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Conformation–Activity Relationship of Tachykinin Neurokinin A (4–10) and of Some [Xaa^8] Analogues

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ABSTRACT: NKA(4–10), the C-terminal heptapeptide fragment (Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2) of tachykinin NKA, is more active than the parent native compound in the interaction with the NK-2 receptor. Substitution of Gly^8 with the more flexible residue β-Ala^8 increases its selectivity with respect to other two known receptors (NK-1 and NK-3), whereas substitution with either D-Ala^8 or GABA^8 deprives the peptide of its biological activity. These findings can be interpreted by a conformational analysis based on NMR studies in DMSO-d_6 and in a DMSO-d_6/H_2O cryoprotective mixture combined with internal energy calculations. NKA(4–10) is characterized by a structure containing a type I β-turn extending from Ser^5 to Gly^8, followed by a γ-turn centered on Gly^8, whereas for [β-Ala^8]NKA(4–10) is possible to suggest a type I β-turn extending from Ser^5 to β-Ala^8, followed by a C_8 turn comprising β-Ala^8 and Leu^9 and by another β-turn extending from β-Ala^8 to the terminal NH_2. The preferred conformation of [β-Ala^8]NKA(4–10) is not compatible with models for NK-1 and NK-3 agonists proposed on the basis of rigid peptide agonists [Levian-Teitelbaum et al. (1989) *Biopolymers 28*, 51–64; Sumner & Ferretti (1989) *FEBS Lett. 253*, 117–120]. The preferred solution conformation of [β-Ala^8]NKA(4–10) may thus be considered as a likely bioactive conformation for NK-2 selective peptides.

Neurokinin A (NKA)[1] is a neuropeptide of sequence His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2, expressed by mammalian neurons (Kimura et al., 1983; Kanagawa et al., 1983) in the same precursor, γ-preprotachykinin A (Nawa et al., 1983), containing the sequences of substance P (SP) and neurokinin B (NKB). The wide spectrum of action of NKA and its potential usefulness in the field of antiasthmatic drugs (Nawa et al., 1984) has stimulated many structure–activity relationship (SAR) investigations (Dion et al., 1987a;

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[1] Abbreviations: Aib, three-letter code for aminoisobutyric acid; β-Ala, β-alanine; BFGS, Broyden–Fletcher–Goldfarb–Shanno algorithm; DMSO-d_6, perdeuteriodimethyl sulfoxide; DQF-COSY, double-quantum-filtered correlation spectroscopy; EM, energy minimization calculations; GABA, γ-aminobutyric acid; GPI, guinea pig ileum; NK-1, substance P receptor; NK-2, NKA receptor; NK-3, NKB receptor; NKA, neurokinin A; NKB, neurokinin B; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RPA, rabbit pulmonary artery; RPV, rat portal vein; SAR, structure–activity relationship; SP, substance P; TMS, tetramethylsilane.

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Table I: Biological Activities[a] of NKA(4–10) (I), [D-Ala[8]]NKA(4–10) (II), [GABA[8]]NKA(4–10) (III), and [β-Ala[8]]NKA(4–10) (IV)

peptide	GPI	RPA	RPV
I	7.43 ± 0.11	8.52 ± 0.14	6.79 ± 0.05
II	<5	6.66 ± 0.09	6.14 ± 0.11
III	6.91 ± 0.18	na[b]	na[b]
IV	6.61 ± 0.24	8.34 ± 0.07	6.01 ± 0.09

[a] pD_2 = −log EC_{50}; each value is the mean ± SEM of 4–6 determinations. [b] na = no activity up to 10 μM.

Rovero et al., 1989a). Studies of fragments have shown that deletions in the C-terminal part lead to almost complete inactivity, but deletion of the first three residues yields a fragment, Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 [NKA(4–10)], that is even more active than the parent native compound in the interaction with the NK-2 receptor (Dion et al., 1987b). Systematic substitution of each residue of NKA with Ala has shown that residues in positions 6 (Phe), 7 (Val), 9 (Leu), and 10 (Met) are essential for activity toward all three typical tachykinin receptors (NK-1, NK-2, and NK-3), whereas other positions can apparently tolerate substitution with Ala without a major loss of activity (Rovero et al., 1989a). Position 8 may play a crucial role since it separates the two pairs of residues most sensitive to substitution. A structural modification in lieu of Gly[8], whose side chain, presumably, is not relevant for surface recognition, may be thought of mainly in structural terms. Substitution with rigidifying residues such as D-Ala, Aib, or Pro does not lead to analogues endowed with outstanding activity and/or selectivity. Surprisingly, substitution of Gly[8] in NKA(4–10) with the more flexible β-Ala[8] residue (Salunke & Vijayan, 1984; Narita et al., 1986) leads to an analogue that is more active than the parent compound and, even more important, is the synthetic tachykinin with the highest known selectivity for the NK-2 receptor (Rovero et al., 1989b). These results pose the question whether the [β-Ala[8]]NKA(4–10) molecule, as a whole, is indeed more flexible than NKA(4–10), a fact that is difficult to reconcile with selectivity. Besides, experimental detection of relevant conformational differences between these two compounds may lead to a better understanding of the conformation–activity relationship of tachykinins and possibly to a more rational drug design of NK-2 agonists and antagonists.

