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Tachykinin NK-1 Receptor Probed with Constrained Analogues of Substance P

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Abstract—The action of rotameric probes introduced either in position 7 or 8 in the sequence of substance P (SP) was investigated, i.e. L-tetrahydroisoquinoleic acid (Tic), L-fluorenylglycine (Flg), L-diphenylalanine (Dip), the diastereoisomers of L-1-indanylglycine (Ing) and L-benz[*f*]indanylglycine (Bfi), the *Z*- and *E*-isomers of dehydrophenylalanine and dehydronaphthylalanine (Δ^ZPhe, Δ^EPhe, Δ^ZNal, Δ^ENal) and L-*o*,*o*′-dimethylphenylalanine (Dmp). The aim of this study was the topographical characterization of the binding subsites of human NK-1 receptor expressed in CHO cells, especially the S_7 and S_8 subsites, corresponding to residues Phe^7 and Phe^8 of substance P. According to the binding potencies of these substituted-SP analogues, the S_7 binding subsite is smaller than the S_8 subsite: the S_7 subsite accepts only one aromatic nucleus, while the S_8 can accommodate three coplanar nuclei altogether. These findings are compatible with the idea that the S_8 binding subsite may reside in the extracellular loops of the hNK-1 receptor. NK-1 agonists bind to human NK-1 receptor and activate the production of both inositol phosphates and cyclic AMP. As already quoted for septide, [pGlu^6, Pro^9]SP(6–11), discrepancies are observed between affinity (K_i) and activity (EC_{50}) values for IPs production. While a weak correlation between K_i and EC_{50} values for IPs production could be found ($r = 0.70$), an excellent correlation could be demonstrated between their affinities (K_i) and their potencies (EC_{50}) for cAMP production ($r = 0.97$). The high potency (EC_{50}) observed for 'septide-like' molecules on PI hydrolysis, compared to their affinity is not an artefact related to the high level of NK-1 receptors expressed on CHO cells since a good correlation was found between EC_{50} values obtained for PI hydrolysis and those measured for spasmogenic activity in guinea pig ileum bioassay ($r = 0.94$). Copyright © 1996 Elsevier Science Ltd

Introduction

Constrained analogues of phenylalanine have been conceptually designed and synthesized for analyzing, in the NK-1 receptor, the binding pockets (S_7, S_8) of Phe^7 and Phe^8, two aromatic residues important for the pharmacological properties of substance P (SP): Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$. An amino acid side chain is generally flexible, its orientation depending on the value of the torsional angle χ_1 which can take three different limit values (Fig. 1). For the past few years, different restrained analogues of amino acids, mainly phenylalanine and tyrosine, have been introduced in the sequences of peptides, i.e. enkephalins,[1–3] somatostatin,[4] angiotensin,[5] α-melanocyte stimulating hormone,[6] to probe the preferred orientation(s) of these aromatic residues once bound to the receptor. In the case of angiotensin, restricting the inherent mobility of one aromatic residue conferred antagonist properties to the modified peptide.[5]

Here, the rotameric probes incorporated in positions 7 and 8 of substance P are either conformationally (**1–8**) or configurationally stable (**9–12**) residues (Fig. 1). The preferred χ_1 orientation(s) of amino acids **1–8** depend(s) on both the local peptide backbone conformation and the C_β substitution while χ_1 orientation of dehydrophenylalanine, ΔPhe, (**9**, **10**) and dehydronaphthylalanine, ΔNal, (**11**, **12**) corresponds to either *E*- or *Z*-orientation close to 180 ° and −60 °, respectively. The binding potencies, for NK-1 specific binding site on rat brain synaptosomes, of substance P analogues incorporating residues **1–3** and **5–10** have already been reported.[7] From these data we concluded that the S_7 binding subsite is small, accommodating only one aromatic nucleus in the *trans* orientation whereas the S_8 binding subsite is large enough to accept one aromatic nucleus in the *trans* orientation and two aromatic nuclei in the *gauche* (−) orientation. However, for some peptides, discrepancies were observed between their binding abilities (IC_{50} values) and their potencies (EC_{50} values), to induce spasmogenic activity on guinea pig ileum longitudinal muscle. This situation was reminiscent of that encountered with a family of peptides, the so-called septide-like molecules, i.e. peptides with low affinity for NK-1

Abbreviations: the abbreviations for the amino acids are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical nomenclature (*Eur. J. Chem.* **1984**, *138*, 9). The symbols represent the L-isomer except when indicated otherwise. For the three code-letters nomenclature of the non-natural amino acids see Fig. 1, and footnote of Fig. 1. The other abbreviations used are as follows: CHO cells, Chinese hamster ovary cells; PI, phosphatidylinositol; cAMP, cyclic adenosine 5′-phosphate; GPI, guinea-pig ileum; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran; LDA, lithium diisopropyl amide; HMPA, hexamethylphosphorous triamide; TFA, trifluoroacetic acid; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; DMF, dimethylformamide; DMSO, dimethylsulfoxide.

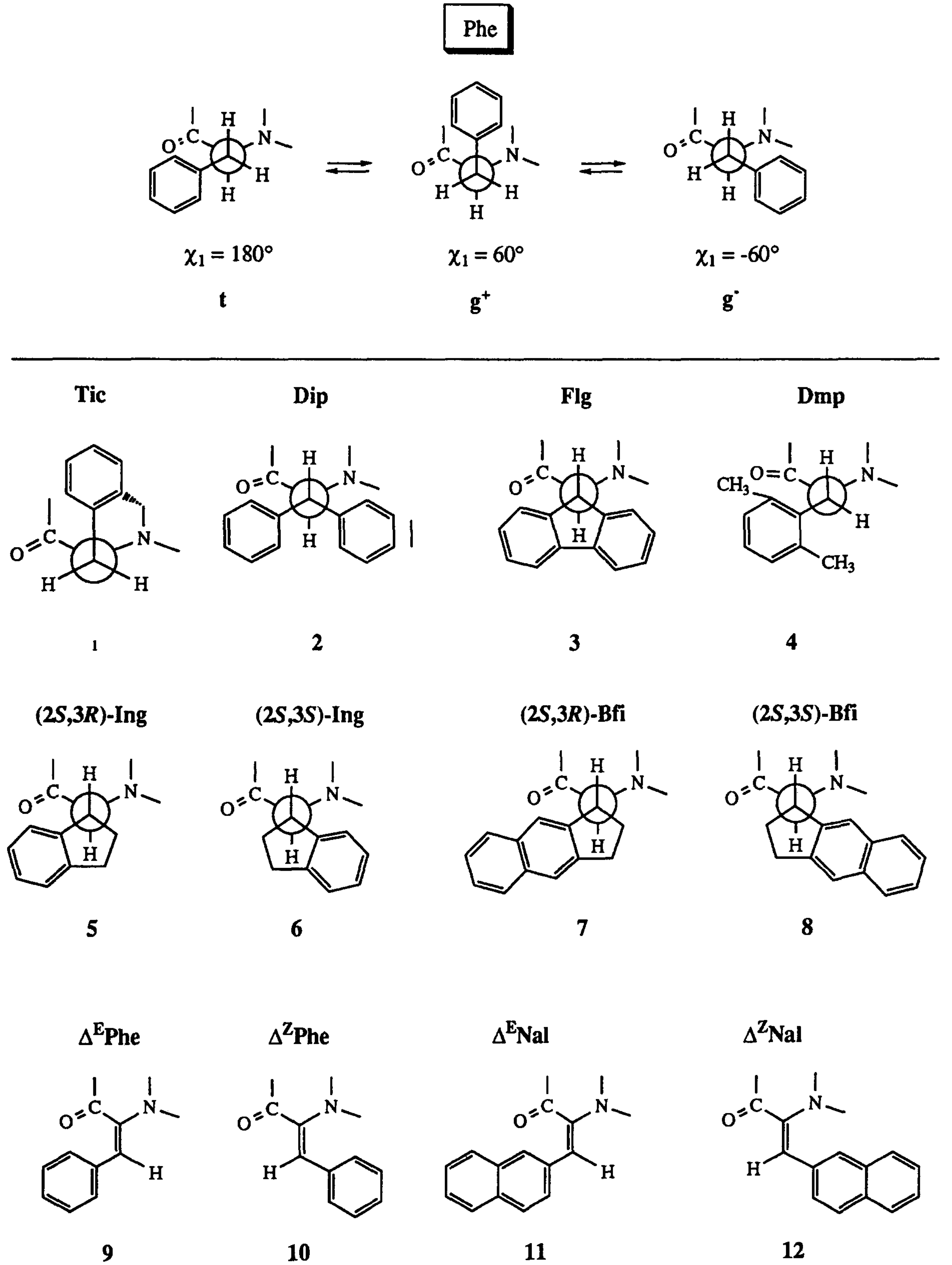


Figure 1. Side-chain orientation for α-amino acids (χ_1 torsional angle) and Newman projections of the conformational probes used in this study. Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Dip: 3,3-diphenylalanine; Flg: 2(9-fluorenylglycine); Dmp: *o,o*′-dimethylphenylalanine; Ing: 2-(1-indanyl)glycine; Bfi: 2-(1-benz[*f*]indanyl)glycine; ΔPhe: dehydrophenylalanine; ΔNal: dehydronaphthylalanine. Amino acids **1–8** have the *S* chirality at the Cα chiral center.

specific binding sites but high potency on NK-1 receptors.[8-11] Therefore, we have now investigated the pharmacological properties of these substance P analogues on CHO cells transfected with the human NK-1 receptor, a monoreceptor preparation, and compared their binding potencies, their abilities to stimulate PI hydrolysis, cAMP accumulation and their EC_{50} values in the guinea pig ileum bioassay.

