A New Class of High Affinity Ligands for the Neurokinin A NK_2 Receptor: $\psi(CH_2NR)$ Reduced Peptide Bond Analogues of Neurokinin A_{4-10}[†,‡]

Scott L. Harbeson,* Scott A. Shatzer, Tieu-Binh Le, and Stephen H. Buck[§]

Marion Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, Ohio 45215

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Analogues of [Leu[10]]NKA_{4-10} were synthesized in which each of the amide bonds was sequentially replaced with the reduced amide $\psi(CH_2NH)$ bond to determine the effect of this structural modification on the antagonism of NKA binding to the HUB NK_2 receptor. [$\psi(CH_2$-NH)[9],Leu[10]]NKA_{4-10} (**6**) retained significant affinity for the NK_2 receptor (IC_{50} = 115 nM) and showed weak partial stimulation of PI turnover (approximately 10–15% of NKA maximum). **6** behaves as a competitive antagonist of NKA-stimulated PI turnover with a pA_2 = 6.7. The secondary amine of the $\psi(CH_2NH)$ moiety of **6** was converted to a tertiary amine by alkylation. This modification was found to have a small effect upon receptor affinity but did result in attenuation of partial agonist activity. A combination of amino acid substitutions and $\psi(CH_2NH)$ alkylation yielded [βAla[8],$\psi(CH_2N(CH_2)_2CH_3)$[9],Phe[10]]NKA_{4-10} (**21**) which has very high affinity for the HUB NK_2 receptor. This compound inhibited [^{125}I]NKA binding with an IC_{50} = 1 nM which is equal to the receptor affinity of NKA. Compound **21** also shows very weak partial agonism of PI turnover (≤5% of NKA maximum) which makes this the most potent member of a new class of NKA ligands: $\psi(CH_2NR)$[9]-NKA_{4-10} analogues which potently antagonize NKA binding and possess minimal partial agonist activity.

Introduction

The tachykinins are a family of peptides containing the C-terminal sequence Phe-Xxx-Gly-Leu-Met-NH_2. The mammalian tachykinins, substance P, neurokinin A, and neurokinin B (Figure 1), are thought to produce most or all of their physiological and pharmacological effects through binding to and activation of NK_1, NK_2, and NK_3 tachykinin receptors, respectively.[1,2] Antagonists of these receptors are starting to appear in the literature (Figure 2), and there is great promise that these antagonists will be useful therapeutic agents for such disorders as asthma, urinary incontinence, gut hypermotility, inflammation, and other peripheral inflammatory conditions.[3-5]

It has been reported that modification of bombesin by introduction of the reduced amide bond at the C-terminus yields a potent bombesin antagonist.[14,15] The $\psi(CH_2NH)$ moiety has subsequently been used to generate antagonists for the gastrin,[16,17] substance P,[18,19] and secretin[20] receptors. Due to the therapeutic potential of selective NKA antagonists, we studied the $\psi(CH_2NH)$ amide bond replacement as a tool for converting NKA_{4-10} into an NK_2

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
substance P

His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
neurokinin A

Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
neurokinin B

Figure 1. Structures of the mammalian tachykinins.

* Author to whom correspondence should be addressed. Present address: Alkermes Inc., 64 Sidney Street, Cambridge, MA 02139.

† This manuscript is dedicated to Professor Ralph Hirschmann on the occasion of his 70th birthday in honor of the invaluable contributions he has made to peptide research and will continue to make to pharmaceutical research.

‡ The abbreviations used for the amino acids follow the IUPAC-IUB Commission on Biochemical Nomenclature guidelines (*J. Biol. Chem.* 1989, *264*, 673–688). Other abbreviations used are: NKA, neurokinin A; Boc, *tert*-butyloxycarbonyl; THF, tetrahydrofuran; DMF, dimethylformamide; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; HOAc, acetic acid; RP-HPLC, reversed-phase high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; AAA, amino acid analysis; PI, phosphatidylinositol; HUB, hamster urinary bladder; SAR, structure–activity relationships; and SEM, standard error of the mean.

§ Present address: Afferon Corp., P.O. Box 35604, Tucson, AZ 85740.

