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Short communication

Structure-activity relationship of neurokinin A(4–10) at the human tachykinin NK_2 receptor: the effect of amino acid substitutions on receptor affinity and function

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Abstract

A structure-activity study of the neurokinin A (NKA) fragment NKA(4–10) was performed to investigate the importance of amino acid residues for receptor efficacy, potency and affinity at the NK_2 receptor in human colon circular muscle. Fourteen analogs of NKA(4–10) were produced with substitutions at positions 4, 5, 7, 9 and/or 10 of NKA. Their potencies were determined by *in vitro* contractile responses and affinities by radioligand binding using [^{125}I]NKA. Functional potency was enhanced 8-fold by single amino acid substitutions with Lys^5 and $MeLeu^9$ but not significantly altered by substitutions Glu^4, Arg^5, His^5 and Nle^{10}. The multiply-substituted analogs [$MeLeu^9$, Nle^{10}]NKA(4–10), [Lys^5,$MeLeu^9$,Nle^{10}]NKA(4–10) and [Lys^5,(Tyr^7),$MeLeu^9$,Nle^{10}]NKA(4–10) displayed 6–9-fold increase in potency. Although [Arg^5,Nle^{10}]NKA(4–10) was similar in potency to NKA(4–10), it was the only analog to show significantly reduced efficacy. All analogs were able to compete fully for [^{125}I]NKA binding. [Lys^5,$MeLeu^9$]NKA(4–10), [$MeLeu^9$,Nle^{10}]NKA(4–10), [Lys^5,Nle^{10}]NKA(4–10) and analogs containing single substitutions with Glu^4, Arg^5, Lys^5 and $MeLeu^9$ displayed significantly higher affinity, whereas those with Nle^{10} and [Glu^4,Nle^{10}] substitutions showed significantly lower affinity than NKA(4–10). There was a positive correlation ($r = 0.63$) between binding affinity and functional potency, which was markedly improved ($r = 0.95$) by removal of three analogs: [Lys^5,$MeLeu^9$,Nle^{10}]NKA(4–10), [Lys^5,Tyr^7,$MeLeu^9$,Nle^{10}]NKA(4–10) and [Lys^5,$Tyr(I_2)^7$,$MeLeu^9$,Nle^{10}]NKA(4–10). These exhibited similar binding affinities to that of NKA(4–10) but were more potent in functional studies, possibly indicating a different mechanism of receptor interaction. In conclusion, substitution of Ser^5 with Lys, and/or *N*-methylation of Leu^9, were the most effective changes to increase functional and binding potency of NKA(4–10) at the human colon NK_2 receptor.

Keywords: Neurokinin A; Structure-activity; Human colon; Circular muscle; Radioligand binding; NK_2 receptor

1. Introduction

NKA (His^1-Lys^2-Thr^3-Asp^4-Ser^5-Phe^6-Val^7-Gly^8-Leu^9-Met^{10}-NH_2) and its truncated form NKA(4–10) are potent spasmogens of human colon circular muscle, an action mediated exclusively via tachykinin NK_2 receptors [1,2]. A high density of NK_2 receptors has been demonstrated in this tissue, using *in vitro* autoradiography and radioligand binding [3,4].

Most structure-activity studies at NK_2 receptors have been carried out using laboratory animals [5–9], although there are considerable species differences in the sequences of NK_2 receptors between species [10], and results obtained in one species cannot necessarily be usefully projected to another. In a previous structure-activity study of NKA(4–10) at human NK_2 receptors [11], we investigated the role of the native residues and of their chirality. We found that the side groups of Asp^4, Val^7, Leu^9, Met^{10} and in particular Phe^6,[1] were essential for activity and that changes in amino acid chirality were detrimental to the binding and functional activity of NKA(4–10). Single substitutions are a useful tool to determine the effect of a change in one amino acid. However, multiple amino acid substitutions may provide information about the interaction of a particular residue with other amino acids in the whole peptide as well as with the receptor protein.

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Abbreviations: NKA, neurokinin A; SR140333, [(*S*) 1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxy phenyl acetyl) piperin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2] octane chloride]; ACh, acetylcholine; BSA, bovine serum albumin.

