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TRANSGLUTAMINASE-SYNTHESIZED SPERMINE DERIVATIVE OF SUBSTANCE P RECOGNIZES RAT PORTAL VEIN NEUROKININ-3 RECEPTORS

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Summary

The effects of the transglutaminase-sinthesized polyamine derivatives of Substance P (SP) have been further characterized by their ability to contract *in vitro* the rat portal vein strip (RPV), a pharmacological preparation particularly rich in NK-3 receptors. The effects of selective agonists of NK-1, NK-2 and NK-3 receptors [Sar^9,$Met(O_2)^{11}$]SP, ß-Ala^8 NKA(4-10), and senktide respectively, were also evaluated by measuring RPV concentration-response curves. Peptide [GR-82334 (NK-1) and MEN-10,376 (NK-2)] and nonpeptide [WIN 51,708 (NK-1) and SR 142801 (NK-3)] NK receptor antagonists were used to confirm the participation of the different NK receptors to contractile response. Our results demonstrated that the spermine derivative of SP (Spm-SP), previously shown to be unable to recognize NK-1 and NK-2 receptors in some bioassays, contracts RPV (EC_{50} = 588 nM) better than the native neuropeptide (EC_{50} = 1120 nM). A pretreatment with thiorphan, an inhibitor of neutral endopeptidases, significantly reduced such a difference. While this inhibitor shifts the SP concentration-response curves to the left (EC_{50} = 720 nM) the action of Spm-SP and [Sar^9,$Met(O_2)^{11}$]SP were completely thiorphan-resistant. In the absence of thiorphan we found the following rank order of potency: senktide>>ß-Ala^8 NKA(4-10)>[Sar^9,$Met(O_2)^{11}$]SP=Spm-SP>SP. Among the mentioned NK receptor antagonists, only the selective NK-3 receptor antagonist, SR 142801, shifted to the right Spm-SP and [Sar^9,$Met(O_2)^{11}$]SP concentration-response curve, showing pK_B values of 5.84 and 5.88, respectively. Therefore, the reported results suggest that the introduction of a Spm moiety into the SP alters the parent peptide molecule by increasing its affinity for NK-3 receptors and/or by preventing its degradation by some proteolytic enzymes.

Key Words: **substance P, portal vein, γ-(glutamyl^5)spermine derivative of SP, NK-3 receptors**

Substance P (SP), first isolated from horse tissues by Von Euler and Gaddum (1), is an undecapeptide of the tachykinin family. Members of this numerous family were isolated from non-mammalian species (e. g. physalaemin, eledoisin and kassinin) (2) whereas SP, neurokinin A (NK A) and neurokinin B (NK B) are the three tachykinins so far evidentiated in mammalian tissue, and called neurokinins (NKs). NKs exert different biological activities (enclosed

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vasodilatation, smooth muscle contraction and gland secretion) which, in most cases, depend upon activation of specific receptors expressed on the membrane of target cells (3). Three receptor types, NK-1, NK-2 and NK-3, have been identified by molecular cloning and sequence analysis (4). NK-1 shows high affinity for SP, NK-2 for NK-A, and NK-3 for NK-B, although it must be remembered that the naturally occurring NKs have high affinity for, and act as full agonists on, the three receptor types and, therefore, show poor selectivity (5). The recognition of biological importance of NKs in regulating several functions has stimulated the interest to investigate the possibility of modulating the action of these peptides not only in physiological but also in pathophysiological conditions, through the development of either specific receptor agonists and antagonists or metabolically stable analogues. The selectivity for each receptor has been markedly improved with agonists obtained by structural modifications of the neurokinin -COOH terminal sequence (6). We have previously demonstrated that SP is an effective substrate *in vitro* of the enzyme transglutaminase (E.C.,2.3.2.13; TGase) (7), only the first of the two glutamine residues of the peptide (Gln^5) being able to covalently link amino donor substrates, such as 1,3-diaminopropane (Dap), spermine (Spm), spermidine (Spd), or 5-mono-dansylcadaverine (Dns). While Dap-, Spd- and Dns- derivatives of SP showed any significant difference as compared to native SP in various biological assays, the γ-(glutamyl^5)Spm derivative of SP (Spm-SP) was found to be completely ineffective in producing *in vitro* some biological effects typical of the parent peptide (i.e. guinea pig ileum and rat stomach and duodenum contractions) (8). On the other hand Spm-SP, as well as SP, was able to elicit rabbit aorta relaxation, to decrease rat arterial blood pressure, and to inhibit collagen-induced platelet aggregation (9). These analyzed Spm-SP effects are known to be mediated mostly by NK-1 and NK-2 receptors; therefore the purpose of this study was to test the capability of Spm-SP to interact with NK-3 receptors. We chose the rat portal vein strip (RPV); it remains the sole isolated preparation containing only NK-3 receptors, thus useful to elucidate whether the Spm attachment to SP also influenced its biological activities related to NK-3 receptor activation. In the same experimental conditions, we also investigated the behaviour of the other SP-derivatives and we tested agonists and antagonists of NKs in order to functionally characterize Spm-SP effects.

