ELSEVIER

Biochemical Pharmacology 63 (2002) 217–224

Biochemical Pharmacology

# Structure–activity studies of bufokinin, substance P and their C-terminal fragments at bufokinin receptors in the small intestine of the cane toad, *Bufo marinus*

Lu Liu, Michael Murray, Elizabeth Burcher*

*School of Physiology and Pharmacology, University of New South Wales, Sydney 2052, NSW, Australia*

Received 7 May 2001; accepted 12 October 2001

**Abstract**

Bufokinin is a substance P-related tachykinin peptide with potent spasmogenic actions, isolated from the intestine of the cane toad, *Bufo marinus*. Bufokinin acts via a tachykinin receptor with similarities to the mammalian $NK_1$ receptor. In this structure–activity study of bufokinin, substance P (SP) and their C-terminal fragments, we have used isolated segments and homogenates of toad small intestine to compare the contractile potencies and abilities to compete for the binding of [$^{125}$I]-Bolton–Hunter bufokinin. In general, potency was very similar in both studies ($r = 0.956$) and was primarily related to peptide length, with the natural undecapeptide tachykinins bufokinin ≈ ranakinin > SP ≈ cod SP ≈ trout SP being most potent. The weakest peptides were [Pro$^9$]SP, BUF(7–11) and SP(7–11). Bufokinin fragments (BUF) were approximately equipotent to the corresponding SP fragments, with only BUF(5–11) showing unexpectedly low binding affinity. Data obtained with SP, bufokinin and fragments were subjected to quantitative structure–activity (QSAR) analysis which demonstrated that molecular connectivity and shape descriptors yielded significant regression equations ($r \approx 0.90$). The predictive capacity of the equations was confirmed using ranakinin, trout SP and cod SP, but not using the synthetic analogs [Pro$^9$]SP and [Sar$^9$]SP. The study suggests that the full undecapeptide sequence of bufokinin is required for optimal activity, with high potency conferred by Lys$^1$, Pro$^2$, Gly$^9$ and probably Tyr$^8$. The finding that receptor–ligand interactions were correlated with the shape descriptor $^2\kappa_\alpha$ and favored by basic and rigid residues at position 1–3 is consistent with an important role of conformation at the N-terminus of bufokinin. 

*Keywords:* Structure–activity; Substance P; Tachykinin receptors; Amphibian; Intestine; QSAR

## 1. Introduction

The tachykinins are an important group of neuropeptides of diverse actions, widely distributed in vertebrates and invertebrates. We have recently isolated a novel tachykinin undecapeptide, bufokinin, from the small intestine of the cane toad, *B. marinus* [1]. In mammals, tachykinins interact with three well characterized receptors, $NK_1$, $NK_2$ and $NK_3$ [2], but studies of their counterparts in nonmammals have been quite limited.

Bufokinin is a potent spasmogen of longitudinal and circular muscle in the toad intestine [3]. Binding and functional studies in toad intestinal smooth muscle have delineated a bufokinin-preferring receptor, which has similarities to the mammalian $NK_1$ receptor [3,4]. This toad tachykinin receptor is coupled to G-proteins and the signal transduction pathways mediating intestinal contractility are linked to phosphoinositol hydrolysis [3,4]. Dense bufokinin-immunoreactive fibers innervate the intestine and binding sites for [$^{125}$I]BH-bufokinin occur over intestinal smooth muscle and blood vessels [5]. Thus, bufokinin appears to be involved in mediating gastrointestinal and cardiovascular functions in the toad.

The tachykinin family is characterized by possession of the carboxyl-terminal pentapeptide sequence –Phe–X–Gly–Leu–Met–$NH_2$, where X is either aromatic (Phe, Tyr) or aliphatic (Val, Ile). Many previous studies have shown the importance of the C-terminus, including the terminal amide, for interaction at the mammalian receptor, whereas the 'inactive' N-terminus serves to distinguish

* Corresponding author. Fax: +61-2-9385-1059.

*E-mail addresses:* lu.liu@unsw.edu.au (L. Liu), e.burcher@unsw.edu.au (E. Burcher).

*Abbreviations:* SP, substance P; NKA, neurokinin A; BH, Bolton–Hunter; QSAR, quantitative structure–activity analysis; BUF, bufokinin C-terminal fragment.

0006-2952/02/$ – see front matter 
PII: S0006-2952(01)00875-9

Table 1
Structures of naturally occurring tachykinins used in this study

| Structural feature | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bufokinin (toad SP) | Lys | Pro | Arg | Pro | Asp | Gln | Phe | Tyr | Gly | Leu | Met | $NH_2$ |
| Ranakinin (frog SP) | Lys | Pro | Asn | Pro | Glu | Arg | Phe | Tyr | Gly | Leu | Met | $NH_2$ |
| Trout SP | Lys | Pro | Arg | Pro | His | Gln | Phe | Phe | Gly | Leu | Met | $NH_2$ |
| Cod SP | Lys | Pro | Arg | Pro | Gln | Gln | Phe | Ile | Gly | Leu | Met | $NH_2$ |
| Mammalian SP | Arg | Pro | Lys | Pro | Gln | Gln | Phe | Phe | Gly | Leu | Met | $NH_2$ |

receptor subtypes and cause different biological actions [2,6,7]. Amphibian tachykinins have quite diverse chemical structures, particularly at the N-terminus [8]. Bufokinin exhibits high affinity at all three tachykinin receptors in the rat [1] and has structural features intermediate between SP and NKA (Table 1). Like SP, bufokinin contains an aromatic residue, $Tyr^8$, at the C-terminal region (corresponding to $Phe^8$ of SP) and has the N-terminal motif Arg/Lys–Pro–X–Pro, a feature also shared by several other SP-related peptides from frog (ranakinin), trout and cod (Table 1). Bufokinin also possesses an acidic residue, $Asp^5$, corresponding to position 4 of NKA, which would account for its high affinity at the rat $NK_2$ receptor [1]. However, there is no evidence to date for an $NK_2$-like receptor in the toad intestine [3].