Here we present a detailed conformational analysis of NKA(4–10) and of three synthetic analogues, [β-Ala[8]]-NKA(4–10), [D-Ala[8]]NKA(4–10), and [GABA[8]]NKA(4–10), based on ^1H NMR studies in two solvent systems, neat DMSO-d_6 and the cryomixture 90% DMSO-d_6 (v/v)/10% H_2O (v/v).

Materials and Methods

All peptides were prepared by conventional solid-phase methods and purified to homogeneity by gel filtration and preparative high-pressure liquid chromatography (Rovero et al., 1987). The biological activity data, summarized in Table I, were evaluated on in vitro tests selective for NK-1, NK-2, and NK-3 receptors, that is, (a) guinea pig ileal longitudinal muscle (GPI) for the NK-1 receptor (Dion et al., 1987), (b) endothelium-deprived rabbit pulmonary artery (RPA) for the NK-2 receptor (D'Orleans-Juste et al., 1985), and (c) rat portal vein (RPV) for the NK-3 receptor (Mastrangelo et al., 1986).

All samples for NMR measurements were approximately 3 mM. ^1H spectra were recorded at 500 MHz on a Bruker WM-500 spectrometer interfaced to an Aspect 2000 computer or at 400 MHz on a Bruker AM-400 spectrometer interfaced to an Aspect 3000 computer and referenced to internal TMS. 1D spectra were typically acquired in quadrature detection. The water signal was suppressed by a low-power selective irradiation in the homogated mode. 2D experiments were performed as follows. For phase-sensitive DQF-COSY (Piantini et al., 1982) and NOESY (Jeener et al., 1979) experiments, 512 experiments with 128 scans of 2048 points were performed over 6014 Hz of spectral width at 500 MHz and over 4500 Hz at 400 MHz. Time domain data matrices were all zero-filled to 4K in both dimensions, yielding a digital resolution of 2.7 Hz/point. Gaussian window functions were applied before transformation in both dimensions. Pure absorption NOESY spectra were obtained with different mixing times with a 10% random variation of mixing time to cancel scalar correlation effects. Values reported in Table IV correspond to a mixing time (τ_m) of 500 ms since at lower values cross peaks were too small for accurate measurements and extrapolation of the ratio NOE/τ_m vs τ_m (Majumdar & Hosur, 1990) showed that none of them is affected by spin diffusion even at the fairly high value of 500 ms. Classification of the effects into three categories (weak, medium, and strong of Table IV) corresponds to ranges 0–3.5 (w), 3.5–6.0 (m), and 6.0–12.5 (s), expressed as percentages with respect to diagonal peak intensity.

Irradiation of the ^1H_2O resonance in 2D experiments was carried out during the relaxation time and, for NOESY, also during the mixing time.

The united-atom (Alagona et al., 1984) and all-atom (Weiner et al., 1986) parametrizations of the AMBER force field were used in a series of energy minimization (EM) calculations. The computational procedure can be divided into the following steps: (i) a starting guess is generated from the NMR data, (ii) a united-atom EM calculation is performed by a quasi-Newton method, the Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm (Broyden, 1970; Fletcher, 1970; Goldfarb, 1970; Shanno, 1970), stopping when the gradient norm is 10^{-2} or less, (iii) nonpolar hydrogen atoms are added to the resulting structure, and an all-atom BFGS EM is run until a gradient norm of 10^{-3} is reached, and (iv) a final refinement is obtained by a full Newton–Raphson minimization, with a convergence criterion on the gradient norm of 10^{-6} or less.

Solvation effects may be relevant in solutions of high polarity, such as the media employed in the present study; in order to account for them, three different approaches were used: (i) a distance-dependent dielectric constant $\epsilon = r$, (ii) a fixed $\epsilon = 10$ value, and (iii) a damped distance-dependent value of $\epsilon = 20r$. The main effect of increasing the value of ϵ is a general increase in the average hydrogen-bond lengths, and consequently, a decrease of their stability, but the folding patterns here proposed are essentially insensitive to ϵ variations, whereas side chains and terminal regions are affected to a larger extent. All final calculations were performed with $\epsilon = 10r$.

