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Conformational Analysis of the C-Terminal Gly-Leu-Met-NH_2 Tripeptide of Substance P Bound to the NK-1 Receptor

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Summary

We examined the effect of simultaneously incorporating proline or proline-amino acid chimeras in positions 9, 10, and/or 11 of substance P, on the affinity for the two NK-1 binding sites and on second-messenger activation. Because these *3*-substituted prolines constrain not only the (ϕ,ψ) values of the peptide backbone, but also the χ space of the amino acid side chain, we were able to gather data on the structural requirements for high-affinity binding to the NK-1 receptor. We were able to confirm that this C-terminal component is crucial and that it should adopt an extended conformation close to a polyproline II structure when bound to the receptor. The partial additivity of these constraints, more specifically, for the NK-1M site, suggests that the peptide backbone flexibility around the hinge-point residue Gly^9 is essential to subtly position crucial side chains.

Introduction

NMR analyses of substance P (SP: H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) in micellar media have demonstrated the presence of pure α helix or equilibria between α helix, 3_{10}-helix, or consecutive β-turns [1–10]. Schwyzer et al. proposed that the highly positively charged N-terminal sequence of SP (address domain) led to an accumulation of SP on the anionic cell surface, which induced an insertion of the hydrophobic message domain in the membrane as an α helix aligned with the lipid chains [11, 12]. From these studies, it has been extrapolated that SP should adopt one of these helical structures when bound to its receptor. However, stabilization of helical structures in short, flexible peptides is a well-known phenomenon readily observed in both micellar media and organic cosolvents, such as trifluoroethanol. A recurrent question remains, whether these media stabilize a preexistent but poorly populated structure in aqueous solution, which represents bioactive conformation, or if they induce helical structures unrelated to the conformation of the ligand bound to its receptor.

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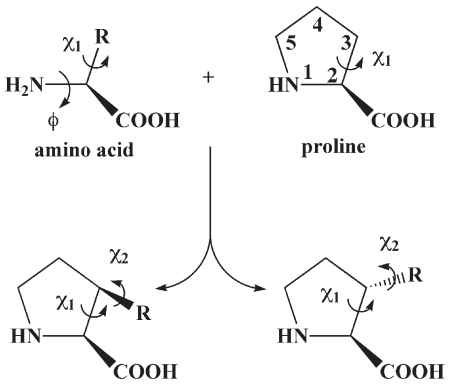
The one-turn helical structure observed for residues Pro^4 to Phe^8 of SP in methanol [13] has been validated by the design of highly potent cyclic analogs of SP [14, 15]. In contrast, the U-turn structure proposed for the C-terminal tripeptide, when in methanol, has been invalidated because none of the substituted SP analogs designed to probe this U-turn conformation turned out to be active [16]. However, structure-activity relationships performed with N-methylated aminoacids and/or proline have suggested that the C-terminal residues of SP adopt a more or less extended conformation, the residue in position 9 (Gly^9) being a hinge between these two domains of SP [16]. More recently, we have shown that the introduction of a *3*-substituted proline (Figure 1) in position 10 or 11 of SP allowed us to define more precisely the ϕ angle (proline constraint) of residues 10 and 11 [17, 18]. This proline scaffold positions not only the peptide backbone, but also the side chain of residues in a predefined orientation. According to the potencies of these substituted SP analogs, the following values for the torsion angles of Leu^{10} and Met^{11} residues, when bound to the NK-1 receptor, can be proposed: $\phi_{10} \approx -60°$; $\psi_{10} \approx 150°$; $\chi_{1(10)} \approx -60°$; $\chi_{2(10)} \approx 180°$; $\phi_{11} \approx -60°$; and $\chi_{1(11)} \approx 180°$. The χ_1 torsion angle of both Leu^{10} and Met^{11} could be determined by comparison of the conformational spaces of corresponding prolinoamino acids, and the χ_2 torsion angle was tentatively proposed, according to energy considerations [17]. The introduction of a single *3*-substituted proline still allows fluctuations around the Ψ torsion angles of residues 9, 10, and 11, preventing the determination of a unique conformation of the peptide backbone. Calculations performed on model dipeptides and previous structure-activity relationships converge to a destabilization of helical and poly γ-turn structures. Because the monosubstituted proline analogs, [Pro^9]SP, [P$^t_3$Leu^{10}]SP, [P$^C_3$Met^{11}]SP, and [P$^t_3$Met^{11}]SP are all potent agonists, there is an additional question as to the additivity of these proline constraints in the C-terminal tripeptide of SP: can all these constraints be simultaneously accepted within the binding site?

To get insight into the binding process, two to three constraints combining Pro, *3*-substituted Pro, or N-methylamino acid have been introduced in the C-terminal tripeptide of SP and SP(6-11), Gly-Leu-Met-NH_2, and the consequences of such modifications have been analyzed in terms of affinity and activity on the two specific binding sites [19, 20] associated with the NK-1 receptor. The structural effects of such constrained amino acids in the SP sequence have also been evaluated on model tripeptides by molecular modeling.

Results

Syntheses of SP and SP(6-11) Analogs Incorporating Two or Three Constrained Residues

The C-terminal-constrained residues were coupled manually to the MBHA resin to ensure complete coupling with a minimum of equivalents used. Three to five



R = -$CH(CH_3)_2$, (2S)-*3*-isopropyl-pyrrolidine-2-carboxylic acid: *3*-prolinoleucine

R = -CH_2SCH_3, (2S)-*3*-methylsulfonylmethyl-pyrrolidine-2-carboxylic acid: *3*-prolinomethionine

Figure 1. Schematic Representation of *3*-Substituted Prolines

equivalents with longer coupling times were needed for full efficiency, as ascertained by the Kaiser test. These peptidyl resins were split for the preparation of the various SP (undeca- and hexapeptides) analogs (Table 1) because only 1–5 mg of material was required for all the pharmacological analyses.

Molecular Modeling of Tripeptides

The conformational space of model tripeptides Ac-Gly-Leu-Met-NH_2 (a), Ac-Pro-Leu-Met-NH_2 (b), Ac-Gly-P^t_3Leu-Met-NH_2 (c), Ac-Gly-Leu-P^c_3Met-NH_2 (d), Ac-Gly-P^t_3Leu-P^c_3Met-NH_2 (e), Ac-Pro-Leu-P^c_3Met-NH_2 (f), Ac-Pro-NMeLeu-P^c_3Met-NH_2 (g), and Ac-Pro-P^t_3Leu-P^c_3Met-NH_2 (h) has been investigated using molecular dynamics at 300 K. Values of χ_1, χ_2, and χ_3 torsion angles correspond to a discrete number of conformations (2 or 3 per dihedral angle), and calculation of minimum-energy conformations enables us to describe the orientation of the side chains. In contrast, ϕ,ψ torsion angles are more variable, and the comparison of the conformational space of the peptide backbone is best achieved by calculating the size of the potential wells around minimum-energy conformations [21]. Thus, conformational spaces of model peptides a–h were probed using 10 ns free dynamics at 300 K, and structures were saved every 10 ps, yielding 1000 conformations for analysis. Starting structures were built in extended conformations, but similar maps were obtained with longer dynamics (up to 24 ns) or from other starting conformations (helical, γ-turn). Ramachandran maps (Figure 2) for residues Gly, Leu and Met in the reference tripeptide (a) are in agreement with those reported for these residues in proteins [22]. As previously reported, γ-turn conformations [18] are excluded for residues preceding a *3*-ProAA (peptides c–h) and helical conformations are destabilized for nonglycine residues preceding a *3*-ProAA (peptides d–h) [23]. The same effects are observed for residue Pro preceding NMeLeu (peptide g).

