similar actions on both non-mammalian and mammalian species (31), it is likely that the sequences of the receptors may also be conserved across different vertebrate groups. However, there is limited
information on tachykinin receptor structure or signal transduction mechanisms in non-mammals. NK₁-like receptors cloned from chicken (GenBank AF131057) and from fugufish Fugu rubripes (GenBank AY327862) show 78% and 65% identity, at the amino acid level, to their human counterpart. In the bullfrog Rana catesbeiana, a SP-preferring receptor isolated from sympathetic ganglion exhibits 70% identity to mammalian NK₁ receptors (32). Across these diverse vertebrate species, cytoplasmic loop 1 and transmembrane regions II and VII are highly conserved (92-100% identity), whereas the extracellular N-terminus and extracellular loops 2 and 3 are the least conserved regions (15-61% identity). Tachykinin-like receptors have also been cloned from several different invertebrate species including the echiuroid worm Urechis uninctus (19) and it is notable that the second messenger systems activated by Urechis receptors (PLC-IP3-calcium signal transduction cascade, cAMP) are the same as those induced in mammalian tachykinin-dependent signaling processes (35, 36).

We have previously isolated a SP-like undecapeptide, bufokinin, from the small intestine of the cane toad, Bufo marinus (6). Bufokinin exhibits high affinity at all three tachykinin receptors in the rat, where it shows even greater affinity than its mammalian counterpart, SP, for the NK₁ receptor (6). Our earlier studies have shown that bufokinin is a potent spasmogen of intestinal smooth muscle and is also a potent vasodilator in the toad (21, 22). Binding and functional studies in toad intestinal smooth muscle have described a bufokinin-preferring NK₁ like receptor which couples to the G-protein phosphoinositol hydrolysis pathway (22). However, this NK₁ like receptor shows negligible affinity for selective NK₁ receptor agonists and antagonists (3, 22), suggesting that key amino acids in the binding domains for these ligands are not well conserved in non-mammals.

From an evolutionary point of view, it is important to gain more information about the tachykinin receptors in non-mammalian species. Amphibians are extant representative of the first terrestrial vertebrates and therefore occupy an extremely important position in
phylogeny. Moreover, amphibians such as the cane toad, *Bufo marinus*, are convenient and low cost experimental animals, extensively used in biological studies. Thus it is necessary to know the extent to which they can serve as models for the study of genetic or physiological processes.

We have therefore extended our earlier pharmacological studies of tachykinin receptors in *Bufo marinus*, to better understand the molecular basis of the biological functions and evolutionary and phylogenetic relationships of tachykinin receptors. In this study, we report the cloning of three NK₁ receptor isoforms, bNK₁-A, bNK₁-B and bNK₁-C¹, from the toad brain and gut, and the tissue distribution and expression level of these isoforms. Their pharmacological profiles were studied in transfected heterologous expression systems.

**MATERIALS AND METHODS**

1. **Isolation of RNA**

Toads of both sexes, weight 150–200 g, were purchased from Mr P. Douch, North Queensland, Australia, and housed in captivity at room temperature for up to a week. Toads were anesthetized by immersion in 0.25% tricaine methanesulfonate (MS222, Sigma), killed by decapitation and their organs were removed. Total RNA was extracted from small intestinal smooth muscle, brain, spinal cord, lung, aorta, atrium, ventricle, bladder, skin, skeletal muscle, stomach, testis and liver using RNAgents® Total RNA Isolation System (Promega) and treated with RNase-Free DNase to remove traces of chromosomal DNA. RT-PCR amplification of the β-actin gene using Access RT-PCR System (Promega) was performed in the presence and absence of AMV reverse transcriptase, in order to monitor the quality of RNA and the presence of DNA contamination. Only high quality and DNA-free RNA samples were used in subsequent studies.
2. Amplification of partial cDNAs by RT-PCR

Total RNA from intestinal smooth muscle and brain was reverse-transcribed and PCR amplified using TM-2 and TM-7 primers (Table 1), which were derived from the conserved transmembrane (TM) II and VII regions of the bullfrog NK₁-like receptor cDNA sequence (32). cDNA was created and amplified as per the manufacturer's instructions (Access RT-PCR System, Promega). The reaction mixture contained 1 µg total RNA, 0.2 µM of each primer, 0.2 mM dNTPs, 1.5 mM MgSO₄, 2.5 U AMV reverse transcriptase and 2.5 U Tfl DNA polymerase. The RT-PCR reaction was performed at 40°C for 45 min, 94°C for 2 min and 40 cycles of 94°C for 30 s, 48°C for 1 min and 70°C for 2 min, followed by one cycle 48°C for 3 min, 70°C for 10 min. PCR products of the expected size (720 bp) were excised, purified and cloned into pGEM-T vector (Promega). Positive clones were sequenced with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