Materials and Methods

Chemicals. SP acetate, TGase purified from guinea pig liver, Dap dihydrochloride, Spm tetrahydrochloride, Dns, Spd trihydrochloride, acetylcholine chloride, bradykinin, U 46619 and thiorphan were supplied by Sigma Chemical Co., St. Louis, MO, USA. [Sar^9 Met $(O_2)^{11}$]SP, β Ala^8 NKA (4-10), senktide, GR 82334, WIN 51708 and MEN 10376 were supplied by Research Biochemicals International, Natick, MA, USA. SR 142801 was kindly provided by Dr. Xavier Emonds-Alt, Sanofi Recherche, Montpellier, France.

TGase-catalyzed synthesis and purification of SP derivatives. Each preparation of γ-(glutamyl^5) Dap, γ-(glutamyl^5) Spd, γ-(glutamyl^5) Spm, and γ-(glutamyl^5) Dns of SP (Dap-SP, Spd-SP, Spm-SP and Dns-SP, respectively) was obtained by incubating at 37°C 100 μg of the native peptide SP with 2 μg of TGase in 0.1 ml 125 mM Tris-HCl buffer (pH 8.0) containing 2.5 mM $CaCl_2$, 10 mM dithiothreitol, and 0.2 M Dap, Spd, Spm or Dns (7-9). After 6-h incubation, 2 μ g of TGase were added, and the reaction was carried on for additional 12 h. A control sample incubated without amines was simultaneously run. At the end of the incubation the reaction mixtures were centrifuged at 10,000 rpm for 10 min, and the supernatants were loaded individually onto a μBondapak C18 column (3.9 x 300 mm; HPLC Beckman System Gold) after addition of 10 μl of acetonitrile containing 1% trifluoroacetic acid. The column was

equilibrated with 0.1 % trifluoroacetic acid/acetonitrile (90:100, vol/vol), and the peptides were eluted by a linear gradient of acetonitrile from 10 to 50% in 30 min (flow rate, 1.0 ml/min). Fractions of 0.2 ml were collected, and the absorbance peaks, eluted at 19.5 (Spm-SP), 20.1 (Spd-SP), 20.9 (Dap-SP), 23.0 (SP), or 27.0 min (Dns-SP), were pooled and evaporated to dryness. The dry samples were dissolved in 100 µl of distilled water, and , after identification of the adducts by a previously reported fast atom bombardment mass spectrometric technique (7-9), samples were stored in aliquots at -20°C until used for biological activity tests.

RPV experimental preparation. Male Wistar rats (300-350g) were stunned and killed by cervical dislocation. After insertion of a PE-50 polyethylene catheter into its lumen, the portal vein was excised and rolled around the catheter on damp filter paper to remove the endothelial cells. It was then placed in Krebs' solution (millimolar composition: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11; pH 7.4) and freed of adhering tissues. The vessel was cut longitudinally to yield a strip approximately 1 cm long. Cotton threads were tied to each strip end, and the strips were placed vertically, by means of stainless two steel wires, into a double-jacketed organ bath containing 10 ml Krebs' solution at 37 °C bubbled with 5% CO_2 in oxygen and connected to an isometric transducer (NARCO F60) coupled to a physiograph (LINSEIS 2046). The strips underwent a resting tension of 0.5 g and were allowed to equilibrate for at least 1 hour before undergoing any pharmacological treatment, with frequent renewal of the bathing medium.

