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ANALYSIS OF TACHYKININ BINDING SITE INTERACTIONS USING CONSTRAINED ANALOGUES OF TACHYKININS

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Besides Substance P (SP) [1], two tachykinins have been isolated from the brain and the spinal cord of mammals, i.e. Neurokinin A (NKA, also named Substance K and Neuromedin L) and Neurokinin B (NKB, also named Neuromedin K) [2–4] (Table 1). Binding studies performed on different preparations and using either tritiated or iodinated analogues of the tachykinins, including Substance P [5–8], Eledoisin [9–11], Kassinin [12], Neurokinin B [13], Neurokinin A [11, 12] and an analogue of NKB [14] have demonstrated the existence of two different high-affinity binding sites in the rat central nervous system, whose endogenous ligands are SP and NKB. Both binding sites have also been found in peripheral tissues [11, 15–24] as well as a third type of binding site presenting a different pharmacological profile [11, 23–24]. Neurokinin A is the most potent endogenous tachykinin interacting with this third subclass of binding sites. A general agreement exists for the occurrence of three types of tachykinin receptors, as evidenced by bioassays on peripheral organs [24–26]. And, they present pharmacological profiles closely related to those observed with the three classes of binding sites. During the "Substance P and Neurokinins" Symposium held in Montréal (Canada, July 1986) a consensus was reached for the designation of these three subtypes of receptors, namely NK1, NK2 and NK3, whose preferred endogenous substrates are SP, NKA and NKB, respectively.

Differences and/or analogies in the three-dimensional structures of Substance P and Neurokinin B have been evaluated by conventional structure–activity relationships and by studying the conformational behaviours, in solution, of the tachykinins and related peptides by nuclear magnetic resonance (NMR).

The final aim of such studies is the design of specific, long-lasting analogues and/or selective antagonists of these peptides for assessing the physiological responses associated with each type of receptor. Some aspects of our researches on tachykinin receptors will be reported.

TACHYKININ BINDING SITES

NK1 binding site (Table 2)

A derivatized analogue of SP, ^{125}I-Bolton Hunter SP (^{125}I-BHSP) and crude synaptosomes from rat brain were used, since previous experiments done at the periphery [15, 16] by other groups, and in the brain in our laboratory [5], on closed systems (i.e. intact cells) were successful. Scatchard analysis indicated the existence of a single population of non-interacting sites, for this ligand, with a high-affinity (K_D = 470 pM) and a low density (B_{max} = 15 fmol/mg protein) on this synaptosomal preparation [7]. SP was the most potent tachykinin in inhibiting ^{125}I-BHSP specific binding and the following rank order of potency, for the various tachykinins, was observed: SP ⩾ PHY ⩾ NKA ⩾ ELE > KAS ⩾ NKB (Table 2) [7, 27]. A good relationship was found between the length of SP C-terminal fragments and their ability to compete with ^{125}I-BHSP. SP was the most active and the deletion of one amino acid from the *N*-terminal residue led to a gradual decrease in binding potency [7]. The potency of a wide variety of synthetic SP-related peptides enabled us to estab-

Table 1. Sequences of the most commonly studied tachykinins

Origin	Peptide	Receptor	Sequence
Mammal	Substance P (SP)	NK1	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Amphibian	Physalaemin (PHY)	NK1	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Mammal	Neurokinin A (NKA)	NK2	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Octopod	Eledoisin (ELE)	NK2/NK3	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Mammal	Neurokinin B (NKB)	NK3	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
Amphibian	Kassinin (KAS)	NK3	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2

Table 2. Binding affinities of tachykinins and SP analogues on ^{125}I-BHSP binding on rat brain synaptosomes (NK1 binding site)

Peptides	IC_{50} (nM)	Relative affinity
SP	0.64	100
NKA	140	0.5
NKB	2200	0.03
PHY	2.4	26
ELE	110	0.6
KAS	1100	0.06
SP(2–11)	4	16
SP(6–11)	710	0.09
SP(7–11)	$>10^3$	—
[pGlu^1]SP	6.3	10
[Met^7]SP	2700	0.02
[Nle^{11}]SP	1500	4.3
SP Free acid	$>10^3$	—