3. Rapid Amplification of 5’- and 3’- cDNA Ends (5’- and 3’-RACE)

Gene specific primers used in 5’- and 3’-RACE were designed, based on the sequence information generated from the partial cDNA sequence. 5’-RACE was conducted as per the previously published method (34). In brief, reverse transcription (RT) occurred in the presence of 1 µg total RNA, 0.2 µM of the gene specific primer 5’GSP, 0.2 mM dNTPs, 1 mM dithiothreitol (DTT) and 200 U SuperScript II reverse transcriptase (Life Technologies) at 42°C for 30 min, followed by 5 cycles at 50°C for 1 min, 53°C for 1 min and 56°C for 1 min. After removal of the RNA by alkaline hydrolysis cleavage with EDTA, an anchor oligonucleotide, DT88, was ligated to the 5’-end of the single-stranded cDNA by T4 RNA ligase. The anchor-ligated cDNA was then amplified by a touchdown PCR using the anchor-specific primer, DT89, and 5’GSP at 90°C for 2 min; followed by 10 cycles at 95°C for 10 s and 70°C for 1 min, reducing one degree per cycle. The initial 5’-RACE PCR product was
then used as template to perform a hemi-nested PCR using primers DT 89 and 5’NGSP, which is internal to the primer 5’GSP. The PCR was set to 90°C for 2 min, followed by 30 cycles of 95°C for 5 s and 68°C for 1 min. A single distinct PCR product (~500 bp) was subsequently purified, cloned and sequenced as mentioned above.

The 3’ end of the full length cDNA was amplified using the 3’RACE System for Rapid Amplification of cDNA Ends Kit (Life Technologies). Total RNA was reverse transcribed using (dT)$_{17}$-adapter primer and SuperScript II reverse transcriptase following the manufacturer’s protocol. The poly(dA)-tailed double strand cDNA was obtained by PCR using the gene-specific primer, 3’GSP and the abridged universal amplification primer (AUAP) at 94°C for 2 min, 30 cycles of 94°C for 30 s, 45°C for 1 min and 70°C for 2 min, and one cycle of 45°C for 3 min, 70°C for 10 min. The hemi-nested PCR was performed based on 100-fold dilution of first round PCR products using primers, 3’NGSP I and AUAP with PCR parameters similar to those described above except that annealing was conducted at 50°C instead of 45°C. Three products of size of ~820 bp, ~730 bp and ~540 bp were generated. The hemi-nested PCR was repeated with primers 3’NGSP II and AUAP. Again three products of ~670, ~580 and ~390 bp were produced. All products were purified, cloned and sequenced as above.

4. Synthesis of open reading frame cDNAs and construction of plasmid DNAs
cDNAs corresponding to the open reading frame (ORF) were synthesized by RT-PCR, using RNA extracted from toad brain. The annealing temperature for RT-PCR reaction was changed from 48°C min to 55°C, otherwise the conditions were identical to those described above (Method section 2). Primers, 5’BFR and 3’BFR-AI were used for synthesis of the bNK$_{1}$-A ORF, which was cloned into mammalian expression vector, pTargetT using a TA cloning kit (Promega). A sense primer, 5’BFR-$M_{lu}I$, and antisense primers, 3’BFR-BI-$S_{al}I$ and 3’BFR-
CI-SalI, were used to perform the amplification of bNK₁-B and bNK₁-C ORF, respectively. The products were digested with Mlu I and Sal I, and cloned into pTargetT. The plasmid DNAs were sequenced to confirm the nucleotide sequence of the inserts and used for expression in COS-7 cells and Xenopus oocytes.

5. Cell line transfection and binding assay

**Transient transfections**: The COS-7 (African, Green monkey kidney fibroblast, SV40 transformed) cell line was revived from liquid nitrogen storage and maintained in complete media consisting of Dulbecco’s Minimal Essential Medium (DMEM, ThermoTrace), 10% fetal calf serum (FCS), penicillin/Streptomycin (50 U/ml), and L-glutamine (2 mM). Cells (1.5 × 10⁵ per well) were seeded in 6 well plates and grown in complete media for 24 h, and then transfected with 2.5 µg plasmid DNAs using 7.5 µl Tfx-20 Reagent (Promega). To maximize plasmid transfer, a second round of transfection was carried out after 6 h, using only half the amount of Tfx-20 and plasmid DNA (38). Transfected cells were cultured for further 48 h before radioligand binding studies were performed.

**Stable transfections**: Transfected COS-7 cell cultures were maintained in complete DMEM media supplemented with 0.5 mg/ml of G-418 (Promega). Cultures were washed with DMEM to remove dead cells and passaged weekly for 4 weeks. Surviving cells were cloned out in 96 well plates, propagated and screened for receptor expression by radioligand binding. Positive clones were seeded in 6 well plates (2-3 × 10⁵ cells per well) and incubated overnight before radioligand binding.

**Radioligand binding**: Radioligand binding studies were performed on monolayer transient or stably transfected COS-7 cells using [¹²⁵I]Bolton-Hunter bufokinin ([¹²⁵I]BH-bufokinin) and a method modified from the membrane binding assay (22). The medium was aspirated and the cells were washed twice with 2 ml Tris-HCl buffer (50 mM, pH 7.4, 25°C) containing bovine
serum albumen (BSA, 1 mg/ml). Cells were incubated with 200-300 pM $[^{125}\text{I}]$BH-bufokinin in incubation buffer containing Tris-HCl (50 mM), BSA (1 mg/ml), MnCl$_2$ (3 mM), and peptidase inhibitors; bacitracin (40 µg/ml), leupeptin (4 µg/ml) and chymostatin (4 µg/ml), in a volume of 0.5 ml (2). Non-specific binding was defined by 1 µM unlabelled bufokinin. After incubation at 25°C for 45 min, cells were rinsed with 3 times × 1 ml wash buffer consisting of Tris-HCl (50 mM), MnCl$_2$ (3 mM) and BSA (0.2 mg/ml) and then solubilized in 1 ml 1M NaOH. The bound radioactivity was quantified in a Wizard gamma counter (78% efficiency). No significant difference was observed for binding to transiently or stably transfected cells.