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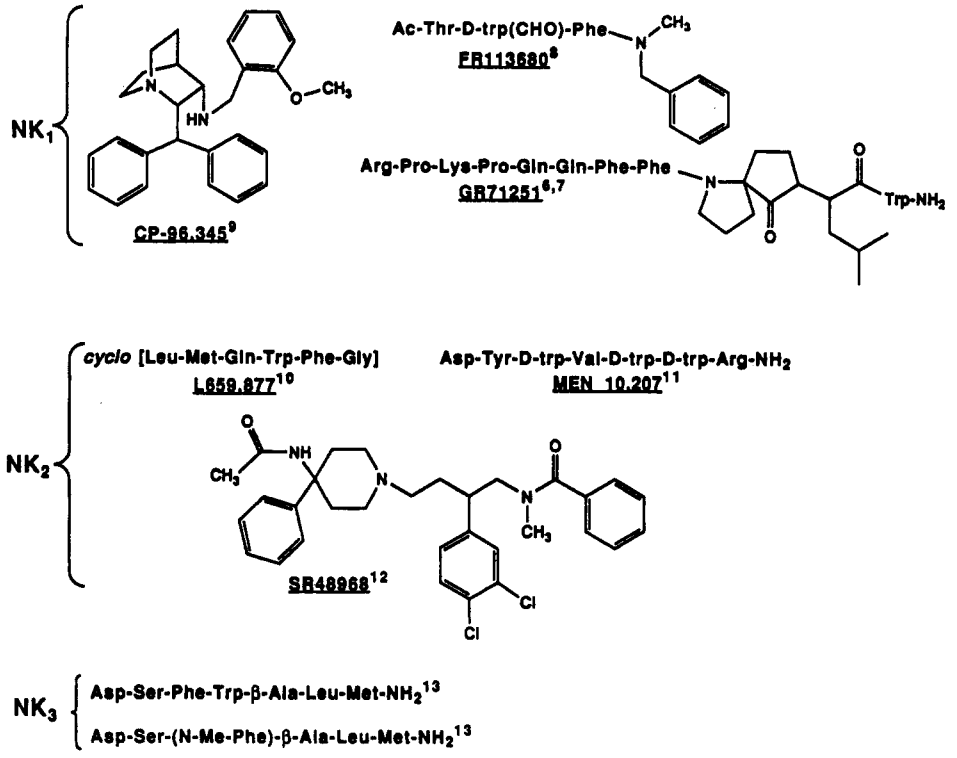

Figure 2. Antagonists of the mammalian tachykinin receptors.

antagonist. In this study, each of the amide bonds of [Leu^{10}]NKA$_{4-10}$ was systematically replaced with the ψ(CH$_2$NH) group and the activity of each pseudopeptide at the hamster urinary bladder (HUB) NK$_2$ receptor was determined.[21a] Although there have been numerous studies on the effects of *N*-alkyl amide bonds upon peptide activity and conformation, there have been no reports of *N*-alkyl reduced amide bonds and the effects of this alkylation upon the activities of ψ(CH$_2$NH)-containing peptides. This report also describes the synthesis of ψ(CH$_2$NR) amide bond replacements and the effects of *N*-alkyl substituents upon the affinity and activity of ψ(CH$_2$NH) analogues of NKA$_{4-10}$.[21b]

Experimental Section

Materials. 4-Methylbenzhydrylamine hydrochloride resin (1.1 mequiv/g) and Boc-protected amino acids were obtained from Peptides International (Louisville, KY). The protecting groups for amino acid side chains were as follows: Asp, cyclohexyl and Ser, benzyl. All reagents and solvents were ACS grade or better and used without further purification. [[^{125}I]iodohistidyl^1]-neurokinin A and [^{3}H]*myo*-inositol were obtained from Amersham.

Amino Aldehydes. The Boc-protected amino aldehydes were prepared by a modification of the method of Fehrentz and Castro.[22] The Boc-amino acids were first reacted with *N,N'*-carbonyldiimidazole (1.1 equiv) in either anhydrous ether or THF for 30 min at room temperature. To this reaction was then added a solution of *N,O*-dimethylhydroxylamine hydrochloride (1.5 equiv) and DIEA (1.5 equiv) in DMF (5 mL). The reaction was stirred at room temperature overnight. The reaction was diluted with ether and washed with 0.5 N HCl, saturated aqueous NaHCO$_3$, and saturated aqueous NaCl. The organic phase was dried over MgSO$_4$, filtered, and concentrated in vacuo to yield the Boc-protected amino acid *N,O*-dimethylhydroxamates. The hydroxamate was then reduced to the corresponding aldehyde with lithium aluminum hydride according to the published procedure. The crude Boc-amino aldehyde was used immediately in the reductive alkylation reaction without further purification. Boc-glycinal was prepared from *N*-Boc-allylamine by ozonolysis followed by reductive cleavage of the ozonide with zinc in acetic acid.[23]

Peptide Synthesis. Peptides were prepared by the solid phase technique using 4-methylbenzylhydrylamine hydrochloride resin (0.5 mmol) with an ABI 430A synthesizer. Amino acid residues were incorporated via preformed symmetrical anhydrides according to standard protocols provided by the manufacturer. The amino acids were added sequentially until the site of the reduced amide bond was reached. At that point, the N-terminal

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Boc group was cleaved with TFA and the resulting trifluoroacetate salt was neutralized with DIEA according to instrument protocol. A solution of the Boc-amino aldehyde (approx. 2.5 equiv) in 1% HOAc/DMF (10 mL) was introduced into the reaction vessel via the resin sampling line, followed by a solution of sodium cyanoborohydride (4 equiv) in DMF (2 mL). The reaction vessel was vortexed for 3 h and drained, and the resin was washed three times with DMF. Conversion of the secondary amine to a tertiary amine was accomplished in an analogous fashion except that the appropriate aldehyde was used in place of the Boc-amino aldehyde. The synthesis of the peptide was then completed using the automated cycles as before.