In this study, we have used both functional and radioligand binding techniques to describe the structure–activity relationships of bufokinin, SP and their C-terminal fragments, to elucidate which amino acids are crucial elements of receptor affinity and efficacy in the toad small intestine. A QSAR approach was used to derive equations that accounted for >80% of the data variance and were used to predict the binding properties of additional-related peptides. Structure–activity relationships of SP and NKA have been investigated extensively in mammals [9], but little work has been carried out in non-mammalian vertebrates. However, non-mammalian tachykinin peptides have proved important in the discovery of mammalian tachykinins and in the development of mammalian tachykinin receptor pharmacology [10]. Further information about phylogenetically older tachykinin receptors may be of relevance to variants described in mammals. This study may contribute to the eventual design of novel ligands useful in therapeutic and/or research fields.

## 2. Methods and materials

### 2.1. Functional studies

Toads of both sexes, weight 150–250 g, were obtained commercially from Douch, North Queensland, Australia and held in captivity at room temperature for up to a month. Toads were killed by pithing. Four segments (10–15 mm) of small intestine from each toad were excised 1 cm proximal to the large intestine. Whole segments were mounted longitudinally in 2 mL organ baths containing toad Mackenzie's solution (composition (mM): NaCl 115, KCl 3.2, $NaHCO_3$ 20, $NaH_2PO_4$ 3.1, $MgSO_4$ 1.4, $CaCl_2$ 1.3 and D-glucose 16.7), maintained at 25° and aerated with 95% $O_2$ and 5% $CO_2$ (carbogen). Muscle activity was recorded isometrically using Grass FTO3C force transducers and recorded on polygraph (Crawford, University of New South Wales). All preparations were set at an initial tension of 1 g and allowed to equilibrate for 60 min. At the beginning of each experiment, a supramaximal concentration of SP (1 μM) was applied to each preparation to define the maximum contraction [3,4]. After thorough washing and re-equilibration, one concentration–response curve to a single tachykinin was obtained in each muscle strip, using discrete additions of peptide [3,4].

Contractile responses of tachykinins were recorded as grams tension and then expressed as a percentage of the maximum (SP) response. The concentration–effect curves were fitted using the non-linear regression analysis program of Graph Pad Prism and the agonist potencies were expressed as $EC_{50}$.

### 2.2. Homogenate binding studies

Membranes were prepared from fresh intestinal smooth muscle, minus mucosa, as described [11]. Membranes were finally suspended in incubation buffer consisting of Tris–HCl (50 mM, pH 7.4, 15°), $MnCl_2$ (3 mM), BSA (0.02%) and the peptidase inhibitor bacitracin (40 μg/mL), established as the appropriate binding conditions for [$^{125}$I]BH-bufokinin in the toad small intestine [3]. The radioiodination of bufokinin with [$^{125}$I]BH reagent (specific activity, 2200 Ci/mmol) and purification by HPLC were carried out following the method described previously [3].

Competition binding assays were performed by incubating membrane aliquots (3% (w/v)) with [$^{125}$I]BH-bufokinin (70 pM) in the presence of varying concentrations of competitors, at 15°. Non-specific binding was determined by 1 μM unlabelled bufokinin. At equilibrium (60 min), the reactions were terminated by immediate filtration through Whatman GF/B glass-fiber filters following by three additional washes with ice-cold wash buffer containing Tris–HCl (50 mM, pH 7.4, 4°), $MnCl_2$ (3 mM) and BSA (0.02%). Filters were presoaked overnight at 4° in 0.1% polyethyleneimine. Radioactivity on the filters was

then quantified in a Wallac Wizard auto-gamma counter (>78% efficiency).

Fitting of binding data to a one or two site model was achieved using the non-linear regression analysis program (Graph Pad Prism, Graph Pad Software Inc.). The inhibition of [$^{125}$I]BH-bufokinin by competing ligands was expressed as $IC_{50}$, defined as the concentration of competitor displacing 50% of specifically bound radioligand. The correlation between functional and binding data was determined by regression analysis. The criterion for statistical significance was set at $P < 0.05$.

### 2.3. Quantitative structure–activity analysis

Molecular connectivity ($\chi$) and shape ($\kappa$) indices were used to analyze the binding data of bufokinin, SP and the bufokinin-truncated analogs (8 peptides) and the functional data of bufokinin, SP and both sets of truncated analogs (12 peptides). Connectivity indices ($^{m}\chi_{t}$) were calculated by established methods [12,13]. Here, $m$ is the number of connected bonds used in the calculation (0–4 in this study) and $t$ is the arrangement of bonds (path, cluster or path/cluster). Thus, in the first-order expression:

$$^{1}\chi = \sum_{z=1}^{n} (\delta_i^v \delta_j^v)_z^{-0.5}$$

where $n$ is the number of single bonds of peptides (for a first-order index) and $i$ and $j$ are the bonded atoms, $\chi$ indices of different order were determined in analogous fashion.