Pharmacology of SP and SP(6-11) Analogs

The affinities of the SP analogs for the two specific binding sites, NK-1M and NK-1m, associated with the human NK-1 receptor, have been measured on transfected CHO cells. The more abundant binding site, NK-1M (85%), is labeled by [^{3}H]SP and is coupled to cAMP production, whereas the less abundant binding site, NK-1m (15%), is labeled by [^{3}H]propionyl[Met(O_2)^{11}]SP(7-11) and is associated with IPs production [19]. The binding affinities, and agonist or antagonist (partial

Table 1. Summary of Peptide Syntheses and Analytical Data

	HPLC *k* (min)[a]				Mass Spectral Analysis $(M+H)^+$	
Peptide	Preparation	Analytical	Yield, %	Purity, %	Calculated	Found
[P^t_3Leu^{10},P^c_3Met^{11}]SP	11.55[b]	7.56[f]	13	>98	1399.77	1400.08[l]
[Pro^9,P^t_3Leu^{10},P^c_3Met^{11}]SP	9.81[b]	8.77[g]	15	>98	1439.80	1439.96[l]
[Pro^9,P^c_3Met^{11}]SP	10.51[c]	6.27[h]	12	>98	1413.78	1413.91[l]
[Pro^9,NMeLeu^{10},P^c_3Met^{11}]SP	13.33[d]	5.05[h]	n.d.[j,k]	>98	1427.79	1427.91[l]
[pGlu^6,Pro^9,P^c_3Met^{11}]SP(6-11)	17.96[e]	12.16[i]	13	>98	790.40	790.47[m]
[pGlu^6,Pro^9,NMeLeu^{10},P^c_3Met^{11}]SP(6-11)	22.16[b]	12.07[i]	4[k]	97	804.41	804.30[m]

[a] HPLC *k* = peptide retention time in solvent system: A (H_2O/0.1% TFA) and B (CH_3CN 60%/H_2O 40%, TFA 0.1%).
[b] Semipreparative HPLC using a linear gradient over 30 min with 30%–70% B.
[c] Semipreparative HPLC using a linear gradient over 30 min with 35%–65% B.
[d] Semipreparative HPLC using a linear gradient over 30 min with 30%–65% B.
[e] Semipreparative HPLC using a linear gradient over 30 min with 40%–65% B.
[f] Analytical HPLC in isocratic mode with 56% B.
[g] Analytical HPLC in isocratic mode with 53% B at 45°C (at room temperature, peak dedoubling probably due to *cis/trans* isomerization of amide bonds).
[h] Analytical HPLC in isocratic mode with 59% B.
[i] Analytical HPLC in isocratic mode with 55% B.
[j] Aggregates in 10% acetic acid. Dissolved in 50% acetic acid for purification.
[k] Another major product was isolated and identified as a Pro-deleted compound (ESI+ M+1 = 707.3 for the hexapeptide analog [pGlu^6, Pro^9, NMeLeu^{10}, P^c_3Met^{11}]SP(6-11)).
[l] Mass spectral analysis performed by MALDI-TOF.
[m] Mass spectral analysis performed by ESI+ analysis.

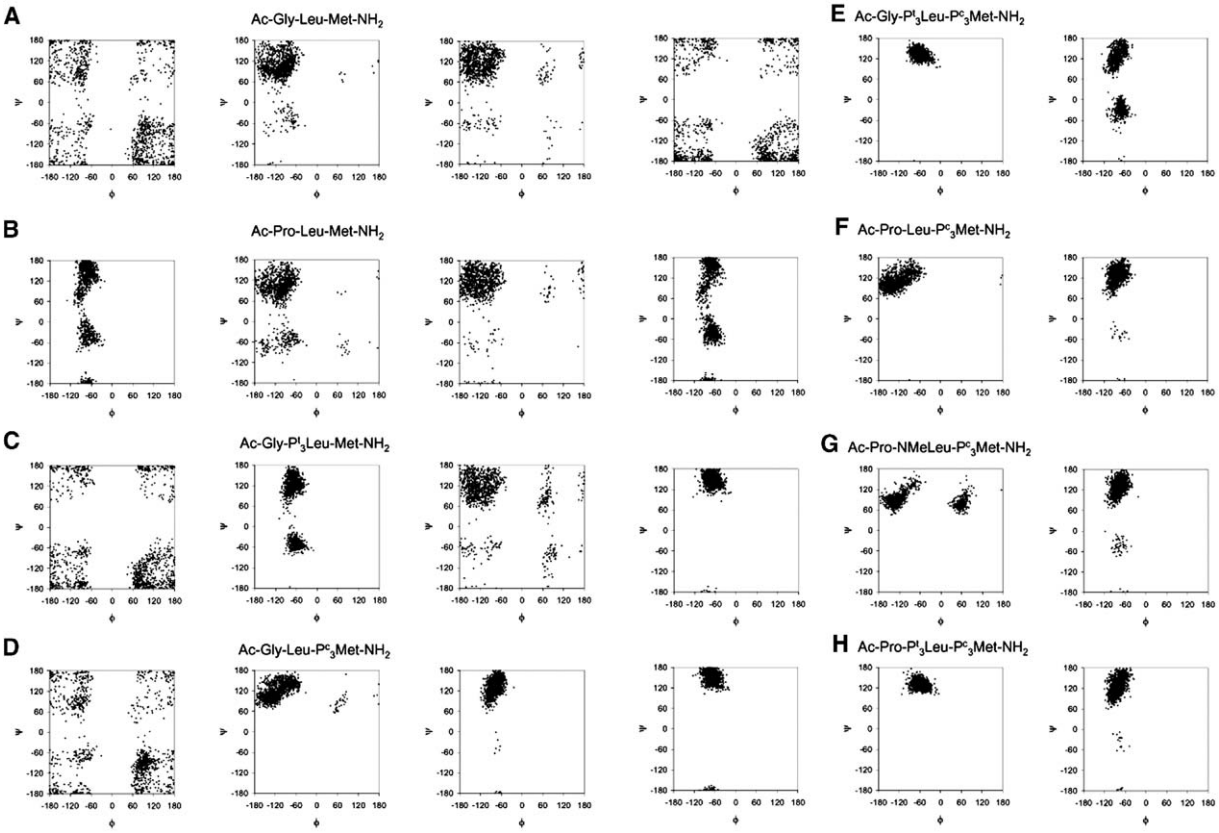


Figure 2. Ramachandran Diagrams of Model Tripeptides Containing Proline or *3*-Prolinoamino Acid

agonist) potencies, expressed as Ki for NK-1M (major site) and NK-1m (minor site) and EC_{50} values (and pK_B values for partial agonists) for the cAMP and IPs pathways, are reported in Table 2. Deviations of the pharmacological behavior of these analogs compared either to SP or [pGlu^6, Pro^9]SP(6-11) were evaluated with Student's t test. Although a factor of two to three was sometimes found to be significant, we have classically considered only a factor of ten or more to correspond to a significant difference in the binding mechanism.

Undecapeptide Analogs Incorporating One Constraint

We have previously shown that Gly^9 can be replaced by a proline [16, 19]. However, proline incorporation in position 10 (Leu) or 11 (Met) was deleterious for both affinities and activities on the two binding sites [16–18]. Reincorporation of one isopropyl side chain on position 3 of the proline scaffold led to [P$^c_3$Leu^{10}]SP and [P$^t_3$Leu^{10}]SP analogs, only the *trans* analog being as potent as SP on the two binding sites [18]. In the same manner, reincorporation of the thioether side chain on the proline scaffold led to the analogs [P$^c_3$Met^{11}]SP and [P$^t_3$Met^{11}]SP being almost as potent as SP on the two binding sites [17].

Undecapeptide Analogs Incorporating Two Constraints

On the NK-1M-specific binding site, [P$^t_3$Leu^{10}, P$^c_3$Met^{11}]SP was a slightly weaker competitor compared to SP (ratio, 3.8), being significantly less potent on the cAMP pathway (EC_{50} = 53 nM) with a decreased efficacy (53%) and being a partial agonist (K_B = 3800 nM). On the NK-1m binding site, [P$^t_3$Leu^{10}, P$^c_3$Met^{11}]SP is an excellent competitor (K_i = 35 pM, 4-fold increase in affinity compared to SP); however its EC_{50} on IPs production is only in the nanomolar range (EC_{50} = 5.4 nM), being 7.7-times less potent than SP and [Pro^9]SP. The double-constrained SP analog [Pro^9, P$^c_3$Met^{11}]SP is a weaker competitor compared to SP on NK-1M-specific binding (ratio, 53) and a partial agonist (EC_{50} = 30 nM, 31% efficacy, K_B = 112 nM) on cAMP production. On the NK-1m-specific binding sites [Pro^9, P$^c_3$Met^{11}]SP is only a slightly weaker competitor (K_i = 0.55 nM), but is as potent as SP and [Pro^9]SP on IPs production (EC_{50} = 1 nM; ratio, 1.4 compared to SP).