[1] Amino acids have been numbered as occuring in the decapeptide NKA.

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Table 1
Functional potencies and binding affinities of substituted NKA(4–10) analogs

No.	Peptide	Functional studies			Radioligand binding		
		$-\log EC_{20}$ ACh ± SEM	Maximum response (10 μM) % ± SEM	R.P.[a]	Slope factor	pIC_{50} ± SEM	R.A.[b]
1	NKA(4–10)	7.34 ± 0.15	60 ± 3.2	100	0.88	8.06 ± 0.03	100
2	[Glu^4]NKA(4–10)	7.67 ± 0.12	59 ± 2.8	210	1.19	8.49 ± 0.07*	270
3	[His^5]NKA(4–10)	6.92 ± 0.07	57 ± 6.0	38	1.08	7.91 ± 0.09	71
4	[Arg^5]NKA(4–10)	7.81 ± 0.18	54 ± 4.6	300	1.01	8.72 ± 0.14**	460
5	[Lys^5]NKA(4–10)	8.25 ± 0.18*	59 ± 8.3	810	1.07	9.02 ± 0.03**	910
6	[MeLeu^9]NKA(4–10)	8.24 ± 0.15*	70 ± 9.3	800	1.17	8.80 ± 0.14**	550
7	[Nle^{10}]NKA(4–10)	6.83 ± 0.17	55 ± 4.4	31	0.95	7.51 ± 0.05**	28
8	[Glu^4,Nle^{10}]NKA(4–10)	6.84 ± 0.26	56 ± 13	32	0.85	7.64 ± 0.08*	38
9	[Arg^5,Nle^{10}]NKA(4–10)	7.32 ± 0.28	40 ± 4.2*	95	0.86	8.21 ± 0.17	140
10	[Lys^5,Nle^{10}]NKA(4–10)	7.89 ± 0.27	55 ± 9.4	355	1.23	8.64 ± 0.08**	380
11	[MeLeu^9,Nle^{10}]NKA(4–10)	8.25 ± 0.11*	73 ± 6.5	810	0.89	8.57 ± 0.02**	325
12	[Lys^5,MeLeu^9]NKA(4–10)	8.00 ± 0.15	63 ± 8.7	450	0.99	8.78 ± 0.10**	525
13	[Lys^5,MeLeu^9,Nle^{10}]NKA(4–10)	8.31 ± 0.18**	74 ± 8.6	930	1.02	8.16 ± 0.07	130
14	[Lys^5,Tyr^7,MeLeu^9,Nle^{10}]NKA(4–10)	8.14 ± 0.12*	63 ± 7.7	630	0.87	7.73 ± 0.11	47
15	[Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]NKA(4–10)	8.01 ± 0.04	64 ± 1.5	470	0.89	7.77 ± 0.15	51

$-\log EC_{20}$ values are determined as 20% of the ACh maximum response. Values represent the mean ± SEM of 4–6 independent experiments. $pIC_{50} = \log IC_{50}$. pIC_{50} values and slope factors represent the mean ± SEM of three determinations in the presence of NK_1 receptor antagonist SR140333 (0.1 μM).

[a] R.P.: relative potency compared with that for NKA(4–10) (100).

[b] R.A.: relative affinity compared with that for NKA(4–10) (100).

* $P < 0.05$.

** $P < 0.01$, compared with NKA(4–10) (one-way ANOVA, Newman–Keuls multiple comparison test).

The aim of this study was to assess the activities of NKA(4–10) heptapeptide analogs with one or more amino acid substitutions in positions 4, 5, 7, 9 and 10 (relative to NKA), in order to examine the influence of single vs. multiple substitutions on affinity and potency of NKA-(4–10) for the tachykinin NK_2 receptor in a natural system such as human colon circular muscle. In the gastrointestinal tract, selective NK_2 receptor antagonists may be useful as therapeutic agents in conditions associated with increased, or exaggerated gut motility, such as diarrhoea and irritable bowel diseases [12]. Results should be applicable to NK_2 receptors in other human tissues since a series of Ala-substituted analogs had identical potencies in human colon and urinary bladder strips [19]. Our current knowledge suggests that there are two isoforms of the NK2 receptor, which differ only by a single amino acid, and are found in jejunum and trachea [13].