Molecular shape indices ($^{m}\kappa_{\alpha}$) were calculated according to Kier [14], where $m$ is the number of bonds and $\alpha$ indicates that modified atom values were used to facilitate the treatment of heteroatoms and non-sp$^3$-hybridised atoms. Thus, 11 parameters were used in the QSAR analyses. The $\chi$ and $\kappa$ values were scaled by a factor of 10 to yield more manageable coefficients in regression equations [15]. The quality of the data fit was evaluated from the correlation coefficient ($r$), the standard deviation from the regression ($s$) and the $F$ ratio of the regression.

### 2.4. Materials

Bufokinin and all bufokinin fragments were custom-synthesized by Auspep. SP, [Sar$^9$]SP, [Pro$^9$]SP and SP fragments were purchased from Auspep. Trout SP and cod SP were gifts from Jensen, University of Göteborg, Sweden and ranakinin was synthesized by Conlon, Creighton University, USA. Bacitracin (zinc salt) was purchased from Sigma. The [$^{125}$I]-Bolton–Hunter reagent (2 mCi/mmol) was purchased from Amrad Pharmacia Biotech. Stock solutions of peptides were prepared in 0.01 M acetic acid containing 1% β-mercaptoethanol and stored in aliquots at −20°, except for BUF(5–11) which was initially dissolved in DMSO.

## 3. Results

### 3.1. Functional studies

All peptides tested behaved as full agonists in inducing contractions of the toad intestinal longitudinal muscle, with maximum responses similar to that of SP (Fig. 1). Bufokinin and ranakinin were the most potent contractile agents. Of the natural undecapeptide tachykinins, bufokinin (toad SP) was 4-, 5.5-, 6- and 10-fold more potent than trout SP, cod SP and mammalian SP, respectively.

BUF(2–11) was 6-fold weaker than bufokinin, whereas BUF(3–11), BUF(4–11), BUF(5–11) and BUF(6–11) all showed similar potency, being 44–83-fold less potent than bufokinin (Table 2). The concentration–response curves for these four fragments were superimposed (Fig. 1A). BUF(7–11) was a much weaker contractile agonist, being three orders of magnitude less potent than bufokinin. The $EC_{50}$ values and potency ratios relative to bufokinin are summarized in Table 2.

SP was 7-, 9-, 3- and 100-fold more potent than its C-terminal fragments SP(4–11), SP(5–11), SP(6–11) and

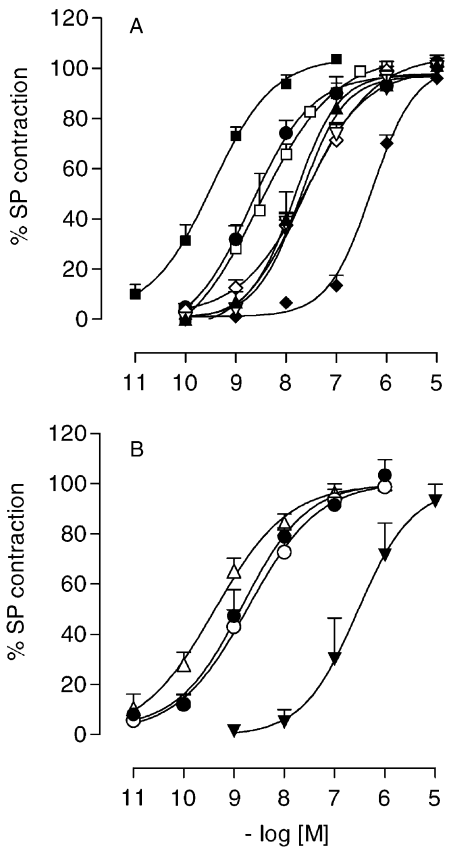


Fig. 1. Concentration response curves of tachykinins and analogs in contracting toad isolated small intestine. Data are expressed as a percentage of the maximal response to SP ($10^{-6}$ M). Each point represents the mean ± SEM of determination in 4–9 animals. (A) Bufokinin (■); BUF(2–11) (●); SP (□); BUF(3–11) (▲); BUF(4–11) (▽); BUF(5–11) (▼); BUF(6–11) (◇) and BUF(7–11) (◆). (B) Ranakinin (△); trout SP (●); cod SP (○); and [Pro$^9$]SP (▼).

Table 2
Potency of tachykinins, fragments and analogs as competitors against [$^{125}$I]BH-bufokinin in toad intestinal membranes and as contractile agents in toad isolated intestinal segments