Results and Discussion

Figure 1 shows a comparison of the partial (amide region) 400-MHz 1D spectra of (I) NKA(4–10), (II) [D-Ala[8]]NKA-(4–10), (III) [GABA[8]]NKA(4–10), and (IV) [β-Ala[8]]NKA-(4–10) in DMSO-d_6. It can be appreciated that the single substitution in position 8 brings about spectral changes reflecting an increased ordering for [β-Ala[8]]NKA(4–10), as suggested by the larger spread of chemical shifts and by the absence of degeneracies in the NH region of this compound with respect to the spectrum of NKA(4–10). The NH resonances of the other two analogues are also better resolved but

Table II: Chemical Shifts and Temperature Coefficients of Amidic Protons of NKA(4–10) (I), [D-Ala^8]NKA(4–10) (II), [GABA^8]NKA(4–10) (III), and [β-Ala^8]NKA(4–10) (IV) in DMSO-d_6[a]

peptide	residue	α	β, β'	γ, γ'	δ, δ'	others	NH
I	Asp^4	4.02	2.53, 2.66				
II	Asp^4	4.33	2.53, 2.70				
III	Asp^4	3.87	2.47, 2.62				
IV	Asp^4	3.70	2.43				
I	Ser^5	4.32	3.54, 3.86			5.01	8.53 (−6.7)
II	Ser^5	4.33	3.52			5.02	8.49 (−5.8)
III	Ser^5	4.29	3.53			5.02	8.45 (−6.0)
IV	Ser^5	4.23	3.50			5.03	8.41
I	Phe^6	4.56	2.84, 3.09				8.19 (−5.6)
II	Phe^6	4.55	2.81, 3.05				8.17 (−4.7)
III	Phe^6	4.54	2.86, 3.08				8.2 (−5)
IV	Phe^6	4.47	2.90, 3.09				8.33 (−6.3)
I	Val^7	4.12	1.99	0.89			8.13 (−10.1)
II	Val^7	4.13	1.98	0.82			7.99 (−7.2)
III	Val^7	4.07	1.95	0.82			8.1
IV	Val^7	4.02	1.98	0.85			8.17 (−9.5)
I	Gly^8	3.73					8.19 (−6.7)
II	Ala^8	4.26	1.19				8.11 (−6.4)
III	GABA^8	2.15	1.62	2.97, 3.12			7.97
IV	β-Ala^8	2.30	3.25				7.95 (−6.7)
I	Leu^9	4.31	1.45	1.61	0.88		8.00 (−6.8)
II	Leu^9	4.24	1.47	1.58	0.83, 0.86		8.10 (−5.5)
III	Leu^9	4.24	1.45	1.61	0.83, 0.89		8.01 (−6.0)
IV	Leu^9	4.18	1.46	1.65	0.85		8.27 (−7.0)
I	Met^{10}	4.25	1.80, 1.98	2.38		2.00	8.00 (−6.8)
II	Met^{10}	4.20	1.82, 1.90	2.36, 2.46		2.00	7.91 (−5.6)
III	Met^{10}	4.24	1.77, 1.94	2.39		2.02	7.95 (−6.0)
IV	Met^{10}	4.2	1.80, 1.93	2.38		2.20	8.02 (−6.5)
I	NH_2						7.05 (−5.0), 7.18 (−5.7)
II	NH_2						7.02 (−5.0), 7.12 (−4.2)
III	NH_2						7.08 (−5.0), 7.27 (−5.0)
IV	NH_2						7.08 (−2.6)

[a] Chemical shifts are in ppm referred to TMS at 297 K; temperature coefficients (in parentheses) are in ppb/K.

have chemical shifts very similar to those of NKA(4–10).

Identification of complete spin systems of all residues of the four peptides was based on DQF-COSY (Piantini et al., 1982) and complemented with NOESY experiments (Jeneer et al., 1979) when ambiguities arose. The identified amino acids were then sequentially ordered by resorting to NOEs between backbone protons and comparison with the known primary sequence. Finally, the pattern of NOEs was interpreted in structural terms.

Table II summarizes all chemical shifts of the four peptides in DMSO-d_6, with the temperature coefficients of the exchangeable protons. Most figures (also those of coupling constants, not shown) are consistent with literature values typical of the so-called random coil conformation (Wüthrich, 1976), as one can expect from a mixture of disordered conformations in fast equilibrium. It may be interesting to note, however, that the temperature coefficients are not uniform; some of them are low enough to hint at the existence of an equilibrium between exposed and hidden NHs, whereas that of the NH of Val^7 is significantly higher than average. This fact may be interpreted as an indication that Val^7 NH senses a major conformational transition in the temperature range examined.