6. Functional expression of receptors in Xenopus oocytes

pTargetT-cDNA constructs were linearised with the restriction enzyme, Not I, and transcribed using the mMessage mMachine In Vitro Transcription kit (Ambion) with the T7 RNA polymerase. Oocytes from *Xenopus laevis* were isolated and injected with cRNA as previously described (37).

Oocytes were placed in a recording chamber and superfused with 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$ 1 mM MgCl$_2$ and 5 mM HEPES, pH 7.5 (ND96) at a rate of 8.5-10 ml/min. Whole cell currents were measured by standard two-electrode voltage-clamp techniques using a Geneclamp 500 amplifier (Axon instruments, Foster City CA., U.S.A.) interfaced with a MacLab2e chart recorder (ADInstruments, Sydney, NSW, Australia) using the Chart software and a Digidata 1200 (Axon Instruments) controlled by an IBM compatible computer using the pClamp software (version 7.0, Axon Instruments). Oocytes were voltage clamped at -50 mV. Peptides (dissolved in 0.05% BSA to minimize adherence to surfaces) were applied at concentrations starting at 10 pM and then increasing by a factor of 10 until an inward current was detected.
The current-voltage relationships for peptide elicited conductances were determined as follows. Steady state current measurements in the absence of peptide were obtained during 200 ms voltage pulses from -30 mV to potentials between -100 and +40 mV in 10 mV steps. These were subtracted from corresponding current measurements in the presence of peptide. The current voltage relationship was measured at both the peak response and after desensitization.

7. Tissue-specific expression of bNK1 receptor mRNA isoforms by RT-PCR

Total RNAs from different tissues were reverse-transcribed with SuperScript II reverse transcriptase using oligo dT adapter (Life Technologies). The reaction was performed in 20 μl volume containing 2 μg total RNA, 0.5 μM (dT)_{17}-adaptor, 0.5 mM dNTPs, 10 mM DTT and 200 U SuperScript II at 42°C for 50 min and heated at 70°C for 15 min. RNA was then removed by digestion with RNase H. One-twentieth of the RT product (equivalent to 100 ng total RNA) from each sample was used for PCR to amplify bNK1-A, NK1-B, NK1-C and β-actin. The sense primer was based on a region common to all three isoforms. The antisense primer, 3’BFR-AII for bNK1-A was based on the unique region of this isoform, and 3’BFR-BII for bNK1-B was chosen from the bNK1-C deletion region, whereas 3’BFR-CII primer for bNK1-C was designed to span the deletion site, so that it could be detected separately. PCR reactions using these primers on plasmids containing individual isoform full length cDNAs showed no cross amplification, confirming that the primers were isoform specific. RT products were PCR amplified using hot start Taq polymerase (Bioline): 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C (for bNK1-A and β-actin) or 60°C for (bNK1-B and bNK1-C) for 1 min and 70°C for 2 min, and a final elongation period at 70°C for 10 min.

8. Statistic analysis
The COS-7 cell line binding data were analyzed using the non-linear regression analysis program of Graph Pad Prism (version 3, Graph Pad Software Inc., San Diego, USA) and expressed as IC50 and 95% confidence intervals of the mean (95% CI). Comparisons of competition curves were carried out using two-way ANOVA statistic analyses.

9. Animal ethics

All procedures were in accordance with the Australian National Health and Medical Research Council “Guidelines for the Prevention of Cruelty to Animals”. These studies were approved by the Animal Ethics Committees of the University of New South Wales (00/110) and Sydney University (K21/5-2001/3/3394).

RESULTS

1. Cloning and identification of three isoforms of bNK1 receptor

Our experimental approach using the RT-PCR and 5’- and 3’-RACE techniques produced five different clones which spanned the ORF locus as well as the 5’ and 3’ untranslated regions of the *Bufo marinus* NK1 receptor (bNK1). Each clone was sequenced in both directions from at least four transformed colonies, generated from different PCR reactions using toad brain and small intestinal RNA extracts as templates. Clone 1 consisted of a 720 bp partial cDNA, which was obtained by RT-PCR using a pair of primers based on the nucleotide sequence of the bullfrog NK1 receptor. This clone included a fragment of receptor protein spanning TM regions II to VII. Clone 2 was a 5’-RACE product of 500 bp, containing the 5’-end of the receptor protein and its untranslated region. 3’-RACE generated three clones, possessing 815, 738, and 545 bp cDNA inserts, respectively. Each of these clones contained individual 3’-end translated and untranslated regions of the receptor gene locus. To exclude potential PCR artifacts, RT-PCR and PCR were conducted with fresh RNA extracts and cDNAs using
several gene-specific primers coding for the 5’ and 3’ ends of the non-translated regions. The existence of three receptor isoforms (bNK₁-A, bNK₁-B and bNK₁-C) was thereby confirmed to be authentic.

The bNK₁-A isoform has the longest cDNA, comprising 1854 nucleotides encoding a 309 amino acid protein; bNK₁-B consists of 1777 nucleotides encoding a protein of 390 amino acids; and bNK₁-C is the shortest cDNA, with 1585 nucleotides encoding a 371 amino acid ORF.