| Peptide | Radioligand binding studies | | | | Functional studies | | |
|---|---|---|---|---|---|---|---|
| | Slope | $IC_{50}$ (nM) | 95% CL | RA[a] | $EC_{50}$ (nM) | 95% CL | RP[b] |
| Bufokinin | 1.09 | 1.7 | 1.5–1.9 | 100 | 0.34 | 0.17–0.70 | 100 |
| BUF(2–11) | 0.91 | 25.7 | 21.7–30.5 | 6.6 | 2.0 | 0.71–5.9 | 17 |
| BUF(3–11) | 0.96 | 101 | 88.0–115 | 1.7 | 15.0 | 8.0–28.2 | 2.3 |
| BUF(4–11) | 0.83 | 141 | 98.2–201 | 1.2 | 19.3 | 11.9–31.1 | 1.8 |
| BUF(5–11) | 0.73 | 356 (H, 72%)[c] | 154–826 | 0.47 | 20.6 | 12.1–35.2 | 1.7 |
| | | 11900 (L, 28%) | 921–154000 | 0.01 | | | |
| BUF(6–11) | 0.90 | 78.0 | 61.8–98.4 | 2.2 | 28.1 | 16.8–47.0 | 1.2 |
| BUF(7–11) | 0.74 | 2780 | 1740–4450 | 0.06 | 503 | 378–668 | 0.07 |
| SP | 0.92 | 10.7 | 8.94–10.7 | 16 | 3.3 | 1.3–8.6 | 9.7 |
| SP(4–11) | | ND[d] | | | 23[e] | 15.4–33.9 | 1.5 |
| SP(5–11) | | ND | | | 29[e] | 19.9–41.7 | 1.2 |
| SP(6–11) | | ND | | | 10[e] | 6.9–14.5 | 3.4 |
| SP(7–11) | | ND | | | 331[e] | 209–525 | 0.1 |
| Ranakinin | 0.94 | 2.8 | 2.2–3.6 | 60 | 0.42 | 0.15–1.2 | 81 |
| Trout SP | 0.91 | 20.8 | 17.4–24.8 | 8.2 | 1.3 | 0.62–2.7 | 26 |
| Cod SP | 0.93 | 14.4 | 12.8–16.2 | 12 | 1.9 | 0.88–4.2 | 18 |
| [Sar$^9$]SP | 0.71 | 180 | 129–252 | 0.94 | 107[e] | 96–120 | 0.32 |
| [Pro$^9$]SP | 0.48 | 937 (H, 40%) | 473–1860 | 0.18 | 285 | 71–1150 | 0.12 |
| | | 39400 (L, 60%) | 16700–93300 | 0.004 | | | |

[a] Ratio of affinity relative to bufokinin.
[b] Ratio of potency relative to bufokinin.
[c] H, high affinity site; L, low affinity site, with percentage of each site, where data were significantly better fitted to a two site model.
[d] Not determined.
[e] Value obtained from [5].

SP(7–11), respectively (Table 2). SP was 32- and 86-fold more potent than its analogs [Sar$^9$]SP and [Pro$^9$]SP, respectively.

### 3.2. Binding studies

The ability of each peptide to inhibit [$^{125}$I]BH-bufokinin binding was determined in toad intestinal membranes (Fig. 2). Of the natural tachykinins, bufokinin was again the most potent competitor, having 1.7-, 6-, 8-, and 12-fold higher binding affinity than ranakinin, mammalian SP, cod SP, and trout SP, respectively.

Bufokinin was 15-, 59-, 83- and 46-fold more potent than BUF(2–11), BUF(3–11), BUF(4–11) and BUF(6–11), respectively. BUF(5–11) was a weak competitor and the data were best fitted to two binding sites of high (72% of sites) and low (28% of sites) affinity. Of all the peptides in this study, this analog was the only one unable to fully compete for binding of the radioligand. BUF(7–11) was very weak, showing 1600-fold reduced affinity.

For SP-related peptides, the potency order in competing for [$^{125}$I]BH-bufokinin binding sites was SP $\gg$ [Sar$^9$]SP = [Pro$^9$]SP. The competition curve for [Pro$^9$]SP was best fitted to two binding sites (Table 2). Only the higher affinity estimate was used in subsequent analyses.

In general, functional potency and binding affinity were increased with increasing length of the molecule (Fig. 3A and B). There was an excellent correlation between functional and binding data. Linear regression analysis showed a positive correlation ($r = 0.956$) between the affinities ($pIC_{50}$) of tachykinins for [$^{125}$I]BH-bufokinin binding site and their potencies ($pD_2$) in contracting the isolated intestine (Fig. 3C). The regression line was displaced towards the $pD_2$ axis, reflecting the generally greater functional potency compared with binding affinity of these peptides.

### 3.3. Quantitative structure–activity analysis

Binding and functional data ($pIC_{50}$, $pD_2$) from bufokinin, SP and their C-terminal fragments were subjected to QSAR analysis. Single variable equations for the interaction of peptides with the bufokinin receptor were determined using molecular connectivity indices (path type $^0\chi - {}^4\chi$, third-order cluster type $^{3C}\chi$ and fourth-order path-cluster type $^{4PC}\chi$) and molecular shape attributes ($^0\kappa_\alpha - {}^3\kappa_\alpha$). Most single variable equations gave similar quality of fit of the binding ($r = 0.87$–$0.91$, $s = 0.43$–$0.51$) and functional data ($r = 0.82$–$0.92$, $s = 0.35$–$0.49$) except for the parameter $^3\kappa_\alpha$ (binding $r = 0.71$, $s = 0.74$; function $r = 0.82$, $s = 0.52$). Adequate information was therefore contained within the zero and first-order functions; equations 1–4 (binding) and 5–8 (function) were derived (Table 3) which accounted for 76–85% of the data variance.