Undecapeptide Analogs Incorporating Three Constraints

These two analogs are weak competitors for [^{3}H]SP-specific NK-1M binding (K_i = 180 and 62 nM, respectively), compared to the nanomolar affinities of SP and [Pro^9]SP. Both analogs are also partial agonists on the cAMP pathway (K_B = 680 and 360 nM, respectively). [Pro^9, P$^t_3$Leu^{10}, P$^c_3$Met^{11}]SP induces, at the highest concentration tested (10^{-5} M), only 16% of the maximal response reached with SP, whereas [Pro^9, NMeLeu^{10}, P$^c_3$Met^{11}]SP induces significant cAMP production (EC_{50} = 35 nM, 50% efficacy). On the NK-1m-specific

Table 2. Affinity and Activity of SP-Constrained Analogs

Peptides	Ki, nM NK-1M	EC_{50}, nM (% Efficacy) cAMP	Ki, nM NK-1m	EC_{50}, nM (% Efficacy) IP
H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly^{9}-Leu^{10}-Met^{11}-NH_2[a]	1.6 ± 0.4	8 ± 2	0.13 ± 0.02	0.7 ± 0.3
[Pro^{9}]SP[a]	1.1 ± 0.1	10 ± 2	0.13 ± 0.02	0.7 ± 0.1
[Pro^{10}]SP	24 ± 2***	375 ± 50***	3.7 ± 0.5**	3.0 ± 1.0
[Pro^{11}]SP[b]	1150 ± 250***	>10,000***	63 ± 6***	56 ± 8**
[P^{c}_{3}Leu^{10}]SP[c]	465 ± 105***	>10,000 (12 ± 3%)*** pK_B = 5.12 ± 0.06	220 ± 30***	260 ± 30 (49 ± 3%)** (No antagonism)
[P^{t}_{3}Leu^{10}]SP[c]	0.86 ± 0.07	39 ± 9*	0.34 ± 0.09	2.1 ± 0.3*
[P^{c}_{3}Met^{11}]SP[b]	2.1 ± 0.1	35 ± 1**	0.08 ± 0.05	0.8 ± 0.1
[P^{t}_{3}Met^{11}]SP[b]	3.0 ± 0.5*	25 ± 2**	0.10 ± 0.007	1.4 ± 0.3
[P^{t}_{3}Leu^{10},P^{c}_{3}Met^{11}]SP	6.0 ± 2.0**	53 ± 3 (53 ± 3%)*** pK_B = 5.42 ± 0.09	0.035 ± 0.015	5.4 ± 0.9**
[Pro^{9},P^{t}_{3}Leu^{10},P^{c}_{3}Met^{11}]SP	180 ± 50***	16 ± 3%*** pK_B = 6.17 ± 0.10	2.7 ± 0.2***	4.7 ± 1.6
[Pro^{9},P^{c}_{3}Met^{11}]SP	85 ± 10***	30 ± 2 (31 ± 4%)** pK_B = 6.95 ± 0.15	0.55 ± 0.1	1.0 ± 0.5
[Pro^{9}, NMeLeu^{10},P^{c}_{3}Met^{11}]SP	62 ± 3***	35 ± 5 (50 ± 2%)** pK_B = 6.44 ± 0.20	0.66 ± 0.15	4 ± 2 (85 ± 5%)
[pGlu^{6}, Pro^{9}]SP(6-11)[a]	490 ± 10	5200 ± 200	2.4 ± 0.5	2.7 ± 0.5
[pGlu^{6}, Pro^{9},P^{c}_{3}Met^{11}]SP(6-11)	>5000***	10 ± 2% at 10^{-5}***	13 ± 4	17 ± 2 (75 ± 3%)**
[pGlu^{6}, Pro^{9}, NMeLeu^{10},P^{c}_{3}Met^{11}]SP (6-11)	>5000***	16 ± 3% at 10^{-5}***	3.5 ± 1	10 ± 2 (75 ± 2%)*

Statistical significance (Student's t test) was evaluated against SP for SP analogs and [pGlu6, Pro9]SP(6-11) for SP(6-11) analogs as: ***$p < 0.001$; **$0.001 < p < 0.01$; *$0.01 < p < 0.05$.
[a] Data already published in [19].
[b] Data already published in [17].
[c] Data already published in [18].

binding sites, [Pro^{9}, P^{t}_{3}Leu^{10}, P^{c}_{3}Met^{11}]SP is a weak competitor (ratio, 1:20 compared to SP, [2.70/0.13]), whereas [Pro^{9}, NMeLeu^{10}, P^{c}_{3}Met^{11}]SP is only a slightly weaker competitor (ratio, 1:5 compared to SP [4.0/0.7]). When IPs production is considered, both analogs are six- to seven-times less potent than SP and [Pro^{9}]SP (EC_{50} = 4.7 and 4.0 nM, respectively). With [Pro^{9}, NMeLeu^{10}, P^{c}_{3}Met^{11}]SP, the maximal response is not attained, IPs production plateau reaching 85% of the response induced by SP and [Pro^{9}]SP.

Hexapeptide Analog Incorporating Two Constraints

C-terminal hexapeptide analogs of SP are micromolar agonists of SP on NK-1M-specific binding sites and on cAMP production [19, 20]. But [pGlu^{6}, Pro^{9}]SP(6-11) is a nanomolar competitor on NK-1m binding sites (K_i = 2.4 nM compared to 0.13 nM for SP and [Pro^{9}]SP) and a potent analog on IPs production (EC_{50} = 2.7 nM), being only four-times less potent than the undecapeptides SP and [Pro^{9}]SP [19, 20]. [pGlu^{6}, Pro^{9}, P^{c}_{3}Met^{11}]SP(6-11) behaves as [pGlu^{6}, Pro^{9}]SP(6-11) on NK-1M and is only a very weak competitor of [^{3}H]propionyl [Met(O_2)^{11}]SP(7-11) (ratio, 1:100 compared to SP, but only 1:5 when compared to [pGlu^{6}, Pro^{9}]SP(6-11)) on the NK-1m binding sites. [pGlu^{6}, Pro^{9}, P^{c}_{3}Met^{11}]SP (6-11) is 24-times less potent than SP and [Pro^{9}]SP, with only 75% of their efficacies on IPs production, but only six-times less potent than [pGlu^{6}, Pro^{9}]SP(6-11).

Hexapeptide Analog Incorporating Three Constraints

Similar to [pGlu^{6}, Pro^{9}, P^{c}_{3}Met^{11}]SP(6-11) double-constrained analog, [pGlu^{6}, Pro^{9}, NMeLeu^{10}, P^{c}_{3}Met^{11}]SP(6-11) is inactive on NK-1M-specific binding sites. However, on NK-1m-specific binding sites, [pGlu^{6}, Pro^{9}, NMeLeu^{10}, P^{c}_{3}Met^{11}]SP(6-11) was a weak competitor (ratio, 1:27 compared to SP and 1:1.5 compared to [pGlu^{6}, Pro^{9}]SP(6-11)). On IPs production, [pGlu^{6}, Pro^{9}, NMeLeu^{10}, P^{c}_{3}Met^{11}]SP(6-11) was 15-times less potent than SP and 3.7-times less potent than [pGlu^{6}, Pro^{9}]SP(6-11), reaching only 75% of the SP maximal response.

Comparative Analysis of the Backbone Conformational Spaces of Model Tripeptides in Connection with High-Affinity and Low-Affinity SP Analogs

The introduction of a proline or a prolinoamino acid limits the ϕ angle to a (–90, –50) interval (peptides b, c, and d; Figure 2). This ϕ angle restriction imposed by a single substitution in the 9–11 segment is well tolerated for both binding sites. The (ϕ,ψ) space of nonglycine preceding residue is also mainly restricted to the upper left quadrant of the Ramachandran diagram (peptide d). Thus, the Gly-Leu-P^{c}_{3}Met-NH_2 analog shows that the double restriction of ψ_{10} and ϕ_{11} is well accommodated in both binding sites. In the double constrained Gly-P^{t}_{3}Leu-P^{c}_{3}Met-NH_2 agonist, three angles are restricted: ϕ_{10}, ψ_{10}, and ϕ_{11}, the conformational space of residue 10 being limited exclusively to the PP_{II} region (peptide e).

Accessible conformations for P^{c}_{3}Met and P^{t}_{3}Leu are identical in peptides e–g (Figure 2). Concerning the position 9, the conformational spaces of peptide b and peptide f are identical. Therefore the lower affinity for NK-1M observed with the [Pro^{9}, P^{c}_{3}Met^{11}]SP analog cannot be explained by a restriction of the conformational space of the C-terminal tripeptide. Because the triple-constrained analogs [Pro^{9}, NMeLeu^{10}, P^{c}_{3}Met^{11}]SP and [Pro^{9}, P^{t}_{3}Leu^{10}, P^{c}_{3}Met^{11}]SP have low affinities

for the NK-1M site, no information can be deduced for ψ_9 angle. This is not the case for the NK-1m site, as both [P^t_3Leu^{10}, P^c_3Met^{11}]SP and [Pro^9, NMeLeu^{10}, P^c_3Met^{11}]SP analogs conserved high affinity. Indeed, the conformational space of peptide g indicates that residue 9 adopts a PP_{II} conformation. As peptides [P^t_3Leu^{10}, P^c_3Met^{11}]SP and [Pro^9, NMeLeu^{10}, P^c_3Met^{11}]SP (model peptides e and g, respectively) have an affinity similar to that of SP on the NK-1m binding site, the lower affinity measured for [Pro^9, P^t_3Leu^{10}, P^c_3Met^{11}]SP (corresponding to model tripeptide h) cannot be explained by restriction of the conformational space of the C-terminal tripeptide in this analog.

Discussion

Comparison between Single and Multiple Constraints

The pharmacological properties of the monosubstituted SP analogs containing either a prolinoleucine or a prolinomethionine, combined for the determination of the minimum-energy conformations of Ac-P^c_3Leu-NHMe and Ac-P^t_3Leu-NHMe, permitted us to identify the side chain orientation of Leu10 (χ_1 *gauche*+, χ_2 *trans*) and Met11 (χ_1 *trans*) in the bioactive conformation of SP [17, 18]. Further calculations indicated that helical conformations were destabilized for the residue preceding a *3*-substituted proline [18], thus showing that the backbone of the C-terminal tripeptide of SP should adopt a rather extended conformation when interacting with the NK-1 receptor.