2. Materials and methods

2.1. Peptides and materials

Fourteen analogs of NKA(4–10) with single or multiple amino acid substitutions in positions 4, 5, 7, 9 or 10 were used in this study (Table 1). These peptides were purchased from Auspep (Australia), synthesised and purified by Peptech (Australia) or were gifts from Dr. S. Lavielle (Université Pierre et Marie Curie, Paris, France). MALDI Mass Spectrometry and analyses of peptide content and amino acids were performed on all analogs. Stock solutions of analogs were prepared in 0.01 M acetic acid containing β-mercaptoethanol (1% v/v), or in dimethylsulphoxide, and stored as aliquots at −20°.

[^{125}I]NKA (2-[^{125}I]iodohistidyl^1)neurokinin A, specific activity 2000 Ci/mmol) was purchased from NEN Life Science Products Inc. (Boston, U.S.A.). The non-peptide NK_1 receptor antagonist SR140333 ([(*S*) 1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxy phenyl acetyl) piperin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2] octane chloride]) was a gift from Sanofi Recherche, Montpellier, France. Acetylcholine (ACh) and the peptidase inhibitors, phosphoramidon, chymostatin, and bestatin were purchased from Sigma (Australia). All other reagents were of analytical grade.

2.2. Specimen collection

Human sigmoid colon was collected from 23 patients (10 females, 13 males), aged 69–84 years, who were undergoing partial colectomy for colon carcinoma (human ethical approval no. 97/139). Patients had not undergone radiation therapy or chemotherapy. Normal colon segments, without any macroscopic signs of inflammation, were taken 10–20 cm from the tumour and placed immediately in ice-cold carbogenated (95% O_2/5% CO_2) Krebs–Henseleit solution for transport to the laboratory. Circular muscle was prepared and frozen as previously described [11].

2.3. Functional studies

Circular muscle strips were suspended in 2 mL organ baths containing carbogenated Krebs–Henseleit solution at 37° under a resting tension of 1 g [11]. Changes in tension were measured isometrically. After equilibration for 60 min, the maximum response of each muscle strip was elicited with ACh (10 mM). Concentration–response curves for NKA(4–10) and analogs were constructed by discrete addition of peptide at 60-min intervals with 3–4-min contact time. The responses were measured as increase in tension and expressed as a percentage of the maximum response to ACh (10 mM). Agonist potencies were expressed as $-\log EC_{20}$ ACh, as not all analogs elicited the same maximum response at the highest concentration tested.

No receptor antagonists or peptidase inhibitors were used in these experiments, since tachykinin-induced contraction of human colon circular muscle is not mediated via NK_1 or NK_3 receptors, and the magnitude or duration of contractile responses to NKA and NKA(4–10) are not enhanced by peptidase inhibitors [1,11].

2.4. Radioligand binding studies

Radioligand binding experiments were performed as previously described [4,11]. Crude membranes (3% w/v) were finally resuspended in incubation buffer containing 50 mM Tris–HCl (pH 7.4, 25°), 3 mM $MnCl_2$, 0.02% w/v BSA, the peptidase inhibitors chymostatin (4 μg/mL), bestatin (10 μM) and phosphoramidon (10 μM), and the NK_1 receptor selective antagonist SR140333 (0.1 μM) and incubated for 60 min at 25° with [^{125}I]NKA (50 pM). Non-specific binding was defined using 1 μM NKA. Receptor binding was terminated by rapid filtration and quantified as described [4]. Three to four independent competition experiments were carried out for each analog (0.1 nM–100 μM). Raw binding data were analyzed using the computer program PRISM v3.0 (GraphPad Software Inc.). Data were analyzed using single and multiple site models. The *F* test was used to determine the best model.