The only way to get more significant conformational indications is from the measurement of nuclear Overhauser effects. This task is generally regarded as difficult or impossible for medium-sized peptides at high field since their correlation time brings about the condition $\omega^2\tau_c^2 = 1$ that makes all NOEs close to zero (Motta et al., 1987). We have been able to show, in the case of enkephalins (Motta et al., 1988), that this condition is not simply due to the rotational reorientation dictated by the molecular weight of these compounds but to the contribution of internal motions. The contribution of these motions can be miminized by high viscosity: it is well known that viscous solvents can influence overall rotational reorientation according to the theory of microviscosity (Gierer & Wirtz, 1953), but in addition, we have observed that the apparent effect on backbone protons is larger than that on side-chain protons (Motta et al., 1987, 1988; Amodeo et al., 1991). Biocompatible media of high viscosity can be assured by the so-called cryoprotective mixtures (Douzou & Petsko, 1984), i.e., mixtures of water and several organic solvents. Accordingly, we ran NOESY spectra of all peptides both in neat DMSO-d_6 and in DMSO-d_6/H_2O cryomixtures, using several compositions in preliminary experiments to find the best compromise between high viscosity and reasonable line widths.

All peptides do give NOEs in neat DMSO-d_6 at room temperature, but in the cases of NKA(4–10), [D-Ala^8]NKA(4–10), and [GABA^8]NKA(4–10) the effects are only sequential, with a number of medium-range or long-range effects insufficient to propose plausible solution structures. The NOESY spectrum of [β-Ala^8]NKA(4–10), on the other hand, shows diagnostically valuable interresidue effects, in particular NH–NH effects for the pairs Phe^6–Val^7, Val^7–β-Ala^8, and Leu^9–Met^{10}.

The contradictory results from chemical shift and coupling constant data (not shown), which point to a mixture of disordered conformations, and Overhauser effects, which hint at the presence of a well-defined folded structure, can be reconciled by the observation that even a modest amount of a folded structure (characterized by short interproton distances) can be evidenced by the strongly nonlinear dependence of NOEs on distances (Temussi et al., 1989; Jardetzky, 1980). Thus, it might be possible to build a fairly detailed model for [β-Ala^8]NKA(4–10) in DMSO but no model can be built for NKA(4–10) at this stage. Accordingly, if we want to compare models of these two peptides to find possible conformational

Table III: Chemical Shifts of Amidic Protons of NKA(4–10) (I) and [β-Ala^8]NKA(4–10) (IV)[a]

peptide	residue	α	β, β′	γ, γ′	δ, δ′	others	NH
I	Asp^4	3.65	2.45				
IV	Asp^4						
I	Ser^5	4.15	3.5				
IV	Ser^5	4.28				5.03	
I	Phe^6	4.5	3.03, 3.07			7.25, 8.6	
IV	Phe^6	4.5	2.96, 3.07			7.22	8.39
I	Val^7	4.0	2.12	0.91			8.47
IV	Val^7	3.98	2.0	0.85			8.12
I	Gly^8	3.81					8.27
IV	β-Ala^8	2.35	3.30				7.98
I	Leu^9	4.2	1.5	1.6	0.8		7.98
IV	Leu^9	4.20	1.5	1.61	0.85, 0.90		8.25
I	Met^{10}	4.2	1.8	2.52, 2.58		2.02	8.1
IV	Met^{10}	4.28				2.20	8.02
I	NH_2						
IV	NH_2						

[a] In the 90/10 (v/v) DMSO-d_6/H_2O cryomixture. Chemical shifts are in ppm referred to TMS at 297 K.

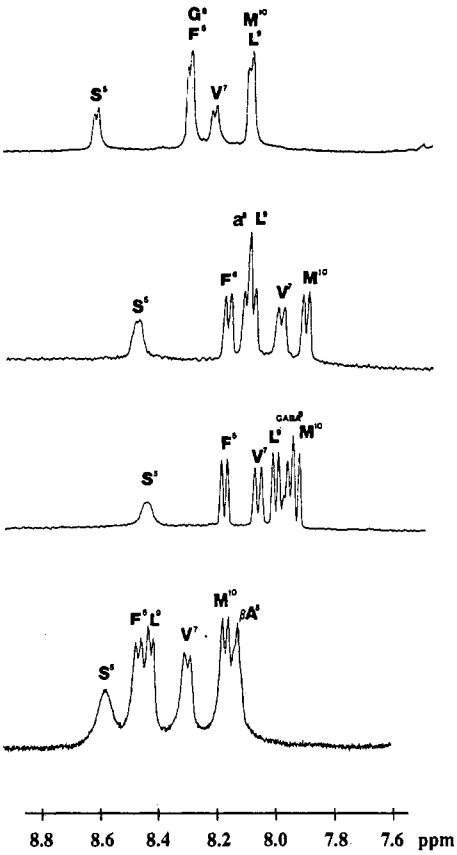


FIGURE 1: Comparison of the partial 400-MHz 1D spectra (amide region) of (I) NKA(4–10), (II) [D-Ala^8]NKA(4–10), (III) [GABA^8]NKA(4–10), and (IV) [β-Ala^8]NKA(4–10) in DMSO-d_6 at 297 K. All samples were approximately 3 mM.