Table 3. Binding affinities of tachykinins and SP fragment on ^{125}I-BHELE binding on rat cortical synaptosomes (NK3 binding site)

Peptides	IC_{50} (nM)	Relative affinity
NKB	5.1	100
NKA	100	6
SP	130	4
PHY	56	9
ELE	14	36
KAS	5.9	86
SP(6–11)	39	13

lish the crucial roles of the arginine guanidinium, the aromatic phenylalanine-7, the sulfur atom of methionine and the C-terminal carboxamide [28]. Binding studies made with crude synaptosomal preparations from different brain structures and autoradiography analysis revealed marked differences in the concentrations of ^{125}I-BHSP binding sites (septum > striatum > hyppocampus, hypothalamus > mesencephalon > cerebral cortex) [29]. A binding site presenting the same pharmacological profile has been evidenced by other groups, using either the same ligand [8, 11, 22] or ^{3}H-SP [6, 19], ^{125}I-Tyr^8] SP [17], ^{3}H-PHY [21] or ^{125}I-PHY [15, 18], in the brain or at the periphery (parotides [16, 18], submaxillary glands [20, 22, 23], pancreatic cells [15, 17, 21], small intestine guinea-pig membranes [19], guinea-pig ileum, colon, jejunum, duodenum membrane preparations [23] and guinea-pig urinary bladder [30]). Recently, we have demonstrated the presence of NK1 binding sites on intact cortical glial cells from newborn mice in primary culture [31]. A good correlation was found between the potency of the tachykinins in inhibiting ^{125}I-BHSP specific binding and their ability to stimulate the phosphatidylinositol turnover. At the periphery only the dog carotid artery bioassay allows discrimination of the various tachykinins and tachykinin fragments in the same way, i.e. SP > NKA > NKB and SP > SP(6–11) [25, 26].

NK3 binding site (Table 3)

Preceding the discovery of NKA and NKB, Eledoisin (ELE) a tachykinin isolated from octopod [32] (Table 1), was shown to be more potent than SP in different bioassays [33–35]. Therefore, ^{125}I-Bolton Hunter Eledoisin, ^{125}I-BHELE, was synthesized for studying its binding to rat cortical synaptosomes. A specific binding for this ligand was demonstrated whose binding characteristics and localization greatly differed from the ^{125}I-BHSP specific binding [9, 36]. NKB was the most potent tachykinin, with an IC_{50} in the nanomolar range, in competing with ^{125}I-BHELE, and the following rank order of potency was observed: NKB ≥ KAS > ELE ≥ PHY ≥ NKA > SP and SP(6–11) > SP. Specific binding was highest in the cerebral cortex and hypothalamus, low in the mesencephalon, septum and substantia nigra and absent in the cerebellum. Comparison of these biochemical and localization data with those obtained with ^{3}H-NKB, which has been synthesized according to a new methodology we have developed [37, 38], led us to postulate that NKB was the endogenous ligand interacting with this NK3 binding site [13]. Similar results were obtained on cerebral cortex membrane preparations using either ^{125}I-BHELE [10, 11, 24] or the Bolton Hunter acylated specific agonist: ^{125}I-Bolton Hunter-NH-Senktide (^{125}I-desamino-3-iodotyrosyl)-[Asp^{5,6}, *N*-methyl Phe^8] SP(5–11) [14]. The distribution of binding sites for ^{125}I-BHELE, ^{3}H-NKB, ^{125}I-BH Kassinin and ^{125}I-BH Neurokinin A was approximately identical throughout the rat brain. In the central nervous system, for the time being, no clear cut evidence exists for the coupling of this NK3 type of binding site to any physiological response. However, at the periphery two bioassays enabling study of the properties of the NK3 type receptor have been described, i.e. the neuronal "SP-N receptor" evidenced on guinea-pig ileum by Laufer *et al.* [39] and the rat portal vein preparation [25, 26].

