Conformational Comparisons of a Series of Tachykinin Peptide Analogs

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Received May 17, 2007

Previous studies have shown differences in the biological activity and the structure of two naturally occurring tachykinin peptides, substance P (SP, RPKPQQFFGLM-NH_2) and ranatachykinin C (RTKC, HNPASFIGLM-NH_2). To further understand the basis for these differences, four analogs that selectively incorporate the amino acid differences between SP and RTKC have been synthesized for study. The four peptide analogs studied have the following amino acid sequences: SP2-11, also known as des-Arg SP (PKPQQFFGLM-NH_2); Q5A-SP (RPKPAQFFGLM-NH_2); Q6S-SP (RPKPQSFFGLM-NH_2); and Q5AQ6S-SP (RPKPAS-FFGLM-NH_2). Nuclear magnetic resonance spectroscopy and molecular modeling calculations were performed on SP, RTKC, SP2-11, Q5A-SP, Q6S-SP, and Q5AQ6S-SP to compare their conformational differences and similarities in the presence of the membrane mimetic system sodium dodecyl sulfate. The molecular modeling data of the analogs Q5A-SP and Q6S-SP show residues 1–3 have a random conformation and residues 4–8 have a helical structure, while the C-terminus contains a poly C_7 conformation that is similar to SP but different from RTKC. The molecular modeling data of the analogs SP2-11 and Q5AQ6S-SP show a continuous helix conformation for residues 4–11 at the C-terminus, which is different from SP but similar to RTKC. These structural differences are related to the functional differences of binding of the peptides at the SP receptor (NK_1).

Introduction

The development of potential therapeutic agents has been the driving force behind innumerable studies of the physiological activities of the tachykinin family of neuropeptides, particularly substance P (SP). SP is found in the mammalian tissue of the central nervous system, skin, lungs, and intestine[1] and plays a role in allergic reactions, cardiovascular control, control of smooth muscle tone, hypertension, inflammation, intestinal contraction, pain, respiratory control, and salivation.[1–6] This health-related impetus has led to investigations of the biologically active conformations of these peptides to formulate a relationship between the structure and the activity between these peptides and their receptors.[7] An assortment of spectroscopic methods has been employed to study the conformation of the tachykinin family of peptides.[1,7–14] SP has been shown to selectively recognize the NK_1 receptor.[1,5] The tachykinin receptors are membrane-embedded G-protein coupled receptors, which eliminates the possibility of investigating the peptide bound to its native receptor due to the large size of the system.[15] It has been known that the membrane itself imposes a conformation onto small peptides in a prebinding event that may represent the biologically active conformation.[16] To preserve the biologically active conformation of the peptide, the membrane is required. Thus, a membrane mimetic system such as the sodium dodecyl sulfate (SDS) micelle is utilized to induce structure on these tachykinin peptides to study their biological significance.[13,17–20] Instrumental to these efforts has been the use of two-dimensional (2D) nuclear magnetic resonance spectroscopy (NMR)[7,13,21] and molecular modeling.

Table 1. Amino Acid Sequences of Tachykinin Peptides and Analogs[a]

SP:	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly Leu-Met-NH_2
RTKC:	His-Asn-Pro-Ala-Ser-Phe-Ile-Gly Leu-Met-NH_2
SP2-11:	Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly Leu-Met-NH_2
Q5A-SP:	Arg-Pro-Lys-Pro-<u>Ala</u>-Gln-Phe-Phe-Gly Leu-Met-NH_2
Q6S-SP:	Arg-Pro-Lys-Pro-Gln-<u>Ser</u>-Phe-Phe-Gly Leu-Met-NH_2
Q5AQ6S-SP:	Arg-Pro-Lys-Pro-<u>Ala</u>-<u>Ser</u>-Phe-Phe-Gly Leu-Met-NH_2

[a] The sequences of the naturally occurring peptides SP and RTKC are shown at the top. Note that in the C-portion of the peptides, the only difference is the substitution of an Ile for Phe in RTKC, a conservative change. More substantive differences that have been examined in the present study are the absence of the N-terminal Arg and replacement of the mid-portion Gln with Ala and Ser. The sequences of the analogs designed to test the effects of these changes on peptide structure are indicated, with the altered residues underlined.

