Further Delineation of the Two Binding Sites (R*\textsubscript{n}) Associated with Tachykinin Neurokinin-1 Receptors Using [3-Prolinomethionine\textsuperscript{11}]SP Analogues* 

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Receptor proteins are found in multiple conformational states with different levels of energy. There is evidence to suggest that seven transmembrane domain receptors can adopt two distinct macrostates, i.e. the inactive R and active R* forms, and a large spectrum of different microstates, i.e. conformational states (1–4). There are also numerous examples that seven transmembrane domain receptors can activate various effector systems, especially in cell lines transfected with receptors, a phenomenon related or not to overexpression (5). This observation has led to the proposal that the so-called active R* form of a receptor may in fact hide multiple conformational states, some of them recruiting specific G proteins via a fruitful interaction (1–4). Most of the current work emphasizes the molecular nature of these R and R* forms of the receptor.

We speculate that if a receptor protein is found in different active three-dimensional structures R*\textsubscript{n} with regard to its interaction with different G proteins, the crucial residues of its ligand (i.e. the so-called pharmacophore) might adopt different orientations when interacting with the different active R*\textsubscript{n} forms of this receptor. This working hypothesis does not foresee at all the recognition mechanism between the ligand and its receptor, i.e. the processes that lead to the selection or induction of particular conformations of both the ligand and the receptor.

The complex formed by substance P ((SP)\textsuperscript{1} Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH\textsubscript{2})\textsuperscript{2} and the human NK-1 tachykinin receptor has been selected for this investigation, as we and others have established that two binding sites associated with this receptor are found both in transfected cell lines (6, 7) and in a mammalian tissue (7). These two specific binding sites are not present in equivalent amounts, the ratio being from 1.5/8.5 in transfected cells (whatever radioligand used) (6, 7) to 4/6 in mammalian tissues (7). A controversy still remains whether these binding sites identified both in binding and second messenger coupling assays are canonical active R* forms, as those determined with constitutive receptors (5, 8). Indeed, although in CHO cells expressing the hNK-1 tachykinin receptor the most abundant site labeled by [\textsuperscript{3}H][Pro\textsubscript{9}]SP is related to cAMP production (9), and the minor one is specifically labeled by [\textsuperscript{3}H][propionyl][Met\textsubscript{O\textsubscript{2}}\textsubscript{11}]SP(7–11) correlates with IP production (7), this hNK-1 tachykinin receptor is, however, also coupled to the activation of at least two other pathways.

The abbreviations used are: SP, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH\textsubscript{2}); CHO, Chinese hamster ovary; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high pressure liquid chromatography; MBHA, o-p-methylbenzyldihydrochloride; h, human; [P\textsuperscript{3}Met\textsubscript{11}]SP, trans-3-prolinomethionine; [P\textsuperscript{3}Met\textsubscript{11}]SP, cis-3-prolinomethionine; P1, phosphatidylinositol; NK-1, neurokinin-1; Bapa, biotinyl-sulfone-5-aminopentanoic acid; pBz, parabenzoyl; Fig, fluorenylglycine; Dip, diphenylalanine; Ing, indanylglycine; Bfi, benz[f]indanylglycine; Tic, tetraphydroisoquinoleic acid; Aib, aminoisobutyric acid; (pB\textsubscript{2},SO\textsubscript{2})Hcy, parabenzoyl homocysteine sulfone; Pen, penicillamine; IP, inositol phosphate.