Results

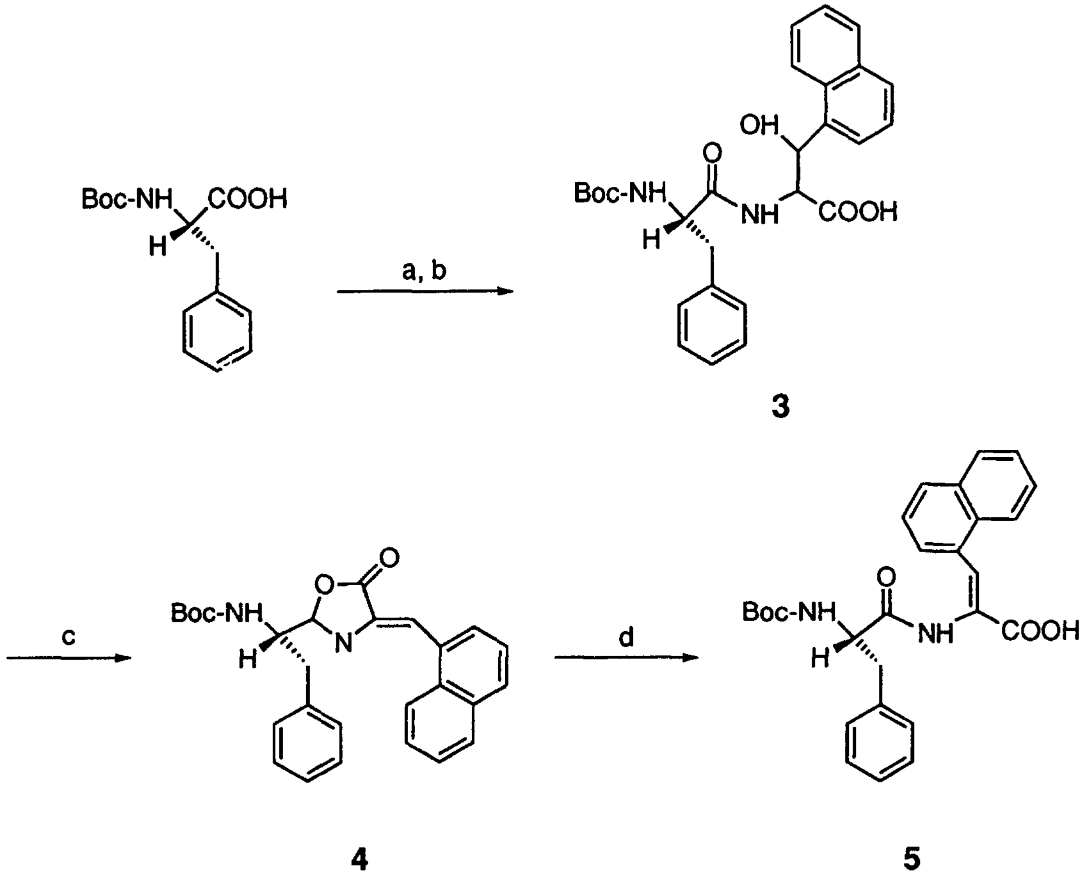
Syntheses

The dehydronaphthylalanine substituted-SP analogues were prepared according to a procedure similar to that described for the synthesis of the dehydrophenylalanine substituted-analogues.[7] The *Z*-dehydronaphthylalanine has been incorporated in the sequence of SP as a dipeptide unit. Boc-Phe-Δ^ZNal-OH, **5**, has been prepared via the corresponding oxazolone **4**, according to the method of Noda et al.[12] (Scheme 1). The corresponding *E*-dehydronaphthylalanine-SP analogue has been obtained after photoisomerization of SP-containing the *Z*-derivative, at 310 nm in methanol, as originally described for enkephalin analogues[13] and as previously reported for *Z*-dehydrophenylalanine.[7]

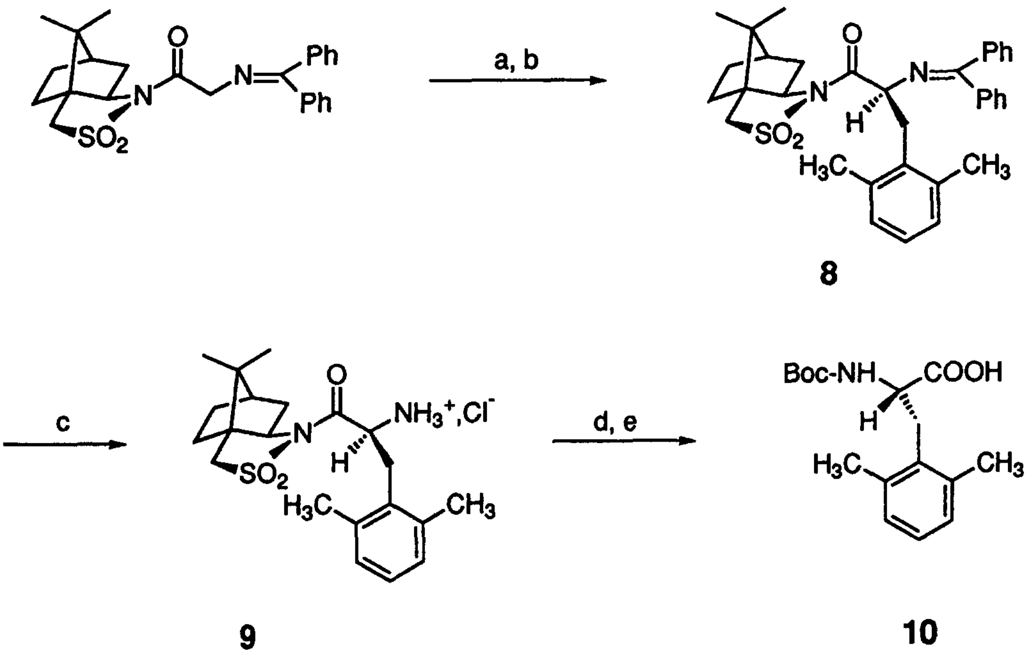
N-tert-(Butyloxycarbonyl)-L-*o*,*o'*-dimethylphenylalanine (**10**) was prepared via an asymmetric alkylation of a Schiff base from a sultam-derived glycinate[7] by 2,6-dimethylbenzyl bromide, with a good yield (71%) and an excellent diastereoselectivity (>97%, as estimated from the NMR analysis) (Scheme 2). Hydrolysis of the alkylated Schiff base **8** yielded the corresponding *N*-aminoacylsultam **9**, the sultam group was then cleaved with LiOH–LiBr–nBu$_4$NBr. The amino acid was isolated and characterized as its *N*-Boc derivative **10**, which was introduced by solid-phase peptide synthesis in the SP sequence.

Binding Data

In position 7, only [(2*S*,3*S*)Ing^7]SP and [Dmp^7]SP presented a binding potency (K_i) for hNK-1 receptor in the nanomolar range comparable to those of SP and [Pro^9]SP, (K_i values of 3.7±0.9 and 2.5±0.4 compared to 1.6±0.4 and 1.1±0.1 nM, respectively) (Table 1). In position 8, [Flg^8]SP, [(2*S*,3*S*)Bfi^8]SP and [Dmp^8]SP were good competitors of [^{3}H][Pro^9]SP specific binding sites on CHO cells expressing hNK-1 receptors, being as potent as SP and [Pro^9]SP (2.1±0.3, 0.45±0.02 and 3.2±0.8 nM, respectively). The binding curves of [Dmp^7]SP and [Dmp^8]SP were shallow with Hill coefficients significantly lower than unity compared to the other peptides, 0.62±0.06 and 0.65±0.05, respectively. Except the disubstituted [(2*S*,3*S*)Ing^7, Flg^8]SP analogue (K_i = 14.3±1.6 nM), all the other peptides were 30- to 300-times less potent than SP in inhibiting [^{3}H][Pro^9]SP



Scheme 1. Synthesis of Boc-Phe-Δ^Z-Nal-OH. (a) iBuOCOCl, NEM, THF, −18 °C; (b) DL-Nal(β-OH), NaOH; (c) AcONa, Ac_2O; (d) LiOH, CH_3CN/H_2O.