Several constraints were introduced simultaneously in the C-terminal tripeptide of SP in order to further define the receptor-bound conformation of Gly-Leu-Met-NH_2. SP analogs [Pro^9]SP, [P^t_3Leu^{10}]SP, [P^c_3Met^{11}]SP (one constraint) and [P^t_3Leu^{10}, P^c_3Met^{11}]SP (two constraints in positions 10 and 11) have a similar affinity when compared to SP for the NK-1M specific binding site. In contrast, peptides [Pro^9, P^c_3Met^{11}]SP (two constraints in positions 9 and 11), [Pro^9, P^t_3Leu^{10}, P^c_3Met^{11}]SP, and [Pro^9, NMeLeu^{10}, P^c_3Met^{11}]SP (three constraints) have a lower affinity than SP for the NK-1M binding site. For the NK-1m binding site, all these peptides have a similar affinity compared to SP, except [Pro^9, P^t_3Leu^{10}, P^c_3Met^{11}]SP, the affinity of which was significantly lower than that of SP. The lower affinities of the most constrained SP analogs do not seem to result from differences in the accessible conformations of the C-terminal tripeptide in these SP analogs, as inferred from comparative analysis of the low-energy conformations of constrained Ac-Gly-Leu-Met-NH_2 model tripeptides. For both NK-1M- and NK-1m-specific binding sites, the common conformational space for high-affinity analogs correspond to the upper left quadrant of the Ramachandran diagram (extended region) and, more specifically, to ϕ,ψ values close to that of PP_{II} conformation ($\phi \sim -78°$, $\psi \sim 149°$) for the three residues of the C-terminal tripeptide (although the ψ_9 value remains ambiguous for the NK-1M site). The lower affinity of the most constrained SP analogs cannot be ascribed to a noticeable change in accessible conformations of the C-terminal part of these peptides, because all these constrained analogs may adopt this extended PP_{II} structure. One possible explanation is that the (ϕ,ψ,χ) angle restrictions are not optimal, and that, in a single constrained analog, these nonoptimal values could be compensated by small deviations around the other nonrestrained torsion angles. Whatever the reason, the bioactive conformation likely corresponds to an extended structure (β/PP_{II}-like). In addition, it should be noted that canonical PP_{II} or β strand structures are quite close to each other, as superimposition yields an rmsd of only 0.13 nm when all heavy atoms are taken in to account (in the Supplemental Data available with this article online). The underlying assumption that compounds of closely related chemical structure bind to the receptor with a common binding process ending in an identical, so-called, "bioactive conformation" is an oversimplification. It is clear, now, that the backbone conformations of different analogs do not have to be strictly identical ("frozen") for successful presentation of the anchoring side chains in a correct three-dimensional orientation for recognition. The receptor may accept slightly different conformations for different ligands when bound to the receptor, and the binding process must be viewed as a dynamic process (multistep and/or sequential) in which mutual adaptation of the two partners (ligand and receptor) cannot be possible if the ligand is too constrained. Therefore, with this latter idea in mind, Ki values indirectly reflect this dynamic process and represent thermodynamic or steady-state values. The affinity value, Ki, a thermodynamic parameter, gives the affinity for the most stable state reached by the competitor/receptor complex (the standard Gibb's free energy $\Delta G^0 = -RT\ \ln Ki$), assuming that equilibrium was reached for both the radioligand used and the competitor tested in the binding assay—differences in Ki values for the two specific NK-1 binding sites could arise from the experimental conditions per se. In the binding assay, equilibrium was reached for the radioligand (in both cases), but the incubation time may have been inadequate for the constrained competitors. Although preincubation time prior to radioligand addition has not been assayed for the analogs described herein, previous attempts with other families of constrained SP analogs have always led to unaltered Ki values.

NK-1M Binding Site Determinants

For the NK-1M-specific binding site, it can be inferred with all the SP analogs so far described that the undecapeptide sequence was required for nanomolar affinity, and that phenylalanine-7, leucine-10, and methionine-11 (or butyl, as in norleucine-11) side chains are needed to retain high affinity. The binding subsites of Phe^7 [24] and Leu^{10} [18] side chains within the NK-1M-specific binding site are rather restricted in volume and flexibility, whereas the subsite for the methionine side chain is larger and more adjustable. Position 9 constitutes a hinge between the N-terminal residues, with the helical core (residues 4–8), and the PP_{II} conformation of the C-terminal dipeptide Leu-Met-NH_2. Depending on the residue in position 9 (Gly, Ala [25], Pro [19], Sip [26], Aib [25]), the three major anchoring points (Phe^7, Leu^{10}, and Met^{11}) of the undecapeptide SP analogs may or may not be correctly positioned within the NK-1M-specific binding site. Indeed, with an aminoisobu-

tyric acid (Aib) in position 9, this most stable conformation cannot be reached within the ligand/receptor complex [25].

The NK-1M-specific binding site accepts two consecutive constraints in positions 10 and 11 ($P^t_3Leu^{10}$ and $P^c_3Met^{11}$, respectively), but two constraints in positions 9 and 11 are not accepted (Pro^9 and $P^c_3Met^{11}$, respectively), although we did not assay a peptide containing the *trans-3*-substituted proline residue. If one assumes that equilibrium was reached for the 9/11-disubstituted competitor, the results suggest that a multistep or sequential binding should occur between the competitor and the receptor. The binding of the isobutyl side chain may happen first to induce mutual adaptation of both the ligand and the protein. When positions 9 and 11 are simultaneously constrained, this mutual adaptation cannot occur for the isobutyl side chain, which has to worm its way into a small binding subsite. Alternatively, if binding of the isobutyl side chain is a "late event" in the binding process (occurring after Phe^7 and the constrained Met^{11} bindings), then mutual adaptation of the Leu^{10} may not be possible within its binding subsite with a constraint in position 9. A fortiori, three consecutive constrained amino acids in the C-terminal tripeptide of SP cannot fit in the NK-1M-specific binding site.

NK-1m Binding Site Determinants

When considering the NK-1m-specific binding site, the relative orientation of the N-terminal residues with respect to the C-terminal dipeptide Leu-Met-NH_2 is not the central question, as the C-terminal hexapeptide exhibits nanomolar affinity [19]. The N-terminal residues of the undecapeptide SP analogs further stabilize the ligand/NK-1 complex, but only by a factor of 10 to 20 [19]. These N-terminal residues may fit within binding subsites, but only the presence and the relative orientation of phenylalanine-7 versus the C-terminal dipeptide is critical. The binding subsite of Phe^7 side chain within the receptor is rather restricted in volume and flexibility, whereas the subsite for the methionine (or butyl, i.e., norleucine-11) side chain is larger and more adjustable. However, in contrast to the NK-1M-specific binding site, the stabilizing interaction of the isobutyl side chain of Leu^{10} is not as important, considering the high affinity of [Gly(ΨCH_2CH_2)Gly^{10}]SP [27]. Surprisingly, [Pro^{10}]SP was a poor competitor for the NK-1m-specific binding site, whereas reintroduction of an isobutyl side chain with a particular geometry, such as in [$P^t_3Leu^{10}$]SP, completely restores the binding potential. Glycine, Pro, and Ala are the best substituents in position 9 to afford correct positioning of the two major anchoring points (Phe^7 and Met^{11}).

The isobutyl side chain is not a key element in the stabilization of the competitor/receptor complex [27]. Indeed, the NK-1m-specific binding site not only accepts two constraints in positions 10 and 11 ($P^t_3Leu^{10}$ and $P^c_3Met^{11}$, respectively), but also in positions 9 and 11 (Pro^9 and $P^c_3Met^{11}$, respectively). The undecapeptide with three consecutive constraints (Pro^9, $NMeLeu^{10}$, and $P^c_3Met^{11}$) can also be accommodated within the receptor (Ki[analog]/Ki[SP] close to 5). The most constrained SP analog, [Pro^9, $P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, has a decreased affinity, but is still in the nanomolar range (Ki[analog]/Ki[SP] close to 20), similar to the affinity of hexapeptide analogs [$pGlu^6$, Pro^9]SP(6-11), septide and [Pro^9, $NMeLeu^{10}$, $P^c_3Met^{11}$]SP(6-11) (the ratio Ki[analog]/Ki[septide] was close to 1). These data suggest that stabilization, by a factor of 10 to 20, of the N-terminal residues is not readily accessible within the most constrained competitor/receptor complex.