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Two binding sites are associated with neurokinin-1 substance P receptors in both transfected cells and mammalian tissues. To further delineate the interactions between the crucial C-terminal methionine of substance P and these two binding sites, we have incorporated newly designed constrained methionines, i.e. (2S, 3S)- and (2S,3R)-prolinomethionines. The potencies of these C terminus-modified SP analogues to bind both sites and to activate phosphatidylinositol hydrolysis and cAMP formation have been measured, together with those of their corresponding sulfoxides and sulfones. The molecular nature of these two binding sites and their selective coupling to effector signaling pathways are discussed in the light of current models of receptor activation. The less abundant binding site is coupled to G\textsubscript{q/11} proteins, whereas the most abundant one interacts with G\textsubscript{i} proteins in Chinese hamster ovary cells transfected with human neurokinin-1 receptors. The specific orientation of the C-terminal methionine side chain imposed by these constraints shows that macroscopically \(\chi_1\) and \(\chi_2\) angles of this crucial C-terminal residue are similar in both binding sites. However, slight but significant variations in the rotation around the C\textsubscript{y}-S bond yield different either stabilizing or destabilizing interactions in the two binding sites. These results highlight the need of such constrained amino acids to probe subtle interactions in ligand-receptor complexes.
ways, i.e. phospholipase A₂ (10) and D (11). Concerning SP, the ligand of the NK-1 receptor, its Phe² and Phe⁸ side chains, and both the aliphatic side chain and carboxamide group of its C-terminal Met¹¹ residue are crucial for spasmodogenic activity (12) and also for the binding and stimulation of second messengers (13–15). Therefore, to further delineate the molecular interactions of this C-terminal amino acid with both binding sites of the human NK-1 tachykinin receptor, we have designed constrained analogues of methionine, i.e. 3-prolinomethionines (Fig. 1) (16, 17). We report herein the binding and activity potencies of a highly homogenous family of SP analogues modified in position 11 with these constrained methionines.

cis- and trans-4-Prolinomethionines (Fig. 2) have previously been described and incorporated in position 11 of SP analogues modified in position 11 with these constrained methionines. cis- and trans-4-Prolinomethionines (Fig. 2) have previously been described and incorporated in position 11 of SP analogues modified in position 11 with these constrained methionines. cis- and trans-4-Prolinomethionines (Fig. 2) have previously been described and incorporated in position 11 of SP analogues modified in position 11 with these constrained methionines. cis- and trans-4-Prolinomethionines (Fig. 2) have previously been described and incorporated in position 11 of SP analogues modified in position 11 with these constrained methionines. cis- and trans-4-Prolinomethionines (Fig. 2) have previously been described and incorporated in position 11 of SP analogues modified in position 11 with these constrained methionines.

![Figure 1](image1.png)  
**Fig. 1.** Schematic representation of chimeric cis- and trans-3-prolinomethionine acids.

![Figure 2](image2.png)  
**Fig. 2.** Schematic representation of methionine, norleucine (Nle), proline, 4-prolinomethionine, and 3-prolinomethionine.

to couple the cis isomer t-butoxycarbonyl-P³Met-OH were unsuccessful. Fmoc-P³Met-OH was introduced manually in a 1:1 ratio on the MBHA resin after activation as an acid fluoride (20, 21). The residual free amino groups on the MBHA-resin were then acetylated. After removal of the Fmoc by piperidine/dimethylformamide, 1:9, all the other t-butoxycarbonyl-amino acids were coupled by the dicyclohexylcarbodiimide-1-hydroxybenzotriazole strategy on the ABI synthesizer. Peptides were cleaved from the resin by anhydrous fluorohydric acid and purified by preparative reverse phase HPLC. The purity of collected fractions was established by analytical HPLC. The sulfoxides were obtained by oxidation of the corresponding crude peptide (2 equivalent NaIO₄ in water, 3 days at 4 °C) and purification by preparative HPLC. NMR analysis establishes the presence (1:1 ratio) of both diastereoisomers. The sulfones were prepared by oxidation of the corresponding crude peptide (3 equivalent H₂O₂, 30% in water, in glacial acetic acid, room temperature for 15 h).

The purity of all peptides was over 98.5%. Matrix-assisted laser desorption ionization-time of flight analysis was performed by Dr. G. Bolbach (UMR 7613, Université P. & M. Curie, Paris). The calculated mass (MH⁺) is 1373.75 for [P³Met¹¹]SP, 1389.89 for [P³Met(O)¹¹]SP, and 1405.84 for [P³Met(O₂)¹¹]SP. Each family of peptides (i.e. sulfide, sulfoxide, sulfone) has been injected in a single run by analytical HPLC for comparison of the elution time (tₑ).