Scheme 2. Synthesis of L-*o*,*o*′-dimethylphenylalanine. (a) *n*-BuLi, THF-HMPA, −78 °C; (b) $(CH_3)_2C_6H_3CH_2Br$, −78 °C→rt; (c) 1 N HCl/Et_2O; (d) LiOH-LiBr-*n*Bu_4NBr, CH_3CN; (e) $(Boc)_2O$.

Table 1. Comparison of affinities and activities of several tachykinin analogues in binding and functional assays on intact CHO cells expressing the human NK-1 receptors and their EC_{50} values in the guinea pig ileum longitudinal muscle bioassay

Peptides		CHO/hNK-1 receptor			Guinea pig ileum
		EC_{50} (nM)[a] cAMP	K_i (nM)[b]	EC_{50} (nM)[a] IPs	EC_{50} (nM)[c]
SP		8±4	1.6±0.4	1.0±0.6	2.5±0.7
[Pro^9]SP		10±2	1.1±0.1	0.8±0.2	1.1±0.2
[pGlu^6, Pro^9]SP (6–11)		5200±200	490±10	2.7±0.5	1.5±0.4
[Tic^7]SP	g$^+$	>10000	365±68	373±111	417±112
[Flg^7]SP	(g$^-$,t)	270±20	95±15	13.5±5.7	9.5±2.1
[Dip^7]SP	(g$^-$,t)	>10000	5000±1500	2000±400 (62%)[e]	226±156
[(2*S*,3*S*)Ing^7]SP	g$^-$	5.3±0.1	3.7±0.9	3.0±0.9	1.2±0.6
[(2*S*,3*R*)Ing^7]SP	t	260±40	85±19	3.2±0.4	6.9±2.8
[Δ^ZPhe^7]SP	‘g$^-$’	>10000	1200±380	530±130	3100±640
[Δ^EPhe^7]SP	‘t’	1700±600	225±53	6.3±1.1	8.4±2.5
[Dmp^7]SP	t	8.2±1.9	2.5±0.4 (0.62±0.06)[d]	0.92±0.08	1.15±0.15
[Tic^8]SP	g$^+$	6000±1000	374±87	193±22	83±17
[Flg^8]SP	(g$^-$,t)	4.8±1.6	2.1±0.3	1.8±0.5	5.7±2.3
[Dip^8]SP	(g$^-$,t)	910±70	350±10	32±13	15±1
[(2*S*,3*S*)Bfi^8]SP	g$^-$	2.9±0.9	0.45±0.02	2.4±0.8	1.7±0.7
[(2*S*,3*R*)Bfi^8]SP	t	145±10	44±6	0.65±0.15	2.8±1.7
[Δ^ZNal^8]SP	‘g$^-$’	7500±1500	400±128	115±8	27±5
[Δ^ENal^8]SP	‘t’	inac.10^{-5} M	2000±700	≅7000	325±170
[Dmp^8]SP	t	6.9±0.7	3.2±0.8 (0.65±0.05)[d]	1.3±0.4	1.15±0.15
[(2*S*,3*S*)Ing^7, Flg^8]SP		27±6	14.3±1.6	6.0±2.5	3.4±1.6
[Dip^8,Pro^9]SP		>10000	162±42	38±11	20±5

SP: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2; septide: pGlu-Phe-Phe-Pro-Leu-Met-NH_2.

[a] For adenylate cyclase activation and PI hydrolysis, concentration–response experiments were conducted on intact CHO cells expressing hNK-1 receptors with an incubation time of 8 min, results are presented as the EC_{50} value and are the mean ± standard error of the mean from at least three experiments done in triplicate.

[b] Binding assays were performed with [^{3}H][Pro^9]SP as described in the Experimental, results are expressed as the K_i value ($0.8 < n_H < 1$) obtained from the Cheng and Prusoff relationship.[17]

[c] Cumulative dose–response curves were obtained, results are presented as the EC_{50} value and are the mean ± standard error of the mean from at least three independent experiments.

[d] *n* Hill, when significantly different from unity.

[e] Maximal response, when significantly different from unity.

specific binding to hNK-1 receptor. [Dip^7]SP, [Δ^ZPhe^7]SP and [Δ^ENal^8]SP were the weakest competitors with $K_i > 1$ μM.

Biological activity on CHO cells transfected with human NK-1 receptors

The results presented as EC$_{50}$ (Table 1) show that these SP analogues can be classified into three groups: (1) peptides with high potencies for stimulating both PI hydrolysis, and cAMP formation: SP, [Pro^9]SP, [(2*S*,3*S*)Ing^7]SP, [Dmp^7]SP, [Flg^8]SP, [(2*S*,3*S*)Bfi^8]SP, [Dmp^8]SP, [(2*S*,3*S*)Ing^7, Flg^8]SP with EC$_{50}$ ranging from 0.8 to 6 nM for PI hydrolysis and from 4.8 to 27 nM for cyclic AMP accumulation. These peptides were also potent inhibitors of [^{3}H][Pro^9]SP specific binding, (see above); (2) peptides with high potencies for activating PI hydrolysis (EC$_{50}$ <40 nM) but weak agonists on cAMP formation (EC$_{50}$ >140 nM) i.e. septide, [Flg^7]SP, [(2*S*,3*R*)Ing^7]SP, [Δ^EPhe^7]SP, [Dip^8]SP, [(2*S*,3*R*)Bfi^8]SP, [Dip^8, Pro^9]SP. This group of peptides were also weak competitors of [^{3}H] [Pro^9]SP specific binding (K_i ranging from 85 to 490 nM); (3) peptides with very low affinity ($K_i > 1$ μM) for [^{3}H] [Pro^9]SP specific binding sites and low or no potency at all for stimulating each of the second messenger pathways ([Dip^7]SP, [Δ^ZPhe^7]SP, [Δ^ZNal^8]SP and [Δ^ENal^8]SP). [Dip^7]SP was less efficient than the other analogues since the maximal response reached by [Dip^7]SP at 10 μM was 62% that of [Pro^9]SP. Thus, peptides from group 1 and group 2 both stimulated PLC pathway whereas only peptides from group 1 were able to activate cAMP formation.

Guinea pig ileum bioassay

All the peptides induced spasmogenic activity of the longitudinal muscle of the guinea pig ileum with EC$_{50}$ values between 1.1. and 20 nM, except three analogues [Dip^7]SP, [Δ^ZPhe^7]SP and [Δ^ENal^8]SP (226±156, 3100±640, 325±170 nM). Considering the classification based on binding and activity on CHO cells transfected with the hNK-1 receptor, these peptides correspond to the third group of peptides.

Potency of the NK-1 antagonist RP 67580

In both CHO cells and guinea pig ileum, the responses of the weak competitors of specific NK-1 binding sites was antagonized by the specific NK-1 antagonist RP 67580 (Table 2). PI hydrolysis induced by [Flg^7]SP, [Δ^EPhe^7]SP and [(2*S*,3*R*)Ing^7]SP, peptides from group 2, was inhibited by RP 67580 with pK_B or pA_2 values similar to those previously obtained for these peptides in the guinea pig ileum bioassay, ranging from 7.0 to 7.5.[7]

Discussion

Depending on the conformation adopted by the constrained amino acid, its side chain will explore different orientations of the space and could be used as either *trans*, *gauche* (+) or *gauche* (−) probe, (Fig. 1), as demonstrated by energy calculations on model peptides.[7] Within a backbone-restricted matrix, the specific orientation of these constrained side-chains is also related to the conformation of the peptide backbone, i.e. the preceding and the following residues. For example, the presence of two turns of helix must prevent a bulky side chain to adopt the *gauche* (+) orientation in the second turn of this helix. Consequently, bulky side chains will be preferentially oriented in the *gauche* (−) and/or *trans* directions, the aromatic nuclei of a bisubstituted C$_\beta$ amino acid such as diphenylalanine (**2**) and fluorenylglycine (**3**) will simultaneously explore the *gauche* (−) and *trans* orientations. From structure–activity relationships and conformational analysis by NMR of substance P analogues,[22–24] the core of substance P has been shown to be folded in either α- or 3$_{10}$-helical structure, the C-terminal tripeptide presenting a 2$_7$ ribbon type structure. Therefore, a Tic residue **1** will disrupt the