The local chemical environment has been shown to influence the conformation of SP in solution.[8,9,12] SP exists as an extended random coil at concentrations in solution of 5.0 mM or less and as an aggregate above 8.0 mM.[8,9,12,22] An α-helical conformation has been observed for SP in SDS in the midregion (PQQFF) of the peptide.[9,12,13] Insertion of the aromatic rings of the F residues of SP into the hydrophobic portion of the SDS micelle[9] has been supported by UV absorption, fluorescence, proton longitudinal relaxation studies, and 2D nuclear Overhauser spectroscopy (NOESY) spectra.[23] The four peptide analogs studied in this research should insert their aromatic rings of the F residues into the hydrophobic portion of the SDS micelle, as seen in SP,[23] since the F residues were not changed in the amino sequence of the analogs (see Table 1). It is postulated that preceding interaction with the receptor, a specific conformation is induced on the neuropeptides backbone and the concentration of the neuropeptide increases near the receptor.

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10.1021/jm070577s CCC: $37.00
Published on Web 12/08/2007

These events are proposed as being vital roles of the membrane toward neuropeptide binding.[16]

In previous studies, we have shown differences in the biological properties of naturally occurring tachykinins isolated from bullfrog when compared to SP.[14] The largest differences were observed between SP and a peptide named ranatachykinin C (RTKC).[14] RTKC has the amino acid sequence of HNPAS-FIGLM-NH_2. The notable differences between SP and RTKC are the absence of the N-terminal R and a replacement of the midregion residues Q5 with A and Q6 with S. Our NMR data have shown that, like SP, RTKC exhibits a helical structure from the midregion to the C-terminus, while exhibiting considerable flexibility in the N-terminal residues, which corresponds well with data published for RTKC (Figure 5c of Perrine et al., 2000).[13,14] This suggests that the differing degree and type of receptor activation seen at the bullfrog substance P receptor[14] and the differences in receptor binding properties seen at rat and human NK1 receptors[24] may result from differences in secondary structure in the C-terminal portions of the peptides.

To test this hypothesis, we have designed four synthetic analogs of SP. The analogs of SP chosen for this study are SP2-11, also know as des-Arg SP, (PKPQQFFGLM-NH_2), Q5A-SP (RPKPAQFFGLM-NH_2), Q6S-SP (RPKPQSFFGLM-NH_2), and Q5AQ6S-SP (RPKPASFFGLM-NH_2). The amino acid sequences from the four analogs as well as that of SP and RTKC are shown in Table 1. These analogs of SP were selected in an attempt to account for the differences observed between SP and RTKC.[14,25] RTKC has no N-terminal R as in SP2-11, has A5 as in Q5A-SP, and S6 as in Q6S-SP, and has both A5 and S6 as in Q5AQ6S-SP instead of Q5Q6 as in SP. Here we describe the relationship between the conformation of a series of SP analogs in SDS to each other, to SP[13] and to RTKC[14] to understand how the amino acid differences affect peptide structure and relate these to the ability of the peptides to displace SP binding to the receptor.

Materials and Methods

Sample Preparation. SP was purchased from Sigma (St. Louis, MO). RTKC, SP2-11, Q5A-SP, Q6S-SP, and Q5AQ6S-SP were custom-designed and synthesized by and purchased from Sigma-Genosys (The Woodlands, TX). Deuterium oxide was purchased from Aldrich Peptides (Milwaukee, WI). SP, RTKC, SP2-11, Q5A-SP, Q6S-SP, and Q5AQ6S-SP were all prepared the same for consistency. A total of 1.8 mg of each peptide was dissolved in 0.1 mL of 10 mM sodium acetate/acetic acid buffer in 90% ^1H_2O and 10% ^2H_2O then added dropwise to 0.5 mL of 150 mM d_{25}-SDS (sodium dodecylsulfate, Cambridge Isotopes, MA) in 90% ^1H_2O and 10% ^2H_2O and buffered to a pH of 4.37, yielding a ~2.5 mM solution. A Denver Instrument Ultra Basic model pH meter was used to measure the pH without correction for the deuterium isotope effect.

NMR and Molecular Modeling. A Bruker AMX-600 spectrometer, operating at a frequency of 600.13 MHz, and a TXI triple resonance (^1H, ^{13}C, ^{15}N) inverse probe was used to collect all NMR data for this research. Each peptide was run under the same conditions and using the same experimental parameters for all 1D and 2D NMR experiments at 300 K. The parameters used for the 1D proton experiment were as follows: 2.0 s presaturation pulse on the ^1H_2O frequency for water suppression, spectral width of 6024.1 Hz, acquired 32 K data points, 0.188 Hz per point digital resolution, 2.72 s of acquisition time, 64 scans, 1.0 Hz line broadening, exponential multiplication, and Fourier transformation.