2.5. Statistics

pIC_{50}, slope factors, $-\log EC_{20}$ ACh and maximum responses were compared statistically using one-way ANOVA followed by Newman–Keuls multiple comparison test, with NKA(4–10) as the parent compound. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Functional studies

All analogs produced concentration-dependent contractions of human colon circular muscle. The maximum response evoked by NKA(4–10) was 60% of that elicited by ACh (Table 1). The $-\log EC_{20}$ ACh of NKA(4–10) was 7.34 ± 0.15; this EC_{20} ACh concentration of peptide produced a contractile response equivalent to 36% of the maximal response evoked by NKA(4–10). There was a trend for analogs containing MeLeu^9 to produce a higher maximum response and for analogs with Nle^{10} to produce a lower maximum response, but these differences were not significant, except for [Arg^5,Nle^{10}]NKA(4–10), where the maximum response was only 66% ($P < 0.05$) of that of NKA(4–10) (Fig. 1, Table 1).

Single amino acid substitutions with Lys^5 or MeLeu^9 significantly increased the potency of NKA(4–10) ($P < 0.05$) by 8-fold, while substitution with Glu^4, Arg^5, His^5 or Nle^{10} produced no significant effect (Table 1). Incorporation of Nle^{10} into [Glu^4]NKA(4–10) produced a significant decrease in potency, compared with [Glu^4]NKA(4–10) ($P < 0.05$). Shallow concentration–response curves were observed for [Arg^5]NKA(4–10), [Lys^5]NKA(4–10), [Arg^5,Nle^{10}]NKA(4–10) and [Lys^5,Nle^{10}]NKA(4–10) (Fig. 1). However, the slopes of these curves were not significantly different from that of NKA(4–10), or from that of a typical sigmoidal dose–response relationship (slope = 0.576) [14].

We also investigated some multiply-substituted analogs (compounds 10–15) of NKA(4–10) which were previously described in animal studies [15,16]. Of these six compounds, [MeLeu^9,Nle^{10}]NKA(4–10), [Lys^5,MeLeu^9,Nle^{10}]NKA(4–10) (selective, potent NK_2 receptor agonist [15]) and [Lys^5,Tyr^7,MeLeu^9,Nle^{10}]NKA(4–10) [16] exhibited significantly enhanced potency (6–9-fold) compared to that of NKA(4–10) (Table 1). For these compounds, no additive effects were observed due to the multiple substitutions compared to corresponding compounds with single substitutions. The potencies of analogs [Lys^5,MeLeu^9]NKA(4–10), [Lys^5,Nle^{10}]NKA(4–10), and [Lys^5,Tyr(I$_2$)^7,MeLeu^9,Nle^{10}]NKA(4–10) were not significantly different from that of NKA(4–10).

3.2. Radioligand binding studies

All analogs were able to compete for the specific binding of [^{125}I]NKA and none of the slope factors differed significantly from unity (Table 1). Single amino acid substitutions with Glu^4, Lys^5, Arg^5, or MeLeu^9 significantly increased the binding affinity compared to that of NKA(4–10) ($P < 0.05$) by 3–9-fold, whereas substitution with Nle^{10} significantly decreased the binding affinity of NKA(4–10) ($P < 0.01$) (Table 1) by a factor of 3.5. The binding affinity of [His^5]NKA(4–10) was not significantly different from that of NKA(4–10).

Some compounds exhibiting multiple amino acid substitutions ([Lys^5,MeLeu^9], [Lys^5,Nle^{10}] and [MeLeu^9,Nle^{10}] analogs) exhibited affinities 3–5-fold greater ($P < 0.01$) than that of NKA(4–10), whereas [Glu^4,Nle^{10}]NKA(4–10) was significantly weaker (2.6-fold;