Analytical HPLC, 5-µm Lichrosorb 10 RP-8 column (Merck) in 0.25 µM triethylamine/acetate buffer, pH 3.0, and acetonitrile (isocratic mode, 21% acetonitrile) is as follows: [P³Met¹¹]SP, tₑ = 13.89 min, MH⁺ (found) = 1373.80; [P³Met(O)¹¹]SP, tₑ = 4.36 min, MH⁺ (found) = 1389.89; [P³Met(O₂)¹¹]SP, tₑ = 5.99 min, MH⁺ (found) = 1405.56; [P³Met¹¹]SP, tₑ = 13.66 min, MH⁺ (found) = 1373.89; [P³Met(O)¹¹]SP, tₑ = 4.60 min, MH⁺ (found) = 1389.74; and [P³Met(O₂)¹¹]SP, tₑ = 6.68 min, MH⁺ (found) = 1405.84.

**Cell Culture—CHO cells expressing hNK-1 receptors were cultured in Ham’s F12 medium supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin, and 10% fetal calf serum. Cultures were kept at 37 °C in a humidified atmosphere of 5% CO₂. Stable transfections were maintained by genetic periodic selection.**

**Binding Assays on CHO Cells—Binding assays were carried out at 22 °C on whole cells in Krebs-Ringer phosphate solution consisting of 120 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, and 15.6 mM NaH₂PO₄, pH 7.2, containing 0.04% bovine serum albumin (w/v), 0.6% glucose (w/v), 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml soybean trypsin inhibitor as described (7). Binding studies were analyzed with the LIGAND program (22).**

**Measurements of Inositol Phosphate and cAMP Formation—Except when mentioned in the text, PI hydrolysis and cAMP accumulation were determined as described previously (23). Curves were fitted using SIGMA PLOT software (Jandel Scientific, Erkrath, Germany).**

**RESULTS**

Eleven SP analogues modified on the C-terminal position have been tested in binding and second messenger formation studies: SP, [Nle¹¹]SP, [Pro¹¹]SP, [Met(O)¹¹]SP, [Met(O₂)¹¹]SP, [P³Met¹¹]SP, [P³Met(O)¹¹]SP, [P³Met(O₂)¹¹]SP, [P³Met¹¹]SP, [P³Met(O)¹¹]SP, [P³Met(O₂)¹¹]SP. Data collected from all these studies are presented in Table I.

Examining first the binding studies, the behavior of [Nle¹¹]SP is identical to SP with 1 nM affinity for [³H]Pro³SP binding
sites and 0.13 nM affinity for [3H]propionyl[Met(O2)11]SP(7–11) binding sites. In contrast to SP and [Nle 11]SP, [Pro11]SP has almost no affinity for [3H][Pro9]SP binding sites (Ki = 1150 nM) and a low affinity for [3H]propionyl[Met(O2)11]SP(7–11) binding sites (Ki, 63 nM). Therefore, we further examined the contribution of the methionine side chain with the cis- and trans-3-prolinomethionine[11]SP analogues. Both [P3Met]11SP and [P2Met]11SP fully recover the binding potency of SP on both binding sites (in competition with [3H][Pro9]SP, Ki values are 3.0 and 2.1 nM, respectively, and with [3H]propionyl[Met(O2)11]SP(7–11), Ki values are 0.1 and 0.08 nM, respectively (Table I).

Although [Nle11]SP retains the activity of SP for NK-1 receptors, as already demonstrated (12), suggesting that the sulfur atom has no role in SP receptor recognition, we had previously shown that the sulfoxides and sulfone analogues of SP or propionyl-SP(7–11) peptides were not recognized in the same way by NK-1 receptors (7). As shown in Table I, [Met(O)11]SP, [P2Met(O)11]SP, and [P3Met(O)11]SP are 30–110-fold weaker competitors than SP at [3H][Pro9]SP binding sites. In contrast with the sulfoxide derivatives, further oxidation of the peptides ([Met(O2)11]SP and [P2Met(O2)11]SP) restores full affinity for [3H][Pro9]SP binding sites, whereas [P2Met(O2)11]SP is still a 5-fold less potent competitor at these binding sites. The results obtained with these oxidized analogues are slightly different at [3H]propionyl[Met(O2)11]SP(7–11) binding sites. The sulfone analogues, [Met(O)11]SP, [P2Met(O)11]SP, and [P3Met(O)11]SP have 4-, 7-, and 60-fold weaker affinity, respectively, than SP for these binding sites. In contrast, the sulfone analogues fully recover SP affinity for [3H]propionyl[Met(O2)11]SP(7–11) binding sites.

