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Quantitative structure–activity analyses of bufokinin and other tachykinins at bufokinin (bNK_1) receptors of the small intestine of the cane toad, *Bufo marinus*

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Abstract

The toad tachykinin, bufokinin (Lys-Pro-Arg-Pro-Asp-Gln-Phe-Tyr-Gly-Leu-Met amide; BUF), acts via tachykinin NK_1-like receptors to contract the intestine of the cane toad, *Bufo marinus*. In this structure–activity study, we used isolated segments of toad small intestine and performed binding studies with [^{125}I] Bolton–Hunter BUF in intestinal membranes to compare the contribution of individual amino acid residues to the potencies of 18 naturally occurring tachykinins and 13 BUF analogs. Potencies were similar ($r = 0.94$) in functional and binding studies, with BUF and ranakinin being most potent. Ranatachykinin A, physalaemin, hylambatin and cod, trout and mammalian SPs exhibited 10–60% of the potency of BUF. The Ala-substituted BUF analogs were 11–60% as potent as BUF in functional studies, with [Ala^2]-BUF and [Ala^4]-BUF the least efficacious, indicating the importance of both proline residues. QSAR equations were developed using 12 connectivity, shape and steric parameters for each of the 7 hypervariable amino acid residues in these peptides. For the binding data, the optimal regression equation explained 81% of the variance, and indicated the importance of the steric function at [Pro^2] and simple connectivity functions at [Gln^6] and [Tyr^8]. The optimal functional regression equation (80% of variance) confirmed the importance of connectivity functions at [Gln^6] and [Tyr^8], as well as the shape of residues [Lys^1] and [Pro^4]. The potencies of most full-length peptides were well predicted using the leave-one-out procedure, as were the potencies of a series of model Ala-substituted BUFs, thus emphasising the potential utility of these equations in the design of new ligands interacting with tachykinin receptors.

Keywords: Structure–activity; Tachykinins; Bufokinin; Substance P; Tachykinin receptors; Non-mammalian; Amphibian; Intestine; Binding; Functional; QSAR

1. Introduction

Tachykinins are a family of peptides that have been found in all vertebrate species studied to date [1]. The C-terminal sequences of tachykinin peptides are relatively conserved, but there is extensive variation in the amino acid composition and chain-length of the N-terminal domain, with many naturally occurring tachykinins being deca- or undecapeptides. Tachykinins are characterised structurally by the presence of the carboxyl-terminal pentapeptide sequence (Phe-*X*-Gly-Leu-Met-NH_2, where *X* is either aromatic Phe, Tyr or aliphatic Val, Ile), with the C-terminal amide essential for biological activity. In mammals, the neuropeptides substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) have been well studied, but elongated forms of NKA (neuropeptide K, neuropeptide γ) exist, as well as the newly described tachykinins, hemokinin and the endokinins, which are of non-neural origin ([2,3] for review). SP, NKA and NKB mediate smooth muscle contraction, glandular and intestinal secretion, endothelium-dependent vasodilatation and plasma extravasation as well as neuronal excitation [4].

Abbreviations: SP, substance P; NKA, neurokinin A; NKB, neurokinin B; BUF, bufokinin; BH, Bolton–Hunter; QSAR, quantitative structure–activity analysis; RTK, ranatachykinin

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In amphibians and other non-mammals, the primary structures of many tachykinins have been described [5], but their actions in their species of origin have been poorly studied.

Our previous studies have described the isolation, distribution and receptor binding characteristics of bufokinin (BUF), an undecapeptide tachykinin from the cane toad, *Bufo marinus* [6,7]. There is extensive innervation of the intestine and vasculature by bufokinin-immunoreactive nerves [8]. Bufokinin causes intestinal contraction, vasodilatation and hypotension and so may be considered as the toad equivalent of mammalian SP [8,9]. In the toad, bufokinin appears to have a role in the cardiovascular and gastrointestinal systems, and would be expected to have actions in the central nervous system. Bufokinin interacts with a receptor (bNK_1), which has similarities with the mammalian NK_1 receptor and exists in three different isoforms that are widely distributed in toad tissues, including the intestine and central nervous system [10].

In a previous study, we used isolated segments and homogenates of toad small intestine to compare the contractile potencies and binding affinities of several tachykinin-related peptides, and described the structure–activity relationships of bufokinin, SP, and their C-terminal fragments [11]. That study suggested that binding affinity and functional potency was primarily related to the length of the peptide molecule and that the complete undecapeptide sequence of bufokinin was required for optimal activity, with high potency conferred by Lys^1, Pro^2, Gly^9 and probably Tyr^8 [11]. Quantitative structure–activity (QSAR) analysis of the data based on equations derived from the whole peptide molecule demonstrated that molecular connectivity and shape descriptors were useful in accounting for the binding and functional potencies of tachykinins [11].

The present study evaluated the interaction of a range of amphibian, fish and mammalian tachykinins with the toad intestinal tachykinin receptor (bNK_1). The present data set also included a series of Ala-substituted BUF peptides and bioactive C-terminal BUF fragments. We initially applied the approach of Liu et al. [11] to the data, but have found that a more detailed QSAR analysis was required, that examined the contribution of individual amino acids to affinity and functional potency at the toad receptor.

2. Methods and materials

2.1. Functional studies

Cane toads (weight 150–250 g, obtained from Peter Douch, North Queensland, Australia) were sacrificed by pithing. Whole segments (length 10–15 mm) of small intestine were taken 1 cm or more proximal to the large intestine, and mounted longitudinally in 2 ml organ baths containing toad Mackenzie's solution, aerated with 95% O_2 and 5% CO_2 at 25 °C, as described [7]. Muscle tension was recorded isometrically using Grass FTO3C force transducers and recorded using Polygraph (Mr. E. Crawford, UNSW, Australia). The preparations were subjected to an initial tension of 1 g and allowed to equilibrate for 60 min, when a supramaximal concentration of mammalian SP (1 μM) was added to each organ bath to define the maximum contraction. After washing and a further 60 min equilibration, a single concentration–response curve to a tachykinin was constructed by discrete addition of peptide, using a 30–60 min concentration cycle with 2–3 min contact time.