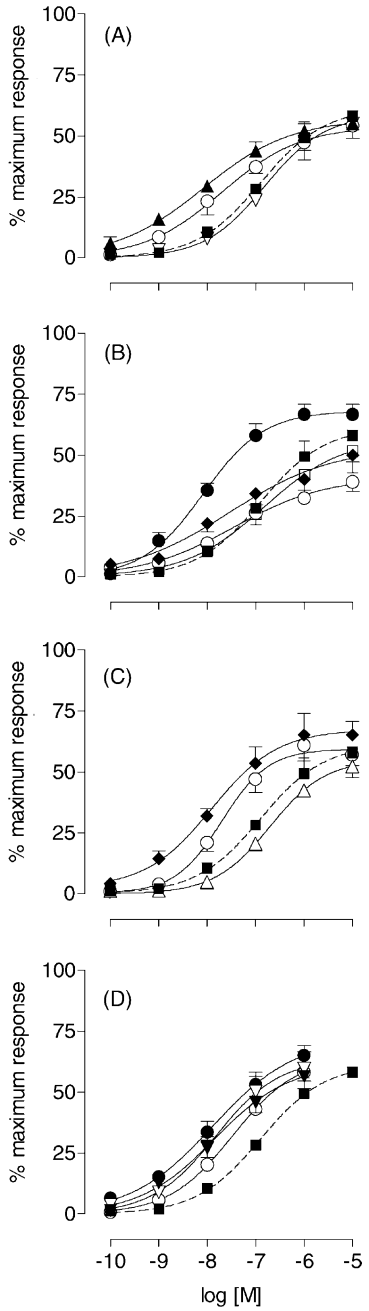

Fig. 1. Concentration–response curves for NKA(4–10) analogs in human colon circular muscle strips. Data points represent means ± SEM of 4–6 independent experiments. Values are expressed as a percentage of the maximum response to ACh (10 mM). No receptor antagonists or inhibitors were used in isolated smooth muscle experiments. (A) NKA(4–10) (■); [Lys^5]NKA(4–10) (▲); [Arg^5]NKA(4–10) (○); [His^5]NKA(4–10) (▽); (B) NKA(4–10) (■); [Glu^4,Nle^{10}]NKA(4–10) (□); [Lys^5,Nle^{10}]NKA(4–10) (◆); [Arg^5,Nle^{10}]NKA(4–10) (○); [MeLeu^9,Nle^{10}]NKA(4–10) (●); (C) NKA(4–10) (■); [Glu^4]NKA(4–10) (○); [MeLeu^9]NKA(4–10) (◆); [Nle^{10}]NKA (4–10) (△); (D) NKA(4–10) (■); [Lys^5,MeLeu^9]NKA(4–10) (▼); [Lys^5,MeLeu^9,Nle^{10}]NKA(4–10) (●); [Lys^5,Tyr^7,MeLeu^9,Nle^{10}]NKA(4–10) (▽); [Lys^5,Tyr(I$_2$)^7, MeLeu^9,Nle^{10}]NKA(4–10) (○).

Table 1). The affinity of [Lys^5,MeLeu^9,Nle^{10}]NKA(4–10) was not significantly different from that of NKA(4–10) (Table 1). Substitution with tyrosine or iodination of tyrosine at position 7 [16] did not significantly alter the

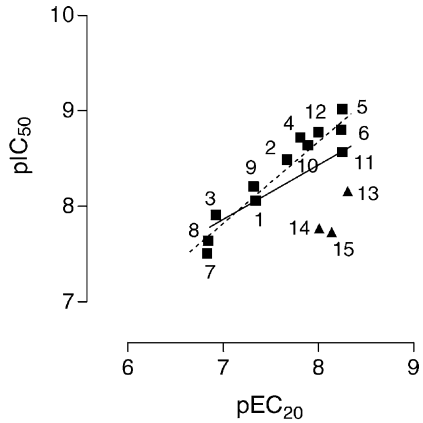

Fig. 2. Correlation of the binding and potency values of NKA(4–10) analogs at the human colon NK$_2$ receptor ($r = 0.63$). Analogs are identified by the number assigned in Table 1. When outlying compounds 13, 14, and 15 were removed from the analysis, there was a very marked improvement of the correlation (dotted line, $r = 0.95$).

binding affinity compared with that of NKA(4–10) or [Lys^5,MeLeu^9,Nle^{10}]NKA(4–10).

3.3. Correlation of binding affinity and functional potencies

Correlation of the binding affinities (pIC_{50}) and potency values ($-\log EC_{20}$) for NKA(4–10) and analogs was moderately good ($r = 0.630$). Three compounds (analogs 13, 14, and 15) were markedly more potent as functional agonists than as binding competitors (Table 1, Fig. 2). When these three compounds were removed from the analysis, the correlation of the remaining 12 compounds was excellent ($r = 0.950$).