Table 2. Comparison of the inhibitory potency of RP 67580 on phosphatidylinositol hydrolysis (CHO cells) and on spasmogenic activity (guinea pig ileum)

Peptides	CHO cells (IPs)		Guinea pig ileum	
	EC$_{50}$ (nM)	RP 67580 pK_B[a] (pA_2)[b]	EC$_{50}$ (nM)	RP 67580 pK_B[a] (pA_2)[b]
SP	1.0±0.6	(7.07±0.14)	2.5±0.7	(7.20±0.10)[c]
[Pro^9]SP	0.8±0.2	(7.00±0.05)[d]	1.1±0.2	6.97±0.07[f]
[pGlu^6, Pro^9]SP (6–11)	2.7±0.5	7.68*±0.08[c,d]	1.5±0.4	(7.60±0.02)[e]
[Flg^7]SP	13.5±5.7	7.54±0.07	9.5±2.1	7.1±0.1 (7.5±0.2)[f]
[Δ^EPhe^7]SP	6.3±1.1	7.51	8.4±2.5	7.15±0.10[f]
[(2*S*,3*R*)Ing^7]SP	3.2±0.4	7.23	6.9±2.8	7.06±0.07[f]

[a] pK_B = log (dose ratio − 1) − log ([RP 67580]), for one concentration of antagonist RP 67580 at 10^{-6} M (IPs) and 10^{-7} M (GPI), two to three independent determinations (run in triplicate for IPs).

[b] pA_2, slope of the Schild plots[34] were not significantly different from unity, two to three independent determinations with [RP 67580] at 5×10^{-8}, 2×10^{-7} and 10^{-6} M (IPs) and 10^{-7}, 3×10^{-7} and 10^{-6} M (GPI).

[c] Insurmountable antagonism K_B* values were derived[15] according to the method of Kenakin.[36]

[d] Data from Sagan *et al.*[15]

[e] Data from Carruette *et al.*[37]

[f] Data from Josien *et al.*[7]

α-helical structure of SP since its aromatic side chain is locked in a *gauche* (+) orientation.[7] The *o,o′*-methyl substituents of L-*o,o′*-dimethylphenylalanine imposes a *trans* orientation of the aromatic nucleus, the helical structure of the preceding residues preventing the free rotation of the aromatic side-chain as deduced from molecular modeling and confirmed by NMR analysis of [Dmp^7]SP (P. Karoyan et al., unpublished data). The preferred orientation(s) of the various phenylalanine constrained analogues (Fig. 1) introduced in positions 7 and 8 are indicated in Table 1.

As previously observed with NK-1 specific binding to rat brain synaptosomes,[7] the S_7 binding subsite is smaller than the S_8 subsite, adopting only one aromatic nucleus, while the S_8 subsite can accommodate altogether three coplanar nuclei, one in the *trans* direction and two in the *gauche* (−) orientation. The large volume available in the NK-1 receptor for substituents in position 8 was rather surprising since the C-terminal residues Phe-Phe-Gly-Leu-Met-NH_2 were supposed to be inserted in the cleft delineated by the seven transmembrane domains according to molecular modelling studies.[25] Therefore, we suspected that the S_8 binding subsite may reside in the extracellular loops. Recent data obtained by photolabeling of NK-1 receptor with [*p*-benzoyl-phenylalanine^8]substance P derivatives have shown that residues from the second extracellular loop were indeed labeled,[26–28] suggesting that the S8 binding site in the hNK-1 receptor do not reside in the transmembrane domain. This result appears more conceivable with the volume calculated from our data[7] for the S_8 binding subsite, i.e. ~240 Å.[3]

The highest affinity of [Δ^EPhe^7]SP vs. [Δ^ZPhe^7]SP for rat brain synaptosomes NK-1 binding sites led us to speculate that (1) the S_7 binding subsite of the rat NK-1 receptor accepts one aromatic probe in the '*trans*' direction and (2) the *gauche* (−) probe [(2*S*,3*S*)Ing^7]SP should rotate and bind as the *trans* rotamer.[7] This interpretation appeared even more speculative with data obtained with the human NK-1 receptors expressed in CHO cells and needed to be further confirmed. Indeed, on the one hand, the affinity of [Δ^EPhe^7]SP for hNK-1 receptor was lower to that measured on rat brain synaptosomes and on the other hand, the affinity of [Δ^ZPhe^7]SP was increased. Altogether the difference between both dehydrophenylalanine substituted-substance P was still significant but too low (4.5-fold) to base a conclusion on reliable results. To address this question L-*o,o′*-dimethylphenylalanine was synthesized and introduced as a *trans* probe of Phe^7 in the sequence of substance P. The high affinity and potencies of [Dmp^7]SP allow us now to conclude that the aromatic nucleus of residue 7 must be in the *trans* direction when bound to the receptor. And as already postulated the high affinity of [(2*S*,3*S*)Ing^7]SP implies that its preferred gauche (−) side chain must rotate inside the protein or alternatively that the protein must quench the minor *trans* rotamer in solution, even if its concentration is low, as shown by NMR analysis[29] of [(2*S*,3*S*)Ing^7]SP. The highest affinity of the *trans* rotamer of [(2*S*,3*S*)Ing^7]SP compared to that of the *trans* probe [(2*S*,3*R*)Ing^7]SP indicates that the cyclopentanyl moiety of the (2*S*,3*S*) diastereoisomer fits better in the S_7 binding subsite than that of the (2*S*,3*R*) isomer.

The human NK-1 receptor transfected in CHO cells activates in the nanomolar range both IPs and cAMP productions, with one order of magnitude in EC_{50} values for cAMP formation. All the peptides listed in Table 1 are agonists, however some discrepancies are observed between K_i and EC_{50} values. For example, despite their low binding potencies, [Flg^7]SP, [(2*S*,3*R*)Ing^7]SP, [Δ^EPhe^7]SP, [Dip^8]SP, [(2*S*,3*R*)Bfi^7]SP and [Dip^8, Pro^9]SP are highly efficient in stimulating PI hydrolysis. This dilemma has been first observed with septide, [pGlu^6, Pro^9]SP(6–11), or C-terminal fragments of substance P and then with undecapeptide analogues of substance P and analogues of neurokinin A.[15] In spite of their lower affinity for hNK-1 receptor, these six constrained peptides evoked a potent stimulation of phosphatidylinositol hydrolysis, with EC_{50} values with one to two orders of magnitude less than their K_i values. This activation is related to the presence of the NK-1 receptor, since no detectable agonist-induced PI hydrolysis occurred in nontransfected CHO cells. Furthermore, RP 67580,[30] an NK-1 specific antagonist, inhibits PI hydrolysis induced by [Pro^9]SP and septide[15] as well as those induced by active peptides with low binding potency, i.e. [Flg^7]SP, [Δ^EPhe^7]SP and [(2*S*,3*R*)Ing^7]SP, Table 2. The pK_B or pA_2 values were close to those previously obtained for [Pro^9]SP and septide.[15] In contrast, these peptides were only weak agonists for cAMP production with EC_{50} values similar to K_i values. In fact, with all the compounds listed in Table 1, the linear regression coefficient found for the correlation between their affinity (K_i) and EC_{50} for IPs production was low ($r = 0.70$, Fig. 2a), whereas an excellent correlation could be demonstrated between their affinities (K_i) and their potencies (EC_{50}) for cAMP production ($r = 0.97$, Fig. 2b). However, the high potency (EC_{50}) observed for some peptides on PI hydrolysis, compared to their affinity, is not an artefact due to an in vitro system expressing a rather high level of hNK-1 receptors (6500 fmol/mg protein). Indeed, a good correlation is found between EC_{50} values obtained for PI hydrolysis and those measured for these peptides in the guinea pig ileum bioassay ($r = 0.94$, Fig. 2c). Similar pK_B or pA_2 values were also obtained in both assays with the NK-1 antagonist RP 67580. Therefore, CHO cells expressing human NK-1 receptor may represent a more convenient or simpler system to analyze the molecular basis for such a phenomenon.