Contractile responses of tachykinins were recorded in grams tension and then expressed as a percentage of the maximum (SP) response. The concentration–response curves were fitted using the non-linear regression analysis program of Graph Pad Prism (Version 3, Graph Pad Software Inc.). The agonist potencies were expressed as EC_{50}. The rank of potency order was statistically analyzed using one-way ANOVA followed by Bonferroni comparison of selected pairs. The statistical significance in potency was set at $P < 0.05$.

2.2. Homogenate binding studies

Membranes were prepared from fresh intestinal smooth muscle, minus mucosa, and finally suspended in incubation buffer consisting of Tris–HCl (50 mM, pH 7.4, 15 °C), $MnCl_2$ (3 mM), bovine serum albumin (BSA, 0.02%) and the peptidase inhibitor bacitracin (40 μg/ml), as described [7]. The radioligand [^{125}I]-Bolton–Hunter (BH) BUF (specific activity 2200 Ci/mmol) was prepared and purified by HPLC following the method described previously [7].

Competition binding assays were performed by incubating membrane aliquots (3%, w/v) with [^{125}I]BH-BUF (70 pM) in the presence of varying concentrations of competitors, for 60 min at 25 °C. Non-specific binding was defined in replicate tubes in the presence of 1 μM unlabelled BUF. Incubations were terminated by filtration using a tissue harvester (Brandel Inc., Gaithersburg, MD, USA) through GF/B filters (Whatman, Maidstone, UK) presoaked overnight at 4 °C in 0.1% polyethyleneimine with three additional washes with ice-cold wash buffer containing Tris–HCl (50 mM, pH 7.4, 4 °C), $MnCl_2$ (3 mM) and BSA (0.02%). Radioactivity on the filters was then quantified in a Packard Minaxi auto-gamma counter (78% efficiency).

For data analysis, fitting of data to a one or two-site model was achieved using the non-linear regression analysis program (Graph Pad Prism, version 3). The inhibition of [^{125}I]BH-BUF by competing ligands was expressed as IC_{50}, defined as the concentration of competitor displacing 50% of specifically bound radioligand. Again, the rank of potency order was statistically analyzed using one-way ANOVA followed by Bonferroni comparison of selected

pairs. The correlation between functional and binding data was determined by regression analysis. The statistical significance in potency was set at $P < 0.05$.

2.3. Quantitative structure–activity analysis

2.3.1. Derivation of parameters used in regression analysis

Molecular connectivity indices ${}^{m}\chi_t$ were calculated by established procedures [12] and employed valence deltas for all atoms other than sp^3-hybridized carbon atoms [13]. Thus, m is the order of the function (the number of bonds over which it was calculated) and t refers to the bond arrangement within the connectivity function (i.e. path, cluster, path/cluster). A first-order connectivity expression is calculated according to the formula:

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

Here, n is the number of individual bond groupings calculated over the structure and i and j are the corresponding atoms. Higher order χ parameters were calculated similarly. χ values were calculated for individual amino acid residues of the tachykinin analogs. Calculation of short intramolecular paths used the side chains of the amino acid, whereas for longer paths, the whole residue including the peptide backbone was used.

Molecular shape indices (${}^{m}\kappa_\alpha$) were calculated according to literature methods [14]. Again, m is the order of the function (the number of bonds over which it was calculated) and α indicates that modified atom values (counts) were used in calculations, which accounts for the presence of atoms other than sp^3-hybridized carbon atoms. κ indices were used to calculate Ξ, the steric descriptor derived by a molecular graph approach, as used in the derivation of χ and κ values. This descriptor was calculated from the following expression that relates zero, first and third order shape (κ) functions ($\Xi = 2^{1}\kappa_\alpha - {}^{0}\kappa_\alpha - {}^{3}\kappa_\alpha$) [15].

By these approaches, seven connectivity parameters, four shape parameters and one steric parameter were derived for non-conserved amino acids in the tachykinin molecule. These were residues A, B, C, D, E, F and G, which corresponded to the amino acids in positions 1–6 and 8 of bufokinin (Table 1). A total of 25 peptides (18 natural tachykinins and 7 tachykinin fragments) were used to derive the equations. Experimental data from hylambatin (which is the only peptide to contain Met instead of Leu at position 10) and the Ala-substituted BUF peptides were excluded from the QSAR analysis.

2.3.2. Derivation of QSAR equations and statistical evaluation

Equations were determined by simple or multiple regression, using the Statview program, for Macintosh computers. The quality of fit of the data to each equation was assessed from the correlation coefficient (r), the standard deviation from the regression (s) and the F ratio. Additional parameters were only included in a regression analysis when justified (t-test of the significance of the new parameter; $P < 0.05$) and a statistical improvement was found (F-test for inclusion).

Optimal regression equations (Eqs. (3) and (8)) were obtained and then further evaluated to ensure that included parameters were not colinear (from correlation matrices). The predictive capacities of the equations were also evaluated by determination of the predictive residual sum of squares (PRESS), the correlation coefficient of predictions (Q), the variance of predictions (Q^2) and the standard deviation of predicted values (S_{PRESS}) [16,17]. Estimates of ligand potency were made from within the optimal equations (pIC_{50calc} and pEC_{50calc}) and by the leave-one-out (LOO) cross-validation approach (pIC_{50LOO} and pEC_{50LOO}). In this approach, each analog is excluded from the regression, the analysis is then performed and the resultant equation is used to calculate the potency of the excluded analog.