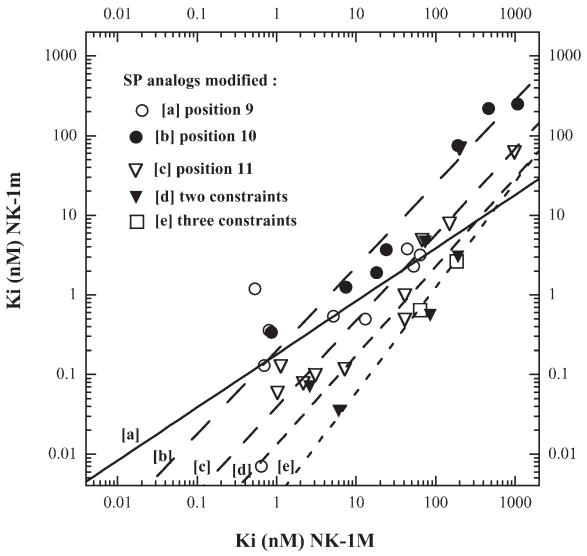


Figure 3. Linear Regression Plot Obtained from NK-1M Versus NK-1m Binding Sites Affinity for Modified Peptide Analogs

Analogs were modified in position 9 ([Pro^9]SP, [Sar^9, $Met(O_2)^{11}$]SP, [Ala^9]SP, [Aib^9]SP, [β^2-$HAla^9$]SP, [β-Ala^9]SP, [(pBz)Phe^9]SP, [Sip^9]SP), position 10 ([αMeLeu^{10}]SP, [β^2-Leu^{10}]SP, [(pBz)Phe^{10}]SP, [$NMeLeu^{10}$]SP, [Pro^{10}]SP, [$P^c_3Leu^{10}$]SP, [$P^t_3Leu^{10}$]SP), position 11 ([αMeMet^{11}]SP, [$Met(O_2)^{11}$]SP, [Pro^{11}]SP, [$P^t_3Met^{11}$]SP, [$P^t_3Met(O)^{11}$]SP, [$P^t_3Met(O_2)^{11}$]SP, [$P^c_3Met^{11}$]SP, [$P^c_3Met(O)^{11}$]SP, [$P^c_3Met(O_2)^{11}$]SP, [Nle^{11}]SP, [(pBz)Phe^{11}]SP, or containing two ([Gly^9(ψCH_2CH_2)Gly^{10}]SP, [Gly^9(ψCH_2CH_2)(S)Leu^{10}]SP, [Gly^9(ψCH_2CH_2)(R)Leu^{10}]SP, [Apa(DLeu$^{9\text{-}10}$)]SP, [$P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, [Pro^9, $P^c_3Met^{11}$]SP), or three constraints ([Pro^9, $P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, [Pro^9, $NMeLeu^{10}$, $P^t_3Met^{11}$]SP).

Discrimination between the Two Binding Sites

Position 9 in SP constitutes a hinge point for recognition discrimination between NK-1M and NK-1m binding sites. Indeed, as shown in the plot of NK-1m versus NK-1M affinities for C-terminal constrained analogs of SP previously or herein described (Figure 3), modifications in positions 10 or 11 led to parallel straight lines shifting toward higher selectivity for the NK-1m binding site, the two or three constraint-containing peptides being the most selective. However with constrained analogs in position 9, the slope of the straight line (Figure 3) is much lower, indicating a more pronounced effect of such modifications on the NK-1M compared to those on the NK-1m recognition process. This residue is also known to be a in a key position to switch from agonist to antagonist behavior [25].

These constrained SP analogs gave the most selective undecapeptide ligands so far described for the NK-1m-specific binding site. [$P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, [Pro^9, $P^c_3Met^{11}$]SP, [Pro^9, $NMeLeu^{10}$, $P^c_3Met^{11}$]SP, and [Pro^9,

P $^{t}_{3}$Leu^{10}, P$^{c}_{3}$Met^{11}]SP are all about 100- to 200-times more selective than any other analog for the NK-1m binding site. [P$^{t}_{3}$Leu^{10}, P$^{c}_{3}$Met^{11}]SP was the best one with a factor of selectivity comparable (around 200-times) to the C-terminal hexapeptide analogs, but with a subnanomolar affinity for the NK-1m-specific binding site (Figure 3). This increase of selectivity came from an increase in affinity for the NK-1m-specific binding site, whereas the affinity for the NK-1M-specific binding site remained unchanged.

Enthalpy and Entropy Effects

If the chemical modifications introduced in the C-terminal region have restricted the C-terminal residues into a conformation mimicking the receptor-bound conformation, then one would have expected to see a significant increase in affinity due to a decrease in the entropy penalty associated with constrained analogs. This was not the case, as only a small (not significant) increase was observed with two SP analogs, [P$^{t}_{3}$Leu^{10}]SP for the NK-1M-specific binding site (Ki[SP]/Ki[analog] = 2) and [P$^{t}_{3}$Leu^{10}, P$^{c}_{3}$Met^{11}]SP (Ki[SP]/Ki[analog] = 4) on the NK-1m-specific binding site. Thus, the decrease in entropy does not compensate for a decrease in enthalpy, which may be tentatively associated with a nonoptimal interaction of one of the major anchoring points (methionine^{11} or isobutyl side chain in position 10 and/or Phe^{7}). Williams et al. have proposed that residual mobility within a ligand/receptor complex must decrease the enthalpy, and that decreasing the flexibility of the anchoring points should conversely increase the enthalpy [28, 29]. According to Hunter and Thomas, an increase in enthalpy may also correspond to a decrease in the number of partially bound states, which are more flexible, and consequently will be associated with a decrease in the entropy of the system, thus tentatively explaining the enthalpy/entropy compensation [30]. Notable is the fact that the solvation/desolvation question (entropy contribution) has not been considered in these studies. This contribution may be important within the NK-1 receptor, as the three major anchoring points are hydrophobic and their binding subsites are expected to be within a hydrophobic environment with "specific" (frozen) water molecules, which have to be displaced (entropy increase) upon binding, as observed, for example, in the growth hormone binding protein [31]. With the undecapeptide SP analogs, one can hypothesize that too many constraints in the C-terminal tripeptide have decreased the number of less efficiently (partly) bound states and thus decrease the entropy of the complex. For the NK-1m-specific binding site, "only" Phe^{7} and Met^{11} side chains are mandatory; thus, less partly (or loosely) bound states may be involved in the binding process compared to the NK-1M-specific binding, for which the helical structure and Phe^{7}, Leu^{10}, and Met^{11} are necessary. One may hypothesize that the entropic loss may be less than that in the NK-1M-specific binding site.

Implications for Current Models of GPCR Activation

A number of kinetic models have been developed to describe the process of agonist activation [32]. In the past few years, studies using fluorescence spectroscopy (receptor-tagged or ligand- and receptor-tagged) have provided insight into the structural changes that occur upon agonist activation. Studies with purified, detergent-solubilized β^2-adrenergic receptor (β^2AR) suggest that the native receptor is a conformationally flexible molecule that exists in at least two predominant conformation substates in equilibrium [32, 33]. Agonists, partial agonists, and neutral antagonists change both the shape of the entire distribution and the population of the conformational states [34–37]. Based on the lifetime of the conformational states, the Kobilka group has proposed a model with multiple agonist-specific receptor states, wherein activation occurs through a sequence of conformational states, from the resting state (R) to an intermediate state (R′) and, finally, the active state (R*) [34–37]. Ki values may not reflect the agonist-induced conformational state (R*) of the receptor if one considers the relative populations of these R, R′, and R* conformational states, unless the affinity of the agonists dramatically increases for the R*. The agonist binding precedes the conformational changes from R′ to R*; the active state is poorly populated even with a saturated concentration of agonist. This R* state appears more flexible than the R and R′ states, and transitions from R′ to R* are relatively rare, high-energy events. It has been shown that the rate of conformational change is relatively slow for the β^2AR (around 300 s), despite a rapid on-rate of agonist binding (t1/2 around 20 s). As such, these authors have proposed a stepwise process for the agonist binding with some interactions occurring rapidly (R to R′); subsequent interactions "cannot form until stochastic conformational fluctuations make the complete binding pocket accessible," leading to the R* state [34–37]. In the case of the isoproterenol/β^2AR complex the energy of binding has been calculated to be 6–10 kcal/mol, in the absence of G-protein. Precoupling of the receptor to the G-protein in intact cells may accelerate the agonist-induced interconversion.

By combining real-time measurements of agonist binding by fluorescence resonance energy transfer and second-messengers responses, the Galzi group has shown that the NK-2 receptor may be sequentially activated, by NKA and analogs, from an R0 (inactive state) to an R1 state (active conformation triggering calcium response) and, with a slower kinetic, to an R2 state (active conformation triggering cAMP response) [38–40]. In the case of the NK-1 receptor, it would be tempting to speculate that the IPs and cAMP production corresponds to the same sequential events. However, this does not seem to be the case, as we have previously shown that the two binding sites do not interconvert, at least within the span of hours that is the time scale used in binding studies [41]. Moreover, the binding kinetics of the two analogs are reversed in intact CHO cells (equilibrium reached in 100 min and 70 min for [^{3}H]SP and [^{3}H]propionyl[Met(O$_2$)^{11}]SP(7-11), respectively) and in membrane homogenates (equilibrium reached in 10 min and 60 min for [^{3}H]SP and [^{3}H]propionyl[Met(O$_2$)^{11}]SP(7-11), respectively) [19, 20].