NK2 binding site (Table 4)

Although specific physiological responses for NKA have been demonstrated in the rat central nervous system; a definite demonstration for the existence of NK2 binding sites remains to be established [40, 41]. Indeed, we were unable to reproduce the specific autoradiographic labelling described by Quirion and Dam in the guinea-pig brain [42], as well as the biochemical data obtained by the same authors on a guinea-pig membrane preparation [43].

Table 4. Binding affinities of tachykinins and SP fragment on ^{3}H-NKA specific binding on rat duodenal membranes (NK2 binding site)

Peptides	IC_{50} (nM)	Relative affinity
NKA	7.4	100
NKB	33	22
SP	210	3.5
PHY	940	0.8
ELE	27	27
KAS	25	30
SP(6–11)	4670	4.6
NKA(4–10)	9.5	78

Also, previous autoradiographic studies performed on the rat brain with ^{125}I-Bolton Hunter NKA, ^{125}I-BHNKA, have evidenced a labelling pattern closely related to those observed with ^{125}I-BHELE, ^{3}H-NKB and ^{125}I-BHKAS. The acylation of NKA by I-Bolton Hunter derivative enhanced markedly the affinity of the parent compound for NK3 type binding site [13]. In order to solve the discrepancies observed in the literature, we have synthesized ^{3}H-NKA according to the procedure we have developed for ^{3}H-SP and ^{3}H-NKB [37, 38]. ^{3}H-NKA incubated in the same conditions as ^{125}I-BHELE and ^{125}I-BHNKA did not reveal any specific binding site in the rat brain. However, at the periphery using ^{125}I-BHNKA and different membrane preparations from the gastrointestinal tractus of the rat and guinea-pig [23], a third subclass of specific tachykinin binding sites has been demonstrated. NKA was the preferred ligand for this NK2 binding site with NKA ≥ KAS, ELE, NKB > SP > PHY and SP > SP(6–11). Similar results were obtained on rat duodenum membranes with ^{3}H-NKA [44] and ^{125}I-BHELE [11]. Different bioassays revealed a similar pharmacological profile, i.e. the contractions of rabbit pulmonary artery [25, 26], rat duodenum [25], rat vas deferens and hamster urinary bladder [45]. Furthermore, in this last preparation a correlation was observed between the potency of the various tachykinins and their ability to stimulate the phosphatidylinositol turnover [46].

CONFORMATIONALLY RESTRICTED ANALOGUES OF TACHYKININS

The determination of the bioaffine conformation of SP has been studied by complementary strategies, i.e. structure–activity relationships and spectroscopic analysis of SP and analogues of SP in different solvents.

Conformation of SP in different solvents studied by NMR

The different criteria used to define, by NMR, the three-dimensional structure of a peptide are reported in Fig. 1. The conformation of a peptide is ascertained by the values of the angles Φ and Ψ, Φ being the angle around the C_α–N bond and Ψ the angle around the C_α–CO bond. The angle Φ is directly related to the value of the $^{3}J_{CH-NH}$ coupling constant and can be determined from a Karplus type relationship and from energy calculations. Thus, the spatial orientation of each CH_α and NH can be deduced. The value of Ψ can only be reached by the nuclear Overhauser effect (NOE), which represents a transfer of magnetisation between two protons which are close enough (~ < 4 Å). The nature of the NOE depends on the angle Ψ, i.e. d_{NN} or $d_{\alpha N}$ type. Finally, the last criterion indicative of a structuration is the behaviour of the amide protons. The presence of hydrogen bonds is an index of the existence of a folded structure for the peptide. The influence of the temperature on the chemical shift of an amide proton and the rate of exchange of this proton with a deuterated solvent are directly related to the NH . . . . . CO bonding. Therefore, after the assignment of all the proton signals of the SP spectrum has been made by conventional 2D-NMR spectroscopy, these different criteria (NOE, δ NH/ΔT and exchange rates of the amide protons) were analyzed.