We have previously interpreted[15] these results as septide and 'septide-like molecules' acting on either a preactivated state of the receptor, (the already isomerized R* state), present in low concentration and whose concentration must be tissue-dependent, or on a truncated NK-1 receptor, whose proteolytic cleavage is tissue-dependent, since septide is not potent in all tissues. The first hypothesis is referred to as 'agonist–

receptor trafficking' in the recent Kenakin's paper dealing with agonist–receptor efficacy which specifically considered the case of COS cells transfected with the rat NK-1 receptor.[31] Nevertheless, the problem of receptor heterogeneity even in COS or CHO cells cannot be rejected, post-translational modification of an initial homogenous receptor population may still occur and it can be speculated as already reported[32,33] that a shorter form of an NK-1 receptor may coexist. Mantyh et al.[34] recently reported differential expression of two isoforms of rat NK-1 receptor in vivo in the parotid and submaxillary gland. In both tissues very high levels of [^{125}I]SP binding sites were found whereas only low levels of NK-1 immunoreactivity could be detected, suggesting that the expression of a short form of rat NK-1 receptor predominates in these tissues, in contrast to the striatum. Therefore, as already concluded by Kenakin,[31] binding studies have to be achieved with radiolabelled NK-1 agonist and septide-like molecule to determine, for both radioligands, the maximal binding capacity (B_{max}), even though different pools of G proteins may however interfere with the interpretation of the binding data. Another key step for a better understanding of this phenomenon would be the use of photolabeled analogues of both types of molecules which will allow identification of both molecular weight(s) of covalently linked protein(s) and labelled residue(s) in the binding sites. Since some of these peptides incorporating new constrained phenylalanine analogues behave as 'septide-like' molecules we can now envisage the design and the synthesis of photolabelled analogues of phenylalanine conferring 'septide-like' behaviour to the parent peptides.

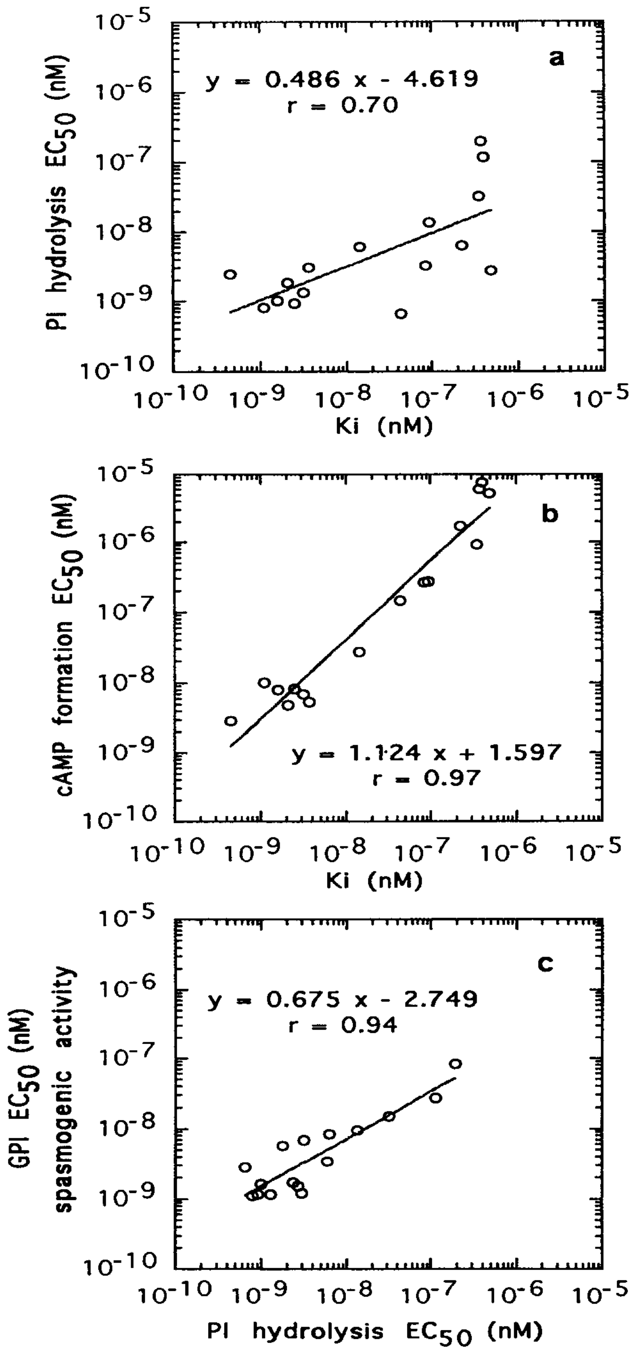


Figure 2. Correlations between the binding affinities (K_i) for [^{3}H][Pro^9]SP specific binding on intact CHO cells transfected with hNK-1 receptors and EC$_{50}$ values for PI hydrolysis (Fig. 2a) or EC$_{50}$ values for cAMP formation (Fig. 2b) or between K_i values and EC$_{50}$ values in guinea pig ileum assay (Fig. 2c). For the correlation analysis, all the active peptides were considered, i.e. 16 compounds for Figs 2(a–c), the analogues inactive in stimulating cAMP formation (K_i>5 µM) were discarded, i.e. [Tic^7]SP, [Dip^7]SP, [Δ^{Z}Phe^7]SP, [Δ^{E}Nal^8]SP, [Dip^8, Pro^9]SP.

Experimental

Chemistry

Melting points were determined on a Kofler melting point apparatus and are uncorrected. NMR spectra were recorded on Jeol NM-3970 (90 MHz), Brucker AC-200 (200 MHz), Brucker ARX-400 (400 MHz) and Jeol GSX400 (400 MHz) spectrometers with chemical shifts expressed in ppm (δ) relative to trimethylsilane or to DMSO. Elementary analyses, performed by the Service Régional de Microanalyses, Paris VI, were within 0.4% of the theoretical values calculated for C, H, and N, as noted. Optical rotations were measured on a Perkin–Elmer 241 polarimeter. Values were obtained from the means of 10-successive 5-s integrations. Analytical TLC was carried out with Merck silica gel 60 F$_{254}$ TLC plates, and compounds visualization was effected with a UV lamp (UV), phosphomolybdic oxidation (PMA) or ninhydrin (after TFA exposure for the *N*-Boc derivative). Column chromatography was performed with Merck silica gel 60, 0.063–0.200 mm (atmospheric pressure) or 0.040–0.063 mm (flash chromatography), and Merck silica gel PF 254, for centrifugal chromatography.

DL-Naphthylserine ethyl ester hydrochloride salt (1). Under argon, to a soln of methyl *N*-(diphenylmethylene) glycine ethyl ester (5 g, 18 mmol) in anhydrous THF (54 mL) and anhydrous HMPA (10.8 mL) was added 2 N LDA (9 mL, 18 mmol) over 5 min, at −78 °C. The mixture was warmed to −40 °C, and naphthylaldehyde (2.48 g, 18 mol) in anhydrous THF was added. The reaction mixture was stirred for 6 h at −78 °C and then quenched with 10% citric acid (50 mL). After dilution with Et_2O (100 mL), the organic layer was extracted and washed with satd NH_4Cl (4 × 75 mL). The imine function was then removed on the crude product. After concn under red. press., the residue was solubilized in Et_2O (30 mL) in the presence of 1 N HCl (30 mL). After stirring for 12 h at 0 °C, the soln was concd and the residual crude solid was recrystallized from warm EtOH affording 2.7 g (50% yield) of a white powder. R_f 0.58 ($CHCl_3$: MeOH, 8:2, UV, PMA); ^1H NMR (90 MHz, DMSO): δ 8.6 (m, 3H, $N\underline{H}_3^+$), 8–7.4 (m, 7H, aromatic) 6.5 (d, 1H, $O\underline{H}$), 5.45 (m, 1H, $C\underline{H}$-OH), 3.9 (q, 2H, $C\underline{H}_2CH_3$), 0.8 (t, 3H, $CH_2C\underline{H}_3$); Anal. ($C_{15}H_{18}NO_3Cl$, 0.5 H_2O) C, H, N calcd (found), C, 59.12 (59.35), H, 5.92, (6.05), N, 4.60 (4.74).