Table 1
Structures of natural occurring tachykinin peptides used in this study[a]

QSAR residue				A	B	C	D	E	F		G			
Amino acid no.				1	2	3	4	5	6	7	8	9	10	11
Amphibian														
Bufokinin				K	P	R	P	D	Q	F	Y	G	L	M
Ranakinin				–	–	N	–	E	R	–	–	–	–	–
RTK-A				–	–	S	–	–	R	–	–	–	–	–
RTK-B					Y	K	S	–	S	–	–	–	–	–
RTK-C					H	N	–	A	S	–	I	–	–	–
Physalaemin				pE	A	D	–	N	K	–	–	–	–	–
Kassinin			D	V	–	K	S	–	–	–	V	–	–	–
Phyllomedusin					pE	N	–	N	R	–	I	–	–	–
Uperolein				pE	–	D	–	N	A	–	–	–	–	–
Hylambatin			D	P	–	D	–	–	R	–	–	–	M	–
[Leu^3, Ile^7]NKA					H	K	L	–	S	–	I	–	–	–
Ranamargarin	D	D	A	S	D	–	A	K	K	–	–	–	–	–
Fish														
Cod SP				–	–	–	–	Q	–	–	I	–	–	–
Trout SP				–	–	–	–	H	–	–	F	–	–	–
Scyliorhinin-I					A	K	F	D	K	–	–	–	–	–
Mammalian														
SP				R	–	K	–	Q	–	–	F	–	–	–
NKA					H	K	T	–	S	–	V	–	–	–
NKB					D	M	H	–	F	–	V	–	–	–
NKA (4–10)								–	S	–	V	–	–	–
Invertebrate														
Eledoisin				pE	–	S	K	–	A	–	I	–	–	–

[a] Amino acid residues identical with those in bufokinin are represented by hyphens.

2.4. Materials

Bufokinin and six C-terminal BUF fragments, ranatachykinin (RTK)-A, RTK-B, RTK-C and [Leu^3, Ile^7]NKA were custom-synthesized by Auspep (Melbourne, Austra-

lia). Ranakinin and six [Ala]-substituted BUF analogs were synthesized by J.M. Conlon. Trout SP and cod SP were gifts from Dr. J. Jensen (Göteborg University, Göteborg, Sweden). Eledoisin, kassinin, NKA, NKA (4–10), NKB, physalaemin, ranamargarin, hylambatin, scyliorhinin I and mammalian SP were purchased from Auspep (Melbourne, Australia), uperolein from American Peptide Company (Sunnyvale, CA, USA) and phyllomedusin from Peninsula Laboratories Inc. (Belmont, CA, USA). Bacitracin (zinc salt) was purchased from Sigma (Sydney, Australia). The [^{125}I]-Bolton–Hunter reagent (2 mCi/mmol) was purchased from Amrad Pharmacia Biotech (Sydney, Australia). Stock solutions of peptides were made in 0.01 M acetic acid containing 1% β-mercaptoethanol and stored in aliquots at −20 °C, except for BUF (5–11) and NKB, which were initially dissolved in dimethyl sulfoxide.

3. Results

3.1. Experimental

3.1.1. Functional studies

All tachykinins and analogs tested behaved as full agonists in inducing contractions of the toad intestinal longitudinal muscle. The size of the maximum response was similar to that of SP, except for the case of NKA (4–10), where a maximum could not be obtained because of its low potency. The data are shown in Fig. 1A–C and Table 2.

Ranakinin was almost equipotent with bufokinin. For the other amphibian tachykinins, which were 5–100-fold weaker than bufokinin, the rank order of potency was physalaemin ≥ RTK-A ≥ uperolein ≈ hylambatin ≥ [Leu^3,Ile^7] NKA ≥ kassinin ≥ phyllomedusin ≈ RTK-C ≈ RTK-B ≥ ranamargarin (Fig. 1).

Other naturally occurring tachykinins were 4–70-fold weaker than bufokinin, with the rank order of potency trout SP ≥ cod SP ≥ mammalian SP ≈ eledoisin (molluscan) ≥ NKA ≥ scyliorhinin I ≥ NKB; NKA, however, was over 550-fold more potent than its fragment, NKA (4–10).

All [Ala]-substituted BUF analogs had fairly similar ability to contract the isolated intestine and were slightly less potent than bufokinin. The potency order was [Ala^5]-BUF ≈ [Ala^6]-BUF ≈ [Ala^1]-BUF ≈ [Ala^3]-BUF ≥ [Ala^2]-BUF ≥ [Ala^4]-BUF. Bufokinin was significantly more potent than [Ala^2]-BUF ($P < 0.05$) and [Ala^4]-BUF ($P < 0.01$, one-way ANOVA). The differences between bufokinin and other [Ala] analogs were not statistically significant.

3.1.2. Binding studies

Using membranes from toad intestinal muscle, the ability of each peptide to inhibit [^{125}I]BH-BUF binding was determined (Table 2). For the amphibian tachykinins, BUF was the most potent competitor, followed by ranakinin ≥ RTK-A ≥ physalaemin ≥ hylambatin > kassinin ≥ uperolein ≥ phyllomedusin ≥ RTK-C ≥ [Leu^3, Ile^7] NKA > RTK-B, which were 2–100-fold weaker than bufokinin. The data for the tetradecapeptide ranamargarin were best fitted to two sites (Table 2).

For other tachykinins, which were 6–40-fold weaker than bufokinin, the potency order was mammalian SP ≥ cod SP ≥ trout SP > scyliorhinin I ≈ eledoisin ≥ NKA ≥ NKB. NKA (4–10) was over 1000-fold weaker than NKA (Table 2).