The three-dimensional structure of SP is strongly influenced by its environment. In water, SP presented a complex equilibrium between different conformers and could not be further analyzed. In dimethylsulfoxide, SP adopted a β-pleated sheet structure. In methanol, a predominant conformation was demonstrated [47] (Fig. 2).

The main features of this three-dimensional structure were (1) the flexibility of the N-terminal

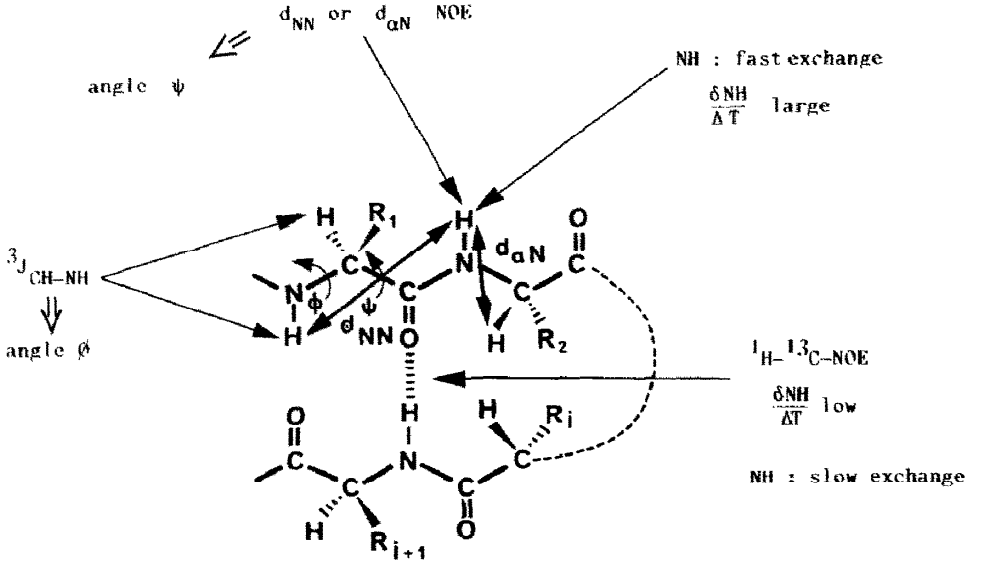

Fig. 1. The different criteria used to determine by NMR the three-dimensional structure of a peptide.

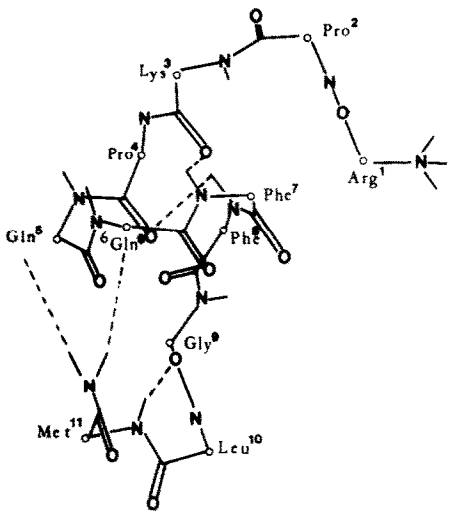

Fig. 2. Preferential conformation of the SP skeleton in methanol [47].

Arg-Pro-Lys, (2) the α-helical structure of the core of SP from Pro^4 to Phe^8 stabilized by two hydrogen bonds involving $NH^7 \rightarrow CO^3$ and $NH^8 \rightarrow CO^4$, and (3) the folding of the C-terminal tripeptide Gly-Leu-Met-NH_2 towards the core of the peptide, allowing the interaction, via hydrogen bonds of the C-terminal carboxamide and the primary amides from both glutamines. Physalaemin, the amphibian tachykinin which, as SP, is a potent NK1 agonist, presented in methanol the same α-helix conformation for the $4 \rightarrow 8$ region stabilized by two hydrogen bonds $NH^7 \rightarrow CO^3$ and $NH^8 \rightarrow CO^4$ and, in that case, by a salt bridge between Asp^3 and Lys^6 [48]. This α-helical structure did not take part in the conformational equilibrium in methanol of NKA, an NK2 agonist with a low potency for NK1 receptor [49]. These results and the binding potencies of SP analogues led us to propose a model for the interaction of SP with NK1 receptor. Assuming that the core of SP is in an α-helicoidal conformation, three major points should be underlined: (1) the NK1 site probably recognized the side of the helix bearing the two side-chains of Phe^7 and Phe^8, (2) the arginine guanidinium interacts with a carboxylate or a phosphate function of the binding site; (3) Gly-Leu-Met-NH_2 is folded towards both glutamines and in the binding site the C-terminal carboxamide should interact with a carboxylate function and the sulfur atom with an electrophile of the binding protein.