With regard to the second messenger assays, it is now well established that NK-1 receptors expressed in CHO cells are highly correlated with their potency to stimulate PI hydrolysis (EC50(1)) and cAMP accumulation (EC50(2)) binding sites. As opposed to [3H][Pro9]SP, the affinity of these 22 analogues for [3H]propionyl[Met(O2)11]SP(7–11) binding sites (from 0.08 to 8 nM), [Pro11]SP being the weaker competitor with an affinity of 63 nM. Previously described correlations between affinity and activity (7, 9) have now been extended to a larger population of substance P analogues (n = 53), including those described in this study. No strong correlation can be found either between their affinities for [3H][Pro9]SP and [3H]propionyl[Met(O2)11]SP(7–11) binding sites (Fig. 3A, r = 0.808) or between their potencies to stimulate PI hydrolysis and cAMP accumulation (Fig. 3B, r = 0.657, inactive analogues with EC50 values >5000 nM were discarded for the correlation analysis). It is also further established that affinity for [3H][Pro9]SP binding sites correlates better with cAMP formation (Fig. 3C, r = 0.922) than with IP formation (Fig. 3D, r = 0.790), as previously quoted (6). Among these 53 analogues, 22 analogues have been screened for their affinity for [3H]propionyl[Met(O2)11]SP(7–11) binding sites. As opposed to [3H][Pro9]SP, the affinity of these 22 analogues for [3H]propionyl[Met(O2)11]SP(7–11) binding sites highly correlates with their potency to stimulate PI hydrolysis (Fig. 3E, r = 0.975) but not cAMP accumulation (Fig. 3F, r = 0.774).

Further analysis of the results obtained in this study with these eleven SP analogues modified on the C-terminal residue leads to another striking feature. The ratios EC50(1)/Ki, which are EC50(1)/cAMP accumulation over Ki, (3H)[Pro9]SP binding sites, are quite homogenous; values varying among themselves only from 1- to 3.5-fold (Table I). In contrast, the ratios

### Table I

**Affinities and potencies of C terminus modified SP analogues**

Affinities of SP peptide analogues for both [3H][Pro9]SP (Ki1) and [3H]propionyl[Met(O2)11]SP(7–11) (Ki2) binding sites and their related potencies to stimulate cAMP accumulation (EC50(1), and PI hydrolysis (EC50(2)). Assays are described under “Experimental Procedures.” All experiments have been done in triplicate in at least three independent experiments. Values presented are the mean ± S.E.

| Peptides | Ki1 (nM) | EC50(1) (% SP max, efficacy) | Ki2 (nM) | EC50(2) (nM) |
|----------|----------|-----------------------------|----------|-------------|
| SP       | 1.3 ± 0.3 | 8 ± 2                        | 0.13 ± 0.04 | 0.7 ± 0.3    |
| [Pro11]SP | 1150 ± 250 | >10,000                      | 63 ± 6    | 56 ± 8      |
| [Nle11]SP | 1.1 ± 0.1 | 15 ± 3                       | 0.13 ± 0.04 | 0.8 ± 0.2    |
| [P3Met]11SP | 3.0 ± 0.5 | 25 ± 2                       | 0.10 ± 0.007 | 1.4 ± 0.3     |
| [P2Met]11SP | 2.1 ± 0.1 | 35 ± 1                       | 0.08 ± 0.005 | 0.8 ± 0.1     |
| [Met(O)11]SP | 40 ± 15  | 240 ± 30                     | 0.50 ± 0.05 | 1.50 ± 0.25   |
| [P2Met(O)11]SP | 40 ± 7   | 545 ± 60                     | 1.00 ± 0.05 | 2.0 ± 0.4     |
| [P3Met(O)11]SP | 145 ± 25  | 3000 ± 400                   | 8 ± 0.5    | 20 ± 2       |
| [Met(O2)11]SP | 0.65 ± 0.25 | 14 ± 2                      | 0.05 ± 0.01 | 1.1 ± 0.1     |
| [P2Met(O2)11]SP | 1.0 ± 0.2 | 17 ± 1                       | 0.06 ± 0.01 | 0.08 ± 0.03   |
| [P3Met(O2)11]SP | 7 ± 1    | 117 ± 8                      | 0.12 ± 0.01 | 1.7 ± 0.2     |