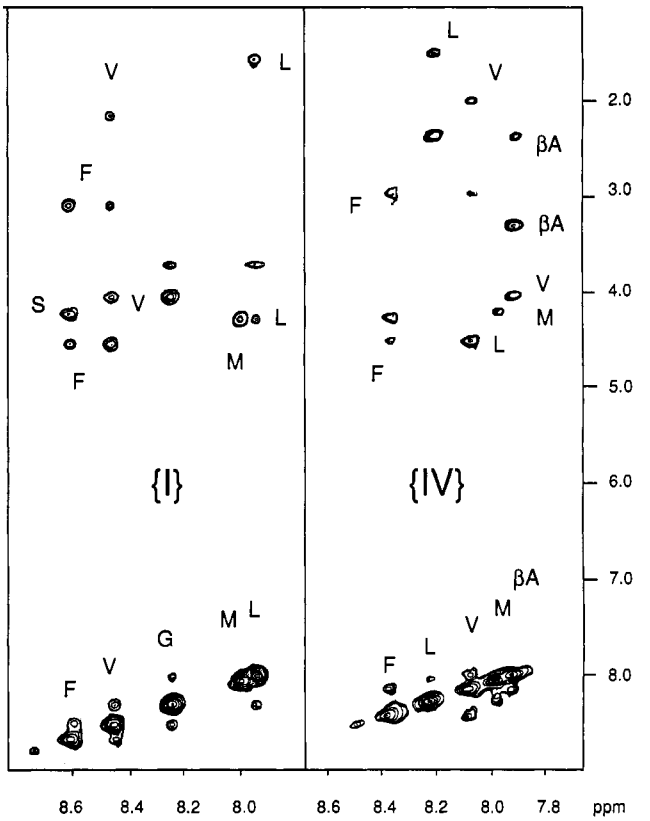


FIGURE 2: Comparison of the low-field regions of the 400-MHz NOESY spectra of NKA(4–10), i.e., peptide I of Table III, and [β-Ala^8]NKA(4–10), i.e., peptide IV of Table III, in a 90/10 (v/v) DMSO-d_6/H_2O cryomixture at 297 K.

causes for the selectivity of [β-Ala^8]NKA(4–10) with respect to NKA(4–10), it is preferable to examine the data in the DMSO-d_6/H_2O cryomixture (vide infra). The studies in the cryomixture have been limited to these two peptides since the other two, even in this medium, have a very low tendency to form well-defined structures.

Figure 2 shows a comparison of the low-field regions of the NOESY spectra of [β-Ala^8]NKA(4–10) and NKA(4–10) in a 90/10 (v/v) DMSO-d_6/H_2O cryomixture; it is possible to see that both peptides give several intra- and interresidue effects. Table III summarizes all chemical shifts of [β-Ala^8]NKA(4–10) and NKA(4–10) in a 90/10 (v/v) DMSO-d_6/H_2O cryomixture, whereas Table IV summarizes NOEs of potential diagnostic value for NKA(4–10) and [β-Ala^8]-NKA(4–10) (with corresponding interatomic distances of backbone atoms in the minimum energy model; vide infra). No measurable cross peak was observed for Ser NH since its resonance, in both peptides, is very broad.

Observation of fairly strong α-CH$_i$–NH$_{i+1}$ effects indicates the presence of extended structures, in agreement with the behavior of chemical shifts, temperature coefficients, and coupling constants. The very presence of NH–NH effects, however, shows that a folded structure (or a family of such structures) is also present and makes it possible to interpret, at least qualitatively, the observed NOEs on the basis of a mixture of a plausible average folded structure and of extended conformers both for [β-Ala^8]NKA(4–10) and for NKA(4–10), provided all NH–NH effects, and possibly also α-CH$_i$–NH$_i$ effects (generally originating from both folded and extended

Table IV: Relevant NOEs of NH Resonances of NKA(4–10) and [β-Ala[8]]NKA(4–10)[a]

	(A) NKA(4–10)				
cross peak	F[6]	V[7]	G[8]	L[9]	M[10]
α_{i-1}	m (2.71)	s (3.60)	s (3.06)	w (2.85, 3.58)	s[b] (2.83)
α_i	m (2.86)	m (2.94)	w[c] (2.90, 2.33)	w (2.89)	s[b] (2.83)
NH_{i-1}	(4.47)	m (2.76)	m (2.69)	w (3.25)	(4.30)
NH_{i+1}	m (2.76)	m (2.69)	w (3.25)	(4.30)	
β_{i-1}		w	w		
β_i	s	w		m	