In order to ascertain the validity of this model, constrained analogues stabilizing the three-dimensional features observed by NMR have been designed. Two types of approaches have been developed: (1) synthesis of cyclic analogues of the tachykinins; and (2) introduction of conformationally restricted amino acids (*N*-methyl amino acids and proline residues).

Conformationally constrained analogues of Substance P

Two groups of cyclic analogues of SP simulating two different conformational criteria of SP have been synthesized. Analogues of Group I were designed in order to mimic the folding of the C-terminal methionine towards the primary amides of glutamines-5 and -6, this type of U-turn has also been deduced from conformational energy calculations. Analogues of Group II should simulate α-helical structure of the $Pro^4 \rightarrow Phe^8$ moiety. The binding affinities have been evaluated on the three radioreceptor assays, namely ^{125}I-BHSP and ^{125}I-BHELE specific bindings on rat brain synaptosomes and ^{3}H-NKA specific binding on rat duodenal membranes. Their agonistic potencies have been evaluated on the guinea-pig ileum bioassay, an organ which has been shown to possess the three subtypes of tachykinin receptors.

Cyclic analogues of Group I (Table 5)

The disulfide bridge cyclisations involving cysteine and/or homocysteine residues in positions 5 and 9, 5 and 10 or 5 and 11 led to analogues with reduced affinity on NK1 specific assay and no affinity on NK2 and NK3 specific binding assays [50]. These observations were in agreement with results obtained by other groups [51]. The subsequent NMR analysis of two potent NK1 agonists [Met^5]SP and [Met^6]SP enabled us to show that the folding of the C-terminal carboxamide was no longer present in [Met^6]SP in methanolic solution [52]. Therefore, the amide bonds observed between the primary amides are not necessary for full binding potency, since [Met^6]SP is as potent as SP, and the C-terminal tripeptide might undergo a translocation when interacting with NK1 binding site.

Influence of conformational constraints in positions 9, 10 *or* 11 *of SP* (Table 6)

Insights into the bioaffine orientation of Gly-Leu-Met-NH_2 were deduced from systematic modifications performed on these amino acids. [Sar^9]SP and all the L-modified analogues at positions 9, 10 or

Table 5. Binding affinities of cyclic analogues of group I on three specific radioreceptor assays and biological activities on guinea-pig ileum

Peptides	^{125}I-BHSP; [NK1] IC_{50} (nM)	^{3}H-NKA; [NK2] IC_{50} (nM)	^{125}I-BHELE; [NK3] IC_{50} (nM)	GPI EC_{50} (nM)
[$Cys^{5,9}$]SP	920	$>10^{+4}$	$>10^{+4}$	100
[$Hcy^{5,9}$]SP	530	$>10^{+4}$	$\sim 10^{+4}$	100
[D-Cys^5,Hcy^{10}]SP	1300	$>10^{+4}$	$\sim 10^{+4}$	2000
[$Cys^{5,11}$]SP	360	$>10^{+4}$	$>10^{+4}$	400
[$Hcy^{5,11}$]SP	270	$>10^{+4}$	$\sim 10^{+4}$	330

Table 6. Binding affinities of SP analogues modified at positions 9, 10 or 11 on three specific radioreceptor assays and biological activities on guinea-pig ileum