RPV experimental procedure. After the equilibration period and before contraction with agonists, RPV were initially challenged with 80 mM KCl as a standard stimulus. Complete removal of functional endothelium was confirmed by observing no dilation in response to bradykinin (100 nM) in preconstricted (U 46619 100 nM) vessels. After washout, replacement with normal medium, and complete relaxation (90 min), SP, SP-derivatives (Dap-SP, Spm-SP, Dns-SP, Spd-SP), [Sar^9 Met $(O_2)^{11}$]SP (NK-1 selective agonist), β Ala^8 NKA (4-10) (NK-2 selective agonist) and senktide (NK-3 selective agonist) concentration-response curves (0.3 nM-10 µM) were obtained. Concentration-response curves to Spm-SP and [Sar^9 Met $(O_2)^{11}$]SP were also performed in presence of thiorphan (1 µM - 30 min before). Experiments were performed in a non cumulative way by consecutive injections of each stimulant at intervals of 20-30 min, in order to avoid desensitization of the tissues. In experiments examining the effects of 1 to 10 µM GR 82334 and WIN 51708, respectively NK-1 selective peptide and non-peptide antagonist, MEN 10376, NK-2 selective peptide antagonist and SR 142801, NK-3 selective non-peptide antagonist, tissues were exposed to the compounds for 30 min before addition of contractile agonists, with the only exception of SR 142801 which was left in the organ bath for 120 min as suggested by Emonds-Alt (10). All antagonists were dissolved in Krebs' solution, excluded SR 142801. It was solubilized in dimethylsulfoxide and further diluted in Krebs' solution (vehicle). Only one agonist concentration-response curve was generated for each tissue.

Data analysis. Results are expressed as the mean ± S.E.M. Agonist potency was calculated on the basis of data from individual vessels and is expressed as EC_{50} (concentration of the agonist needed to produce 50% of the maximal response). The concentration ratio (CR) is the ratio of the EC_{50} values in the presence or absence of the antagonist. pK_B values (i.e. -log dissociation constant) was calculated as follows: log [concentration of antagonist]/(CR-1) (11). $P < 0.05$ was taken to reflect a significant difference (unpaired Student's *t* test).

Results

Contractile effects of SP derivatives and NK receptor agonists on RPV. SP assayed at doses comprised between 0.3 nM and 10 μM evoked a concentration-dependent increase in the force of the RPV spontaneous phasic contractions. Large phasic contractions of low frequency persisted for 4 - 5 min upon washout of SP high concentrations (1-10 μM). The SP-induced increase in tension was calculated as the difference between basal amplitude of the spontaneous rhythmic activity and agonist-stimulated amplitude (EC_{50} values = 1120 nM). Concentration-response curves of Dap-SP, Spd-SP and Dns-SP were not significantly different from that obtained with the parent peptide. Only Spm-SP showed a greater affinity for rat portal vein NK-3 receptors with an EC_{50} value of 588 nM (Tab. 1). A pretreatment (30 min before) with thiorphan significantly increased the effects of SP but not that of Spm-SP and [Sar^9 Met $(O_2)^{11}$]SP. This inhibitor shifted the SP concentration-response curve to the left showing an EC_{50} value of 720 nM (Fig. 1). Senktide, an NK-3 receptor selective agonist, induced concentration-dependent contractile responses of RPV with an EC_{50} value of 17 nM. β -Ala^8 NKA (4-10) and [Sar^9 Met $(O_2)^{11}$]SP (NK-2 and NK-1 receptor selective agonists respectively) showed much lesser affinity for NK-3 receptors (EC_{50} value of β-Ala^8 NKA (4-10) = 131 nM; EC_{50} value of [Sar^9 Met $(O_2)^{11}$]SP = 665 nM) (Fig. 2).

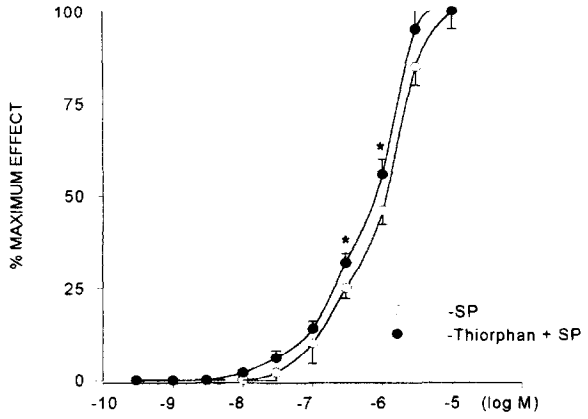

Fig. 1

Concentration-response curve for substance P in rat portal vein strips, in the absence (O) and after a 30 min incubation with thiorphan 1 μM (●). * P <0.05.