	(B) [β-Ala[8]]NKA(4–10)				
cross peak	F[6]	V[7]	β-Ala[8]	L[9]	M[10]
α_{i-1}	s (2.34)	s (3.59)	s (2.36)	s (2.30, 3.55)	(3.57)
α_i	m (2.84)	(2.93)	m[c] (2.65, 3.52)	w (2.78)	m (2.95)
NH_{i-1}	(4.35)	s (2.75)	w (3.85)	(3.88)	w (2.99)
NH_{i+1}	s (2.75)	w (3.85)	(3.88)	w (2.99)	
β_{i-1}		w			
β_i	m	w	s	m	

[a] In the 90/10 (v/v) DMSO-d_6/H_2O cryomixture at 297 K; mixing time 500 ms. Numerical values have been arbitrarily classified into three categories, w (weak), m (medium), and s (strong). No measurable cross peak was observed for Ser NH since its resonance, in both peptides, is very broad. Corresponding interatomic distances of backbone atoms in the minimum energy model are reported in parentheses. [b] Owing to accidental superposition these effects stem from L and M α's. [c] Partially obscured by the residual water resonance.

structures), are self-consistent, i.e., provided that they can be attributed to a single structure of relatively low internal energy.

The NH–NH effects between Phe[6] and Val[7] and between Val[7] and Gly[8] suggest, for NKA(4–10), a structure containing a type I β-turn extending from Ser[5] to Gly[8], followed by a γ-turn centered on Gly[8] (that accounts for the weaker effect between Gly[8] and Leu[9]), whereas for [β-Ala[8]]NKA(4–10) it is possible to suggest a type I β-turn extending from Ser[5] to β-Ala[8], followed by a C_8 turn comprising β-Ala[8] and Leu[9] and by another β-turn extending from β-Ala[8] to the C-terminal NH_2. These structures may not be univocal, since they are based on a limited number of interatomic distances of the central residues of the sequences, but it is possible to test their validity by internal energy calculations and, most of all, by checking their consistency with biological activity.

The models obtained by energy minimization are shown in Figure 3. They are similar to but not identical with the topological conformers suggested by NOEs, as can be seen