Apart from [Ala^5]-BUF, which was 10-fold weaker, the [Ala]-substituted BUF analogs were all about 3–4-fold weaker than BUF. The potency order was bufokinin > [Ala^1]-BUF ≈ [Ala^4]-BUF ≈ [Ala^3]-BUF ≈ [Ala^6]-BUF ≈ [Ala^2]-BUF > [Ala^5]-BUF ($P < 0.001$).

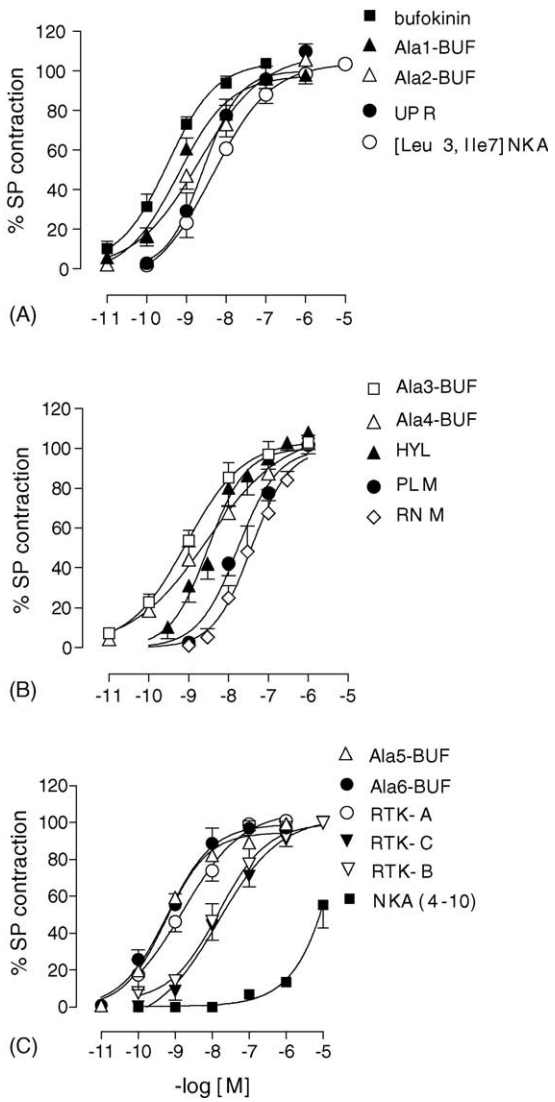

Fig. 1. Concentration response curves to tachykinins and analogs in toad isolated intestine. Each point represents the mean ± S.E.M. of determination in 4–9 animals. UPR, uperolein; HYL, hylambatin; PLM, phyllomedusin; RNM, ranamargarin; RTK, ranatachykinin.

Table 2
Potency of tachykinins and analogs as competitors against BH-bufokinin in toad intestinal membranes and as contractile agents in toad isolated intestinal segments

Peptide	Radioligand binding studies			Functional studies	
	Slope	IC_{50} (95% CL, nM)	R.A.	EC_{50} (95% CL, nM)	R.P.
Bufokinin[a]	1.09	1.7 (1.5–1.9)	100	0.34 (0.17–0.70)	100
Ranakinin[a]	0.94	2.8 (2.2–3.6)	60	0.42 (0.15–1.2)	81
RTK-A	0.99	4.3 (3.4–5.3)	40	1.7 (0.19–14.6)	20
Physalaemin[b]	0.96	6.7 (5.4–8.2)	25	1.5 (0.77–3.1)	23
Substance P[a]	0.92	10.7 (8.94–10.7)	16	3.3 (1.3–8.6)	9.7
Hylambatin[c]	0.88	9.3 (7.5–11.3)	18	3.3 (0.86–12.6)	10
Cod SP[a]	0.93	14.4 (12.8–16.2)	12	1.9 (0.88–4.2)	18
Kassinin[b]	0.97	17.8 (16.1–19.6)	9.5	8.2 (4.8–14.2)	4.1
Uperolein	0.95	20.4 (18.6–22.5)	8.3	3.2 (1.5–6.8)	11
Trout SP[a]	0.91	20.8 (17.4–24.8)	8.2	1.3 (0.62–2.7)	26
Ranamargarin	0.69	(1.8–6.5) (H, 32%)	49	36.6 (17.8–75.4)	0.93
		71.6 (52.6–97.6) (L, 68%)	2.4		
Phyllomedusin	0.93	22.2 (18.3–27.0)	7.6	14.3 (7.5–27.2)	2.4
RTK-C	0.91	27.0 (23.2–36.6)	6.3	14.7 (5.3–40.9)	2.3
[Leu^3, Ile^7]NKA	0.96	32.2 (28.4–36.6)	5.3	4.6 (1.9–11.2)	7.4
Scyliorhinin I[b]	0.93	35.3 (29.0–42.9)	4.8	18.6 (8.9–38.9)[a]	1.8
Eledoisin[b]	1.06	40.6 (34.2–48.2)	4.2	3.7 (3.3–4.2)[a]	9.2
Neurokinin A[b]	0.81	57.8 (43.5–76.9)	2.9	14.2 (9.5–30.2)	2.4
Neurokinin B[b]	1.00	77.5 (70.5–85.3)	2.2	24.2 (11.6–50.5)	1.4
RTK-B	0.99	154 (137–174)	1.1	15.1 (6.21–36.5)	2.2
BUF (2–11)[a]	0.91	25.7 (21.7–30.5)	6.6	2.0 (0.71–5.9)	17
BUF (3–11)[a]	0.96	101 (88.0–115)	1.7	15.0 (8.0–28.2)	2.3
BUF (4–11)[a]	0.83	141 (98.2–201)	1.2	19.3 (11.9–31.1)	1.8
BUF (5–11)[a]	0.73	356 (154–826) (H, 72%)	0.47	20.6 (12.1–35.2)	1.7
		11920 (921–154200) (L, 28%)	0.01		
BUF (6–11)[a]	0.90	78.0 (61.8–98.4)	2.2	28.1(16.8–47.0)	1.2
BUF (7–11)[a]	0.74	2780 (1740–4450)	0.06	503 (378–668)	0.07
NKA (4–10)	0.86	60250 (19470–186400)	0.003	7886 (5070–12270)	0.004
[Ala^1]-BUF[c]	0.92	4.4 (3.8–5.1)	39	0.66 (0.24–1.8)	52
[Ala^2]-BUF[c]	1.01	7.4 (6.9–8.1)	23	2.1 (0.92–4.8)	16
[Ala^3]-BUF[c]	0.95	6.4 (5.5–7.5)	26	0.89 (0.55–1.4)	38
[Ala^4]-BUF[c]	0.98	5.1 (4.7–5.7)	33	3.0 (0.98–9.2)	11
[Ala^5]-BUF[c]	0.98	16.6 (14.3–19.3)	10	0.57 (0.26–1.3)	60
[Ala^6]-BUF[c]	0.99	6.6 (6.0–7.2)	26	0.61 (0.29–1.2)	56