Fig 1 shows the complete sequences of the full length cDNAs and the deduced amino acid sequences for the three toad NK₁ receptor isoforms. These isoforms share a common nucleotide sequence between the 5’-untranslated region to the guanine at position 1219, but downstream regions are unique to each receptor. At position 1220, bNK₁-A contains a stop codon, which results in a highly truncated carboxyl-terminus for this receptor, extending only three amino acids beyond TM VII. The immediate 3’-untranslated region of mRNA encoding for bNK₁-A also differs from those of bNK₁-B and bNK₁-C. The stop codon of bNK₁-B occurs at position 1462, forming the longest carboxyl terminus, which is 81 amino acids longer than bNK₁-A. The nucleotide sequence of bNK₁-C is identical to that of bNK₁-B except for a 192 bp deletion between positions 1272 and 1463. This resulted in a frame shift that gave rise to another ORF of 137 bp in bNK₁-C and an alternative C-terminus to bNK₁-B. Thus, the first 309 amino acids are common to all three receptor isoforms, which then differ in their intracellular carboxyl-terminal regions. A comparison of amino acid sequences of toad, bullfrog and human is shown in Fig 2. The phylogenetic tree constructed from the amino acid sequences of tachykinin receptors using the ClustalX analysis program revealed that the three toad tachykinin receptor isoforms identified in this study belonged to the NK₁ receptor group (Fig 3).
2. Ligand binding assay

Competition binding experiments using $[^{125}\text{I}]$BH-bufokinin were carried out using COS-7 cells transfected with ORF cDNAs, in order to confirm that the cDNA clones isolated in the present study encoded proteins which are able to interact with tachykinins. All three cell lines expressing bNK$_1$-A, bNK$_1$-B and bNK$_1$-C, respectively, bound with high affinity to $[^{125}\text{I}]$BH-bufokinin. The rank order of affinity in competing for $[^{125}\text{I}]$BH-bufokinin binding was, for bNK$_1$-A: bufokinin $>>$ SP $\geq$ NKA; for bNK$_1$-B: bufokinin $>$ SP $>$ NKA and for bNK$_1$-C: bufokinin $>$ NKA $\geq$ SP (Fig 4). Bufokinin was almost equipotent ($\text{IC}_{50}$, 0.6-0.8 nM) in all three isoforms, although these showed different affinities for SP and NKA (Table 2). SP and NKA were very weak competitors for bNK$_1$-A; SP showed highest affinity for bNK$_1$-B whereas NKA showed highest affinity for bNK$_1$-C (Table 2). In most cases, the slope factors of competition curves in all isoforms were below unity (0.5-0.7, data not shown), suggesting binding to more than one site.

When the binding data from the present study were compared to the data obtained in homogenate binding in the toad small intestine using the same radioligand (22), differences were noted (Table 2). Although the affinity order in the small intestine binding and in a functional study using isolated longitudinal ileal segments (Table 2) was bufokinin $>$ SP $>$ NKA, similar to that for bNK$_1$-B, a major discrepancy was observed with NKA. NKA was 34 - 42 fold weaker than bufokinin in the native tissue compared to 228-fold weaker in COS-7 cells expressing bNK$_1$-B.

3. Functional expression in Xenopus oocytes

Application of bufokinin, SP and NKA to oocytes expressing bNK$_1$-B and bNK$_1$-C generated current responses (Fig 5A), but no responses were detected in oocytes expressing bNK$_1$-A. The minimal doses required to elicit a response for the three peptides were bufokinin (0.1
nM), SP (1 nM) and NKA (10 nM) for both bNK₁-B and bNK₁-C. The current response showed significant desensitization in the continued presence of the peptide and subsequent applications of the same dose of peptide generated greatly attenuated responses (see Fig 5A), which precluded obtaining accurate dose responses. The marked desensitization of the current measured in the continued presence of agonist is characteristic of activation of the endogenous calcium dependent chloride channel (24). The reversal potential of the conductance was -20 mV, whether measured at the peak response or after desensitization (Fig 5B), which corresponds with the reversal potential of chloride ions in *Xenopus* oocytes. Application of the chloride channel blocker, niflumic acid, prior to bufokinin application greatly attenuated the current response to the subsequent bufokinin application (Fig 5C).

### 4. Tissue-specific expression of bNK₁ receptor mRNA isoforms

RT-PCR analysis was carried out to determine the expression of the three isoform mRNAs in a number of toad tissues, using unique primers (Table 1). As expected, the control β-actin mRNA was ubiquitously expressed in all tissues examined (Fig 6). Expression of the individual receptor isoforms varied between tissues. The bNK₁-B mRNA was detected in all tissues, being the predominant isoform, except in the skeletal muscle, where bNK₁-A showed the highest expression. The bNK₁-A and bNK₁-C mRNAs were also found in a wide range of tissues, although bNK₁-A was barely detected in liver and aorta, and bNK₁-C was not detectable in aorta, atrium and skin. On the whole, the bNK₁ receptor gene was expressed abundantly in the spinal cord, small intestine, skeletal muscle, lung and brain. A substantial expression was also seen in the stomach and testis. Low expression was observed in aorta, atrium, ventricle, bladder, skin and liver (Fig 6).
DISCUSSION