Peptides	^{125}I-BHSP; [NK1] IC_{50} (nM)	^{3}H-NKA; [NK2] IC_{50} (nM)	^{125}I-BHELE; [NK3] IC_{50} (nM)	GPI EC_{50} (nM)
[Ala^{9}]SP	1.3	1200	$>10^{+4}$	2.0
[D-Ala^{9}]SP	550	—	$>10^{+4}$	180
[Sar^{9}]SP	3.1	2900	110	2.4
[Pro^{9}]SP	2.9	$>10^{+4}$	$>10^{+4}$	2.5
[D-Pro^{9}]SP	670	140	$>10^{+4}$	100
[Me-Leu^{10}]SP	7.4	$>10^{+4}$	370	2.0
[Pro^{10}]SP	14	$>10^{+4}$	$>10^{+4}$	2.9
[Me-Met^{11}]SP	5.5	4900	1700	3.5
[Pro^{11}]SP	420	$>10^{+4}$	6000	25

11 presented either a high potency, i.e. [L-Ala^{9}]SP, [Pro^{9}]SP or a reduced affinity (between 5% and 12% the potency of SP), i.e. [Me-Leu^{10}]SP, [Pro^{10}]SP and [Me-Met^{11}]SP. Amongst these analogues [Pro^{11}]SP was the only poor inhibitor of specific ^{125}I-BHSP binding. Interestingly, among these analogues two of them, i.e. [Pro^{9}]SP and [Pro^{10}]SP could be considered as specific NK1 agonists. From the conformational point of view, these constrained *N*-methylated and prolyl peptides favoured a more or less extended conformation for the bioaffine conformation of the C-terminal tripeptide (i.e. a polyl-prolyl type structure) [53, 54].

Cyclic analogues of Group II (Table 7)

The cyclisations involving the residues 3 → 6, 4 → 7 and 5 → 8 were considered in order to constrain and hence stabilize the α-helical postulated conformation of residues 4 to 8 of SP and PHY, and mimic the salt bridge between Asp^{3} and Lys^{6} in the sequence of PHY. The cyclisation of SP between the positions 3 and 6 was the only one leading to potent agonists of tachykinins, the L-Cys^{3} → L-Cys^{6} analogue being more potent than the D-Cys^{3} → L-Cys^{6} peptide. [Cys^{3,6}]SP was almost as potent as SP in inhibiting specific ^{125}I-BHSP binding on rat brain synaptosomes and as active as NKB in inhibiting ^{125}I-BHELE specific binding, whereas SP is a very weak substrate for NK3 binding site. However, [Cys^{3,6}]SP was a poor agonist for NK2 binding site, with an IC_{50} value in the micromolar range [50]. In order to increase the selectivity of the cyclic analogue, a few modifications were performed in position 8 (in which the nature of the amino acid is distinct from one tachykinin to the other) and in positions 9 and 10 (which in the sequence of SP conferred a NK1 selectivity to the corresponding analogues). The introduction of a tyrosine in position 8, as in PHY, yielded the most potent compound, on both NK1 and NK3 binding sites, [Cys^{3,6}, Tyr^{8}]SP

Table 7. Binding affinities of cyclic analogues of group II on three specific radioreceptor assays and biological activities on guinea-pig ileum

Peptides	^{125}I-BHSP; [NK1] IC_{50} (nM)	^{3}H-NKA; [NK2] IC_{50} (nM)	^{125}I-BHELE; [NK3] IC_{50} (nM)	GPI EC_{50} (nM)
[D-Cys^{5},Cys^{8}]SP	200	$>10^{+4}$	1400	70
[D-Cys^{4},Cys^{7}]SP	1700	$>10^{+4}$	$>10^{+4}$	100
[D-Cys^{3},Cys^{6}]SP	20	2900	72	4
[Cys^{3,6}]SP	1.3	5200	6.4	2.5
[Cys^{3,6},Tyr^{8}]SP	0.42	5000	3	2.2
[Cys^{3,6},Val^{8}]SP	1.6	63	1.6	2.8
[Cys^{3,6},Tyr^{8},Ala^{9}]SP	0.67	4500	41	1.6
[Cys^{3,6},Tyr^{8},Pro^{9}]SP	3.1	$>10^{+4}$	970	2.1
[Cys^{3,6},Tyr^{8},Pro^{10}]SP	5.1	9200	1600	2.3