R.A.: ratio of affinity relative to bufokinin; R.P.: ratio of potency relative to bufokinin.
[a] Data from [11].
[b] Data from [7].
[c] Data from these peptides were not used to derive the QSAR equations. H, high affinity site; L, low affinity site, with % of each site, where data were significantly better fitted to a two-site model.

3.1.3. Correlation of functional and binding data

Functional and binding data were generally matched. Linear regression analysis showed a positive correlation ($r = 0.93$) between the affinities (pIC_{50}) of tachykinins for [^{125}I]BH-BUF binding site and their potencies (pEC_{50}) in contracting the isolated intestine (Fig. 2).

3.2. Quantitative structure–activity analyses

3.2.1. Derivation of regression equations

A range of approaches was taken in the derivation of regression equations using connectivity (χ) and shape (κ) descriptors based on chemical graphs [12,14] and conventional physicochemical parameters [18,19]. The derivation of parameters that represented the entire molecule resulted in an unsuccessful QSAR (data not shown), so all subsequent equations used substituents at individual amino acid residues. By this approach, QSAR equations were derived that accounted for most of the data variance in terms of connectivity and shape descriptors at specific residues. Because the use of zero order and higher order connectivity or shape descriptors often yielded similar analyses, the descriptors of lowest order were used to facilitate interpretation. In contrast, the use of Hansch-type physicochemical parameters describing substituent hydrophobic effects (π), electronic effects (σ) and steric effects (molar refractivity) was largely unsuccessful (data not shown).

Table 3 shows that the optimal regression equation (Eq. (3), Table 5) accounts for 81% of the variance in the binding data in terms of $G\chi_0$, $F\chi_1$ and $B\Xi$, which indicates the importance of simple connectivity functions at the G and F residues (Tyr^8 and Gln^6 in bufokinin) and the steric function at residue B (Pro^2 in bufokinin). The buildup of

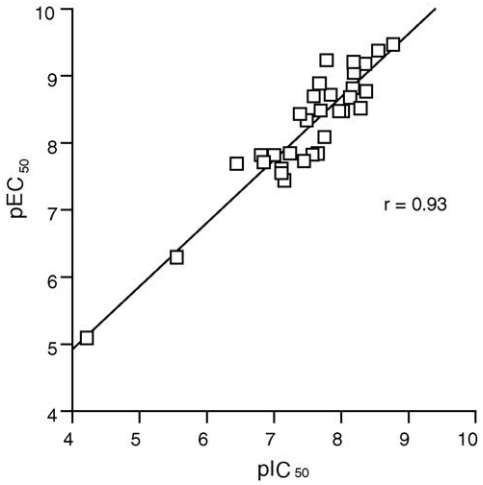

Fig. 2. Correlation of binding data (expressed as pIC_{50}) and functional data (expressed as pEC_{50}) from Table 2 determined by regression analysis ($r = 0.93$, $P < 0.0001$).

this regression is also shown in Table 3, the inclusion of all parameters was highly significant. Inclusion of functions at other residues (A, C, D or E) did not significantly increase the explained data variance. In support of this, the parameters in Eq. (3) (Table 5) were not colinear because the correlation matrix indicates clearly that all parameters were unrelated (r values not significant; Table 4). Also shown in Table 5 is the predictive capacity of Eq. (3) (Table 5), as reflected by the large values of Q and Q^2. From the leave-one-out cross validation procedure, it was found that the potencies of most analogs were well predicted.

Table 3 also presents the optimal QSAR equation for the pEC_{50} data (Eq. (8)). Eq. (8) accounts for 80% of the variance in these data in terms of $G\chi_0$, $F\chi_{3C}$, $D\kappa_1$ and $A\kappa_2$, which indicates the importance of connectivity functions at the G and F residues (Tyr^8 and Gln^6 in bufokinin) and the shapes of residues D and A (Pro^4 and Lys^1 in bufokinin). The regression buildup is shown in Table 3; inclusion of all parameters was statistically justified. Inclusion of functions at other residues (B, C or E) did not significantly increase the explained variance. The parameters in Eq. (8) (Table 5) are not colinear, as indicated by the values in the correlation matrix (Table 4). The predictive capacity of Eq. (8) (Table 5) (Q and Q^2) was strong (Table 5). Thus, the potencies of most tachykinins and analogs were well predicted using the LOO procedure.