***N-tert*-Butyloxycarbonyl-Phe-DL-Nal(β-OH)-OEt** (**2**). To a soln of Boc-Phe-OH (506 mg, 1.9 mmol) and *N*-ethylmorpholine (241 μL, 1.9 mmol) in DMF (25 mL) was added isobutylchloroformate (250 μL, 1.9 mmol), at −18 °C under N_2. After 10 min stirring a soln of **1** (521 mg, 1.73 mmol) and *N*-ethylmorpholine (220 μL, 1.73 mmol) was introduced at −18 °C. The reaction mixture was stirred for 30 min at −18 °C and then for 1 h at room temperature. The soln was concd to remove DMF, diluted with AcOEt (40 mL) and washed with aq 10% citric acid, water, aq 5% $NaHCO_3$, to provide after drying ($MgSO_4$) and concd in vacuo, 850 mg (97% yield) of a white powder. R_f 0.4 (cyclohexane : AcOEt, 6:4, UV, PMA); ^1H NMR (400 MHz, $CDCl_3$): δ 7.8–7.15 (m, 12H, aromatic), 6.7 (m, 1H, $N\underline{H}$Nal) 5.29 (m, 1H, $NCHC\underline{H}OH$), 4.95 (m, 2H, $NC\underline{H}CHOH + N\underline{H}Phe$), 4.4 (m, 1H, CHα Phe), 4.05 (m, 2H, $OC\underline{H}_2CH_3$), 3.55 (m, 1H, $O\underline{H}$), 3.05 (m, 2H, $C\underline{H}_2$β Phe), 1.35 (s, 9H, Boc), 1.0 (t, 3H, $OCH_2C\underline{H}_3$); Anal. ($C_{29}H_{34}N_2O_6$) C,H,N calcd (found), C, 68.69 (68.64), H, 6.76 (6.76), N, 5.52 (5.54).

Boc-Phe-DL-Nal(β-OH)-OH (**3**). A soln of **2** (2.18 g, 4.33 mmol) in acetone (10 mL) and 1 N NaOH (7 mL) was stirred for 2 days at room temperature. After concn under reduced pressure, the residue was dissolved in H_2O and acetic acid was added until pH 4.0. Addition of Et_2O induced precipitation of **3** which was collected by filtration and further purified by centrifugal chromatography ($CHCl_3$: MeOH : AcOH, 8:2:0.02) to yield **3** as a white powder (1.5 g, 73% yield). R_f 0.19 ($CHCl_3$: MeOH, 8:2, UV, PMA); ^1H NMR (90 MHz, CD_3OD): δ 7.8–6.8 (m, 12H, aromatic), 5.15 (m, 1H, $C\underline{H}OH$), 4.7 (m, 1H, $C\underline{H}$α Nal), 4.2 (m, 1H, CHα Phe), 2.8–2.3 (m, 2H, $C\underline{H}_2$β Phe), 1.1 (s, 9H, Boc).

Boc-Phe-Δ^ZNal-azlactone (**4**). A soln of **3** (1 g, 2.09 mmol) and freshly fused AcONa (210 mg, 2.56 mmol) in acetic anhydride (20 mL) was stirred, under argon, for 24 h at room temperature. The reaction mixture was poured into crushed ice, diluted with 5% $NaHCO_3$ (40 mL) and CH_2Cl_2 (50 mL), triturated and filtered. After decantation, the organic layer was washed with 5% $NaHCO_3$ (3 × 40 mL) and dried ($MgSO_4$). The solvent was removed in vacuo and the residue crystallized from warm CH_2Cl_2, to afford 680 mg (73% yield) of a pale yellow powder. Mp 204 °C; $[\alpha]_D^{20}$ −23 (*c* 1, $CHCl_3$); R_f 0.18 (cyclohexane : AcOEt, 9:1, UV, PMA); ^1H NMR (400 MHz, $CDCl_3$): δ 8.33 (m, 2H, aromatic Nal), 7.85 (m, 3H, aromatic Nal), 7.55 (m, 2H, aromatic Nal), 7.37 (s, 1H, $C{=}C\underline{H}$), 7.31–7.2 (m, 5H, aromatic Phe), 5.07 (m, 1H, $C\underline{H}$α Phe), 4.91 (broad peak, $N\underline{H}$ Phe), 3.34–3.31 (AB, 2H, $C\underline{H}_2$β Phe) 1.46 (s, 9H, Boc). Anal. ($C_{27}H_{26}N_2O_4$) C, H, N, calcd (found), C, 73.28 (72.84), H, 5.82 (5.94), N, 6.33 (6.59).

Boc-Phe-Δ^ZNal-OH (**5**). To a mixture of **4** (782 mg, 1.76 mmol) in CH_3CN (25 mL) was added LiOH (63.4 mg, 2.64 mmol) in H_2O (12 mL) and the reaction was stirred for 45 min at room temperature. After concn in vacuo, the remaining soln was diluted with H_2O (60 mL) and washed with Et_2O (2 × 50 mL). The aq layer was then acidified with aq 10% citric acid (120 mL) and quickly extracted with AcOEt (4 × 50 mL). The combined organic layers were washed (H_2O) and dried ($MgSO_4$) to provide, after evapn of the solvent under red. press. and crystallization of the residue from warm $CHCl_3$, 670 mg (85% yield) of a white powder. Mp 202 °C; $[\alpha]_D^{20}$ 139 (*c* 1, *N*-methylpyrrolidone); R_f 0.43 ($CHCl_3$: MeOH : AcOH, 8:2:0.05, UV, PMA); ^1H NMR (400 MHz, DMSO): δ 9.75 (s, 1H, $COO\underline{H}$), 8.28 (s, 1H, $C{-}C\underline{H}$), 8–7.13 (m, 12H, aromatic), 4.36 (m, 1H, $C\underline{H}$α Phe), 3.09–2.83 (AB, 2H, $C\underline{H}_2$β Phe), 1.32 (s, 9H, Boc); Anal. ($C_{27}H_{30}N_2O_6$, 0.5 H_2O) C, H, N calcd (found), C, 67.78 (67.83), H, 5.85 (5.98), N, 5.85 (5.75).

2,6-Dimethylbenzyl alcohol (**6**). To a soln of 2,6-dimethyl benzoic acid (5 g, 33.3 mmol) in THF (20 mL) and $B(OMe)_3$(10 mL), BMS (18.3 mL, 36.6 mmol) was slowly added under argon at 0 °C. The reaction mixture was stirred for 3 days at room temperature and then quenched with MeOH (15 mL). After concn in vacuo the residue was washed with Et_2O to provide a white powder (4.5 g, 100% yield) which was used without further recrystallization. R_f 0.30 (cyclohexane:AcOEt, 8:2, UV, PMA); ^1H NMR (200 MHz, $CDCl_3$): δ 7.08–6.97 (m, 3H, aromatic), 4.69 (s, 2H, $C\underline{H}_2$), 2.31 (s, 6H, $C\underline{H}_3$).

2,6-Dimethylbenzyl bromide (**7**). Phosphorous tribromide (1.22 mL, 12.9 mmol) was added to a suspension of **6** (3.8 g, 25.7 mmol) in anhydrous CH_2Cl_2 (25 mL). After stirring for 16 h at room temperature, the mixture was washed with cold water (2 × 50 mL) and cold 10% aq ammoniac (2 × 10 mL). The organic layer was dried ($MgSO_4$) and concd under red. press., to provide a white liquid which was distilled (Teb: 70 °C, 1 mm Hg), affording colorless crystals (5 g, 98% yield). Mp <48 °C; R_f 0.49 (cyclohexane, UV, PMA); ^1H NMR (400 MHz, $CDCl_3$): δ 7.16–7.13 (t, 1H, H *para*), 7.08–7.06 (d, 2H, H *meta*), 4.61 (s, 2H, $C\underline{H}_2Br$), 2.45 (s, 6H, CH_3 *ortho*). Anal. ($C_9H_{11}Br$) C, H, N calcd (found), C, 54.29 (54.15), H, 5.56 (5.52).

***N*-(Diphenylmethylene)-L-*o*,*o*′-dimethylphenylalanine sultam** (**8**). Under argon, to a soln of *N*-(diphenylmethylene)glycine sultam (3.3 g, 7.6 mmol) in anhydrous THF (25 mL) and HMPA (8 mL) was added, over 2 min, at −78 °C, 1.6 N *n*-BuLi in hexane (5.3 mL, 8.4 mmol). The reaction mixture was stirred 5 min at this temperature and 2,6-dimethylbenzyl bromide (1.67 g, 8.4 mmol) in anhydrous THF (6 mL) was injected dropwise. After 15 min at −78 °C and 2 h at room temperature, the mixture was quenched with a few drops of acetic acid. After evapn in vacuo the residue was dissolved in Et_2O (50 mL) and washed with satd NH_4Cl (3 × 50 mL). Subsequent drying ($MgSO_4$) and concn under red. press. afforded **8** which was crystallized in Et_2O to provide 3 g (71% yield) of a white powder. Mp 186 °C; $[\alpha]_D^{20}$ 43 (*c* 2, $CHCl_3$); R_f 0.11 (cyclohexane:AcOEt, 9:1, UV ninhydrin); ^1H NMR (200 MHz, $CDCl_3$): δ 7.62–7.58 (m, 2H, aromatic), 7.33–7.22 (m, 6H, aromatic), 6.93–6.77 (m, 5H, aromatic), 5.20–5.12 (AB, 1H, $C\underline{H}\alpha$), 3.90–3.84 (AB, 1H, $C\underline{H}NSO_2$), 3.30–2.95 (AB, 2H, $C\underline{H}_2\beta$), 3.28 (AB, 2H, $C\underline{H}_2SO_2$), 2.07 (s, 6H, *o,o'*CH_3), 1.96–1.92 (m, 1H, $SO_2NC\underline{H}CH_2$), 1.77–1.70 (m, 3H, $SO_2NCHCH_2C\underline{H}C\underline{H}_2C\underline{H}_2$), 1.57 (m, 1H, $SO_2NCHC\underline{H}_2$), 1.36–1.17 (m, 2H, $SO_2NCHCH_2CHC\underline{H}_2C\underline{H}_2$), 0.82 (s, 3H, $C\underline{H}_3$), 0.64 (s, 3H, $C\underline{H}_3$); Anal. ($C_{34}H_{38}N_2O_3S$) C, H, N, calcd (found), C, 73.61 (73.70), H, 6.90 (7.03), N, 5.04 (5.01).