4. Discussion

Although numerous studies have identified the structural requirements of NKA(4–10) for interaction at animal NK$_2$ receptors [5–9], few studies have investigated its structure-activity relationship at the human NK$_2$ receptor [11,17,18]. The results of this study suggest that the affinity and potency of NKA(4–10) is influenced by the hydrophilicity and charge carried by residues at positions 4 and 5. At position 4, the presence of acidic residues, Asp (native) or Glu maintained or increased the potency of NKA(4–10), whereas substitution with the neutral residues Ala and Gln was reported to reduce potency [5,6,11]. At position 5, substitution of the neutral native Ser with the positively charged Lys caused parallel substantial increases in binding affinity and potency at the human colon NK$_2$ receptor, with Arg^5 also causing increased affinity, whereas substitution with hydrophobic His had no significant effect. Thus, as at the rabbit pulmonary artery [15] and rat fundus NK$_2$ receptor

[9,16], a positively charged hydrophilic residue in position 5 of NKA(4–10) is preferred, which may interact with an adjacent negative charge on the NK_2 receptor protein [9]. However, both Arg^5 and some Lys^5 containing analogs had a tendency to produce shallow concentration–response curves, an effect not reported at rat NK_2 receptors [7–9], which might indicate a different mechanism of action from NKA(4–10) and the other analogs at the human NK_2 receptor.

At position 9, *N*-methylation of Leu produced an analog that was significantly more potent in binding and functional studies than NKA(4–10). Although an increased efficacy of [$MeLeu^9$]NKA(4–10) was earlier reported in rabbit pulmonary artery [16], this enhanced potency and trend towards an increase in efficacy of analogs with *N*-methyl leucine at position 9 has not been previously noted at the human NK_2 receptor.

At position 10, Met can be successfully replaced with the isosteric Nle to increase NK_2 receptor selectivity in several animal species [6]. However, this leads to 2–30-fold [6,7] decrease in functional potency in rabbit and rat compared with the decapeptide NKA. In the present study, this substitution produced an analog that was 3-fold less potent than NKA(4–10), with binding affinity identical to that found in a study using the cloned human NK_2 receptor [17]. Furthermore, introduction of Nle^{10} caused a decrease (up to 7-fold) in the binding affinities and functional potencies of analogs [Glu^4,Nle^{10}]NKA(4–10), [Arg^5, Nle^{10}]NKA(4–10), and [Lys^5,Nle^{10}]NKA(4–10), compared with their single substituted parent peptide. Thus, the human NK_2 receptor prefers the native Met at position 10, where the non-oxidised sulphur may participate in hydrogen bonding.

For NKA(4–10) and analogs 2–12, the binding affinities of analogs were slightly higher than their observed potencies. However, for [Lys^5,$MeLeu^9$,Nle^{10}]NKA(4–10) (analog 13, selective NK_2 receptor agonist [15]), [Lys^5,Tyr^7, $MeLeu^9$,Nle^{10}]NKA(4–10) (analog 14) and [Lys^5,$Tyr(I_2)^7$, $MeLeu^9$,Nle^{10}] NKA(4–10) (analog 15, selective NK_2 receptor radioligand [16]), the opposite was observed. This discrepancy may suggest that these analogs interact with the binding domain of the human receptor in a different manner compared to that of analogs 2–12. It was of interest that the binding affinities of these three analogs significantly increased in the absence of the NK_1 receptor antagonist SR140333 [19].

In conclusion, substitution of Ser^5 with basic residues, and/or *N*-methylation of Leu^9, were the most effective changes to increase functional and binding potency of NKA(4–10) at the human colon NK_2 receptor. For most analogs, there was a good correlation between binding and functional values. While there was a trend for basic residues to increase the potency of NKA(4–10) analogs, there was an associated decrease in efficacy, suggesting a different mechanism of action not previously reported at non-human NK_2 receptors [6–9,15]. In this study, small differences from results obtained in other species were evident, emphasising the importance of human studies for the development of therapeutic agents.

Acknowledgments

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