3.2.2. *Identification of analogs not well predicted by QSAR equations*

Although the principal QSAR equations were highly significant, data from several tachykinin analogs were not well fitted by the analyses. Fig. 3A shows the relationship between pIC_{50calc} and pIC_{50obs} (Eq. (3)) and it is apparent that potencies of most of the peptides were distributed near the diagonal, which is consistent with a high quality relationship. However, the calculated potencies of three peptides (BUF (2–11), NKA (4–10) and uperolein) were underestimated by 3–9-fold by Eq. (3) (Table 5) and the potencies of three tachykinins (bufokinin, kassinin and eledoisin) were overestimated by 3–4-fold. Fig. 3B indicates the corresponding relationship between pIC_{50LOO} and pIC_{50obs} and demonstrates that the predictive power of Eq. (3) (Table 5) was also good. From this analysis, the same analogs as well as RTK-A were poorly predicted by the analysis (varied from observed values by 3–6-fold). However, the potency of NKA (4–10) was overestimated by 34-fold, which suggests that additional structural features, possibly a decreased interaction with critical receptor residues, detract from the anticipated potency of this truncated analog.

Fig. 3C shows the relationship between pEC_{50calc} and pEC_{50obs} (Eq. (8), Table 5), which is also a high quality relationship. The calculated potencies of two analogs (BUF (5–11) and NKA) were underexplained by Eq. (8)

Table 3
Buildup of regression equations showing parameter coefficients derived by multiple regression

Binding data (pIC_{50})

Equation	Intercept	$G\chi_0$	$F\chi_1$	$B\Xi$	r	r^2	P
1	6.48 (0.30)			5.43 (1.51)	0.60	36	0.002
2	5.01 (0.39)		6.20 (1.36)	5.36 (1.11)	0.82	67	<0.0001
3	1.94 (0.82)	5.10 (1.27)	5.56 (1.06)	6.42 (0.89)	0.90	81	<0.0001
4	2.87 (1.04)	5.50 (1.60)	3.70 (1.34)	5.99 (1.13)	0.82	68	<0.001

Functional data (pEC_{50})

Equation	Intercept	$G\chi_0$	$F\chi_{3C}$	$D\kappa_1$	$A\kappa_2$	r	r^2	P
5	6.74 (0.30)			5.24 (1.08)		0.71	50	<0.0001
6	6.60 (0.25)			4.48 (0.91)	4.06 (1.14)	0.83	69	<0.0001
7	4.76 (0.84)	3.08 (1.36)		4.62 (0.84)	3.62 (1.07)	0.86	75	<0.0001
8	4.08 (0.83)	3.28 (1.25)	43.5 (19.1)	4.14 (0.79)	3.14 (1.00)	0.90	80	<0.0001
9	4.19 (1.18)	2.69 (1.78)	23.0 (27.2)	3.52 (1.13)	4.15 (1.42)	0.79	63	0.004

r: linear correlation coefficient; r^2(%): percentage of data variance explained by the regression equation; P: level of significance of regression. Values in parenthesis are standard deviations of parameter estimates. Criteria for inclusion of a parameter are: improvement in F ratio for inclusion and t-test on each parameter.

Table 4
Squared correlation matrices for optimal regression equations

Eq. (3)	pIC_{50} as a function of $G\chi_0$, $F\chi_1$ and $B\Xi$		
	$G\chi_0$	$F\chi_1$	$B\Xi$
$G\chi_0$		0.141 (2.00)	−0.291 (8.50)
$F\chi_1$			0.014 (0.02)
$B\Xi$			

Eq. (8)	pEC_{50} as a function of $G\chi_0$, $F\chi_{3C}$, $D\kappa_1$ and $A\kappa_2$			
	$G\chi_0$	$F\chi_{3C}$	$D\kappa_1$	$A\kappa_2$
$G\chi_0$		−0.041 (0.17)	−0.033 (0.11)	0.169 (2.87)
$F\chi_{3C}$			0.314 (9.84)	0.260 (6.79)
$D\kappa_1$				0.237 (5.63)
$A\kappa_2$				

Table shows r values and r^2 values (%; in parenthesis) for the linear correlation of parameters.

Table 5
Predictive ability of optimal regression equations

Equation	Q	Q^2	S_{PRESS}	PRESS
3	0.83	69	0.572	6.86
8	0.80	63	0.609	7.42

The statistical parameters Q, Q^2, S_{PRESS} and PRESS are defined in Section 2; [16,17].

(Table 5) by 3–4-fold and the potencies of three analogs were overexplained by 4–10-fold (BUF (4-11), BUF (3–11) and NKA (4–10)). Fig. 3D shows the relationship between pEC_{50LOO} and pEC_{50obs} which again indicates that the predictive power of Eq. (8) (Table 5) is strong. The same five analogs as well as BUF (6–11) and ranamargarin were poorly predicted by the analysis (varied from observed values by 5–8-fold). Again, it was noteworthy that the potency of NKA (4–10) was overestimated by 36-fold. Indeed, this analog was consistently less potent than predicted by the present QSAR analysis.

3.2.3. Predicted potencies of Ala-substituted BUF analogs

Table 6 contains the predicted potencies of Ala-substituted BUF analogs based on the optimal regression equations. From Eq. (3) (Table 5), the calculated values pIC_{50} for four of the six analogs were quite close to the observed potencies (within a factor of two), although the calculated binding potency of [Ala^1]-BUF was about seven-fold greater than predicted and the calculated binding potency of [Ala^6]-BUF was about five-fold greater than predicted. Similarly, from Eq. (8) (Table 5), the calculated functional potency of [Ala^1]-BUF was about six-fold greater than predicted, but the remaining analogs were well predicted by the equation (within about 2.5-fold of the observed values). From these findings it is clear that the derived QSAR equations are robust and that their predictive values extends to atypical tachykinin peptides.