In this study, we report the isolation from toad brain and small intestine of three different isoforms of an NK\(_1\)-like receptor. At the amino acid level, the three isoforms differ only at the intracellular C-terminus. Isoform A has 70% identity to the truncated mammalian NK\(_1\) receptor (311 amino acids), and B shows moderate (~67%) levels of identity with the full length human, rat and guinea pig NK\(_1\) receptor (407 amino acid) and the chicken NK\(_1\) receptor (69%), with greatest similarity to the bullfrog receptor (85%). Thus, bNK\(_1\)-A and bNK\(_1\)-B are counterparts of the mammalian truncated and full-length NK\(_1\) receptors. In contrast, bNK\(_1\)-C is a new isoform with a unique intracellular C-terminus, showing only 21% identity to bNK\(_1\)-B and 17% to the full length human NK\(_1\) receptor in the C-terminal region. The counterparts of bNK\(_1\)-C have not been reported in any other species to date.

All tachykinin receptors are encoded by a five-exon gene structure interrupted by four introns and are likely to have evolved from a common ancestral gene (12, 14, 19, 20, 33). The phylogenetic analysis indicates that the separation of NK\(_2\) from the NK\(_1\)/NK\(_3\) cluster occurred in an earlier evolutionary period than the split of NK\(_1\) and NK\(_3\) into two different genera (27, Fig 3). The short forms of human and guinea-pig NK\(_1\) receptors (1, 9) and bNK\(_1\)-A terminate three residues after TMVII, and are truncated at the exact position where intron D is inserted between exon 4 and exon 5 (Fig 7). The cDNA encoding bNK\(_1\)-C is created by a 192-bp nucleotide deletion of the bNK\(_1\)-B cDNA within the exon 5 region, causing a frame shift. Since there is no current evidence for the existence of multiple genes encoding the NK\(_1\) receptor in the same species, it is probable that the multiple isoforms of the toad NK\(_1\) receptor are derived from a single gene and undergo pre-mRNA alternative splicing.

This study provides direct evidence, at a molecular level, for sequence conservation and divergence between amphibian and mammalian receptors, and may explain some specific characteristics of the toad receptor. The three residues (Glu78 in TM II, and Tyr205 and
Tyr216 in TM V regions) required for activation of the human NK1 receptor (17, 18) are fully conserved in amphibian receptors (see Fig 2). Another four residues (Asn85, Asn89, Tyr92 and Asn96) in TMII, which are also fully conserved in amphibian receptors, were suggested to be important for conformational changes of the mammalian receptor (29). Other fully conserved residues (Asn23 and Phe25) at the N-terminus are important for agonist binding (10, 11, 18). Therefore, the conservation of these key amino acids in toad NK1 receptor would explain the high affinity of bufokinin at mammalian NK1 receptors (7) and the potency of mammalian and other native amphibian tachykinins in the toad (3, 22). On the other hand, synthetic analogues of SP such as [Pro9]SP, [Sar9]SP, and [Sar9,Met(O2)11]SP showed weak or negligible ability to bind and activate the toad NK1 receptor (3, 22), and this may result from substitution of mammalian Gln24 and Gln165 by Pro23 and Leu164 in the toad receptor protein. Other residues occurring in mammalian receptors (refer Fig. 2) but not conserved in toad or bullfrog are important in the binding of non-peptide NK1 antagonists (5, 10, 18), which are potent in mammals but ineffective in the toad (3, 22). Thus, during evolution, the sequences of tachykinin receptors in modern amphibians and mammals have diverged from that of the ancestral tachykinin receptor, and conservation of sequences such as those involved in mammalian non-peptide antagonist binding has not conferred survival value.

All three isoforms had broadly similar distribution patterns, with quantitative differences. The high level of expression in toad small intestine matches our earlier binding and functional data showing high potency NK1 receptors responding to bufokinin (22). Since all three isoforms are densely expressed in the small intestine, our earlier data probably represents interactions at the three isoforms, if not affinity states. As in mammals (2) and other vertebrates (8), there was very high expression in brain and especially spinal cord. The endogenous ligand for these toad receptor(s) is presumably bufokinin (mammalian SP is absent), although the origin of the peptide, whether neuronal or endocrine, is not clear.
Bufokinin might be released from nerve varicosities in these organs, since we have previously reported the distribution of immunoreactive bufokinin in nerves in toad intestine, bladder, around blood vessels, and in endocrine cells in the intestinal mucosa (21). The presence of all three isoforms in the lung was somewhat unexpected, given our previous study showing lack of immunoreactive bufokinin in this organ in the toad (21). Furthermore, high levels of expression in the skeletal muscle, particularly of bNK1-A, are surprising, and further studies would be required to demonstrate the histological location of the receptor protein. It is possible that the receptor(s) is located on vascular endothelial cells as shown in previous autoradiographic studies of mesenteric vessels (21), since both skeletal muscle and lung are well endowed with capillaries. The role that NK1 receptor might play in the testis is less clear, although tachykinins have been shown to modulate the functions of several cell types in the mammalian testis (7). The development of oligonucleotide probes and antibodies specific for each isoform would be important tools to ascertain their functional significance in different tissues and locations.