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As in binding studies, reintroduction of the methionine side chain on the pyrrolidine ring of proline leads to [P3Met]11SP and [P2Met]11SP, both being able to stimulate IP formation (EC50 values of 1.3 and 0.8 nM, respectively) and cAMP accumulation (EC50 values of 25 and 35 nM, respectively) with potencies similar to SP. As with [Nle11]SP, [P2Met]11SP maximal response for cAMP formation only reaches 70% of that obtained with SP. The sulfoxide analogues [Met(O)11]SP, [P2Met(O)11]SP, and [P3Met(O)11]SP are 5-, 3-, and 28-fold less potent than SP to stimulate IP formation, and 30-, 68-, and 375-fold less potent than SP to stimulate cAMP accumulation. Finally, data obtained with the sulfone analogues are also parallel with their binding potencies; [Met(O2)11]SP and [P2Met(O2)11]SP are as potent as SP when stimulating IP formation and cAMP accumulation. [P3Met(O2)11]SP is equipotent to SP to stimulate PI hydrolysis but 15-fold less potent than SP to stimulate cAMP accumulation. Altogether these results show a strong parallelism between the affinity and activity potencies of these analogues. Except for [Pro11]SP and [P2Met(O)11]SP, which have low affinity, all these peptides present high to moderate affinity (from 1 to 40 nM) for [3H][Pro9]SP binding sites. In contrast with [3H][Pro9]SP binding sites, all these analogues retain high affinity for [3H]propionyl[Met(O2)11]SP(7–11) binding sites (from 0.08 to 8 nM), [Pro11]SP being the weaker competitor with an affinity of 63 nM.

Previous correlations between affinity and activity (7, 9) have now been extended to a larger population of substance P analogues (n = 53), including those described in this study. No strong correlation can be found either between their affinities for [3H][Pro9]SP and [3H]propionyl[Met(O2)11]SP(7–11) binding sites (Fig. 3A, r = 0.808) or between their potencies to stimulate PI hydrolysis and cAMP accumulation (Fig. 3B, r = 0.657, inactive analogues with EC50 values >5000 nM were discarded for the correlation analysis). It is also further established that affinity for [3H][Pro9]SP binding sites correlates better with cAMP formation (Fig. 3C, r = 0.922) than with IP formation (Fig. 3D, r = 0.790), as previously quoted (6). Among these 53 analogues, 22 analogues have been screened for their affinity for [3H]propionyl[Met(O2)11]SP(7–11) binding sites. As opposed to [3H][Pro9]SP, the affinity of these 22 analogues for [3H]propionyl[Met(O2)11]SP(7–11) binding sites highly correlates with their potency to stimulate PI hydrolysis (Fig. 3E, r = 0.975) but not cAMP accumulation (Fig. 3F, r = 0.774).
**FIG. 3.** Correlations between affinities and activities by linear regression analysis with 22 (A, E, and F) or 53 (B, C, and D) substance P peptide analogues. A, log \( K_i \) ([H]proplyl[Met(O2)11]SP(7–11)) versus log \( K_i \) ([H][Pro9]SP); B, log EC50 (phospholipase C) versus log EC50 (cAMP); C, log EC50 (cAMP) versus log \( K_i \) ([H][Pro9]SP); D, log EC50 (phospholipase C) versus log \( K_i \) ([H][Pro9]SP); E, log EC50 (phospholipase C) versus log \( K_i \) ([H][Met(O2)11]SP(7–11)); F, log EC50 (cAMP) versus log \( K_i \) ([H][Met(O2)11]SP(7–11)).