The zero-order connectivity function describes the arrangement of atoms within a structure rather than their

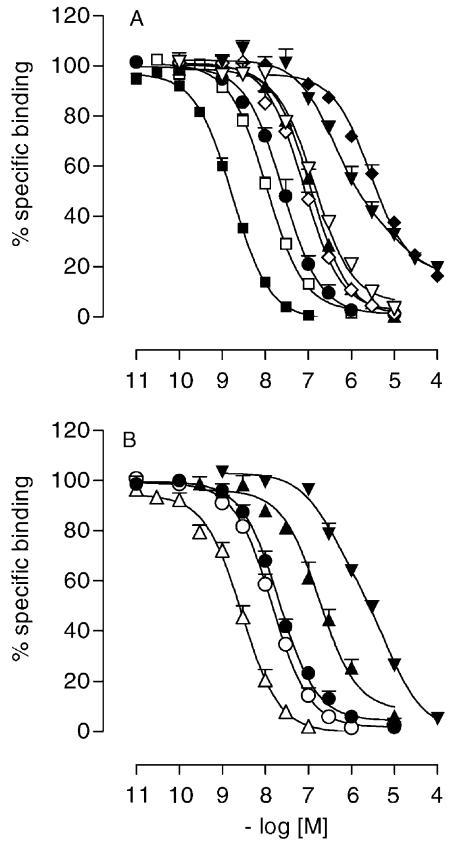


Fig. 2. Competition curves of tachykinins and analogs for [$^{125}$I]BH-bufokinin (70 pM) binding to toad intestinal membranes. Each point represents the mean ± SEM of duplicate determination in 3–6 animals. (A) Bufokinin (■); SP (□); BUF(2–11) (●); BUF(6–11) (◇); BUF(3–11) (▲); BUF(4–11) (▽); BUF(5–11) (▼) and BUF(7–11) (◆). (B) Ranakinin (△); cod SP (○); trout SP (●); [Sar$^9$]SP (▲) and [Pro$^9$]SP (▼).

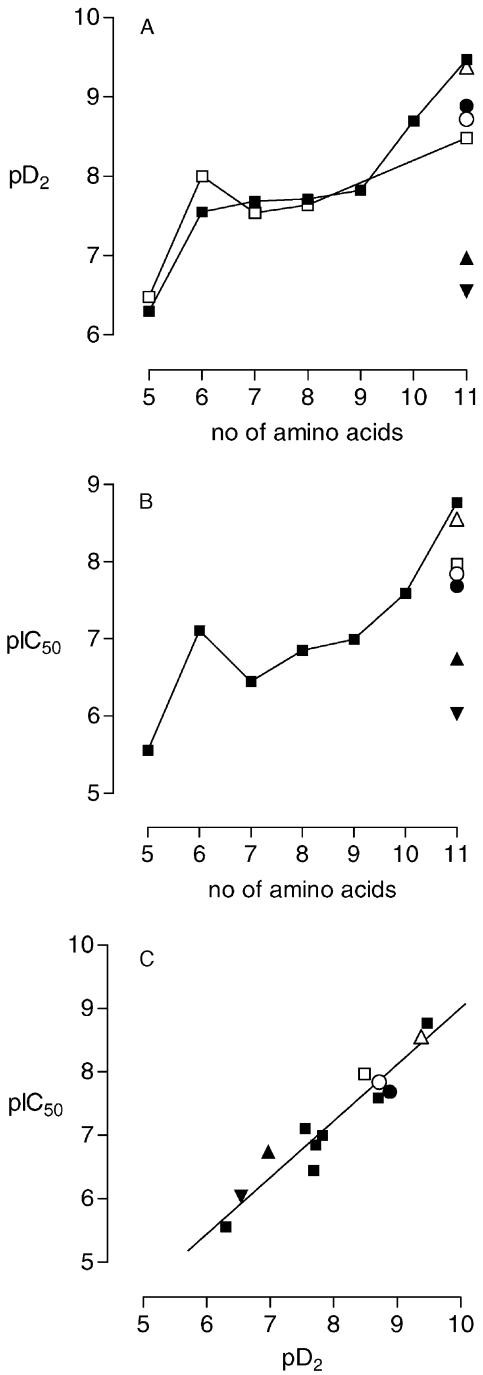


Fig. 3. (A) Functional potency ($pD_2$) and (B) binding affinity ($pIC_{50}$) of tachykinins and analogs, plotted against peptide length. (C) Correlation of binding and functional potency, $r = 0.956$. Bufokinin and C-terminal fragments (■); SP and C-terminal fragments (□); ranakinin (△); trout SP (●); cod SP (○); [Pro$^9$]SP (▼); [Sar$^9$]SP (▲).

bonding. The positive coefficient of $^0\chi$ in equations 1 and 5 indicates the relationship between potency and increases in the number of atoms in the SP and BUF analogs. There is likely to be an optimal number of amino acid residues in these peptides beyond which potency declines, but that point was not attained within the present data set.

The $^1\chi$ parameter apparently relates to hydrophobic character [16] and the second-order shape parameter describes the branching within a molecule [13]. The values of the connectivity and shape parameters in this study were calculated over the entire peptide structure. Thus, a minimal hydrophobicity may be required for the optimal interaction of SP and bufokinin with the toad receptor, but electronic and topological properties are also important.