L-*o,o'*-Dimethylphenylalanine sultam hydrochloride salt (9). To a soln of **8** (2.5 g, 4.5 mmol) in THF (20 mL) was added at 0 °C 1 N HCl (6.75 mL). The reaction was stirred at 0 °C for 4 h. After concn under red. press., the residue was triturated with Et_2O and filtered to provide 2 g (87% yield) of a white powder. Mp 208 °C; $[\alpha]_D^{20}$ 26.4 (*c* 1, MeOH); R_f 0.32 ($CHCl_3$, UV, ninhydrin); ^1H NMR (400 MHz, CD_3OD): δ 7.04–6.97 (m, 3H, aromatic), 4.74–4.70 (m, 1H, $C\underline{H}\alpha$), 3.92–3.89 (m, 1H, $SO_2NC\underline{H}CH_2$), 3.66–3.56 (AB, 2H, $C\underline{H}_2SO_2N$), 3.55–3.21 (AB, 2H, $C\underline{H}_2\beta$), 2.37 (s, 6H, *o,o'*$C\underline{H}_3$), 1.99–1.67 (m, 5H, $SO_2NCHC\underline{H}_2C\underline{H}C\underline{H}_2C\underline{H}_2$), 1.45–1.19 (m, 2H, $SO_2NCHCH_2CHC\underline{H}_2C\underline{H}_2$), 0.89 (s, 3H, CH_3), 0.40 (s, 3H, CH_3); Anal. ($C_{21}H_{31}N_2O_3SCl$, 0.5 H_2O) C, H, N, calcd (found), C, 57.79 (57.36), H, 7.39 (7.46), N, 6.42 (6.22).

***N*-(*tert*-Butyloxycarbonyl)-L-*o,o'*-dimethylphenylalanine (10).** To a soln of **9** (1.2 g, 3.1 mmol) in CH_3CN (15 mL), LiOH (294 mg, 12.3 mmol), LiBr (1.33 g, 15.3 mmol) and nBu_4NBr (395 mg, 1.22 mmol) were added. The reaction mixture was stirred for 1 h at room temperature, the mixture was then diluted with H_2O (15 mL) and 1 N HCl (15 mL) and extracted with CH_2Cl_2 (2 × 20 mL) to allow the sultam recovery. Water was evapd and the crude product was solubilized in EtOH (15 mL). The pH was adjusted to 8–9 with 5% $NaHCO_3$ and di-*tert*-butyldicarbonate (1.05 g, 4.6 mmol) was added. After stirring for 15 h at room temperature and filtration (Celite) the solvant was removed under red. press. The crude product was purified by flash chromatography ($CHCl_3$:MeOH:AcOH, 8:2:0.02) to provide 692 mg of a white powder (77% yield). Mp 122–124 °C; R_f 0.26 ($CHCl_3$:MeOH:AcOH, 8:2:0.02, UV, ninhydrin); $[\alpha]_D^{20}$ −25 (*c* 1, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$): δ 12.58 (br peak, 1H, $COO\underline{H}$), 7.46–7.42 (d, 1H, NH *cis–trans* isomerism), 7.04 (s, 3H, aromatic), 5.20–5.15 (d, 1H, NH *cis–trans* isomerism), 4.6 (m, 1H, $C\underline{H}\alpha$), 3.23–3.15 (m, 2H, $C\underline{H}_2\beta$), 2.43 (s, 6H, *o,o'*$C\underline{H}_3$), 1.36–1.07 (2s, 9H, Boc *cis–trans* isomerism). Anal. ($C_{16}H_{23}NO_4$) C, H, N calcd (found), C, 65.50 (65.43), H, 7.90 (7.95), N, 4.79 (4.91).

Peptide synthesis

Peptide synthesis was carried out on a 0.1 mmol scale on an ABI Model 431A peptide synthesizer starting from a *p*-methylbenzhydrylamine resin (MBHA resin, typical substitution between 0.65 and 0. 75 mmol per g of resin). All *N*α-Boc-amino acids, in 5- or 10-fold excess, were assembled using dicyclohexylcarbodiimide and hydroxybenzotriazole as coupling agents. After removal of the last *N*α-Boc-protecting group, the resin was dried in vacuo. The peptide–resin was transferred into the Teflon vessel of a HF apparatus, and the peptide was cleaved from the resin by treatment with 1.5 mL anisole, 0.25 mL dimethyl sulfide, and 10 mL anhydrous HF per gram of peptide–resin for 30 min at −20 °C and 30 min at 0 °C. After evapn in vacuo of the HF and the solvents, the resin was first washed three times with Et_2O and then subsequently extracted three times with 10% AcOH. Lyophilization of the extract gave crude product containing mainly, as estimated by HPLC, the expected peptide and its sulfoxide as side-products. Peptides were purified by preparative reverse-phase HPLC with an Applied Biosystems apparatus, using a 10 × 250-mm Brownlee column packed with Aquapore octyl, 300 Å pore size. The separation was accomplished with various acetonitrile gradients in aq 0.1% TFA at a flow rate of 6 mL/min with UV detection fixed at 220 nm. Before pooling, the collected fractions were checked for purity by analytical HPLC, performed on a Water Associates apparatus coupled to a D-2000 chromato-integrator (Merck). The separation was accomplished on a Lichrospher 100 RP-8e column (Merck) in isocratic mode (0.25 M triethylammonium phosphate buffer, pH 3.0, and acetonitrile) at a flow rate of 1.5 mL/min with UV detection fixed at 210 nm.

With the exception of $[Dmp^7]SP$, $[Dmp^8]SP$, $[\Delta^E Nal^8]SP$ and $[\Delta^Z Nal^8]SP$ the physicochemical characteristics of all the peptides used in the present investigation, and synthesized in the laboratory, have already been reported.[7]

$[Dmp^7]SP$: electrospray: *m/z* $(M+H)^+$ 1374, $[\alpha]_D^{20}$ −59 (*c* 0.46, 10% acetic acid), HPLC: Lichrosorb RP8-e, 1.5 mL/min, 210 nm, analytical HPLC, isocratic 29.6% CH_3CN in triethylammonium phosphate buffer, pH 3.0 (TEAP buffer), R_f 8.99 min, purity 100%.

$[Dmp^8]SP$: electrospray: *m/z* $(M+H)^+$ 1374, $[\alpha]_D^D$ −76 (*c* 0.46, 10% acetic acid), analytical HPLC, isocratic, 24.6% CH_3CN in TEAP buffer, R_f 8.50 min, purity 100%.

Photoisomerization of [Δ^ZNal^8]SP, preparation of [Δ^ENal^8]SP[7,12,13]

In a thermostated reactor fitted with an argon tube, a solution of already purified [Δ^ZNal^8]SP (20 mg) in deaerated MeOH (10 mL) was irradiated with a UV lamp at $\lambda = 310$ nm, for 15 min at 15 °C. This procedure was repeated twice, the combined crude products were diluted with H_2O, concd and lyophilized in the dark. The peptides were separated by preparative HPLC, the crude mixture consisted of [Δ^ZNal^8]SP, and [Δ^ENal^8]SP in a 1:4 ratio. Purification by preparative HPLC: linear gradient CH_3CN from 25 to 55% in 30 min. Final purity of the pooled fractions was ascertained by analytical HPLC (see above).