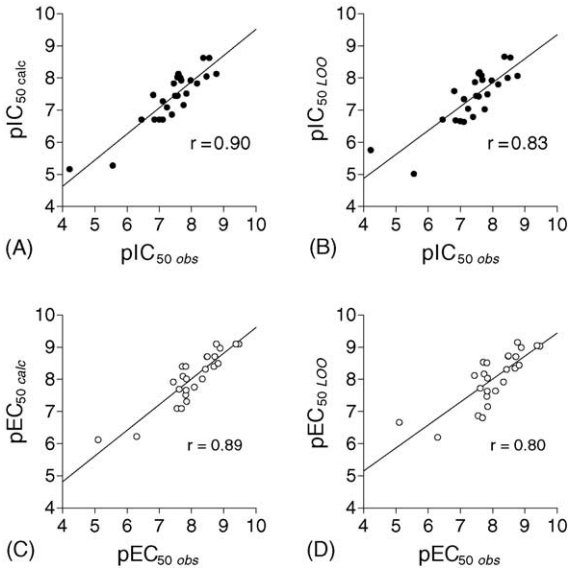

Fig. 3. Correlations of predicted binding (pIC_{50}) and functional (pEC_{50}) data, calculated from the QSAR analyses, with the corresponding experimental data (pIC_{50obs} and pEC_{50obs}). (A), relationship between pIC_{50calc} (calculated using Eq. (3)) and pIC_{50obs} for tachykinins as binding competitors ($r = 0.90$, $P < 0.0001$). (B), relationship between pIC_{50LOO} (calculated as described in Section 2) and pIC_{50obs} for tachykinins as binding competitors ($r = 0.83$, $P < 0.0001$). (C), relationship between pEC_{50calc} (calculated using Eq. (8)) and pEC_{50obs} for tachykinins as functional agonists ($r = 0.89$, $P < 0.0001$). (D), relationship between pEC_{50LOO} (calculated as described in Section 2) and pEC_{50obs} for tachykinins as functional agonists ($r = 0.80$, $P < 0.0001$).

4. Discussion

The present study utilized a structure–activity approach to quantitate the relationships between amino acid residues of naturally occurring tachykinins and BUF analogs, for interaction with the bNK_1 receptor in the toad small intestine. In our previous study with bufokinin, SP and their C-terminal fragments [11], the peptides were treated as single structures, for which connectivity and shape descriptors were calculated. In the present study, which included a more diverse range of amphibian, fish, mammalian and molluscan tachykinins, we first tested the applicability of the regression equations derived from the original analyses [11]. However, this approach proved unsuccessful (not shown) and the current subsequent analyses considered each amino acid separately.

Highly significant equations were obtained ($r^2 \geq 80\%$) that described binding affinity and functional potency of tachykinins and related analogs in terms of QSAR parameters from individual amino acids. The significance of connectivity functions ($G\chi_0$, $F\chi_1$ and $F\chi_{3C}$), shape ($D\kappa_1$ and $A\kappa_2$) and steric parameters ($B\Xi$) in the receptor–ligand interactions were established by the optimal equations. Coefficients of all variables were positive, indicating that these contribute favorably to binding and functional effects of the peptides at the tachykinin receptor.

Table 6
Predicted vs. observed potencies of Ala-substituted bufokinin analogs

	pIC_{50} observed	pIC_{50} predicted	Difference
Binding data analog			
[Ala^1]	8.357	7.524	0.863
[Ala^2]	8.131	8.128	0.003
[Ala^3]	8.194	8.128	0.066
[Ala^4]	8.292	8.128	0.164
[Ala^5]	7.780	8.128	0.348
[Ala^6]	8.180	7.472	0.708
	pEC_{50} observed	pEC_{50} predicted	Difference
Functional data analog			
[Ala^1]	9.180	8.403	0.777
[Ala^2]	8.678	9.097	0.419
[Ala^3]	9.051	9.097	0.046
[Ala^4]	8.523	8.197	0.326
[Ala^5]	9.244	9.097	0.147
[Ala^6]	9.215	9.097	0.118

Residues at positions G and F (Tyr^8 and Gln^6 in bufokinin) appeared very important for both binding and functional potency. Lower order connectivity functions have been related to the molecular properties of hydrophobicity and polarisability [20]. This suggests that partitioning of these key residues into lipophilic regions of tachykinin receptors may produce more effective analogs. Increasing numbers of atoms and bonds in residue G promotes both binding and function. Most of the test peptides possessed a Tyr^8 at residue G and several others also had amino acids with larger side chains, such as Phe, Ile or Val. Replacement of Phe^8 in mammalian SP with Tyr^8 alters the conformation of the peptide in DMSO, water and lipid bilayers [21]. Val^8 has been proposed as an important residue in distinguishing between SP-like and NKA/NKB-like activity [22].

For the functional data, the importance of the χ_{3C} parameter at residue F suggests that clustering within the amino acid side chain may also promote receptor interactions [13]. Twelve of the peptides possessed a Gln^6 residue. Indeed, the addition of a Gln residue at the N-terminus of BUF (7–11) increased the affinity for the toad NK_1 receptor 18- and 36-fold in functional and binding studies, respectively, suggesting that carboxamide side chain of Gln^6 residue may have a crucial role in ligand interactions with this receptor. Although the native toad tachykinin bufokinin was the most potent peptide, as expected, several other amphibian tachykinins such as ranakinin, RTK-A and physalaemin displayed appreciable potency in both binding and functional studies, yet deviated in amino acid sequence from bufokinin at several key residues, including F. These tachykinins contained other large and flexible amino acids, such as Arg and Lys, at this position.