All isoforms expressed in COS-7 cells showed high affinity for [125I]BH-bufokinin, and displayed similar high affinity for the endogenous bufokinin, although bNK1-A lacks any C-terminal sequence. This suggests that interaction of bufokin in with the toad NK1 receptor does not involve the C-terminal region(s). However, the binding profiles of SP and NKA were very different at the three isoforms, with particularly low affinity at bNK1-A. Since these isoforms vary only at the intracellular tail region, this C-terminal divergence must underlie the quantitative differences in binding affinity for SP and NKA. The shallow competition curves and the strikingly dissimilar binding profiles for each isoform in our cell line binding studies may reflect the occurrence of various affinity states caused by coupling to different G-proteins (16) or even the same G-protein(s) in different ways. Alternatively, it is now accepted that different ligands can induce various conformations of tachykinin NK1 and NK2 receptors (27).
Although bufokinin bound with equal affinity for all three isoforms, these were not equally active in the *Xenopus* oocyte expression system. Thus, the short isoform, bNK₁-A, was inactive, as shown for mammalian truncated NK₁ receptors expressed in heterologous systems (9, 30), whereas bNK₁-B and bNK₁-C produced increases in chloride current in response to tachykinin stimulation. For bNK₁-B, there was an excellent correlation between functional and binding data, whereas for bNK₁-C there was a discrepancy between potencies of SP and particularly NKA. High affinity agonist binding does not necessarily correlate with the ability of the receptor to promote agonist-induced functional responses, particularly in heterologous expression systems. In this respect, signal transduction systems may be important. Further studies to determine the signal transduction pathways utilized by these isoforms will be of considerable interest. Little is known about G-proteins in amphibia or the regions of the intracellular domains of GPCRs with which they interact.

In *Xenopus* oocytes, stimulation of bNK₁-B and bNK₁-C was followed by rapid desensitization to subsequent applications of tachykinins. This suggests that bNK₁ receptors couple through G-proteins to the calcium-dependent chloride channel, and this was confirmed by our studies with niflumic acid (Fig 5C). Similar biphasic chloride current responses have been observed for stimulation of dopamine D1 receptors expressed in *Xenopus* oocytes and have been attributed to receptor coupling to stimulation of cAMP production and release of calcium from calcium stores, both of which may lead to activation of the calcium-dependent chloride channel (28). The mechanism of desensitization in mammalian systems involves agonist-induced phosphorylation of Ser/Thr residues at the C-terminus by a receptor-specific kinase, allowing β-arrestin to bind and cause receptor internalization (23, 26, 39). Since these residues are found in the appropriate cytoplasmic domains of bNK₁-B and bNK₁-C receptors (Fig 1), desensitization mechanisms in the toad NK₁ receptor appear similar to those described in mammals (30).
The role of the short isoform bNK₁-A is unclear. As in the toad, there is widespread expression of both long and truncated forms of NK₁ receptor in the human brain and peripheral tissues (4). The lack of ability to produce a functional response in the *Xenopus* oocyte system could be due to the truncated C-terminus inducing a differently folded protein tertiary structure, thus causing abnormal trafficking, whereas receptor protein folding might occur normally in native *Bufo* systems. Alternatively, bNK₁-A might require a specific G-protein, which does not exist in *Xenopus* oocytes. Insights into the role of bNK₁-A may be obtained from studies with truncated splice variants of other GPCRs and ion channel receptors. In many cases, these truncated protein molecules appear to act as binding, non-signalling receptors on their own, and exert functions only when forming heterodimeric complexes with other variants. The truncated toad NK₁ receptor may be a non-functional receptor itself, but heterodimerize with the full-length isoform to regulate receptor activation.

In summary, this study is the first evidence for the existence of a naturally occurring truncated form (bNK₁-A) of NK₁ like receptor in non-mammalian species and is also the first identification of a unique third tachykinin receptor isoform (bNK₁-C) in any species. Thus, bNK₁-A and bNK₁-B are the counterparts of the mammalian truncated and long form of NK₁ receptor, whereas the mammalian equivalent of bNK₁-C is unknown and it may be redundant or have undergone mutation in other vertebrates. Our study suggests that the alternative splicing of NK₁ receptor has been conserved under the selection of evolutionary pressure. Receptors found in lower animals undoubtedly have counterparts with important functions in humans. The identification of these new receptor isoforms and their specific pharmacological properties may provide novel insights into the modulation of tachykinin receptor activities and provide important clues for a better understanding of the phylogenetic origin of the tachykinin family.
GenBank Access No: bNK₁-A (BFR-A), AF289083; bNK₁-B (BFR-B), AF416731 and bNK₁-C (BFR-C), AF482695.
ACKNOWLEDGEMENTS

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GRANTS

This study was funded by the Australian Research Council.
REFERENCES


Fig 1. Comparison of nucleotide sequences and amino acid sequences of the toad NK₁ receptor isoforms, showing (A) the common and (B) the divergent nucleotide and amino acid sequences for bNK₁-A, bNK₁-B and bNK₁-C. Amino acids are shown by single-letter code. The cDNA of bNK₁-A has a unique 3’ untranslated region. The nucleotide sequences of bNK₁-B and bNK₁-C are identical except for a 192-bp deletion in bNK₁-C (indicated by boxed nucleotide sequences), which results in an alternative carboxyl terminus due to a frame shift.