EC50/Ki, which are EC50/2 (IP formation) over \( K_i \) ([H][proplyl][Met(O2)11]SP(7–11) binding sites) are not constant, variations between values spread out from 1- to 25-fold (Table I). With the exception of [P3MetO11]SP, these EC50/2/\( K_i \) ratios are close to unity for analogues that have low affinity (≤40 nM) for [H][Pro9]SP binding sites but still have high affinity for [H][proplyl][Met(O2)11]SP(7–11) binding sites. The most extreme case is [Pro11]SP. Although this analogue has no affinity (\( K_i = 1150 \) nM) for [H][Pro9]SP binding sites and a low affinity (\( K_i = 63 \) nM) for [H][proplyl][Met(O2)11]SP(7–11) binding sites, its EC50/2/\( K_i \) ratio value is 0.9, suggesting a strict relationship between binding and activity parameters. We suspected that the EC50/2 of compounds with high affinity for [H][Pro9]SP binding sites may be underestimated, if this more abundant site is not (or weakly) coupled to IP production. It was therefore of interest to investigate whether the potency to stimulate IP formation could be modulated according to the relative amounts of both binding sites (7) and the agonist tested.

Thus, we tested, in PI hydrolysis and cAMP accumulation measurements, the influence of the cell number variation, that is the ratio between the relative site numbers and the amount of agonist on the concentration-response curves. For that purpose [Pro11]SP with an EC50 value for IP formation identical to [H][Pro9]SP depending on the number of cells and thus on the ratio of receptor numbers versus the amount of agonist (EC50 = 1.4 ± 0.3 nM with the lowest number of cells used, that is 2.10^4 and 5.10^5 cells/well favoring [Pro9]SP binding sites and EC50 = 0.46 ± 0.16 nM with the highest number of cells used, that is 10^6 and 3.10^6 cells/well, exacerbating [H][proplyl][Met(O2)11]SP(7–11) binding sites). In contrast to [P3MetO11]SP, parallel experiments done with [Pro11]SP, devoid of affinity for [H][Pro9]SP binding sites, did not present any significant shift in the concentration-response curves, according to assay conditions (Fig. 4). These results establish that the most abundant binding site is not coupled to IP production, otherwise this coupling is far less efficient than the interaction between the minor site and \( \gamma_{11} \) proteins. Therefore, the correlation between the affinity for the minor binding site labeled with [H][proplyl][Met(O2)11]SP(7–11) and the potency to stimulate PI hydrolysis is even better when only analogues with low affinity (\( K_i > 50 \) nM) for the most abundant binding sites labeled with [H][Pro9]SP are used for linear regression analysis (Fig. 3E, \( r = 0.975 \) compared with Fig. 5B, \( r = 0.990 \)). Even though peptides with high affinities (\( K_i < 50 \) nM) for [H][Pro9]SP also interact with the minor site, no correlation exists between their potency to stimulate PI hydrolysis and their affinity for the major site labeled with [H][Pro9]SP, Fig. 5A.

**DISCUSSION**

This study further supports the concept of the multiplicity of receptor conformations associated with the hNK-1 tachykinin receptor. It has recently been reported that hNK-1 receptor transfected in CHO cells directly activates \( \gamma_{11} \), \( \gamma_s \), and \( \gamma_{0} \) proteins (24) and that there is no cross-talk between cAMP accumulation and IP formation (23, 25, 26). Therefore, it is likely that EC50 values of all SP analogues measured in this study arise from direct NK-1 receptor/G protein coupling.

Two theoretical models, based upon the two-state allosteric ternary complex model (27) accounting for promiscuity or multiple receptor-effector coupling, could depict results reported herein. In the three-state model of agonist action, the receptor exists under one inactive (R) and at least two active (R* and R**) states, each active state coupling to a single G protein type (1, 2). This model predicts that different agonists would have different affinity for R* and R** and therefore that the associated pharmacology with both effector pathways could be different. A particular agonist (A) acts to displace the receptor equilibrium from unoccupied (R, R*, and R**) to occupied (AR, AR*, and AR**) forms (1). Thus, all the occupied forms are enriched according to the affinity constant of the agonist for the three different receptor states R, R*, and R** (1). As there is a finite number of receptors, this model assumes that in terms of efficacy, the two pathways are dependent on one another, which is definitely not the case with the hNK-1 tachykinin receptor. Of the fifty-three tachykinin analogues already tested, some of them (represented by [Nle11]SP in this study) have an efficacy in cAMP accumulation response lower than others, but this efficacy is not correlated to a higher efficacy in...
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IP formation response. Thus, there is no mutual depletion of active receptor states, as expected from the model. In the isolated pathways of the three-state model, which may be applied when a receptor is coupled to one G protein in an assay system and a different one in another assay system, the R* and R** states are no independent of one another. Under these conditions, one agonist may exhibit different efficacy and potency orders between the two pathways. Data presented herein better fit these isolated pathways of the three-state model. Indeed, the maximal binding capacities differ for the two binding sites, favoring the existence of these independent R* and R** states for the hNK-1 tachykinin receptor. However, working with CHO cells transfected with a single cDNA of human NK-1 receptors, this model would thus imply the hypothetic existence of two distinct receptors or the compartmentalization of receptor signaling pathways that may artificialy create conditions in which two pathways could operate in an isolated manner (28–30). But, it has to be mentioned that identical affinity could be measured for [Pro9]SP and propionyl[Met(O2)11]SP (7–11) versus both [H]Pro9SP and [H]propionyl[Met(O2)11]SP (7–11) in binding assays at 37 °C (40-min incubation) or 4 °C (180-min incubation), indicating that desensitization and internalization processes should not play a major role in the differential pharmacology associated with these two binding sites (data not shown).