Although the molecular basis of these two specific binding sites is unknown, a few conclusions on their respective activation patterns may be drawn from these

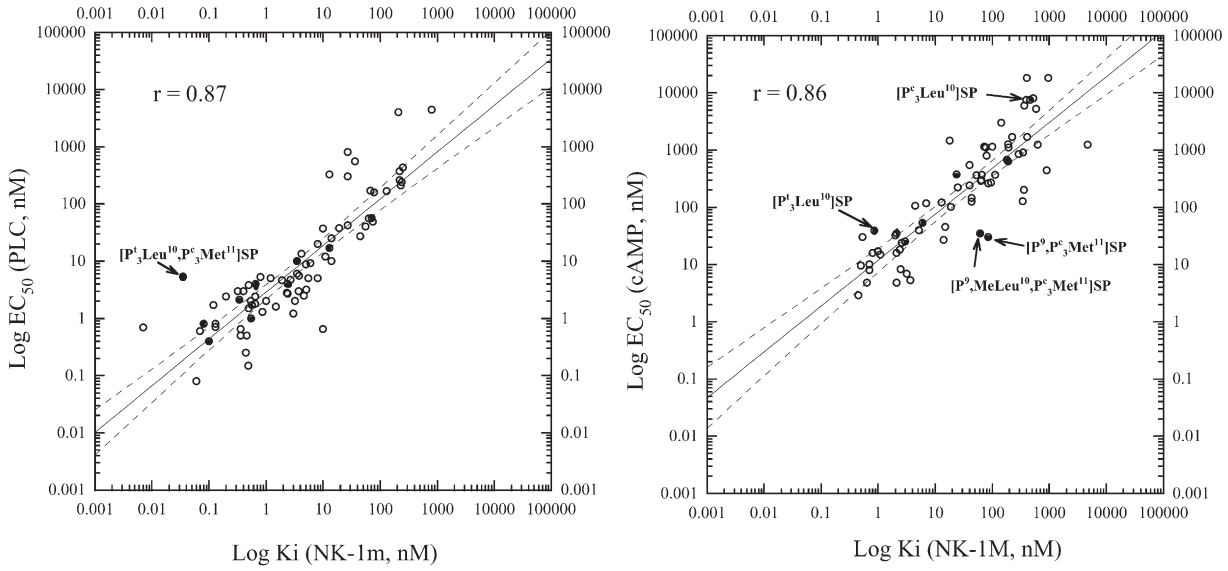


Figure 4. Linear Regression Plot with 95% Confidence Interval of Ki (NK-1m) versus EC_{50} (PLC) and Ki (NK-1M) versus EC_{50} (AC) Obtained from 103 analogs (data not shown) synthesized and pharmacologically tested in our laboratory.

proline-substituted SP analogs. All the nanomolar or subnanomolar competitors of the NK-1m-specific binding site display expected EC_{50} values for IPs production within the 95% confidence interval, except [P^t_3Leu^{10}, P^c_3Met^{11}]SP (Figure 4). The most selective ligand among the constrained analogs reported herein is clearly seen out of the correlation of Ki (NK-1M) and EC_{50} (cAMP) (Figure 4). If the Ki value reflects the (R) and/or (R′) conformational states, the subsequent conformational change to (R*) is less efficient, and the binding energy is not transformed into an energy of activation for the conformational rearrangement within the complex. When considering the NK-1M-specific binding site, all the analogs with two or three constraints are partial agonists (i.e., [P^t_3Leu^{10}, P^c_3Met^{11}]SP, [Pro^9, P^c_3Met^{11}]SP, [Pro^9, P^t_3Leu^{10}, P^c_3Met^{11}]SP, and [Pro^9, NMeLeu^{10}, P^c_3Met^{11}]SP). Correlations are observed between Ki (NK-1M) values and EC_{50} (cAMP) values for two of them, [P^t_3Leu^{10}, P^c_3Met^{11}]SP and [Pro^9, P^t_3Leu^{10}, P^c_3Met^{11}]SP; but [Pro^9, P^c_3Met^{11}]SP and [Pro^9, NMeLeu^{10}, P^c_3Met^{11}]SP are clearly outside the 95% confidence interval of the correlation (Figure 4). However when their pK_B values were taken into account, these discrepancies were not so obvious (data not shown in Figure 4). Neutral antagonists have been obtained by restricting the flexibility around position 9 of SP with a β-II′ turn. Partial agonism has been observed with some SP analogs bearing an aromatic substituent on the C-terminal residue [42, 43]. The [P^t_3Leu^{10}, P^c_3Met^{11}]SP, [Pro^9, P^c_3Met^{11}]SP, [Pro^9, P^t_3Leu^{10}, P^c_3Met^{11}]SP, and [Pro^9, NMeLeu^{10}, P^c_3Met^{11}]SP analogs show that there is no need for either an aromatic substituent or a β-II′ turn structure [44, 45] to confer partial antagonist properties on SP undecapeptide [46]. There is no clear relationship between the agonist activity (either EC_{50} values or the plateau reached) and the antagonist potency (pK_B). Thus, additional constraints in the C-terminal SP tripeptide may eliminate some partially bound states within the ligand/receptor complex, which are normally involved in the productive (activation of second messenger pathways) transconformation of the receptor.

In conclusion, it has been clearly established with these proline-substituted SP analogs that the two specific binding sites associated with the NK-1 receptor recognize the C-terminal tripeptide of SP, Gly-Leu-Met-NH_2, in an extended conformation (close to PP_{II}). Additionally, as proposed for the NK-2 tachykinin receptor and the β^2-AR, the binding of SP and the amino acids constituting its pharmacophore should be sequential, involving mutual adaptation and partially bound states.

Significance

The NK-1 receptor-bound conformation of SP (H-RPK PQQFFGLM-NH_2) had been established as a highly flexible N-terminal domain with a core helical structure (residues 4–8) and crucial residues in positions 7, 10, and 11. However, there are divergent views on whether there is a helical or a more or less extended C-terminal domain. To study the C-terminal structure of SP, *cis* and *trans* prolinoleucine and prolinomethionine (P_3Leu, P_3Met) were introduced at positions 10 and 11 of SP. These amino acid chimeras, combining the proline constraint (ϕ angle value) with the side chain of an amino acid, are useful for exploring the χ space required for ligand/receptor interaction. The use of such constrained analogs allowed the determination of the *gauche+* (χ_1) and *trans* (χ_2) orientation of the Leu^{10} side chain and the *trans* orientation (χ_1) of the Met^{11} side chain. In this study, two or three prolinoamino acid chimeras were thus incorporated in to the SP sequence to observe the effects of additional constraints on the biological activity of the resulting

analogs. Results have shown that, although describing similar conformational spaces, no more than two constraints were tolerated by the NK-1 receptor in positions 10 and 11. The nonadditivity of these constraints suggests that peptide backbone flexibility is essential to precisely position the crucial recognition side chains. These analogs were also useful for the discrimination of recognition specificity between the two binding sites associated with the NK-1 receptor. Indeed, the NK-1m binding site can accommodate more constraints than can that of NK-1M. In addition, position 9 in SP was found to constitute a hinge point for recognition discrimination between the two binding sites. Altogether, these multiconstrained analogs favor the structurally similar PP_{II} or β strands as the conformational organization for the C-terminal part of SP.