The putative seven transmembrane domains (TM I to TM VII) (underlined), the three cytoplasmic loops (C₁, C₂ and C₃) and extracellular loop (E₁, E₂ and E₃) are shown. Also indicated are potential N-glycosylation sites (▲), which are important in determining the correct distribution of the receptor in the cell and for controlling receptor expression. Two cysteine residues (●) which are involved in disulfide bonds and form part of the agonist binding pocket are conserved in the bNK₁ receptors. A conserved cysteine residue (●) described as a palmitoylation site, to stabilize the conformation of a GPCR, is observed in bNK₁-B and bNK₁-C. Putative protein phosphorylation sites identified using the PROSITE database are also shown: all bNK₁ receptor isoforms contain a potential protein kinase C (PKC) site (Θ) within the cytoplasmic loop III (Ser-233). Additional PKC sites are present at the C-terminus of bNK₁-B (Ser-336, Thr-373 and Ser-386) and bNK₁-C (Thr-338 and Ser-344). Potential protein kinase A sites (●) were found at the C-terminal regions of bNK₁-B (Ser-378) and bNK₁-C (Ser-342).

Fig 2. Alignment of amino acid sequences for the bNK₁-B isoform with the bullfrog (32) and human NK₁ receptors. Those amino acid residues conserved in all species are boxed. The transmembrane domains (TM I to TM VII) are shown by horizontal bars; C₁-C₃ indicates cytoplasm loops 1-3, and E₁-E₃, extracellular loops 1-3. Residues involved in NK₁ receptor
activation in human (Glu78, Tyr205 and Tyr216) are denoted by ⊕; high affinity SP binding sites in human (Asn23, Gln24, Phe25 and His108) are denoted by ⊖; residues (Asn85, Asn89, Tyr92 and Asn96), denoted by ▲, are important for conformational changes of the human receptor; residues involved in non-peptide antagonist binding in human (Ser169, His197 and His265) are denoted by ▼; residues involved in both agonist and non-peptide antagonist binding in human (Gln165, Tyr287) are denoted by ⊗ (5, 10, 11, 17, 18, 29).

**Fig 3.** Phylogenetic tree of tachykinin receptors was constructed by alignments of amino acids using the neighbor joining (NJ) method of ClustalX program. The robustness of the branches is indicated by bootstrap scores at nodes. Scale bar represents an amino acid replacement distance. GenBank accession numbers of amino acid sequences are shown in brackets.

**Fig 4A-C.** Competition binding of bufokinin, SP and NKA against $[^{125}\text{I}]$BH-bufokinin in COS-7 cells expressing (A) bNK$_1$-A, (B) bNK$_1$-B and (C) bNK$_1$-C. Data represent the mean ± s.e.m. of duplicate determination in 3 – 5 different experiments. (A), bufokinin showed significantly higher affinity than SP and NKA ($P < 0.001$), but there was no difference between SP and NKA ($P = 0.48$); (B), bufokinin showed higher affinity than SP and NKA ($P < 0.001$), and SP showed higher affinity than NKA ($P < 0.05$); (C) bufokinin showed higher affinity than NKA and SP ($P < 0.01$), and the difference between NKA and SP was not significant ($P = 0.27$). Two-way ANOVA was used for the statistic analyses.

**Fig 5. A.** Representative current trace from a *Xenopus* oocyte expressing bNK$_1$-C. Bufokinin applied for 1 min at 0.1 nM gave a very small response, whereas a concentration of 1 nM elicited a much larger current. Response to the second application at 1 nM was greatly
reduced, indicating desensitization. **B.** The current–voltage relationship was measured at both the peak response (○) and after desensitization (●) following application of 1 nM bufokinin. The reversal potential of the conductance was -20 mV for both measurements. **C.** The current–voltage relationship was measured 2 s after initial current deflection following application of 1 nM bufokinin (BFK) (○) and after co-application of 1 nM bufokinin and 50 µM niflumic acid (BFK + NFA) (●).

**Fig 6.** Representative gels showing expression of each toad NK₁ receptor isoforms in different toad tissues. β-actin expression in the same tissue was used as an internal control. Two to four other experiments for each isoform gave similar results.

LU, lung; AO, aorta, AT, atrium; V, ventricle; BL, bladder; BR, brain; SC, spinal cord; SK, skin; SI, small intestine; SkM, skeletal muscle; ST, stomach; TE, testis; LI, liver.

**Fig 7.** Schematic diagram of the gene encoding toad NK₁ receptor isoforms. Analysis of the genomic sequences of mammalian and invertebrate tachykinin receptors (12, 14, 19, 20, 33) indicates that all tachykinin receptor proteins are encoded by a five-exon gene structure (Exon 1-Exon 5) interrupted by four introns (Intron A-Intron B, refer bNK₁-B). The truncation of bNK₁-A occurs at the position where intron D is inserted, implying that during pre-mRNA splicing, an intron sequence may be retained which results in an insertion of a premature termination codon. The cDNA encoding bNK₁-C is created by a 192-bp nucleotide deletion of the bNK₁-B cDNA within the exon 5 region, causing a frame shift. The transmembrane domains (TM I to TM VII) are shown by solid dark box; numbers indicate amino acid position at which splicing sites insert. The codes for translation initiation (ATG) and termination (TGA) are also indicated.
Table 1. Primers used in RT-PCR and 5’ and 3’-RACE for sequencing and expression analysis of toad NK₁ receptor isoforms