Peptide Cleavage and Global Deprotection. Cleavage of the peptide from the resin support and removal of the protecting groups was accomplished with anhydrous hydrogen fluoride containing anisole (HF–anisole–resin 10 mL:1 mL:1 g) at 0 °C for 45 min. The HF was removed in vacuo, and the residue was stirred in ether and suction-filtered to yield a solid. The crude peptide was extracted from the solid with 30% aqueous acetic acid and isolated by lyophilization.

Purification. The crude peptides were purified by semi-preparative RP-HPLC with a Vydac C18 column (10 μm, 2.2 × 25 cm) eluting with a linear acetonitrile gradient containing 0.1% TFA (v/v). Purifications were performed on a Waters Delta Prep 3000 equipped with a Waters 490E multiwavelength detector, a Goerz Metrawatt SE400 four-channel recorder, and an Isco Foxy fraction collector. The fractions corresponding to the major product peak were analyzed by analytical HPLC and those fractions of desired purity were pooled and lyophilized to yield the final product. Analytical RP-HPLC was performed with a Vydac C18 column (5 μm, 4.6 × 250 mm) using the same linear acetonitrile gradient as used on a preparative scale on a Waters 600 HPLC equipped with a Waters 712 WISP, a Waters 481 LC spectrophotometer, and a Waters 740 data module.

Characterization. Peptide purity was checked by analytical HPLC in two systems: aqueous acetonitrile containing either 0.1% TFA (v/v) or 0.5 mM tetrabutylammonium phosphate. Fast atom bombardment mass spectrometry (FAB-MS) was performed on a ZAB2-SE reverse geometry, double focusing mass spectrometer (VG Analytical, Ltd.). The spectrometer is equipped with a cesium ion gun operated at 20–25 kV. The ion source accelerating potential was held constant at 8 kV. The matrix sources used for analysis were either glycerol–thioglycerol or *m*-nitrobenzyl alcohol. Peptides were hydrolyzed in vacuo with 6 N HCl (110 °C) using a Waters Pico-Tag workstation. Hydrolyzates were analyzed using a Beckman 6300 amino acid analysis system.

Biological Assays. Receptor binding assays were performed with minced hamster urinary bladder tissue which had been homogenized in and washed with Tris buffer.[24,36] Filtration binding assays were performed in Tris buffer at room temperature using [[^{125}I]iodohistidyl^{1}]neurokinin A according to previously published procedures.[24,25,36] Chopped bladder tissue was labeled for 1 h at 37 °C with [^{3}H]*myo*-inositol, and phosphatidylinositol (PI) turnover was determined by quantitating total inositol phosphates by a modification of the published procedure[26] in which the assay is conducted at room temperature in Krebs–HEPES (20 mM) buffer and the ion exchange columns are eluted sequentially with water (10 mL), 5 mM disodium tetraborate–60 mM sodium formate (5 mL) and 1 M ammonium formate–0.1 M formic acid (10 mL).

Results and Discussion

Chemistry. The methodology for the introduction of the ψ(CH₂NH) moiety as an amide bond replacement in a peptide backbone is well established in the literature.[27,28] Using these procedures, the replacement of each of the amide bonds in [Leu^{10}]NKA$_{4-10}$ was accomplished to provide the ψ(CH₂NH) analogues shown in Table I. The only problematic replacement anticipated was the N-terminal Asp-Ser sequence. Selective formation of the α-carboxaldehyde of a β-ester derivative of Boc-L-aspartic acid is a difficult synthetic manipulation. This problem was avoided, however, by using succinic semialdehyde in the reductive alkylation reaction to provide **11** as the final product. It is known that succinyl-NKA$_{5-10}$ retains significant activity;[29] therefore, **11** is a logical alternative to the Aspψ(CH₂NH)Ser compound. The very poor affinity of **11** for the NK$_2$ receptor showed that further synthetic efforts toward the Aspψ(CH₂NH)Ser analogue were not justified.

Table I. IC$_{50}$ Values for Competitive Inhibition of [^{125}I]NKA Binding to Hamster Urinary Bladder