Experimental Procedures

Peptide Syntheses

$P^t_3Leu^{10}$ and $P^c_3Met^{11}$ have been synthesized as described previously [17, 18]. All Boc-amino acids were purchased from Senn Chemicals Int. (Cachan, France). [$P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, [Pro^9, $P^c_3Met^{11}$]SP, [Pro^9, $P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, [Pro^9, $NMeLeu^{10}$, $P^c_3Met^{11}$]SP, [$pGlu^6$, Pro^9, $P^c_3Met^{11}$]SP(6-11), and [$pGlu^6$, Pro^9, $NMeLeu^{10}$, $P^c_3Met^{11}$]SP(6-11) were prepared by solid-phase peptide synthesis on an α-*p*-methylbenzhydrylamine resin (MBHA resin, substitution: 0.73 mmol/g of resin). In all cases, the three C-terminal residues were coupled manually to the resin. Nonnatural amino acids Fmoc-P^c_3Met, Boc-P^t_3Leu, Boc-P^c_3Leu, Boc-pGlu, and Boc-NMeLeu [47, 48] were assembled in a 3- or 5-fold excess using HBTU as coupling reagent. Other Nα-Boc-amino acids were assembled in a 10-fold excess using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole as coupling reagents. For peptides [$P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP and [Pro^9, $P^c_3Met^{11}$]SP, synthesis was performed on a 0.2 mmol scale and the resin was split in two after coupling of $P^t_3Leu^{10}$. For peptides [Pro^9, $P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, [Pro^9, $NMeLeu^{10}$, $P^c_3Met^{11}$]SP, [$pGlu^6$, Pro^9, $P^c_3Met^{11}$]SP(6-11), and [$pGlu^6$, Pro^9, $NMeLeu^{10}$, $P^c_3Met^{11}$]SP(6-11), synthesis was performed on a 0.3 mmol scale and the resin was split in half after coupling of $P^c_3Met^{11}$. Then, after coupling of Pro^9, the two sets of resin were split in half again to synthesize undecapeptide and hexapeptide analogs. In a typical manual procedure, the following steps were performed in each cycle, with coupling efficiency monitored with the Kaiser test: (1) activation of the Nα-Boc-amino acid in NMP with the coupling reagents DCC/HOBt or HBTU (1 eq) and DIEA (2 eq) for 5 min; (2) introduction to the reaction vessel, and mixing for 4 to 16 hr; (3) washings with CH_2Cl_2, MeOH, and CH_2Cl_2; (4) monitoring the completion of the reaction with the Kaiser test; (5) Boc removal for 30 min with 50% TFA in CH_2Cl_2 and 1 mg indole/100 ml of solution (or 20% piperidine for Fmoc-P^c_3Met); (6) washings with CH_2Cl_2; (7) neutralization with 10% DIEA in CH_2Cl_2; and (8) washings with CH_2Cl_2, MeOH, and CH_2Cl_2. For the undecapeptides [$P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, [Pro^9, $P^c_3Met^{11}$]SP, [Pro^9, $P^t_3Leu^{10}$, $P^c_3Met^{11}$]SP, and [Pro^9, $NMeLeu^{10}$, $P^c_3Met^{11}$]SP, the remaining couplings were carried out on an ABI Model 431A peptide synthesizer. For the hexapeptides [$pGlu^6$, Pro^9, $P^c_3Met^{11}$]SP(6-11) and [$pGlu^6$, Pro^9, $NMeLeu^{10}$, $P^c_3Met^{11}$]SP(6-11), all residues were coupled manually. After removal of the last Nα-Boc-protecting group, the resin was dried in vacuo. The peptidyl resin was transferred into the Teflon vessel of an HF apparatus and the peptide was cleaved from the resin by treatment with 1.5 ml of anisole, 0.25 ml of dimethyl sulfide, and 10 ml of anhydrous HF per gram of peptide resin for 1 hr at 0°C. After evaporation in vacuo of HF and the solvents over 2 hr, the resin was first washed three times with Et_2O and then subsequently extracted three times with 10% AcOH. After lyophilization of the extract, the crude peptide was purified by preparative reverse-phase HPLC with a Dionex apparatus, using a 7.8–300 mm, C_8 (7 μm) SymmetryPrep column. The separation was accomplished using various acetonitrile gradients in aqueous 0.1% TFA (see Table 1) at a flow rate of 6 ml/min, with UV detection fixed at 220 nm. Before pooling, the purity of collected fractions was analyzed by analytical HPLC (Waters Associates). The separation was performed on a 4 mm × 250 mm, C_8 (10 μm) Lichrosphere 100 RP column (Merck) in isocratic mode (see Table 1 for solvent systems) at a flow rate of 1.5 ml/min with UV detection fixed at 220 nm. Further information on synthesized peptides are summarized in Table 1.

Molecular Mechanics Calculation

Model tripeptides Ac-Gly-Leu-Met-NH_2, Ac-Pro-Leu-Met-NH_2, Ac-Gly-P^t_3Leu-Met-NH_2, Ac-Gly-Leu-P^c_3Met-NH_2, Ac-Gly-P^t_3Leu-P^c_3Met-NH_2, Ac-Pro-Leu-P^c_3Met-NH_2, Ac-Pro-NMeLeu-P^c_3Met-NH_2, and Ac-Pro-P^t_3Leu-P^c_3Met-NH_2 were built with all the peptide bonds in a *trans* conformation using the InsightII package (Accelrys Inc.). Molecular mechanics calculations were performed with the Discover program and CFF91 Forcefield. The electrostatic potential was calculated in vacuo with a distance-dependent dielectric screening of 4·r. Structures were minimized using steepest descent and conjugate gradient algorithms until the gradient was less than 0.001 kcal · mol^{-1}. Structures were gradually heated from 50 K to 300 K at 50 K intervals, in six steps of dynamics of 5 ps, with a time-step of 1 fs. Then, the conformational space was generated by 10 ns free dynamics at 300 K with a time-step of 1 fs. Structures were saved every 10 ps, yielding 1000 conformations for analysis [18, 21, 22].

Binding Assays/Second Messenger Pathways

CHO cells expressing 6 pmol of human NK-1 receptors per mg of membrane proteins were used. For [^{3}H]SP (120 Ci.$mmol^{-1}$) binding assays, 5 × 10^3 cells per well were seeded, and 5 × 10^4 cells per well were needed for [^{3}H]propionyl[$Met(O_2)^{11}$]SP(7-11) (100 Ci·$mmol^{-1}$) binding experiments [19]. Time incubations at 22°C were 100 min and 80 min, respectively, in Krebs-phosphate buffer [19, 20]. For second-messengers experiments, 10^5 CHO cells were labeled for 24 hr with [^{3}H]inositol (0.5 μCi/well) or [^{3}H]adenine (0.2 μCi/well) [19, 20]. Linear regressions between affinity and activity were obtained with 103 analogs of SP (list not shown), with a 95% confidence interval.

Supplemental Data

There is a supplemental figure (Figure S1) available at http://www.chembiol.com/cgi/content/full/12/5/555/DC1/.

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References

1. Sumner, S.C., Gallagher, K.S., Davis, D.G., Covell, D.G., Jernigan, R.L., and Ferretti, J.A. (1990). Conformational analysis of the tachykinins in solution: substance P and physalaemin. J. Biomol. Struct. Dyn. *8*, 687–707.
2. Grdadolnik, S.G., Mierke, D.F., Byk, G., Zeltser, I., Gilon, C., and Kessler, H. (1994). Comparison of the conformation of active and nonactive backbone cyclic analogs of substance P as a tool to elucidate features of the bioactive conformation: NMR and molecular dynamics in DMSO and water. J. Med. Chem. *37*, 2145–2152.
3. Young, J.K., Anklin, C., and Hicks, R.P. (1994). NMR and molecular modeling investigations of the neuropeptide substance P in the presence of 15 mM sodium dodecyl sulfate micelles. Biopolymers *34*, 1449–1462.
4. Keire, D.A., and Kobayashi, M. (1998). The orientation and dynamics of substance P in lipid environments. Protein Sci. *7*, 2438–2450.
5. Cowsik, S.M., Lucke, C., and Ruterjans, H. (1997). Lipid-induced conformation of substance P. J. Biomol. Struct. Dyn. *15*, 27–36.
6. Wymore, T., and Wong, T.C. (1999). Molecular dynamics study