Three SP-related peptides from different species and two synthetic analogs were evaluated as ligands of the toad bufokinin receptor to test the predictive power of the equations (Table 1). Equations 1–8 accurately predicted the potency of trout SP, cod SP and ranakinin (frog SP) within a factor of two (Fig. 4). The exception to these findings was that equations 1 and 8 (Table 3) provided a 4-fold overestimate and 3-fold underestimate, respectively, of the potencies of trout SP, and equations 4 and 8 provided a 3-fold underestimate of the potencies of ranakinin. The slight underestimate of the potencies of ranakinin and trout SP might be due to more complex interactions than those reflected by the estimated $^2\kappa_\alpha$ value. However, equations 1–8 overestimated the potencies of the SP analogs [Sar$^9$]SP and [Pro$^9$]SP by a factor of 17–333 (Fig. 4).

Table 3
Regression analysis describing of the binding and functional effects of bufokinin and SP and analogs at the toad bufokinin receptor

| Equation | Intercept | $r$ | $r^2$ (%) | $s$ | $F$[a] |
|---|---|---|---|---|---|
| Binding data ($n = 8$) | | | | | |
| (1) 0.75 $^0\chi$ (0.15)[b] | 3.98 | 0.89 | 79 | 0.47 | 23.5 |
| (2) 1.21 $^1\chi$ (0.25) | 4.16 | 0.89 | 79 | 0.48 | 22.6 |
| (3) 0.19 $^0\kappa_\alpha$ (0.04) | 4.57 | 0.89 | 79 | 0.49 | 21.7 |
| (4) 0.10 $^2\kappa_\alpha$ (0.20) | 3.83 | 0.91 | 84 | 0.43 | 30.7 |
| Functional data ($n = 8$) | | | | | |
| (5) 0.70 $^0\chi$ (0.12) | 5.05 | 0.88 | 77 | 0.43 | 33.9 |
| (6) 1.13 $^1\chi$ (0.20) | 5.21 | 0.87 | 76 | 0.45 | 31.3 |
| (7) 0.18 $^0\kappa_\alpha$ (0.03) | 5.61 | 0.87 | 76 | 0.44 | 32.2 |
| (8) 1.09 $^2\kappa_\alpha$ (0.14) | 4.69 | 0.92 | 85 | 0.35 | 57.6 |

[a] All $F$ ratios significant ($P < 0.001$).
[b] Standard error of the coefficient.

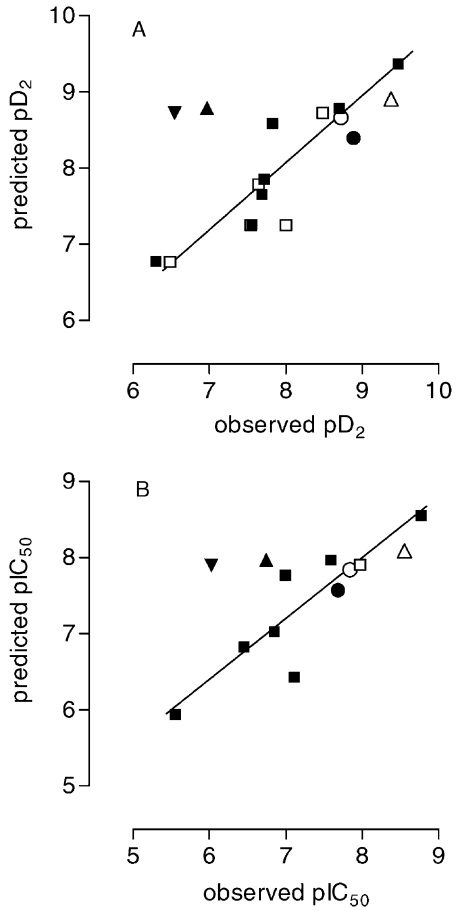


Fig. 4. Validation of QSAR analysis. (A) Observed $pD_2$ values plotted against predicted $pD_2$ values, using equation 4 (Table 3). Linear regression ($r = 0.901$) based on bufokinin, SP and both sets of fragments ($n = 12$). (B) Observed $pIC_{50}$ values plotted against predicted $pIC_{50}$ values, using equation 8 (Table 3). Linear regression ($r = 0.888$) based on bufokinin, SP and bufokinin fragments ($n = 8$). Bufokinin and C-terminal fragments (■); SP and C-terminal fragments (□); ranakinin (△); trout SP (●); cod SP (○); [Pro$^9$]SP (▼); [Sar$^9$]SP (▲).

## 4. Discussion

The present study describes structure–activity relationships at the bufokinin receptor in the toad small intestine by examining the potencies of SP-like tachykinins from toad (bufokinin), frog (ranakinin), mammal, cod and trout, and the fragments of bufokinin and SP. In general, the potency observed in binding studies was very similar to that seen in functional studies (Fig. 3C), suggesting that the binding site corresponds to the functional receptor. The toad receptor showed a marked preference for bufokinin and ranakinin, with the next most potent group consisting of the three other undecapeptides (mammalian, cod and trout SP) and BUF(2–11). Although the QSAR equations gave good predictions for potency of frog (ranakinin), trout and cod SP, the potencies of the two SP analogs were not well predicted. Selective and highly potent agonists for the mammalian $NK_1$ receptor have been produced by methylation of Gly$^9$ (to yield Sar$^9$) [6] or replacement with Pro$^9$ [17]. However, the atypical [Sar$^9$]SP and the conformationally restrained [Pro$^9$]SP demonstrated 100–1000-fold diminished potencies at the toad bufokinin receptor. These data suggest that the toad tachykinin receptor cannot accommodate any side chain at residue 9, as discussed in our previous studies [3,4].