In the model assuming agonist trafficking of receptor signals, an agonist selectively leads to the coupling of the receptor to one G protein over another through particular conformations of the receptor (3, 4). In this model, the receptor protein adopts a gaussian distribution of multiple conformations, which is specifically shifted by agonists to enrich particular states characterized by their relative affinity for G proteins (3, 4). It is also assumed that different agonists may differentially activate signaling pathways not only in terms of realized efficacy but also of potential efficacy (31). For the hNK-1 tachykinin receptor, it has to be noticed that the ratio between EC50 values of different agonists to stimulate IP production. Therefore, it might be more judicious to associ-
ate these two binding sites with their fruitful interaction with G protein, i.e. the less abundant binding sites with G

Whether these two binding sites represent, in view of the current models of receptor activation, different conformational states of the same receptor in very slow equilibrium (>120 min, the time course of binding studies) or distinct receptor proteins differently coupled to G proteins as suggested from other studies (34–36) is a question that remains to be addressed.

Whatever the explanation may be, the highly homogenous family of peptides modified at the C-terminal methionine led us to conclude that slight, but significant, differences exist in the orientation of the sulphur substituents (Cγ–S rotation), in the two binding sites. This conclusion arises from comparison of the potencies (EC50(1), and EC50(2)) of the two constrained 3-prolinomethionine analogues [P3

The pseudorotation cycle of the five-membered pyrrolidine led to a fluctuation of the x1 angle between −90° and −150° for the 3S-isomer and +90° and +150° for the 3R isomer of 3-prolinomethionine. The two extreme values (−150° and +150°) are close to that of the trans rotamer (t, 180°) for the corresponding nonconstrained amino acid (Fig. 7). The two other values of x1 for the prolinomethionine (−90° and +90°) are close to the gauche(−) rotamer (g−, −60°) and gauche(+) rotamer (g+, +60°), respectively, for methionine (Fig. 7). With a prolinomethionine substituted in position 4, the x1 value can only oscillate between −30° and +30°, values close to the g− and g+ rotamer for methionine (Fig. 7). These rotamers are excluded in the hNK-1 tachykinin receptor, because SP(6–11) analogues substituted with both diastereoisomers of 4-prolinomethionine were completely inactive in guinea pig ileum bioassay (18), a tissue already depicted for the presence of the two binding sites (37). The high potencies of [P3

The x2 angle depends on the destabilizing gauche interaction developed in the three orientations (−60°, +60°, 180°) of the ligand. For the diastereoisomer [P3

FIG. 7. Side chain orientation (x1 and x2 torsional angles) and Newman projections for methionine, trans- and cis-4-prolinomethionines, trans-3-prolinomethionine, and cis-3-prolinomethionine.

Rotation around the Cγ–S bond controls the spatial distribution of the methyl and the lone pairs (sulfides) or the oxygen atoms (sulfoxides, sulfones). These orientations can be deduced from comparison of the potencies of the sulfoxide and sulfone analogues with those of SP for the two signaling pathways, because each peptide [Met(O)11]SP, [P3

In conclusion, these constrained prolinomethionines turned out to be valuable tools to highlight the importance of the methionine in the SP-receptor complex and to analyze the conformational states of the hNK-1 tachykinin receptor.

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