A homonuclear Hartmann–Hahn (HOHAHA) experiment was acquired at 300 K, 305 K, 310 K, and 315 K, and the 300 K HOHAHA data were used and reported because they resulted in the best resolution. The parameters used for the homonuclear Hartmann–Hahn experiment were as follows: total spin-lock mixing time of 70 msec with a MLEV-17 mixing sequence having a 2.5 msec trim pulse at the start and end of the MLEV-17 sequence, 1.0 s presaturation pulse on the ^1H_2O frequency for water suppression, spectral width of 6172.8 Hz in both domains, 3.086 Hz per point digital resolution (f_2) and 6.173 Hz per point digital resolution (f_1), 0.1659 s of acquisition time, 96 scans with 64 dummy scans for thermal equilibrium, 2 K time-domain data points for 1 K t_1 values of 96 scans, zero-filled to 2 K × 1 K, and processing with QSINE in both dimensions.

The parameters used for the NOESY experiment were as follows: presaturation pulse of 1.5 s for solvent suppression, 200 msec mixing time, spectral width of 6,172.8 Hz in both domains, 3.086 Hz per point digital resolution (f_2) and 6.173 Hz per point digital resolution (f_1), 0.1659 s of acquisition time, 32 scans with 16 dummy scans for thermal equilibrium, 2 K time-domain data points for 1 K t_1 values of 32 scans, zero-filled to 2 K × 1 K, and processing with QSINE in both dimensions. Simulated annealing calculations were carried out according to protocols already published.[13]

Receptor Binding: Competition Radioligand Binding. CHO cells stably transfected with cDNA encoding rat NK_1 were used to determine the binding properties of the peptides. Bolton-Hunter [^{125}I]Lys_3-SP (NEN, Boston, MA) at a concentration of 50 pM and increasing amounts, 10^{-11} to 10^{-5} M, of unlabeled peptide analog or SP, were used in the binding assays. The methods have been previously described in detail.[26] Briefly, CHO cells expressing rat NK_1 were incubated with radiolabeled SP and unlabeled peptide for 2 h at 4 °C, washed to remove any unbound ligand, and collected and the radiation was counted using a Packard Cobra II series autogamma counter (Packard, Meriden, CT).

Specific binding was determined and the data were normalized as the ratio of bound to free (B/B_0) for each unlabeled peptide, where B is specifically bound [^{125}I]Lys^3-SP in the absence of unlabeled peptide and B_0 is specifically bound [^{125}I]Lys^3-SP in the presence of 1 μM unlabeled peptide. Data were plotted as the competitor concentration vs fraction specific binding and the IC_{50} determined from these plots in GraphPad Prism. Each data point represented at least four replicates.

Results

NMR Assignments. Natural abundance sequence-specific proton assignments were accomplished using techniques developed by Wuthrich.[27,28] These assignments were obtained by the interactive interpretation of the 2D-HOHAHA and 2D-NOESY spectra. This process will be explained here using the peptide SP2-11. First, spin systems are identified in the fingerprint region of the HOHAHA spectrum, as shown in Figure 1. Each amino acid gives a characteristic pattern in this experiment that allows for the identification of the spin system. In some cases (K3, G9 and L10), it is possible to make specific assignments because these amino acids are unique in these peptides. However, it is impossible to distinguish Q from M and which spin system belongs to which F. The two spin systems for P were identified in the alkyl region (data not shown), however, they were not assigned to P2 or P4 specifically using just the HOHAHA data. The 2D-NOESY (200 msec mixing time) data provide NOE correlations that are essential for sequence-specific assignments. An amide proton will show correlations to the other protons in its spin system and correlations to some of the protons in the previous amino acids spin system, which is illustrated in Figure 2 for G9. The amide proton of G9 shows a through space correlation to its own alpha protons as well as the alpha and two beta protons of F8, assigning one of the F spin systems specifically to F8. This process is continued for all of the amino acids, resulting in sequence-specific assignments. The specific chemical shift assignments of all four peptide analogs, SP, and RTKC are provided in Table 1 of the Supporting Information. NMR data