Apart from ranakinin, which had additional substitutions (Arg$^3$ → Asn, Asp$^5$ → Glu, Gln$^6$ → Arg), the residues in positions 1 and 3 were the basic Arg or Lys (Lys$^1$, Arg$^3$ in bufokinin, trout SP and cod SP, and Arg$^1$, Lys$^3$ in mammalian SP), which could be considered interchangeable. Hence, for these four natural tachykinins, important variations occurred only at position 5 (polar residue) and position 8 (aromatic residue). The greater potency of bufokinin (and ranakinin) compared with mammalian SP indicates that Asp$^5$ (or Glu$^5$) and/or Tyr$^8$ are favorable residues for interacting with the bufokinin receptor. In many amphibian tachykinins, Tyr$^8$ is a commonly conserved residue [8] important for binding to the amphibian $NK_1$-like receptors. Hydrogen bonding might occur between the hydroxyl group of Tyr$^8$ and the toad receptor whereas such an interaction would not be possible with the Phe$^8$ residue of SP. The QSAR analysis is consistent with these possibilities since the peptides containing the tyrosyl-*p*-hydroxylated residue contain a greater number of atoms and bond paths than those in which this hydroxyl group was absent (SP series). The Asp$^5$ residue in bufokinin may

form a salt bridge with $Lys^1$ (or $Arg^3$) as proposed for physalaemin [18], which may stabilize the peptide in a conformation favorable for receptor interaction.

For naturally occurring tachykinins and bufokinin fragments, potency in both functional and binding studies was related to the peptide length (Fig. 3A and B). This was also a general conclusion from the QSAR analysis, showing a positive coefficient of the zero-order connectivity function $^0\chi$, which describes the arrangement of atoms within a structure rather than their bonding. Truncations or other variations at the N-terminus may have been made in less important regions, since the peptides in the present study were still able to compete fully with radioligand for the binding site (except for BUF(5–11)) and all peptides were able to elicit a maximum response.

In the bufokinin series, binding potency and functional potency fell substantially, first with loss of $Lys^1$ (BUF(2–11)) and then $Pro^2$ (BUF(3–11)). However, further truncation of the bufokinin molecule did not lead to significant further decreases in potency until the pentapeptide BUF(7–11). Although this fragment was more than 1000-fold weaker than the parent undecapeptide bufokinin, both BUF(7–11) and SP(7–11) were still full agonists. This finding corresponds to the observation in mammals that SP(7–11) was the minimum sequence to maintain significant biological activity [19]. BUF(7–11) and SP(7–11) exhibited similar $EC_{50}$ values, indicating that their one structural difference ($Tyr^8$ or $Phe^8$), appeared to have no influence on functional potency of the pentapeptide agonist. Apart from SP(6–11), which was more potent than BUF(6–11), the fragments of bufokinin and SP showed remarkably similar functional potency. On the basis of the present data, it appears that the preference of the toad receptor for bufokinin compared with mammalian SP was seen only for the undecapeptide.

Most SP-like peptides found in vertebrates possess a proline residue at position 4 [6,8,9]. Thus, our finding that addition of $Pro^4$ to the C-terminal sequence in BUF(4–11) did not improve the activity compared to BUF(5–11) and BUF(6–11) is unexpected. At the rat $NK_1$ receptor, $Pro^4$ has been demonstrated to be important in conferring high binding affinity [20]. In our functional study, the additions of Asp, Pro–Asp or Arg–Pro–Asp residues at the N-terminus of BUF(6–11) to form BUF(5–11), BUF(4–11) and BUF(3–11) did not significantly influence potency, compared with BUF(6–11). Similar results were seen with the SP fragments. Does this suggest that $Arg^3$, $Pro^4$ and $Asp^5$ may not be critical residues? The observation that ranakinin was almost equipotent with bufokinin would support this hypothesis. The substantially increased affinity achieved by the addition of $Lys^1$ and $Pro^2$ residues suggests that the bufokinin fragments may not interact with the receptor in the same way as their parent undecapeptide and that the full sequence may modify the conformation of the carboxyl-terminal sequence.

Although there was an excellent correlation between binding potency and functional potency for these peptides, some anomalous results were obtained with BUF(5–11) and BUF(6–11). Virtually identical in functional studies, BUF(5–11) was 5-fold weaker as a binding competitor than the shorter BUF(6–11), and was unable to fully compete for the [$^{125}$I]BH-bufokinin binding site, with the data indicating binding to more than one site. This observation differs from the general trend towards increased potency with increased peptide length. Since BUF(5–11) was initially dissolved in DMSO, this solvent might change the conformation of the peptide, although DMSO alone up to 3% did not affect BH-bufokinin binding. An alternative explanation is that a negatively charged residue, $Asp^5$, at the N-terminus may be unfavorable in interacting with the hydrophobic membranes.

The QSAR analysis accounted for the binding and functional potencies of the SP and bufokinin series in terms of the number and nature of atoms in the peptide structure as well as its shape. In addition to individual residues discussed, the QSAR analysis highlights the importance of topology, hydrophobicity and branching [13,16] in binding and functional interactions with the toad receptor. Thus, a minimal hydrophobicity may be required for the optimal interaction of SP and bufokinin with the toad receptor, but electronic and topological properties are also important. It is noteworthy that Horwell *et al*. [21] identified similar properties that contributed to biological activity in a series of tachykinin $NK_1$ receptor antagonists, using 3D-QSAR.