[Δ^ZNal^8]SP: electrospray: *m/z* $(M+H)^+$ 1396, $[\alpha]_D^{20}$ -48 (*c* 0.46, 10% acetic acid), analytical HPLC, isocratic 29.6% CH_3CN in TEAP buffer, R_f 7.70 min, purity 99.7%.

[Δ^ENal^8]SP: electrospray: *m/z* $(M+H)^+$ 1396, $[\alpha]_D^{20}$ -24 (*c* 0.46, 10% acetic acid), analytical HPLC, isocratic, 29.6% CH_3CN in TEAP buffer, R_f 5.01 min, purity 99.0%.

Drugs

Reagents and materials were obtained from the following source [11-^{3}H][Pro^9]SP (65 Ci/mmol) was synthesized at CEA, Saclay (France), according to Chassaing et al.,[14] *myo*-[2-^{3}H]inositol (17 Ci/mmol) and [2-^{3}H]adenine (26 Ci/mmol) were from Amersham Corp. (U.S.A.) and Du Pont de Nemours (NEN Products, France), respectively. Cell culture medium Ham F-12, fetal calf serum (FCS), 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), penicillin, streptomycin were from GIBCO (France). 3-Isobutyl-1-methylxanthine (IBMX), bacitracin, lithium chloride (LiCl) was from Sigma (U.S.A.). Bovine serum albumin (BSA) was from Calbiochem (France, Biochem). AG 50W-X4 Dowex resin in the hydrogen form (200–400 mesh), AG 7 neutral alumina resin (100–200 mesh) and AG 1-X8 Dowex resin in the formate form (200–400 mesh) were from Bio-Rad laboratories (U.S.A.). Aquasol-2 was from Du Pont de Nemours (France). Cell culture plastic ware were from NUNC (Denmark).

Cell culture

CHO-K_1 cells expressing the human NK-1 receptor were cultured in Ham-F-12 medium supplemented with 10% FCS, 100 IU/mL penicillin, 100 IU/mL streptomycin, 25 mM HEPES, 1.2% $NaHCO_3$ at 37 °C in a humidified atmosphere of 5% CO_2. The clone used in this study expresses high levels of hNK-1 receptor (6500 fmol/mg protein).[15]

Binding assays[15]

CHO cells expressing the human NK-1 receptor were seeded in 24-well plates (10^4 cells/well) 24 h before performing the binding assays. Cells were first washed three times for 10 min with 0.5 mL Krebs–phosphate buffer (in mM: NaCl 120; KCl 4.8; $CaCl_2$ 1.2; $MgSO_4$ 1.2 and NaH_2PO_4 15.6, pH 7.2) containing BSA (0.4 mg/mL), bacitracin (0.03 mg/mL) and glucose (6 mg/mL) as described.[16] The incubation for 100 min at room temperature (22 °C) with 0.3–0.7 nM [^{3}H][Pro^9]SP (in 0.2 mL Krebs–phosphate buffer) was conducted in either the presence or absence of varying concentrations of each agonist tested. The incubation was stopped by discarding the supernatant, washing the cells three times with 0.5 mL of cold (4 °C) Krebs–phosphate buffer, and detaching cells with 0.1% Triton X100 (0.5 mL) containing BSA (1 mg/mL). All determinations were performed in triplicate. Non specific binding was determined in the presence of 1 μM [Pro^9]SP. K_i values were calculated using the equation $K_i = IC_{50}/[1+(L/K_d)]$, where L is the concentration of the radioligand and K_d its dissociation constant.[17] Protein concentration in CHO cells was estimated by the Bradford method.[18]

Measurements of phosphatidylinositol hydrolysis

PI hydrolysis was measured as described previously[19] with slight modifications.[15] CHO cells expressing the human NK-1 receptor were seeded in 24-well plates ($\approx 5 \times 10^4$ cells) 48 h before performing the measurements. Cells were labeled with [^{3}H]inositol (0.5 μCi per well) for 24 h at 37 °C. Cultures were washed three times with Krebs–phosphate buffer (in mM: NaCl, 120; KCl, 4.8; $CaCl_2$, 1.2; $MgSO_4$, 1.2 and NaH_2PO_4, 15.6, pH 7.2) containing BSA (0.4 mg/mL), bacitracin (0.03 mg/mL) and glucose (6 mg/mL). The reaction was started by replacing the Krebs–phosphate buffer with 0.5 mL of fresh buffer containing 10 mM LiCl and test reagents. For dose–response studies, incubations with agonist or agonist/antagonist were carried out at 37 °C for 8 min, no preincubation with the antagonist was made before the addition of the agonist. Incubations were terminated by adding 0.5 mL Triton X-100 (0.1%) to each well for 30–60 min, cell lysates and supernatant were then transferred into glass tubes. To each tube, 1 mL chloroform:methanol (1:2) was first added and then 10 min later, 0.5 mL of chloroform. After centrifugation (3000 rpm for 5 min), 1 mL of the upper phase diluted with water (4 mL) was passed through an AG1-X8 (200–400 mesh, formate form) column. The initial eluate containing free [^{3}H]inositol was collected into vials. Columns were washed with 0.1 M formic acid (8 mL) and [^{3}H]inositol phosphates were eluted with 5 mL of 1 M ammonium formate:0.1 M formic acid. Radioactivity in the eluates was determined after addition of Aquasol-2. Results are the mean ± standard error of the mean of at least two independent experiments done in triplicate.

Measurements of cAMP formation

Cyclic AMP levels were estimated as previously reported[20] with modifications.[15] CHO cells expressing the human NK-1 receptor were seeded in 24-well plates ($\approx 5\times 10^4$ cells per well) 48 h before the adenylate cyclase assay. Cells were labeled with [^{3}H]-adenine (0.2 μCi per well) for 2 h at 37 °C. Cultures were washed three times with Krebs–phosphate buffer as described for PI hydrolysis measurements. The reaction was started by replacing the Krebs–phosphate buffer with 0.5 mL of fresh buffer containing 1 mM IBMX and test reagents. For dose–response experiments, incubation with agonist were carried out at 37 °C for 8 min. Incubations were terminated by adding 0.5 mL trichloroacetic acid (TCA) (10%) to each well. After 30–60 min incubation, cells were harvested with the supernatant and transferred into glass tubes. To each tube, 100 μL of a solution containing 3 mM cAMP, 3 mM ATP and 1% SDS in 50 mM Tris–HCl pH 7.4, were added. After centrifugation (3000 rpm/10 min), the supernatant (1 mL) diluted with water (3 mL) was passed through an AG 50W-X4 (200–400 mesh, hydrogen form) column. The initial eluate containing [^{3}H]ATP and [^{3}H]adenine was collected into vials. The AG 50W-X4 columns were then eluted with water (7 mL) on AG 7 neutral alumina columns. [^{3}H]cAMP was eluted from the AG 7 columns by adding 0.1 M imidazole pH 7.4 (4 mL). Radioactivity in the eluates was determined after addition of Aquasol-2. Results are the mean ± standard error of the mean of at least two independent experiments done in triplicate.

Guinea pig ileum bioassay

Male albino guinea pigs (Charles River, 300–350 g) were stunned and bled. The terminal portion of the ileum was used and 2 cm long strips were suspended in a 30 mL isolated organ bath (Ugo Basile) containing oxygenated (95% O_2, 5% CO_2) Tyrode solution at 37 °C coupled to a channel recorder (Gemini 7070, Ugo Basile). The composition of Tyrode solution was (mM) NaCl: 308, KCl: 11.3, $CaCl_2$: 2.2, $MgCl_2$ $6H_2O$: 1.0, $NaHCO_3$: 5.5, glucose: 12. After an equilibration period of 60 min, the strips were exposed to a maximally effective concentration of [Pro^9]SP (25 nM) at 15–20 min intervals until two reproducible responses were obtained. Then, the agonist to be tested was added cumulatively, i.e. the concentration in the bath was increased by a factor of 2 whenever a steady response to the previous concentration was reached. For each agonist, at least three different cumulative concentration–reponse curves were obtained. pK_B values (pK_B = log (dose ratio − 1) − log ([RP 67580]) were estimated for one concentration of the antagonist RP 67580 preincubated for 5 min before the first addition of agonist. As the dose ratio was close to 2, pK_B values were close to pA_2 values. The pA_2 values were obtained by regression of Schild plots.

Analysis of results

Saturation binding was analyzed using the program LIGAND.[21] IC_{50} values were obtained from the Hill regression plot. K_i values were obtained from the Cheng–Prusoff equation $K_i = IC_{50}/(1 + [L]/K_d)$.[17] All values in text and tables are means ± standard error of the mean. For dose–response curves in presence of antagonist, the Schild transformation was used to determine pK_B and pA_2 values when the antagonism was surmountable.

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