Table 8. Binding affinities of conformationally constrained analogues of tachykinins on three radioreceptor assays and biological activities on guinea-pig ileum

Peptides	^{125}I-BHSP; [NK1] IC_{50} (nM)	^{3}H-NKA; [NK2] IC_{50} (nM)	^{125}I-BHELE; [NK3] IC_{50} (nM)	GPI EC_{50} (nM)
[Cys^{2,5}]NKB	3400	750	6.9	2.1
[Me-Phe^{8}]SP	440	1310	18	2.3
[Me-Val^{7}]NKB	9600	440	1.5	2.9
[Pro^{7}]NKB	4200	$>10^{+4}$	13	3.1

being active in the nanomolar range on both sites. The replacement of the aromatic residue by the aliphatic valine, present in the sequence of NKA and NKB, provided the less selective compound [55]. [Cys^{3,6}, Val^{8}]SP was active in the nanomolar range on NK1 and NK3 specific binding sites and its IC_{50} value on NK2 binding site was 63 nM. Selective analogues for the NK1 binding site were obtained after modifications at positions 9 and 10; [Cys^{3,6}, Tyr^{8}, Pro^{9}]SP and [Cys^{3,6}, Tyr^{8}, Pro^{9}]SP being potent in the nanomolar range on NK1 binding site and in the micromolar range on NK2 and NK3 binding sites (Table 7) [54].

The NMR studies of the potent NK1 and NK3 agonist [Cys^{3,6}, Tyr^{8}]SP, in methanol, revealed the presence of a β-turn III which stabilized an α-helix for the $4 \rightarrow 8$ sequence, via the formation of three hydrogen bonds starting from NH Cys^{6}, NH Phe^{7} and NH Tyr^{8}. The α-helical structure observed for SP and PHY has been rigidified, as expected, since in contrast to SP, two of these hydrogen bonds were still observed in dimethylsulfoxide, i.e. NH Cys^{6} and NH Phe^{7}. In water, a β-turn structure inside the 14-membered disulfide ring could still be postulated. The three-dimensional structure of the C-terminal tripeptide, which was at the origin of the selectivity, was strongly affected by changing the hydrophobicity of the medium. Thus, a few conclusions may be drawn: (1) the fitting of the aromatic side-chain of the phenylalanine-7 residue should be similar in NK1 and NK3 binding sites. (2) the C-terminal tripeptide must adopt in [Cys^{3,6}, Tyr^{8}]SP the conformations fitting into both the NK1 and NK3 binding sites. The conformational constraint introduced in the core of the tachykinin must therefore influence the degree of flexibility of Gly-Leu-Met-NH_2, since SP and NKB are relatively specific ligands for the NK1 and NK3 sites, respectively. The different orientations of the C-terminal tripeptide should be influenced by the particular orientation of the amino acid in position 8 [56]. Indeed, *N*-methylation of the residue 8, as in [*N*-MePhe^{8}]SP was associated with a drastic loss in potency for the NK1 binding site and a concomitant increase in the NK3 binding potency [57].

Conformationally constrained analogues of Neurokinin B

From the results previously observed, two strategies have been developed: firstly, cyclisation of NKB, secondly, introduction of conformationally constrained amino acids (*N*-methyl amino acids and proline residues) into the sequence of NKB.