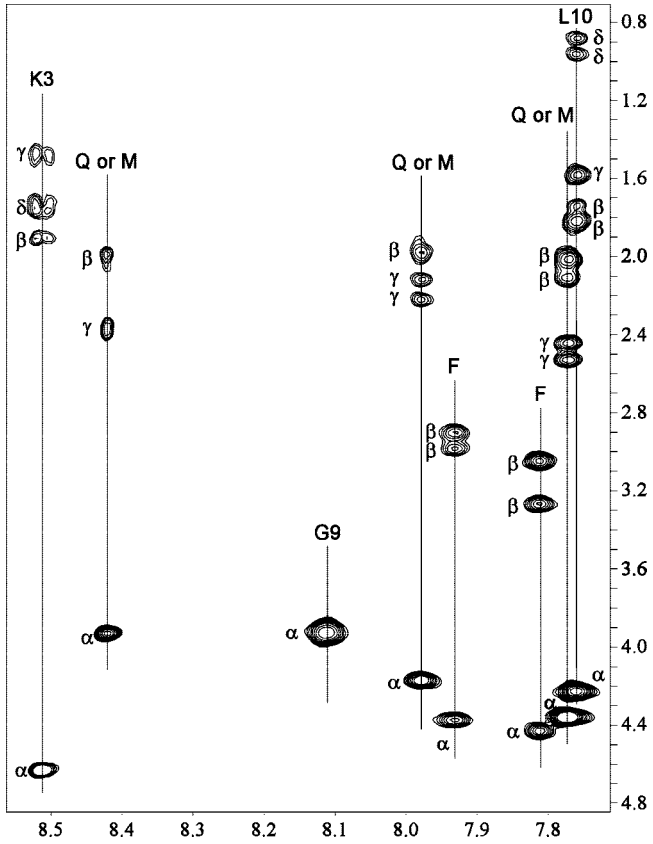

Figure 1. Fingerprint region of the HOHAHA of SP2-11 showing tentative amino acid assignments.

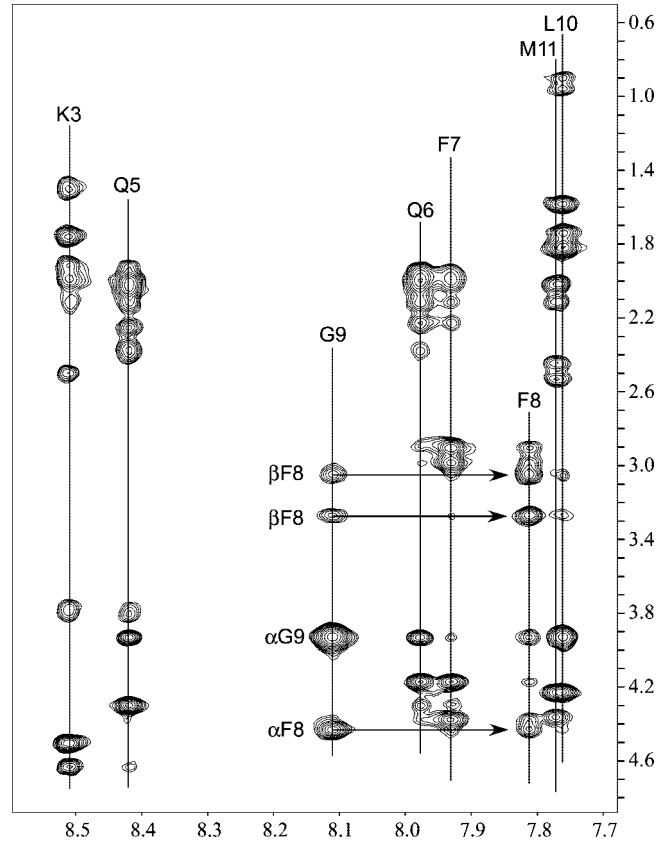

Figure 2. Fingerprint region of the NOESY of SP2–11 showing specific amino acid assignments and illustrating the i to $i - 1$ correlations of G9 to the α and βs of F8.

on SP and RTKC were also interpreted and used for molecular modeling to confirm that our data were consistent with the previously published data.[13,14]