peptide	structure	IC$_{50}$, nM ± SEM
1	substance P	148 ± 19
2	neurokinin A	1.0 ● 0.2
3	neurokinin B	18 ± 4
4	Asp-Ser-Phe-Val-Gly-Leu-Met-NH$_2$ (NKA$_{4-10}$)	6 ± 1
5	Asp-Ser-Phe-Val-Gly-Leu-Leu-NH$_2$	136 ± 20
6	Asp-Ser-Phe-Val-Gly-Leuψ(CH$_2$NH)Leu-NH$_2$	115 ± 8
7	Asp-Ser-Phe-Val-Glyψ(CH$_2$NH)Leu-Leu-NH$_2$	>10^4
8	Asp-Ser-Phe-Valψ(CH$_2$NH)Gly-Leu-Leu-NH$_2$	5662 ± 333
9	Asp-Ser-Pheψ(CH$_2$NH)Val-Gly-Leu-Leu-NH$_2$	>10^4
10	Asp-Serψ(CH$_2$NH)Phe-Val-Gly-Leu-Leu-NH$_2$	>10^4
11	Sucψ(CH$_2$NH)Ser-Phe-Val-Gly-Leu-Leu-NH$_2$	1652 ± 175
12	Asp-Ser-Phe-Val-Gly-Leuψ(CH$_2$NCH$_3$)Leu-NH$_2$	193 ± 14
13	Asp-Ser-Phe-Val-Gly-Leuψ(CH$_2$NCH$_2$CH$_3$)-Leu-NH$_2$	277 ± 20
14	Asp-Ser-Phe-Val-Gly-Leuψ(CH$_2$N(CH$_2$)$_2$CH$_3$)-Leu-NH$_2$	103 ± 13
15	Asp-Ser-Phe-Val-Gly-Leuψ(CH$_2$N(Iva))Leu-NH$_2$	162 ± 21
16	Asp-Ser-Phe-Val-Gly-Leuψ(CH$_2$NH)Met-NH$_2$	50 ± 4
17	Asp-Ser-Phe-Val-Gly-Pheψ(CH$_2$NH)Leu-NH$_2$	>10^4
18	Asp-Ser-Phe-Val-Gly-Leuψ(CH$_2$NH)Phe-NH$_2$	9 ± 2
19	Asp-Ser-Phe-Val-βAla-Leuψ(CH$_2$NH)Leu-NH$_2$	108 ± 8
20	Asp-Ser-Phe-Val-β-Ala-Leuψ(CH$_2$NH)Phe-NH$_2$	10 ± 1
21	Asp-Ser-Phe-Val-β-Ala-Leuψ(CH$_2$N(CH$_2$)$_2$CH$_3$)-Phe-NH$_2$	1 ± 0.2

The formation of diastereomeric peptides due to apparent racemization at the α-carbon of the Boc-amino aldehyde component was observed in several cases. An important factor in the observed racemization was the conditions for the reductive alkylation reaction. If the Schiff base was allowed to form for 1 h prior to addition of the sodium cyanoborohydride, both **6** and **8** were isolated as approximately 1:1 mixtures of diastereomers. Formation of the Schiff base in the presence of sodium cyanoborohydride reduced the amount of diastereomeric peptide to 5–8%. In the case of **6** and **8**, the diastereomers were separated by RP-HPLC and shown to be identical by AAA and FAB-MS. However, with **6**, only the major diastereomer showed affinity for the NK$_2$ receptor; both peptides were substrates for aminopeptidase M, indicating that they were diastereomers and not α- and β-aspartyl peptide isomers.[30]

The ψ(CH₂NCH₃) reduced amide bond analogue **12** was easily prepared by reductive alkylation of *N*-methyl-L-

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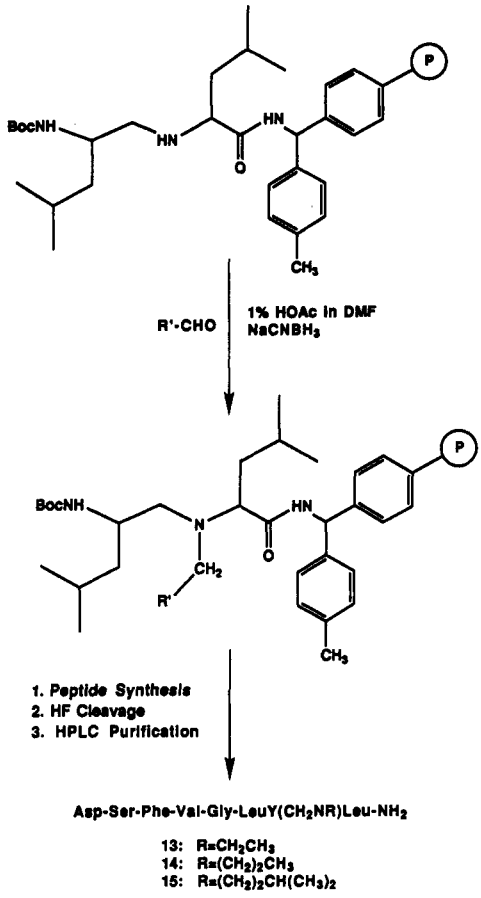
Scheme I. Synthesis of *N*-Alkyl $\psi(CH_2NR)$ Analogues of [Leu[10]]NKA_{4-10}



Asp-Ser-Phe-Val-Gly-LeuY(CH_2NR)Leu-NH_2

13: R=CH_2CH_3
14: R=$(CH_2)_2CH_3$
15: R=$(CH_2)_2CH(CH_3)_2$

leucyl-4-methylbenzhydrylamine resin with Boc-L-leucinal. Although others have reported significant (40%) diastereomer formation during the reductive alkylation of the secondary amine of L-proline with Boc-L-phenylalaninal,[31] we did not observe any contaminating diastereomer of **12** by analytical RP-HPLC (two solvents systems). It is very unlikely that HPLC in two different solvent systems would have failed to detect a significant diastereomeric impurity in **12**; therefore, the reductive alkylation reaction appears to have proceeded without significant racemization. The discrepancy between our observations and the previous report is most likely due to the different reactants: Boc-Phenylalaninal versus Boc-L-leucinal and L-proline versus *N*-methyl-L-leucine.