Table 1

Contractile potency (expressed as EC_{50} values) of substance P (SP) and its derivatives [1,3-diaminopropane SP (Dap-SP), spermidine SP (Spd-SP), spermine SP (Spm-SP) and 5-mono-dansylcadaverine SP (Dns-SP)] on isolated rat portal vein.

DRUGS	EC_{50} (μM)
SP	**1.120 ± 0.09**
Dap-SP	**1.230 ± 0.08**
Spd-SP	**1.320 ± 0.15**
Spm-SP	**0.588 ± 0.04***
Dns-SP	**1.160 ± 0.12**

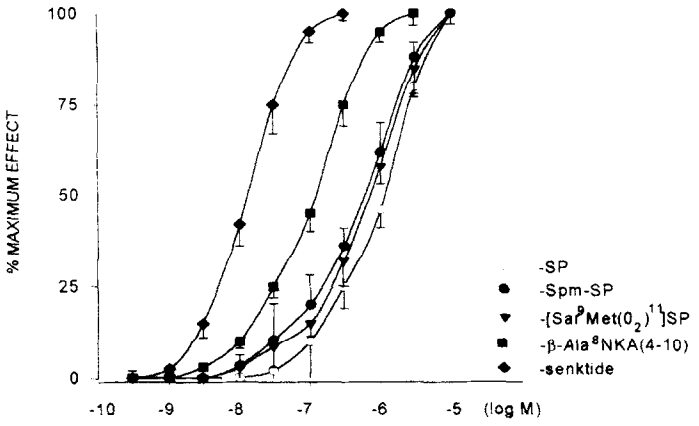

Fig. 2

Contractile effects of substance P (SP; O), γ-glutamyl^5-spermine SP (Spm-SP, ●), [Sar^{9}Met(O$_2$)^{11}] SP (▼), β-Ala^8 NKA (4-10) (■) and senktide (◆) on rat portal vein strips. Each value is the mean ± S.E.M. of at least 5 experiments.

Table 2

Comparison of the antagonist activity (expressed as pK_B values) of GR 82334, MEN 10376 and SR 142801 on the rat portal vein contractile response induced by the NK receptor agonists and γ-(glutamyl^5)spermine derivative of SP (Spm-SP).

AGONIST	pK_B values		
	GR 82334	**MEN 10376**	**SR 142801**
[Sar^9,Met(O$_2$)^{11}]SP	inactive at 3 μM	-	5.88 (1 μM)
β-Ala^{8}NKA (4-10)	-	inactive at 3 μM	-
Senktide	-	-	7.18 (1 μM)
Spm-SP	inactive at 3 μM	inactive at 3 μM	5.84 (1μM)

Effects of NK receptor selective antagonists on the RPV contractile response induced by NK receptor agonists and Spm-SP. RPV contractile response to both Spm-SP and senktide was analyzed in the presence of GR 82334 and WIN 51708 (NK-1 receptor selective antagonists), MEN 10376 (NK-2 receptor selective antagonist), or SR 142801 (NK-3 receptor selective antagonist). WIN 51708, a non peptide product reported as an NK-1 selective antagonist (12) slightly antagonized Spm-SP, β-Ala^8 NKA (4-10) and senktide-induced contraction of rat portal vein. Our findings, also reported by others (13), lead us to believe that WIN 51708 interacts with NK receptors at least in a non selective manner and, therefore we omited its results (Tab. 2). 10 μM MEN 10376 and GR 82334 reduced, even if not significantly, the effects of all tested agonists and impaired smooth muscle contractility (data not shown). Moreover, only SR 142801 was able to significantly shift to the right either Spm-SP and senktide or [Sar^9 Met (O$_2$)^{11}]SP concentration response curves (Fig. 3 and 4). The pK_B values were 5.84 for Spm-SP, 5.88 for [Sar^9 Met (O$_2$)^{11}]SP and 7.18 for senktide (Tab. 2).