Structure Calculations. NOEs from the NOESY experiment at 200 msec mixing time were used for structure calculations. Figure 3 shows a graph of the sequential and medium range structural NOEs used in the molecular modeling calculations. The total NOEs for each peptide are given in Table 2. Each NOE was converted over to a $^1H-^1H$ distance range and classified as strong (1.9–2.7 Å), medium (1.9–3.3 Å), and weak (1.9–4.0 Å) based on the NOE intensity.[29] The total number of NOEs for each analog peptide correlated well with the previously published SP data of Young et al.[13] and RTKC data of Perrine et al.,[14] thus, enumeration of specific NOEs is not made because no significance is added to the literature. More NOEs were obtained for SP because it had the least amount of overlap between resonances. Only unambiguous NOEs were used in initial structure calculations. As calculations proceeded, some NOEs were clarified and put into the next calculation. During the calculation process, any NOEs that violated their distance range were moved to the next largest distance range for the next calculation. The dihedral angles φ and Ψ were obtained for some residues using the TALOS program[30] using $^1H^\alpha$ chemical shifts. These angles were restrained by ±20°, around the average angle predicted from TALOS. TALOS was used because dihedral angles from coupling constants are difficult to obtain on these peptides because they are tumbling at the rate of the micelle in which they are inserted, leading to extremely broad line widths. The variation in the dihedral angle was increased by 10° for any violations. Structure calculations continued until no NOE was violated by more than 0.5 Å and no dihedral angle was violated by more than 5°. Once a good set of NOEs and dihedral angles were obtained, 50 structures were generated for final structural and statistical analysis. Figure 4 shows 10 randomly selected structures for SP, RTKC, and the analogs superimposed on themselves from residues 4–11 (which is 3–10 for RTKC), with the statistics for these superimpositions given in Table 2.

Comparison to SP and RTKC. The structure calculated for SP during these studies was identical to the previously published NMR structure, which is helical for residues 4–8, a poly C_7 structure at the C-terminus (residues 9–11), and unstructured at the N-terminus (residues 1–3).[13] The structure calculated for RTKC during these studies was similar to the previously published NMR structure, showing a helical conformation for residues 3–10.[14] The structure of analogs Q5A-SP and Q6S-SP are very similar to SP (see Figure 5). This is supported by identical NOEs and dihedral angles obtained for these peptides. Some of the NOEs were not identified for the two analogs due to overlap of signals. These peptides have a 0.42 Å (Q5A) and 0.58 Å (Q6S) backbone superimpose from residues 4–11 onto the SP structure.

The peptides SP2-11 and Q5AQ6S-SP resulted in structures that differed from SP at G9. The SP2-11 structure superimposes (residues 4–11) to 1.01 Å into the SP structure, while the Q5AQ6S-SP structure was 2.10 Å (see Figure 5). Figure 6 shows that these differences arise from a single NOE, which was from the amide ^1H of G9 to the alpha ^1H of residue 6 in both the SP2-11 and Q5AQ6S-SP peptides; however, this NOE was not observed for SP, Q5A-SP, or Q6S-SP. This NOE has a weak intensity in the SP2-11 data and a medium intensity in the Q5AQ6-SP data. This NOE helps to define a continuous helix from residues 4–11 for the Q5AQ6S-SP structure, while SP2-11 results in a structure that is between the SP structure and that of Q5AQ6S-SP. There is a comparable NOE from the amide