Conversion of the secondary amine of the $\psi(CH_2NH)$ moiety to the tertiary amine $\psi(CH_2NR)$, where R is not methyl, was accomplished by a second round of reductive alkylation as shown in Scheme I. Although previous work on $\psi(CH_2NH)$ analogues of peptides indicated that alkylation of the reduced amide bond nitrogen may be possible,[32] this is the first report, to our knowledge, of the synthesis of N-alkylated reduced amide bond containing peptides. Although it has been reported that the $\psi(CH_2NH)$ moiety is very resistant to acylation,[27,33] this secondary amine was reactive to α-unsubstituted aldehydes. Under the usual conditions of the reductive alkylation reaction a crude product mixture was obtained in which the major component was the desired product. It is also of interest that although the synthesis of the $\psi(CH_2NH)$ moiety produced compound **6** containing from 5 to 8% of a contaminating diastereomer, there was no indication of any diastereomeric contaminants in **13**, **14**, and **15** (R = ethyl, propyl, and isovaleryl, respectively) which were derived from the same reaction sequence as **6**. Whereas the diastereomers of **6** were very difficult to resolve by HPLC, we propose that the *N*-alkyl substituent has a marked effect upon the HPLC retention times of the diasteromers resulting in a greater separation. The 5–8% diastereomer impurity is then easily removed during purification. Currently, this methodology appears to be limited to α-unsubstituted aldehydes since we were unable to obtain any desired product when benzaldehyde was used in the reductive alkylation of the $\psi(CH_2NH)$ moiety. This failure is probably due to steric hindrance of the Schiff base formation, but the limitations on this reaction will need to be studied further.

Table II. Amino Acid Analyses of $\psi(CH_2NR)$ Analogues of NKA_{4-10}

peptide	Asp	Ser	Phe	Val	Gly	Leu	% peptide
6[a]	1.08	1.01	1.01	0.89	1.02	–	58.0
7[b]	1.06	0.75	1.01	0.96	–	1.01	50.6
8[a]	0.98	0.90	0.76[c]	–	–	2.02	75.3
9[b]	1.00	0.85	–	–	0.40[c]	2.00	62.7
10[a]	1.01	–	–	0.93	1.02	2.05	58.2
11[a]	–	–	0.96	0.98	1.03	2.03	86.6
12[a]	1.01	0.89	0.98	1.00	1.02	–	53.5
13[a]	1.03	0.93	0.98	0.94	1.05	–	69.0
14[b]	1.07	1.01	0.99	0.92	1.02	–	65.1
15[b]	1.06	1.00	0.99	0.95	1.01	–	56.4
16[a]	1.22	0.88	0.99	0.98	1.03	–	50.6
17[b]	1.15	1.09	1.03	0.94	1.04	–	62.6
18[b]	1.03	0.93	0.99	0.97	1.01	–	60.9
19[b]	1.05	0.99	0.95	0.51[d]	–	–	56.1
20[e]	1.01	0.93	0.98	0.60[d]	–	–	63.7
21[b]	1.02	0.93	0.98	0.60[d]	–	–	63.0

[a] 24-h hydrolysis with 6 N HCl. [b] 48-h hydrolysis. [c] Low due to adjacent reduced dipeptide. [d] Low values, see Results and Discussion in text. [e] 72-h hydrolysis.

Analytical. The peptides prepared were characterized by AAA (Table II) and FAB-MS (Table III). In agreement with a previous report,[33] several peptides in Table II gave low values for residues adjacent to the Xxx-$\psi(CH_2NH)$-Yyy moiety. In the case of **9**, proton NMR was used to confirm that glycine was present in an equimolar ratio to the other residues. The other problem sequences were those containing the β-Ala[8] residue (compounds **19**, **20**, and **21**). In all three compounds, Val[7] was consistently low after 48-h hydrolysis and in one experiment only reached a value of 0.79 after 113-h hydrolysis. This problem appears to be sequence-specific and not indicative of a deletion impurity in the peptides since there is no evidence for this by FAB-MS or analytical HPLC. The expected $(M + H)^+$ value from FAB-MS was observed for each compound (Table III). The purity of each peptide was determined by analytical RP-HPLC in two solvent systems (Table III). All compounds were greater than or equal to 98% pure, but in those examples where purity is less than 98%, FAB-MS did not show any impurities, and

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Table III. HPLC and Mass Spectral Analyses of $\psi(CH_2NR)$ Analogues of NKA_{4-10}

peptide	system I[a]		system II[b]		FAB-MS (M + H)$^+$	
	t_R, min	% purity	t_R, min	% purity	found	calcd
6	20.8[c]	98	20.0[d]	91	735	735
7	18.4[e]	98	19.3[f]	96	735	735
8	19.5[g]	95	22.8[g]	91	735	735
9	17.0[h]	98	20.0[h]	91	735	735
10	23.2[i]	94	21.4[i]	92	735	735
11	22.0[j]	98	20.7[j]	96	720	720
12	19.9[g]	98	26.7[g]	97	749	749
13	22.6[e]	98	26.1[f]	98	763	763
14	24.6[k]	98	22.9[l]	98	777	777
15	18.4[m]	98	23.3[n]	98	805	805
16	16.6[g]	98	22.4[g]	98	753	753
18	21.4[k]	98	19.4[o]	98	769	769
19	21.0[k]	98	24.4[p]	98	749	749
17	21.2[k]	98	26.2[p]	87	769	769
20	20.1[q]	98	23.3[j]	98	783	783
21	23.2[o]	98	23.4[l]	98	825	825