In conclusion, the full undecapeptide sequence of bufokinin is required for optimal activity, with high potency conferred by $Lys^1$, $Pro^2$, $Gly^9$ and probably $Tyr^8$. However, the toad receptor was able to tolerate polar substituents at $Arg^3$, $Asp^5$ and $Gln^6$ without great loss of potency. The presence of positive charges and one or two proline residues at the N-terminus is likely to modify the conformation of the ligand in order to optimize its interaction with the agonist binding domain of the receptor. The present QSAR analysis focusing on connectivity and shape descriptors has provided good but not universally correct predictions of binding and functional potencies of tachykinins and analogs. It may be desirable to refine any future analysis in order to assess the relative contributions of the individual residues.

## Acknowledgments

This study was supported by the Australian Research Council. We thank J. Jensen, University of Göteborg, Sweden, for donation of cod and trout SP.

## References

[1] Conlon JM, Warner FJ, Burcher E. Bufokinin: a substance P-related peptide from the gut of the toad, *Bufo marinus* with high affinity but low selectivity for mammalian tachykinin receptors. J Peptide Res 1998;51:210–5.

[2] Mussap CJ, Geraghty DP, Burcher E. Tachykinin receptors: a radioligand binding perspective. J Neurochem 1993;60:1987–2009.
[3] Liu L, Warner FJ, Conlon JM, Burcher E. Pharmacological and biochemical investigation of receptors for the toad gut tachykinin peptide bufokinin in its species of origin. Naunyn-Schmiedeberg's Arch Pharmacol 1999;360:187–95.
[4] Burcher E, Warner FJ. Tachykinin receptors in the small intestine of the cane toad (*Bufo marinus*): a radioligand binding and functional study. Naunyn-Schmiedeberg's Arch Pharmacol 1998;357:692–700.
[5] Liu L, Shang F, Perry MA, Comis A, Burcher E. Bufokinin: immunoreactivity, receptor localization and actions in intestine and mesenteric circulation of the toad. Peptides 2000;21:1345–54.
[6] Regoli D, Drapeau G, Dion S, Couture R. New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. Trends Pharmacol Sci 1988;9:290–5.
[7] Schwyzer R. Membrane-assisted molecular mechanism of neurokinin receptor subtype selection. EMBO J 1987;6:2255–9.
[8] Severini C, Salvadori S, Guerrini R, Falconieri-Erspamer G, Mignogna G, Erspamer V. Parallel bioassay of 39 tachykinins on 11 smooth muscle preparations structure and receptor selectivity/affinity relationship. Peptides 2000;21:1587–95.
[9] Harbeson SL, Rovero P. Structure–activity relationships of agonist and antagonist ligands. In: Buck SH, editor. The tachykinin receptors. Totowa (NJ): Humana Press, 1994.
[10] Maggio JE, Mantyh PW. History of tachykinin peptides. In: Buck SH, editor. The tachykinin receptors. Totowa (NJ): Humana Press, 1994.
[11] Burcher E, Buck SH, Lovenberg W, O'Donohue TL. Characterization and autoradiographic localisation of multiple tachykinin binding sites in gastrointestinal tract and bladder. J Pharmacol Exp Ther 1986;236:819–31.
[12] Kier LB, Hall LL. Molecular connectivity in chemistry and drug design. New York: Academic Press, 1976.
[13] Kier LB. Molecular connectivity as a descriptor of structure for SAR analyses. In: Yalkowsky SH, Sinkula AA, Valvani SC, editors. Physical chemical properties of drugs. New York: Marcel Dekker, 1980. p. 277–319.
[14] Kier LB. Indexes of molecular shape from chemical graphs. Med Res Rev 1987;7:417–40.
[15] Martini R, Murray M. Rat hepatic microsomal aldehyde dehydrogenase: identification of 3- and 4-substituted aromatic aldehydes as substrates of the enzyme. Chem Res Toxicol 1996;9:268–76.
[16] Kier LB, Murray WJ, Hall LL. Molecular connectivity. 4. Relationship to biological activities. J Med Chem 1975;18:1272–4.
[17] Lavielle S, Chassaing G, Julien S, Marquet A, Bergstrom L, Beaujouan JC, Torrens Y, Glowinski J. Specific recognition of SP or NKB receptors by analogues of SP substituted at positions 8 and 9. Eur J Pharmacol 1986;125:461–2.
[18] Chassaing G, Convert O, Lavielle S. Conformational analogy between substance P and physalaemin. Biochim Biophys Acta 1986;873:397–404.
[19] Bury RW, Mashford ML. Biological activity of C-terminal partial sequences of substance P. J Med Chem 1976;19:854–6.
[20] Cascieri MA, Huang RR, Fong TM, Cheung AH, Sadowski S, Ber E, Strader CD. Determination of the amino acid residues in substance P conferring selectivity and specificity for the rat neurokinin receptors. Mol Pharmacol 1992;41:1096–9.
[21] Horwell DC, Howson W, Higginbottom M, Naylor D, Ratcliffe GS, Williams S. Quantitative structure–activity relationships (QSARs) of N-terminus fragments of NK1 tachykinin antagonists: a comparison of classical QSARs and three-dimensional QSARs from similarity matrices. J Med Chem 1995;38:4454–62.