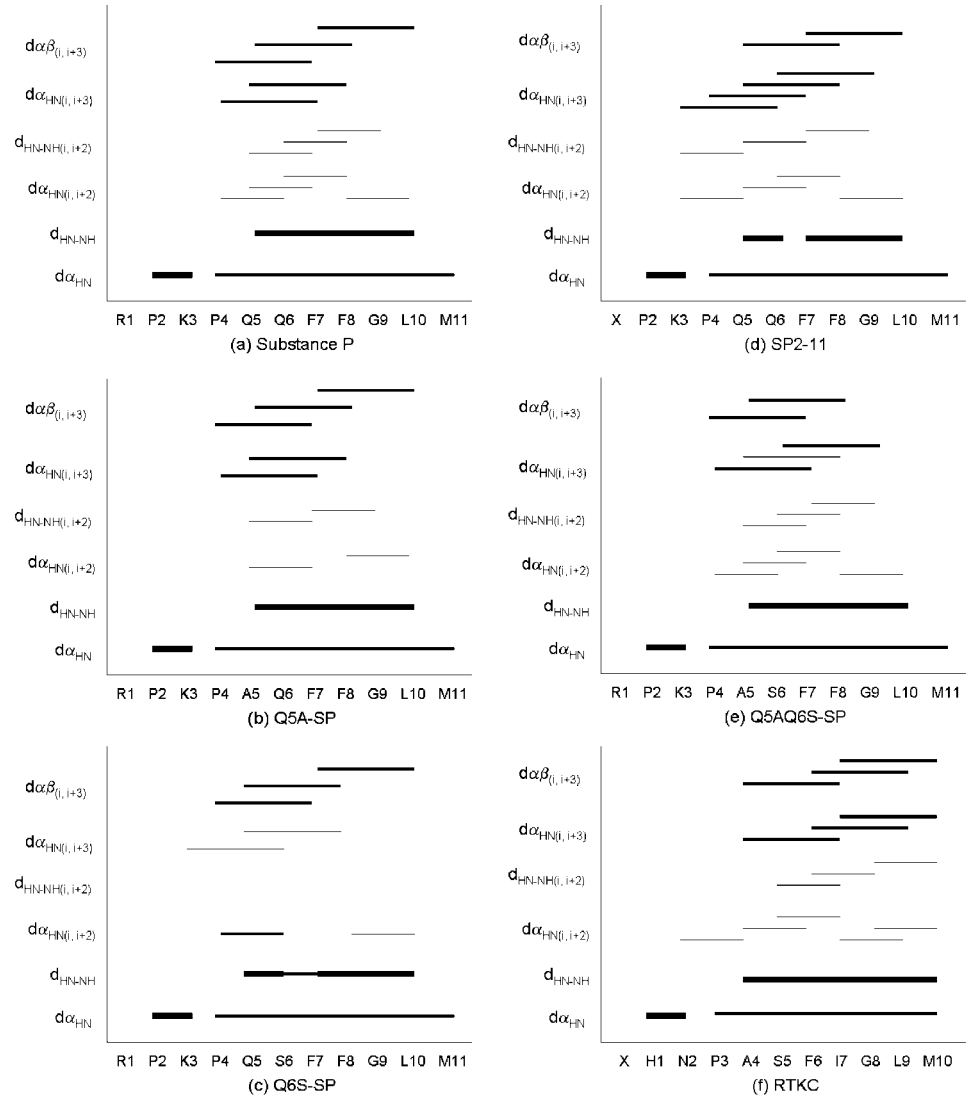

Figure 3. Graph of sequential and medium range NOEs for (a) SP, (b) Q5A-SP, (c) Q6S-SP, (d) SP2-11, (e) Q5AQ6S-SP, and (f) RTKC. The increasing thickness of the line represents the increasing strength of the NOE (thinnest = weak to thickest = strong).

Table 2. Experimental Constraints and Statistics of the Peptide Structures Calculations

	SP	SP2–11	Q5A	Q6S	Q5AQ6S	RTKC
total NOEs	101	77	78	75	78	78
intra residue (*i* to *i*)	36	32	30	32	32	28
inter residue (*i* to *i* ± 1)	45	27	31	29	28	30
medium (*i* to *i* ± 4)	20	18	16	14	18	20
dihedral angles, φ and Ψ	13	12	13	13	13	13
energy (kcal/mol)	108.93 ± 9.81	84.37 ± 2.82	90.73 ± 3.42	86.86 ± 1.60	91.09 ± 9.81	87.77 ± 2.31
backbone superimpose (residues 4–11, Å)	0.34 ± 0.10	0.35 ± 0.13	0.36 ± 0.09	0.40 ± 0.08	0.47 ± 0.12	0.32 ± 0.12

^{1}H of G8 to the alpha ^{1}H of S5 of RTKC. This suggests that these two peptides (SP2-11 and Q5AQ6S-SP) are approaching a conformation closer to RTKC. In fact, these peptides have more helical characteristics in residues 9–11 than SP, Q5A-SP, and Q6S-SP; however, they do not form as tight of a helix as RTKC.

Radioligand Competition Binding. The ability of each tachykinin peptide to compete with SP binding to the rNK1R was measured in competition binding studies. The competition binding curves for SP and RTKC have previously been determined[14] and will be presented here for comparison with the analogs. The analogs bind to the rNK1R in a concentration-dependent manner and displace [^{125}I]-SP with similar maximum effects. The competition binding curves for all of the ligands were best fit by a one-site binding model (Hill coefficient = 1). The rank order of IC_{50} values (nM) is SP (0.4) ≥ Q5A-SP (0.7) > Q6S-SP (4.1) ≥ SP2–11 (4.6) > Q5AQ6S-SP (21.0) > RTKC (32.6). The mean ± sem of the fits of the competition binding curves are shown in Table 3.