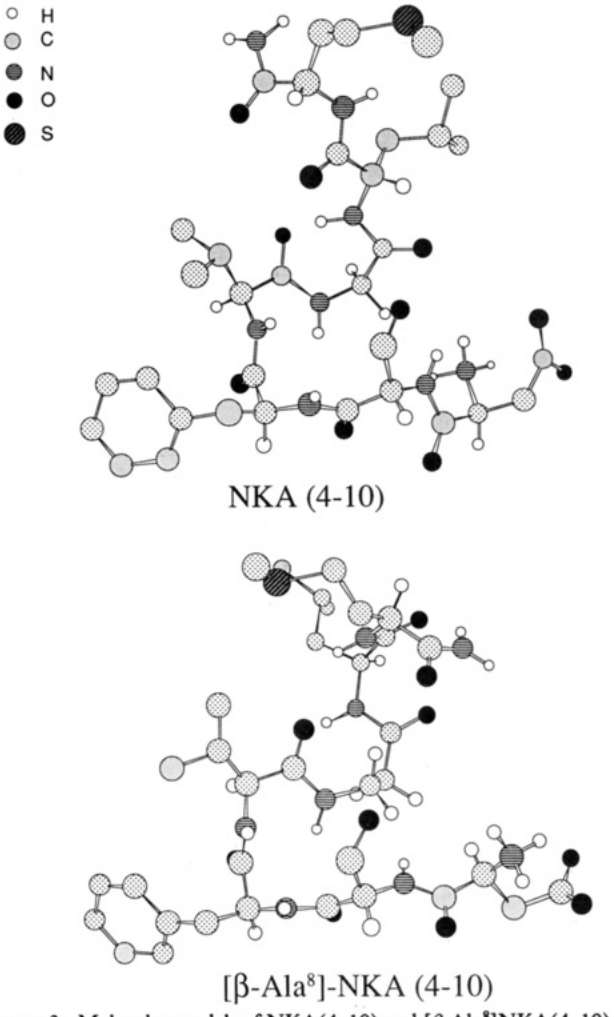


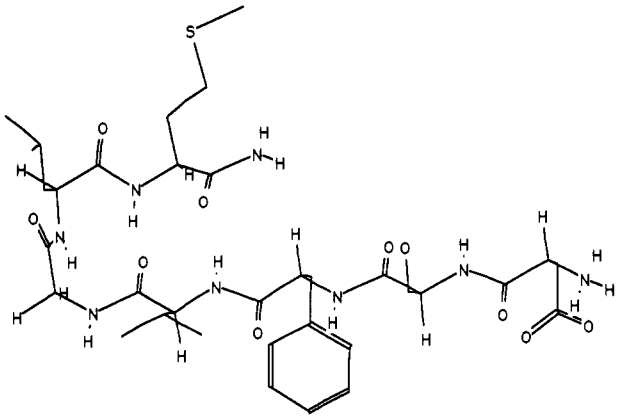
FIGURE 3: Molecular models of NKA(4–10) and [β-Ala[8]]NKA(4–10) obtained from a combination of NOEs and internal energy (MM) calculations. NKA(4–10) is characterized by a type I β-turn from Ser[5] to β-Ala[8] and a γ-turn centered on Gly[8]. [β-Ala[8]]NKA(4–10) is characterized by a type I β-turn extending from Ser[5] to β-Ala[8], followed by a C_8 turn comprising β-Ala[8] and Leu[9] and by another β-turn extending from β-Ala[8] to the C-terminal NH_2.

from the comparison of their internal coordinates summarized in Table V. The internal coordinates of N- and C-terminal residues are most affected by the energy minimization procedure, whereas those of central residues that give rise to

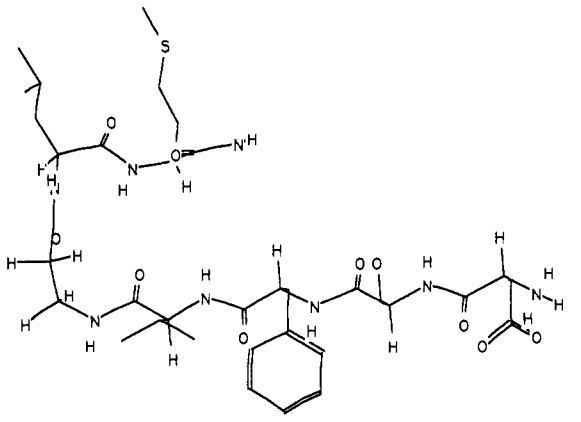
Table V: Relevant Internal Coordinates of the Molecular Models of NKA(4–10) and [β-Ala[8]]NKA(4–10)[a]

	I				F			
residue	φ	ψ	χ_1	χ_2	φ	ψ	χ_1	χ_2
				(A) NKA(4–10)				
Asp		180	180	180		−38	−21	−82
Ser	180	180	180		−68	−162	18	
Phe	−60	−30	180	90	−66	−54	179	73
Val	−90	0	180		−95	19	−31	
Gly	77	−65			70	−45		
Leu	−60	−30	180	180	−158	−62	−112	−118
Met	−90	0	180	180	−105		−83	110
				(B) [β-Ala[8]]NKA(4–10)				
Asp		180	180	180		123	−26	−76
Ser	180	180	180		−139	−163	20	
Phe	−60	−30	180	90	−72	−43	179	74
Val	−90	0	180		−99	31	−57	
β-Ala[b]	90	120	−90		−97	110	−88	
Leu	−60	−30	180	180	−82	−35	−163	−157
Met	−90	0	180	180	−52		−84	110

[a] I results from the NMR data; F was obtained by refinement of I by means of internal energy calculations. [b] The torsion angles of β-Ala under the headings φ and ψ refer to the first and last bonds of the sequence (NH–CH_2–CH_2–CO) of backbone atoms, whereas that under the heading χ refers to the torsion angle centered around the central CH_2–CH_2 bond.



NKA (4-10)



[β-Ala^8]-NKA (4-10)

FIGURE 4: Schematic molecular models of NKA(4–10) and [β-Ala^8]NKA(4–10) according to the conformation proposed by Levian-Teitelbaum et al. (1989) for NK1 agonists. The type I β-turn from Val^7 to Met^{10} is possible for NKA(4–10) but is prevented for [β-Ala^8]NKA(4–10) by the lengthening of the Met^{10} NH → Val^7 CO and of the Val^7 NH → Met^{10} CO hydrogen bonds induced by β-Ala^8.

observable backbone NOEs are conservative, even if no distance constraint was explicitly used in the calculations. Table IV shows that NH–NH and α-CH$_i$–NH$_i$ distances of the models are consistent with the observed NOEs, whereas α-CH$_i$–NH$_{i+1}$ distances are too long to account for the effects observed. In fact, it is expected that the main contributions to α-CH$_i$–NH$_{i+1}$ effects come from extended conformers; owing to the intrinsically disordered nature of these conformers, no explicit attempt was made to simulate the corresponding NOEs, as well as those involving side chains.