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
<th>Sequence position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers used in sequencing:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM-2 (sense)</td>
<td>ATGAGGACTGTCACCAACTATTT</td>
<td>637-659&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TM-7 (antisense)</td>
<td>AATAAATGATGGGGTTGTACAT</td>
<td>1363-1342&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DT88 anchor</td>
<td>GAAGAGAAGTTGGAATGGCGTTTTGG</td>
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</tr>
<tr>
<td>DT89</td>
<td>CCAAAACGCGATTTCCACCTTCCTTC</td>
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</tr>
<tr>
<td>5’GSP (antisense)</td>
<td>ATGAGTCCATAGTACCAGTG</td>
<td>596-576</td>
</tr>
<tr>
<td>5’NGSP (antisense)</td>
<td>AAATAGTGGTGACAGTCTCAT</td>
<td>497-475</td>
</tr>
<tr>
<td>Adapter primer</td>
<td>GGCCACGCGTGACTAGTAC(dT)&lt;sub&gt;17&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>AUAP</td>
<td>GGCCACGCGTGACTAGTAC</td>
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<tr>
<td>3’GSP (sense)</td>
<td>CGCAAGGTTGGAAGATGATG</td>
<td>1021-1041</td>
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<tr>
<td>3’NGSP I (sense)</td>
<td>ATGATCGTGGTAGTTTCGACA</td>
<td>1039-1059</td>
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<tr>
<td>3’NGSP II (sense)</td>
<td>CGTGTGGAGATGAGCTCCACC</td>
<td>1160-1182</td>
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<tr>
<td>Primers used in amplifying the coding regions of cDNAs:</td>
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<td></td>
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<tr>
<td>5’BFR (sense)</td>
<td>GCCACCATGAATTCTACCACAGAAT</td>
<td>289-312</td>
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<td>3’BFR-AI (antisense)</td>
<td>CCTACCTGTCATTTCCACAGA</td>
<td>bNK₁-A 1223-1200</td>
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<td>5’BFR-MluI (sense)</td>
<td>CCACGCGTCATGAATTCTACCACAG</td>
<td>291-309</td>
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<td>3’BFR-BI-SalI (antisense)</td>
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<td>3’BFR-CI-SalI (antisense)</td>
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<td>Primers used in tissue expression analysis:</td>
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<tr>
<td>5’BFR (sense)</td>
<td>GCCACCATGAATTCTACCACAGAAT</td>
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<td>3’BFR-AII (antisense)</td>
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<tr>
<td>β-actin fp (sense)</td>
<td>ACGGGGTCACCCACACTGTGC</td>
<td>543-563&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>β-actin rp (antisense)</td>
<td>CTAGAAGCATTGGCGTTGGAC</td>
<td>1201-1181&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a</sup> positions are relative to bullfrog NK₁-like receptor cDNA sequence (GenBank accession No. U67736)

<sup>b</sup> positions are relative to human β-actin cDNA sequence (GenBank accession No. NM001101).
Table 2. IC$_{50}$ values of tachykinins in competing for [125I]BH-bufokinin binding in COS-7 cells expressing toad NK$_1$ isoforms, in comparison with data obtained in toad small intestine.

<table>
<thead>
<tr>
<th>Tachykinin</th>
<th>bNK$_1$-A</th>
<th>bNK$_1$-B</th>
<th>bNK$_1$-C</th>
<th>Small intestine binding$^a$</th>
<th>Small intestine functional$^{a,b}$</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM) (95% CI) n</td>
<td>Ratio</td>
<td>IC$_{50}$ (nM) (95% CI) n</td>
<td>Ratio</td>
<td>IC$_{50}$ (nM) (95% CI) n</td>
</tr>
<tr>
<td>Bufokinin</td>
<td>0.66 (0.24-1.8) 4</td>
<td>100</td>
<td>0.60 (0.19-1.9) 4</td>
<td>100</td>
<td>0.75 (0.25-2.2) 5</td>
</tr>
<tr>
<td>SP$^c$</td>
<td>910 (240-3470) 3</td>
<td>0.07</td>
<td>7.3 (1.3-41) 5</td>
<td>8.2</td>
<td>30 (8.6-99) 4</td>
</tr>
<tr>
<td>NKA$^d$</td>
<td>1660 (690-3980) 3</td>
<td>0.04</td>
<td>137 (28-660) 4</td>
<td>0.43</td>
<td>8.1 (2.2-30) 4</td>
</tr>
</tbody>
</table>

95% CI, 95% confidence interval of the IC$_{50}$ estimation.

n, numbers of experiments performed.

Ratio, IC$_{50}$ relative to bufokinin.

$^a$ data from Liu et al., 1999 (23).

$^b$ functional data are from longitudinal segments of intestine.

$^c$ SP showed significantly higher affinity for isoform B than for C ($P < 0.05$) and lower affinity for isoform A ($P < 0.001$ compared to B and C). Two-way ANOVA was used for the statistic analyses.

$^d$ NKA showed significantly higher affinity for isoform C than for B ($P < 0.05$) and lower affinity for isoform A ($P < 0.01, 0.001$ compared to B and C, respectively). Two-way ANOVA was used for the statistic analyses.
Fig 2.
Fig 3
Fig 4.
Fig 5.
Fig 6.
Fig 7.