Discussion

The conformation of a series of SP analogs has been examined to further understand the differences between SP and another naturally occurring tachykinin peptide RTKC. The major differences between these peptides are the presence or absence of an N-terminal Arg residue and the change of two critical Gln residues in the middle of SP to an Ala and a Ser, as in RTKC.

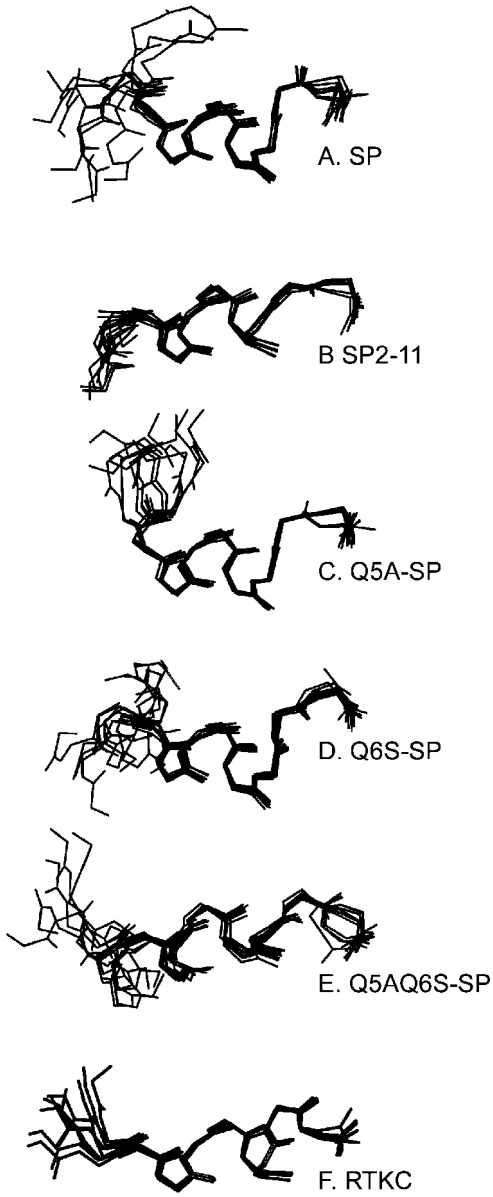

Figure 4. Backbone superimpose (residues 4–11) of the lowest energy structures of SP and analogs.

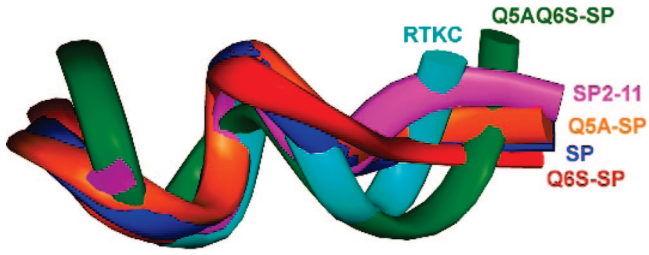

Figure 5. Backbone of residues 4–11 (residues 1–3 not shown), of the analogs, and of RTKC superimposed onto the substance P structure.

The NMR structure for SP is helical from residues 4–8, a poly C_7 structure at the C-terminus (residues 9–11), and unstructured at the N-terminus (residues 1–3).[13] The peptides where the Arg residue is absent (SP2-11) and where both Gln are changed to Ala and Ser (Q5AQ6S-SP) resulted in structures that differed from SP in that Q5AQ6S-SP has a continuous helix from residues 4–11, while SP2-11 results in a structure that is between that of the SP structure and that of the Q5AQ6S-SP