[a] Solvent A: 0.1% TFA in distilled H_2O; solvent B: 0.1% TFA in 60% acetonitrile/40% distilled H_2O; 1 mL/min; linear gradient (25 min) and then isocratic elution (5 min). [b] Solvent A: 0.5 mM tetrabutylammonium phosphate in distilled H_2O; solvent B: 0.5 mM tetrabutylammonium phosphate in 60% acetonitrile/40% distilled H_2O; 1 mL/min; linear gradient (25 min) and then isocratic elution (5 min). [c] 80% A–50% A. [d] 70% A–50% A. [e] 80% A–20% A. [f] 70% A–15% A. [g] 75% A–10% A. [h] 70% A–10% A. [i] 80% A–10% A. [j] 70% A–30% A. [k] 75% A–35% A. [l] 55% A–15% A. [m] 60% A–20% A. [n] 50% A–20% A. [o] 65% A–25% A. [p] 70% A–40% A. [q] 70% A–20% A.

the contaminating peaks observed by RP-HPLC are assumed to be diastereomers. This was shown conclusively in the case of **6** by isolating the impurity and characterizing it by AAA and FAB-MS.

Biology. Receptor Binding. As can be seen from the competitive binding assay data in Table I, sequential replacement of the amide bonds of $[Leu^{10}]NKA_{4-10}$ with the $\psi(CH_2NH)$ moiety drastically reduces receptor affinity in all cases except for **6**. Although the affinity of **6** is at least 100× less than NKA at the NK_2 receptor, it is equipotent to $[Leu^{10}]NKA_{4-10}$ (**5**). The fact that **6** and **5** bind with equal affinity shows that introduction of the $\psi(CH_2NH)$ amide bond replacement has no effect upon ligand affinity.

Further inspection of the data in Table I shows that previous SAR studies of NKA are also applicable to **6**. NKA_{4-10} binds with greater affinity than $[Leu^{10}]NKA_{4-10}$ and this same result is seen with **16** where Leu^{10} is replaced with methionine of the natural sequence and results in enhanced binding. It has been shown that replacement of Gly^8 of NKA_{4-10} with β-Ala can result in an enhancement of activity at the NK_2 receptor.[29,34] In the rat vas deferens, $[\beta\text{-}Ala^8]NKA_{4-10}$ is 8.5× more active than NKA_{4-10} whereas in rabbit pulmonary artery, this compound is equipotent to NKA_{4-10}. $[\beta\text{-}Ala^8]NKA_{4-10}$ does show enhanced selectivity for NK_2 receptors versus either NK_1 or NK_3 receptors. When Gly^8 of **6** is replaced with $\beta\text{-}Ala^8$ to give **19**, essentially no increase in receptor affinity is seen in agreement with the previous observation for $[\beta\text{-}Ala^8]\text{-}NKA_{4-10}$ in rabbit pulmonary artery. Although Phe^{10} analogues of NKA and NKA_{4-10} have not been reported, it has been shown that substitution with Phe at the C-terminus of a series of NK_2 antagonists based upon the NKA_{4-10} sequence does increase binding affinity.[35] It is not surprising, therefore, to see that **18** shows an increase in binding over **6**. However, the analogous substitution of Phe for Leu^9 abolishes affinity. On the basis of these results, compound **19** was prepared in which Gly^8 and Leu^{10} were simultaneously replaced by β-Ala and Phe, respectively. The $\beta\text{-}Ala^8$ analogue **19** showed no increase in binding with respect to compound **6**, and the Phe^{10} analogue **18** showed a 13× increase in receptor affinity. When these two modifications were combined to give **20**, the NK_2 affinity was statistically identical to **18**. Again, the β-Ala residue at position 8 has no effect upon affinity. Compound **20** still requires testing in other NK_2 tissues to determine whether affinities depend upon the tissue under study.

The other structural modification studied was alkylation of the $\psi(CH_2NH)$ secondary amine and the effect of this modification upon affinity and activity. As shown in Table I, the alkyl substituent on the $\psi(CH_2NH)$ secondary amine of **6** was systematically varied from H to methyl (**12**), ethyl (**13**), propyl (**14**), and isovaleryl (**15**). The affinity of these analogues for the NK_2 receptor versus **6** decreased 1.7× and 2.4× for the methyl and ethyl groups, respectively. However, for **14**, where R is propyl, the affinity is equal to **6**, indicating a favorable interaction of the longer propyl substituent with the receptor. As shown by **15**, further increasing the steric bulk of the propyl substituent by addition of methyl groups does not further enhance receptor binding. In the case of **21**, introduction of the propyl substituent onto the $\psi(CH_2NH)$ nitrogen did increase receptor affinity. This observation is contrary to the previous one in which the $\psi(CH_2NR)$ *N*-propyl substituent on compound **6** yielded **14** with equal receptor affinity. Compound **21** shows a 10× increase in affinity versus **20** and a greater than 100× increase in receptor affinity with respect to **6**. Apparently, either the $\beta\text{-}Ala^8$ or Phe^{10} modification allows a bound conformation for **21** which results in a favorable interaction of the propyl group with the receptor and enhances binding with respect to **20**. The same modification of **6** to yield **14**, however, did not increase affinity which points out the importance of $\beta\text{-}Ala^8$ and Phe^{10}.