It can be appreciated that insertion of β-Ala^8 between Val^7 and Leu^9, although obviously not consistent with a classical γ-turn, does not change the overall shape of the NKA(4–10) conformation; in fact, insertion of the additional CH$_2$ group is still compatible with a hydrogen bond between Val^7 CO and Leu^9 NH. Even more interesting is the fact that substitutions of Gly^8 with D-Ala^8 or GABA^8 are not compatible with the conformation of [β-Ala^8]NKA(4–10) shown in Figure 3, a fact that may account for the poor activity of these compounds and that supports the explanation of the higher selectivity of [β-Ala^8]NKA(4–10). Strictly speaking, differences in solution structures for the various analogues may not be indicative, per se, of ability of the receptor to recognize the peptide. It is certainly possible that the receptor induces a conformation different from those observed in solution, but it has been pointed out (Schwyzer, 1987) that the extremely low values of active concentration of neuropeptides require a partial ordering of the structure of the peptide prior to receptor interaction. Accordingly, different conformational preferences in solution constitute a useful indication of different biological activity. Usually, it is difficult to find *any* difference in physicochemical properties among agonists with very similar chemical constitution but different biological properties. The conformational differences found among the NKA agonists (with different activity) in the present study look like a promising starting point for further conformation–activity studies of all tachykinins.

Finally, it is worth mentioning that the models of Figure 3 are not compatible with conformations of typical NK-1 and NK-3 agonists proposed on the basis of solution studies on selective NK-1 and NK-3 peptides more rigid than NKA(4–10) (Levian-Teitelbaum et al., 1989; Sumner & Ferretti, 1989). As an example, Figure 4 shows the schematic models of the structures of NKA(4–10) and [β-Ala^8]NKA(4–10) consistent with the model of NK-1 agonist proposed by Levian-Teitelbaum et al. (1989). It can be appreciated that substitution of Gly^8 with β-Ala^8 prevents formation of a turn centered on residues 7, 8, 9, and 10 (corresponding to residues 8, 9, 10, and 11 of the agonist of Levian-Teitelbaum et al.) owing to a complete destabilization of two hydrogen bonds. This observation substantiates our hypothesis on the conformational origin of the selectivity of [β-Ala^8]NKA(4–10) with respect to NKA(4–10).

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Registry No. [β-Ala^8]NKA(4–10), 122063-01-8; [D-Ala^8]NKA(4–10), 136237-50-8; [GABA^8]NKA(4–10), 122063-09-6.

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A cAMP-Binding Ectoprotein in the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: Purified plasma membranes from the yeast *Saccharomyces cerevisiae* bind about 1.2 pmol of cAMP/mg of protein with high affinity (K_d = 6 nM). By using photoaffinity labeling with 8-N_3-[^{32}P]cAMP, we have identified in plasma membrane vesicles a cAMP-binding protein (M_r = 54 000) that is present also in *bcy1* disruption mutants, lacking the cytoplasmic R subunit of protein kinase A (PKA). This argues that it is genetically unrelated to PKA. Neither high salt, nor alkaline carbonate, nor cAMP extract the protein from the membrane, suggesting that it is not peripherally bound. The observation that (glycosyl)phosphatidylinositol-specific phospholipases (or nitrous acid) release the amphiphilic protein from the membrane, thereby converting it to a hydrophilic form, indicates anchorage by a glycolipidic membrane anchor. Treatment with N-glycanase reduces the M_r to 44 000–46 000 indicative of a modification by N-linked carbohydrate side chain(s). In addition to the action of a phospholipase, the efficient release from the membrane requires the removal of the carbohydrate side chain(s) or the presence of high salt or methyl α-mannopyranoside, suggesting complex interactions with the membrane involving not only the glycolipidic anchor but also the glycan side chain(s). Topological studies show that the protein is exposed to the periplasmic space, raising intriguing questions for the function of this protein.

In the yeast *Saccharomyces cerevisiae*, cAMP[1] is known to influence a number of cellular processes and to link them to the nutritional situation of the cell. Among them are storage carbohydrate metabolism (Pall, 1981; Thorner, 1982), cell size regulation and cell cycle progression (Matsumoto et al., 1985; Baroni et al., 1989), sporulation (Matsumoto et al., 1983a,b), and transcription (Merino et al., 1989). Control of these functions is thought to be achieved through the activation of the well-characterized cytoplasmic cAMP-dependent protein kinases (Beebe & Corbin, 1986) in response to the modulation

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[1] Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; C (R) subunit, catalytic (regulatory) subunit; DTT, dithiothreitol; EDTA, ethylendiaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GPI, glycosylphosphatidylinositol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; MES, 2-(*N*-morpholino)-ethanesulfonic acid; α-MMP, methyl α-mannopyranoside; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; N-glycanase, peptide N-glycohydrolase F; PEG, poly(ethylene glycol); (G)PI-PLC (D), (glycosyl)phosphatidylinositol-specific phospholipase C (D); PKA, cAMP-dependent protein kinase; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; TX-114, Triton X-114, poly(ethylene glycol) mono(octyl phenyl ether).

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