Cyclic analogue of NKB (Table 8)

The potency of $3 \rightarrow 6$ disulfide bridged analogues of SP suggested that the NK1 and NK3 binding sites might recognize similar three-dimensional structures of SP and NKB, the selectivities being ensured by specific interactions between the different side-chains of the tachykinins and the binding sites. Indeed, the reintroduction of the amino acids belonging to the sequence of NKB yielded a potent and selective NK3 agonist. [Cys^{2,5}]NKB inhibited the specific ^{125}I-BHELE binding on rat cortical synaptosomes with an IC_{50} value in the nanomolar range, whereas micromolar concentration was needed to

Table 9. Summary of the different features of the three tachykinin binding sites

Characteristics	NK1	NK2	NK3
Radioligand used in this study	^{125}I-BHSP or ^{3}H-SP	^{3}H-NKA or ^{125}I-BHNKA	^{125}I-BHELE or ^{3}H-NKB
Tachykinins potencies	SP ⩾ PHY ⩾ NKA > ELE >KAS ⩾ NKB	NKA ⩾ KAS, ELE, NKB > SP > PHY	NKB ⩾ KAS > ELE ⩾ PHY > NKA ⩾ SP
	SP > SP(6–11)	SP > SP(6–11)	SP(6–11) > SP
Specific agonists			
This study	[Pro^{9}]SP, [Pro^{10}]SP		[Cys^{2,5}]NKB
	[Cys^{3,6},Tyr^{8},Pro^{9}]SP		[Pro^{7}]NKB
	[Cys^{3,6},Tyr^{8},Pro^{10}]SP		
Other groups	[pGlu^{6},Pro^{9}]SP(6–11)*	NKA(4–10)†	[pGlu^{6},(R)Gly[ANC-2]]SP(6–11) succinyl [Asp^{6},MePhe^{8}]SP(6–11) [MePhe^{7}]NKB(4–10)‡

* SP specific agonist on NK1 specific bioassay. When tested on NK1 radioreceptor assay with ^{125}I-BHSP as a ligand the IC_{50} value is in the micromolar range and no selectivity was observed [61, 24].
† NK2 specific agonist on NK2 specific bioassay and ^{3}H-NKA specific binding [62].
‡ NK3 specific agonist on NK3 specific bioassay, no binding data available [58].

inhibit ^{125}I-BHSP specific binding. Moreover, in contrast to NKB, [Cys^{2,5}]NKB is a very weak agonist for the NK2 binding site [55].

Influence of conformationally constrained residue at position 7 of NKB (Table 9)

Since *N*-methylation of phenylalanine-8 in the sequence of SP led to a potent NKB agonist, the influence of such a modification in the sequence of NKB was analyzed, as well as the introduction of a proline residue in this position. These conformational constraints yielded potent NK3 agonists, [Me-Val^7]NKB and [Pro^7]NKB. [MeVal^7]NKB was five times more potent than NKB in inhibiting specific ^{125}I-BHELE binding on rat cortical synaptosomes and ten times less active in inhibiting specific ^{3}H-NKA binding on rat duodenal membranes. [Pro^7]NKB constituted a selective NK3 agonist, completely devoid of any potency on NK2 radioreceptor assay. The influence of *N*-methylation has also been observed by Laufer *et al.* with shorter fragments of SP, the modified SP(6–11) analogue, Senktide, i.e. succinyl-Asp-Phe-MePhe-Gly-Leu-Met-NH_2 being a potent and selective NK3 agonist [14]. Recently, Regoli *et al.* have shown that Asp-Phe-MePhe-Gly-Leu-Met-NH_2 was also a potent and selective NK3 agonist [58].

The major findings of this study are the design of linear and cyclic specific analogues of NK1 and NK3 specific binding sites. A summary of these results are reported in Table 9. These compounds are [Pro^9]SP, [Pro^{10}]SP and the cyclic peptides [Cys^{3,6}, Tyr^8, Pro^9]SP and [Cys^{3,6}, Tyr^8, Pro^{10}]SP for the NK1 binding site and [Pro^7]NKB, [Cys^{2,5}]NKB for the NK3 binding site. These peptides should be particularly useful in designing the characteristics of the pharmacological profiles of NK1, NK2 and NK3 receptors since, on the one hand cyclic analogues of linear peptides should be more resistant to proteolytic degradation and on the other hand the angiotensin-converting enzyme type degradation of SP [59] must be abolished by proline residue in positions 9 or 10 while the cleavage by the endopeptidase EC-24.11 (also named "enkephalinase") of NKB must be inhibited in the case of [Pro^7]NKB [60].

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