An understanding of the interactions of these ligands with the receptor is beyond the scope of this report. Currently, there is no firm data regarding the solution conformation of NKA and nothing is known about the bound conformation of the ligand at the NK_2 receptor. Molecular modeling could be used to predict possible active conformations of NKA and the $\psi(CH_2NR)$ pseudopeptides; however, the flexibility of these compounds makes these types of studies questionable as to their relevance. Future studies will be directed toward developing a model of the NK_2 receptor based upon recent reports on the structural features of bacteriorhodopsin.[39] The introduction of constraints into the compounds will greatly limit the possible conformations and permit more relevant models

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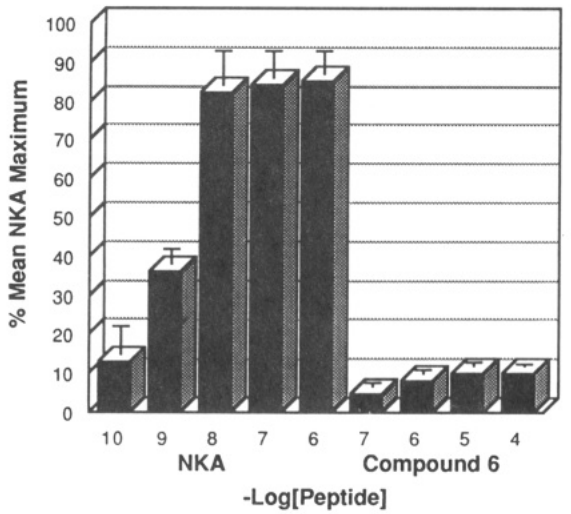

Figure 3. The effects of NKA and compound **6** on PI turnover in HUB. Values are mean ± SEM for seven (NKA) and six (compound **6**) experiments. In a typical experiment, the PI turnover ratio (inositol phosphates cpm/extraction organic phase cpm) was 0.21 ± 0.01 for basal and 1.60 ± 0.12 for maximum stimulation (1 μM NKA).

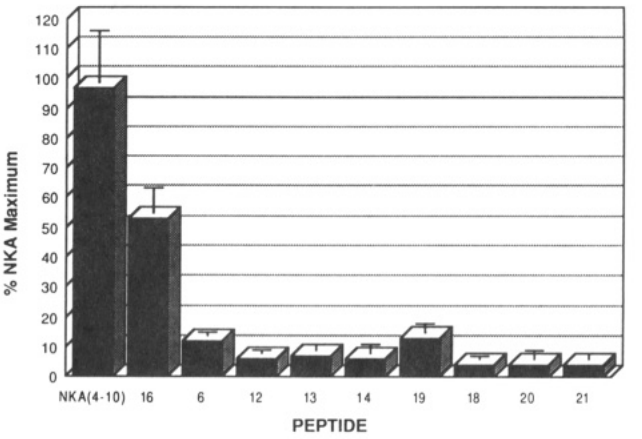

Figure 4. Phosphatidylinositol turnover in minced HUB tissue. Values are mean ± SEM of at least two experiments in triplicate. Values are for 10 μM concentration of each peptide, the highest concentration that was tested. In a typical experiment, the PI turnover ratio (inositol phosphates cpm/extraction organic phase cpm) was 0.1 ± 0.01 for basal and 0.94 ± 0.1 for maximum stimulation (1 μM NKA).

of the solution conformations. These can then be used with the receptor model to arrive at a proposed bound conformation of the ligands at the receptor.

Receptor Activity. Those compounds which showed affinity for the receptor were then evaluated for agonist activity according to their ability to stimulate PI turnover in HUB tissue. [^{3}H]Inositol was used in the assay, and the incorporation of radiolabeled compound into inositol phosphates was measured. The base line level was determined by measuring PI turnover in the absence of the receptor agonist, NKA. NKA was then used to generate the dose–response curve which gave a value for the maximum PI turnover response to this agonist. The agonist activity of the compounds was then expressed as an EC_{50} for a full agonist or as a percent maximum stimulation for a partial agonist.