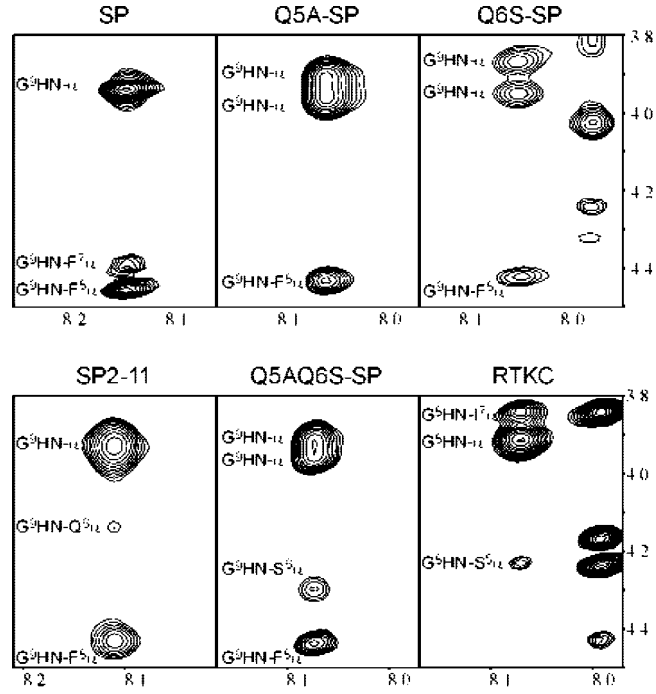

Figure 6. Spectra of NOEs are shown from HN to α for G9 of SP and analogs (G8 for RTKC).

Table 3. IC_{50} Values for Competition Binding at the NK_1 Receptor for Tachykinin Peptides and Analogs[a]

peptide	pIC_{50}	sem
SP	9.39	0.14
Q5A-SP	9.13	0.09
Q6S-SP	8.39	0.07
SP2–11	8.33	0.04
Q5AQ6S-SP	7.68	0.04
RTKC	7.49	0.09

[a] The data are shown as the mean $pIC_{50} \pm$ sem fron competition binding of unlabeled peptides with labeled SP; *n* values range from 6 to 12 for each data point; GraphPad Prism software (GraphPad Software Inc., San Diego, CA) was used to fit the competition binding curves to the Hill equation to determine the mean and sem; and none of the Hill coefficients were significantly different from 1.

structure (see Figure 5). Conversely, the NMR structure of analogs Q5A-SP and Q6S-SP are both unstructured at the N-terminus (residues 1–3), helical from residues 4–8, and a poly C_7 structure at the C-terminus (residues 9–11). Both analogs Q5A-SP and Q6S-SP have NMR structures very similar to SP shown through the excellent superimposition of residues 4–11 onto the SP structure (see Figure 5). These structural observations made through the NMR and molecular modeling studies correlate well with the biological studies.

The effects of these amino acid changes on peptide structure can be rationalized with the binding data. The Q5A-SP analog has a structure similar to SP and only a slightly increased IC_{50} of 0.7 nM. Thus, changing the amino acid at this position from polar to small hydrophobic does not have a great effect on binding or structure. SP2–11 and Q6S-SP have an order of magnitude higher IC_{50} than SP, 4.6 and 4.1 respectively. For SP2–11, the structure deviated by 1.01 Å from SP; therefore, there could be a dual effect for this peptide. This dual effect first involves a slight change in the overall structure and second, because R1 is not present at the N-terminus in SP2-11 then contacts of R1 with the NK1 receptor are missing for SP2-11 but present in SP. It is believed that at the N-terminal, R may have a charge interaction with an extracellular loop of the NK_1 receptor. Saebo et. al[31] have shown using molecular modeling

that there could be three hydrogen bonds from Q7 of the NK_1 receptor to R1 of SP. The change in IC_{50} for Q6S-SP can be completely attributed to the amino acid change because the structure was similar to SP. Most likely, the side chain -NH_2 of Q6 forms hydrogen bonds with the NK_1 receptor that cannot be maintained by the -OH group of S.

The most dramatic change in IC_{50} was obtained for Q5AQ6S-SP, which was 21.0 nM. This increase could be explained by a combination of effects. First, the structure showed a significant deviation of 2.10 Å from SP and the C-terminal residues form a helix instead of the poly C_7 structure of SP. Molecular modeling calculations by Saebo et. al[31] have also shown that there may be two hydrogen bonds from N14 of the NK_1 receptor to M11 and the terminal amine of SP.[30] The helical structure at the C-terminus of Q5AQ6S-SP would displace M11 away from these hydrogen bonds. There would also be a loss of possible hydrogen bonds to Q6 for this analog like Q6S. It does appear that the Q5AQ6S-SP analog may be acting similar to RTKC instead of SP because the IC_{50} and structure are closer to RTKC than SP.

In conclusion, the data show that the differences in binding activity can be attributed to two major differences in the peptides. Part of this difference is accounted for by the absence of the N-terminal Arg in RTKC. However, most of the difference is attributable to the alteration in the midregion of the peptides. Changing the Gln in SP to Ala and Ser alters the helical structure of the C-terminus of the peptide, which likely leads to its altered binding affinity.

Supporting Information Available: Chemical shift assignments of SP and analogs. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM070577S