of substance P peptides in a biphasic membrane mimic. Biophys. J. *76*, 1199–1212.
7. Wymore, T., and Wong, T.C. (1999). Molecular dynamics study of substance P peptides partitioned in a sodium dodecylsulfate micelle. Biophys. J. *76*, 1213–1227.
8. Lee, S., Suh, Y.H., Kim, S., and Kim, Y. (1999). Comparison of the structures of beta amyloid peptide (25–35) and substance P in trifluoroethanol/water solution. J. Biomol. Struct. Dyn. *17*, 381–391.
9. Qi, X.F., Zhorov, B.S., and Ananthanarayanan, V.S. (2000). CD, ^{1}H NMR and molecular modeling studies of the interaction of Ca^{2+} with substance P and Ala7-substance P in a nonpolar solvent. J. Pept. Sci. *6*, 57–83.
10. Auge, S., Bersch, B., Tropis, M., and Milon, A. (2000). Characterization of substance P-membrane interaction by transferred nuclear Overhauser effect. Biopolymers *54*, 297–306.
11. Schwyzer, R., Erne, D., and Rolka, K. (1986). Membrane structure of Substance P: prediction of preferred conformation, orientation and accumulation of substance P on lipid membranes. Helv. Chim. Acta *69*, 1789–1797.
12. Schwyzer, R. (1987). Membrane-assisted molecular mechanism of neurokinin receptor subtype selection. EMBO J. *6*, 2255–2259.
13. Chassaing, G., Convert, O., and Lavielle, S. (1986). Preferential conformation of substance P in solution. Eur. J. Biochem. *154*, 77–85.
14. Ploux, O., Lavielle, S., Chassaing, G., Julien, S., Marquet, A., d'Orleans-Juste, P., Dion, S., Regoli, D., Beaujouan, J.C., Bergstrom, L., et al. (1987). Interaction of tachykinins with their receptors studied with cyclic analogs of substance P and neurokinin B. Proc. Natl. Acad. Sci. USA *84*, 8095–8099.
15. Convert, O., Ploux, O., Lavielle, S., Cotrait, M., and Chassaing, G. (1988). Analysis of tachykinin-binding site interactions using NMR and energy calculation data of potent cyclic analogs of substance P. Biochim. Biophys. Acta *954*, 287–302.
16. Lavielle, S., Chassaing, G., Ploux, O., Loeuillet, D., Besseyre, J., Julien, S., Marquet, A., Convert, O., Beaujouan, J.C., Torrens, Y., et al. (1988). Analysis of tachykinin binding site interactions using constrained analogs of tachykinins. Biochem. Pharmacol. *37*, 41–49.
17. Sagan, S., Karoyan, P., Chassaing, G., and Lavielle, S. (1999). Further delineation of the two binding sites (R*(n)) associated with tachykinin neurokinin-1 receptors using [*3*-prolinomethionine(11)]SP analogs. J. Biol. Chem. *274*, 23770–23776.
18. Quancard, J., Karoyan, P., Sagan, S., Convert, O., Lavielle, S., Chassaing, G., and Lequin, O. (2003). Characterization of the bioactive conformation of the C-terminal tripeptide Gly-Leu-Met-NH_2 of substance P using [*3*-prolinoleucine10]SP analogues. Eur. J. Biochem. *270*, 2869–2878.
19. Sagan, S., Beaujouan, J.C., Torrens, Y., Saffroy, M., Chassaing, G., Glowinski, J., and Lavielle, S. (1997). High affinity binding of [^{3}H]propionyl-[Met(O_2)^{11}]substance P(7–11), a tritiated septide-like peptide, in Chinese hamster ovary cells expressing human neurokinin-1 receptors and in rat submandibular glands. Mol. Pharmacol. *52*, 120–127.
20. Sagan, S., Chassaing, G., Pradier, L., and Lavielle, S. (1996). Tachykinin peptides affect differently the second messenger pathways after binding to CHO-expressed human NK-1 receptors. J. Pharmacol. Exp. Ther. *276*, 1039–1048.
21. Chalmers, D.K., and Marshall, G.R. (1995). Pro-D-NMe-amino acid and D-Pro-NMe-amino acid: simple, efficient reverse turn constraints. J. Am. Chem. Soc. *117*, 5927–5937.
22. Laskowski, R.A., Mac Arthur, M.W., Moss, D.S., and Thornton, J.M. (1993). Procheck: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. *26*, 283–291.
23. MacArthur, M.W., and Thornton, J.M. (1991). Influence of proline residues on protein conformation. J. Mol. Biol. *218*, 397–412.
24. Josien, H., Lavielle, S., Brunissen, A., Saffroy, M., Torrens, Y., Beaujouan, J.C., Glowinski, J., and Chassaing, G. (1994). Design and synthesis of side-chain conformationally restricted phenylalanines and their use for structure-activity studies on tachykinin NK-1 receptor. J. Med. Chem. *37*, 1586–1601.
25. Sagan, S., Milcent, T., Ponsinet, R., Convert, O., Tasseau, O., Chassaing, G., Lavielle, S., and Lequin, O. (2003). Structural and biological effects of a beta2- or beta3-amino acid insertion in a peptide. Eur. J. Biochem. *270*, 939–949.
26. Cavelier, F., Marchand, D., Martinez, J., and Sagan, S. (2004). Biological activity of silylated amino acid containing substance P analogues. J. Pept. Res. *63*, 290–296.
27. Lavielle, S., Chassaing, G., Brunissen, A., Rodriguez, M., Martinez, J., Convert, O., Carruette, A., Garret, C., Petitet, F., Saffroy, M., et al. (1993). Importance of the leucine side-chain to the spasmogenic activity and binding of substance P analogues. J. Pept. Protein Res. *42*, 270–277.
28. Williams, D.H., Stephens, E., and Zhou, M. (2003). How can enzymes be so efficient? Chem. Commun. *16*, 1973–1976.
29. Williams, D.H., O'Brien, D.P., Sandercock, A.M., and Stephens, E. (2004). Order changes within receptor systems upon ligand binding: receptor tightening/oligomerisation and the interpretation of binding parameters. J. Mol. Biol. *340*, 373–383.
30. Hunter, C.A., and Thomas, S. (2003). Cooperativity, partially bound states, and enthalpy-entropy compensation. Chem. Biol. *10*, 1023–1032.
31. Clackson, T., and Wells, J.A. (1995). A hot spot of binding energy in a hormone-receptor interface. Science *267*, 383–386.
32. Kenakin, T. (2004). Principles: receptor theory in pharmacology. Trends Pharmacol. Sci. *25*, 186–192.
33. Gether, U., and Kobilka, B.K. (1998). G protein-coupled receptors: II. Mechanism of agonist activation. J. Biol. Chem. *273*, 17979–17982.
34. Ghanouni, P., Gryczynski, Z., Steenhuis, J.J., Lee, T.W., Farrens, D.L., Lakowicz, J.R., and Kobilka, B.K. (2001). Functionally different agonists induce distinct conformations in the G protein coupling domain of the β2 adrenergic receptor. J. Biol. Chem. *276*, 24433–24436.
35. Ghanouni, P., Steenhuis, J.J., Farrens, D.L., and Kobilka, B.K. (2001). Agonist-induced conformational changes in the G-protein-coupling domain of the beta2 adrenergic receptor. Proc. Natl. Acad. Sci. USA *98*, 5997–6002.
36. Swaminath, G., Xiang, Y., Lee, T.W., Steenhuis, J., Parnot, C., and Kobilka, B.K. (2004). Sequential binding of agonists to the β2 adrenoceptor: kinetic evidence for intermediate conformational states. J. Biol. Chem. *279*, 686–691.
37. Peleg, G., Ghanouni, P., Kobilka, B.K., and Zare, R.N. (2001). Single-molecule spectroscopy of the β2 adrenergic receptor: observation of conformational substates in a membrane protein. Proc. Natl. Acad. Sci. USA *98*, 8469–8474.
38. Vollmer, J.Y., Alix, P., Chollet, A., Takeda, K., and Galzi, J.L. (1999). Subcellular compartmentalization of activation and desensitization of responses mediated by NK2 neurokinin receptors. J. Biol. Chem. *274*, 37915–37922.
39. Palanche, T., Ilien, B., Zoffmann, S., Reck, M.P., Bucher, B., Edelstein, S.J., and Galzi, J.-L. (2001). The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. J. Biol. Chem. *276*, 34853–34861.
40. Lecat, S., Bucher, B., Mely, Y., and Galzi, J.-L. (2002). Mutations in the extracellular amino-terminal domain of the NK2 neurokinin receptor abolish cAMP signaling but preserve intracellular calcium responses. J. Biol. Chem. *277*, 42034–42048.
41. Kim, H.R., Lavielle, S., and Sagan, S. (2003). The two NK-1 binding sites are distinguished by one radiolabelled substance P analogue. Biochem. Biophys. Res. Commun. *306*, 725–729.
42. Ward, P., Ewan, G.B., Jordan, C.C., Ireland, S.J., and Hagan, R.M. (1990). Potent and highly selective neurokinin antagonists. J. Med. Chem. *33*, 1848–1851.
43. Lavielle, S., Brunissen, A., Carruette, A., Garret, C., and Chassaing, G. (1994). Highly potent substance P antagonists substituted with β-phenyl- or β-benzyl-proline at position 10. Eur. J. Pharmacol. *258*, 273–276.
44. Poulos, C., Antoniou, M., Patacchini, R., Maggi, C.A., and Rovero, P. (1994). Synthesis and biological activity of NK1 tachykinin antagonists not containing D-residues. Neuropeptides *26*, 55–57.
45. Karagiannis, K., Stravropoulos, G., Poulos, C., Jordan, C.C., and Hagan, R.M. (1993). Synthesis of a potent antagonist of substance P by replacing the CH_2SCH_3 and the α-carboxamide groups of the methionine at [Orn^6]-SP(6–11) by benzyl ester groups. Int. J. Pept. Protein Res. *42*, 565–569.

46. Sachon, E., Girault-Lagrange, S., Chassaing, G., Lavielle, S., and Sagan, S. (2002). Analogues of substance P modified at the C-terminus which are both agonist and antagonist of the NK-1 receptor depending on the second messenger pathway. J. Pept. Res. *59*, 232–240.
47. Karoyan, P., and Chassaing, G. (1997). Asymmetric synthesis of (2S,3S)- and (2S,3R)-*3*-prolinomethionines: *3*-methylsulfonylmethyl-pyrrolidine-2-carboxylic acids. Tetrahedron Asymmetry *8*, 2025–2032.
48. Karoyan, P., Quancard, J., Vaissermann, J., and Chassaing, G. (2003). Amino-zinc-enolate carbometalation reactions: application to ring closure of terminally substituted olefin for the asymmetric synthesis of cis- and trans-*3*-prolinoleucine. J. Org. Chem. *68*, 2256–2265.