A typical dose–response for NKA is shown in Figure 3. In this experiment, NKA stimulated PI turnover with an EC_{50} value of 2 nM. Also shown in Figure 3 is the dose–response curve for compound **6**. This compound behaves as a very weak partial agonist as indicated by a maximal response which is only approximately 10% of the NKA response. In HUB, this very weak partial agonism results in compound **6** behaving as a competitive antagonist of NKA-stimulated PI turnover with a $pA_2 = 6.7$.[22]

The ability of each of the compounds to stimulate PI turnover is represented in Figure 4. Values are reported as the percent of maximum response elicited by NKA (100%). Basal PI turnover in the absence of any stimulation is represented as 0%. As can be seen, all of the NKA_{4-10} analogues cause some stimulation of PI turnover but in the cases of compounds **12**, **13**, **14**, **18**, **20**, and **21**, this stimulation is very weak and is, at best, only 5% of the maximum PI response. The observation that these compounds both competitively inhibit [^{125}I]NKA binding to the HUB NK_2 receptor and stimulate the PI response in this tissue very weakly shows that $\psi(CH_2NR)^9$ analogues of NKA_{4-10} are a new class of NKA ligands with high affinity and very weak partial agonist activity.

It is noteworthy that certain structural modifications attenuate partial agonist activity. Introduction of the $\psi(CH_2NH)^9$ moiety into NKA_{4-10} to give **16** decreased the maximal PI response but **16** still retains significant (approximately 50%) partial agonism. When the C-terminal Met of **16** is replaced with Leu (compound **6**), the partial agonist activity is further decreased to approximately 10%. Conversion of the secondary amine of the $\psi(CH_2NH)$ group of **6** to a tertiary amine results in further attenuation of the partial agonism. Compound **14** represents the best compound in the $Leu^9\psi(CH_2NR)Leu^{10}$ series since it has a receptor affinity which is equal to **6** but shows weaker partial agonism than **6**. Replacement of Gly^8 in **6** with β-Ala to yield **19** has no effect upon either receptor affinity or agonist activity. However, when Leu^{10} of **6** is replaced by Phe (**18**), there is a marked increase in receptor affinity and a very low level of partial agonism. In agreement with the previous observations, replacement of Gly^8 in **18** with β-Ala produced **20** which was no different from **18** in affinity and activity. The best overall compound is **21**. This compound has very high affinity for the HUB NK_2 receptor and, as can be seen in Figure 4, has almost negligible partial agonist activity.

There are several possible explanations for the persistent weak partial agonism which has been observed. It is not surprising that the introduction of the $\psi(CH_2NR)$ moiety into the C-terminus of NKA has a dramatic effect upon activity. Previous reports have demonstrated that the C-terminus of the tachykinins is critical for activity.[29,40,41] At this point, it is still unclear as to whether this effect is conformational, electronic, or both. The replacement of the planar amide bond with the tetrahedral and more flexible $\psi(CH_2NR)$ moiety obviously increases the conformational freedom of the ligand and may allow a bound conformation which fails to activate the receptor. The

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weak agonism may be due to a small population of ligands bound in an agonist conformation. The introduction of the alkyl substituents onto the $\psi(CH_2NH)$ group may introduce further steric constraints and decrease the number of ligands which can bind in an agonist conformation. The replacement of the amide bond with the $\psi(CH_2NH)$ group also introduces a basic nitrogen into the peptide backbone. The protonation state of this nitrogen may determine whether the ligand is bound in an agonist (neutral) or antagonist (charged) conformation. The attenuation of partial agonism upon introduction of alkyl substituents onto the $\psi(CH_2NH)$ nitrogen may simply be due to a shift in the pK_a of this amine. Attempts to determine a pK value by NMR for the $\psi(CH_2NH)$ amine in 6 were prevented by the poor solubility of the compound. A complex interplay of conformation and charge effects could also be at work here; more detailed studies are required before a reasonable explanation can be proposed. Finally, the weak partial agonism observed could simply be due to activation of other receptors, such as NK_1, at high concentrations of these compounds. This question could be answered using cell cultures which express only the NK_2 receptor class.

Further studies on compound 21 are currently in progress. Its activity in other tissues will be of interest since 6 behaves as an antagonist in HUB but acts as a full agonist in guinea pig trachea and rabbit pulmonary artery.[3,36] This discrepancy may be due to receptor subtypes[37,38] and it will be of interest to learn if 21 exhibits the same anomalous behavior. The high receptor affinity of 21 for the HUB NK_2 receptor should also be observed in more therapeutically relevant receptor models such as the human jejeunal and tracheal NK_2 receptors.

Conclusions

Sequential replacement of each of the amide bonds in [Leu^{10}]NKA_{4-10} yielded [$\psi(CH_2NH)^9$,Leu^{10}]NKA_{4-10} (6) with significant affinity for the HUB NK_2 receptor and very weak partial agonist activity. N-Alkylation of the $\psi(CH_2NH)^9$ moiety resulted in an attenuation of the partial agonist activity. Replacement of Leu^{10} in 6 with Phe yielded 18 with enhanced receptor affinity (IC_{50} = 9 nM). The most potent compound was obtained by incorporating both the Phe^{10} and $\psi(CH_2NH)$ alkylation modifications into the same molecule to yield 21 with an affinity for the HUB NK_2 receptor equal to NKA. These *N*-alkyl $\psi(CH_2NR)$ analogues of NKA_{4-10} represent a new class of partial agonists/antagonists at the HUB NK_2 receptor and may be useful in the design of antagonists for other tachykinin receptors.