The shape properties of the residues at positions D and A (Pro^4 and Lys^1 in bufokinin) were important to the receptor interaction underlying the functional data, but not to the binding interaction. Shape effects are related to the number of atoms in a functional group and their relationship to each other (i.e. bonding and whether this gives rise to branched or long-chain substituents). Increasing the order of the shape descriptor produces a decline in the value of the shape attribute with linear analogs but may increase with highly substituted or cyclic systems, whereas branching decreases the value of a shape descriptor of a particular order. The positive coefficient for the shape descriptors at residues D and A indicates that lower branching nature enhances potency. The finding that most of the functionally potent compounds were bufokinin-like, in that they had a proline at position D, is consistent with the importance of the shape attribute of this residue.

Shape encodes different information from steric factors. The steric effect of isomeric compounds is roughly similar but their shapes are quite different, explaining the marked loss of potency when D-amino acids are substituted for L-amino acids [23]. The graph-based steric parameter Ξ is a composite of shape descriptors as (defined in the Section 2) and is correlated significantly with Taft's steric constant [15]. The advantage of this graph-based parameter is that it reflects the intrinsic properties of the functional group and is not dependent on measurement. In the present study, the steric effect at residue B (Pro^2 in bufokinin) was significant in binding interactions. The positive value of the coefficient again indicates that increasing steric nature enhances receptor interactions. This appears important since all but one of the 10 most potent peptides had a Pro^2 residue.

The [Ala]-substituted BUF tachykinin analogs were prepared to evaluate, in a systematic fashion, the role of physicochemical properties at individual residue positions in the peptide. Somewhat surprisingly, the relative potencies of these derivatives in binding and functional interactions were similar. Ala^2 and Ala^4 were the weakest of all the Ala peptides suggesting that replacement of these prolines is more deleterious to activity than replacement of residues at positions 1, 3, 5 or 6. It is clear, however, that the QSAR-predicted potencies were generally in accord with the observed potencies of the analogs. The exceptions were the [Ala^1]-BUF and [Ala^6]-BUF analogs. The [Ala^1]-BUF analog exhibited greater than anticipated potency in binding (seven-fold) and functional (six-fold) studies. It is conceivable that the reason for this may lie in the effect of replacing the large basic lysine residue with a small neutral alanine residue at position A. Position A was most critical for functional activity (as a consequence of its shape attribute) and the adjacent position B was important for receptor binding (as a consequence of its steric effect). The conformation of the [Ala^1]-BUF analog could be significantly different from bufokinin itself around positions 1 and 2. The potency of the [Ala^6]-BUF analog in receptor binding was five-fold greater than predicted by Eq. (3) (Table 5). The replacement of the large polar glutamine residue in the endogenous peptide with the smaller neutral alanine may have diminished the contribution of the con-

nectivity function at position 6 ($F\chi_1$ in Eq. (3), Table 5) and impaired the ligand–receptor binding interaction. As regards [Ala^5]-BUF, replacement of the polar aspartic acid had a markedly more deleterious effect on binding than on functional potency, even though the potencies of this analog were reasonably well predicted by the optimal equations. Many of the most potent peptides (in the binding assay) possessed an acidic residue (mainly Asp) at position 5, even though this apparent preference was not a feature of the optimum QSAR equation, Eq. (3) (Table 5).

Hylambatin and ranamargarin are somewhat atypical. Hylambatin does not share the common C-terminal pentapeptide, but possesses a Met^{10} residue instead of Leu^{10} residue. Hylambatin was almost equipotent with physalaemin in our binding and functional experiments, in agreement with previous studies showing that effects of these peptides on smooth muscle contraction, vasodilatation and salivary secretion of rat, guinea pig and rabbit were nearly indistinguishable [24]. The substitution of Met^{10} for Leu^{10} does not alter the hydrophobicity of the C-terminus, which seems to be the more important property of this residue. On the other hand, ranamargarin, whose C-terminal pentapeptide is conserved, but which has a somewhat elongated N-terminus with a number of acidic residues, was rather weak, similar to several BUF truncated analogs. Ranamargarin possesses few of the key amino acids demonstrated by QSAR analysis to be important for interaction with the bNK_1 receptor. It was the only native tachykinin to show two-site bindings, displaying unexpected high affinity interaction with a minor population of (probably non-bNK_1) binding sites, which appear unrelated to smooth muscle functional responses.

Most tachykinin-related QSAR studies have investigated interactions of NK_1 receptor antagonists, rather than peptide agonists, at the human receptor [25,26]. Such antagonists have recently shown promise in the treatment of disorders such as anxiety, depression and in cisplatin-induced emesis [27]. There are relatively few peptide QSARs reported in the literature. This is most likely due to the complexity of ligand–receptor interactions, especially for small peptide ligands, such as the tachykinins, in which each residue position may have a role in receptor binding interactions. However, Gupta et al. showed a correlation of renin–receptor binding with Kier-like parameters in a series of chain-modified peptides [28].

In summary, the present study did not attempt to define how structural modification at specific residues influenced tachykinin receptor binding potency. Rather it attempted to account for the interactions of heterologous tachykinins with the toad intestinal receptor. The hydrophobicity and polarisability of residues F and G appeared important for efficient interactions between the ligands and the receptor (as revealed by strong correlations with connectivity functions describing the residues). In addition, shape and steric properties of residues D, E and G emerged as significant positive effectors of tachykinin ligand interactions with the bNK_1 receptor. The present equations provide a quantitative approach to describe the relationship between biological function and binding activity with the molecular properties of each amino acid of the tachykinin peptide, and thus offer insights into the design of new peptide mimetics that interact with the intestinal receptor. Comparative studies with other tachykinin receptors from different species will provide pharmacophoric information on the similarities and differences between the receptors.

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