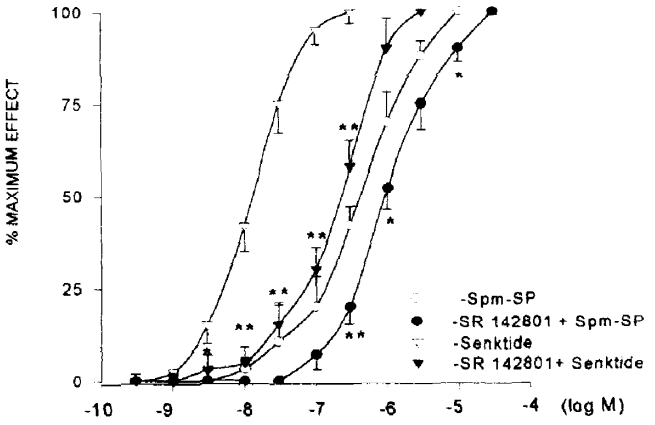

Fig. 3

Effect of SR 142801 (1 μM) on the concentration-response curves to γ-glutamyl^5-spermine SP (Spm-SP; O) and senktide (∇)-induced contraction of rat portal vein strips. Strips were incubated either with vehicle (hollow symbols) or with vehicle + SR 142081 (filled symbols). Data are expressed as percent of maximal tension (mean ± S.E.M. of at least 4 experiments) induced by agonist alone. * $P < 0.05$, ** $P < 0.01$.

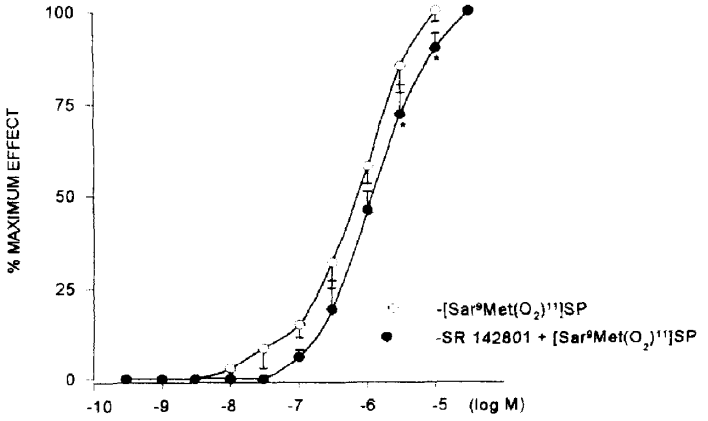

Fig. 4

Concentration-response curve for [Sar^{9}Met$(O_2)^{11}$] SP in rat portal vein strips, in the absence (O) and after a 120 min incubation with SR 142801 1 μM (●). * $P < 0.05$.

Discussion

Longitudinal smooth muscle of the rat portal vein is known to be sensitive to neurokinin B (14) which produces a selective venoconstriction by activating specific NK-3 receptors (15). NK-3 receptors widely occur in the central nervous system while their expression in the peripheral tissues is much more limited; the guinea pig ileum (16) and rat portal vein (14) are notable exception in this respect. However, since no potent and selective NK-3 antagonists have been decribed for quite a long time, these findings remained largely speculative. The recent studies on SR 142801, a potent and selective NK-3 receptor antagonist (10), prompted us to ascertain if the various γ-(glutamyl^5)amino derivatives of SP, synthesized *in vitro* in the presence of TGase, could produce a contractile response of the rat portal vein and if SR 142801 could be able to antagonize this effect. Our study demonstrated that Spm-SP recognizes NK-3 receptors occurring on the rat portal vein, showing a greater potency than native SP and the other SP-derivatives tested. Among the three neurokinin-selective agonists assayed, Spm-SP effectiveness resulted similar to that exhibited by [Sar^9 Met $(O_2)^{11}$]SP. It is noteworthy that this known NK-1 selective agonist (17) is able to contract rat portal vein. Previous studies (13, 18) reported [Sar^9 Met $(O_2)^{11}$]SP to be inactive in RPV preparation. In these papers it is not clear if the endothelium layer of rat portal vein was removed or not. In the latter case part of contractile response elicited by [Sar^9 Met $(O_2)^{11}$]SP could be masked by activation of endothelial NK-1 receptors, responsible for smooth muscle relaxation. Furthermore, only SR 142081 was able to antagonize Spm-SP as well as [Sar^9 Met $(O_2)^{11}$]SP mediated rat portal vein contractions, yielding a pK_B value of 5.84 and 5.88, respectively, whereas both NK-1 and NK-2 receptor antagonists (GR 82334 and MEN 10376) resulted completely ineffective.

The insertion in the SP molecule of a large size hydrophilic compound, like Spm, at the Gln^5 level alters the affinity of the neuropeptide for its receptors either because the hydrophobic region of the middle part of SP (4-8 sequence) is directly involved in the recognition (19 - 21) of the receptor binding sites, or because the TGase-catalyzed structural modification of SP could affect or prevent the degradation of the neuropeptide by some proteolytic enzymes (Fig. 5).

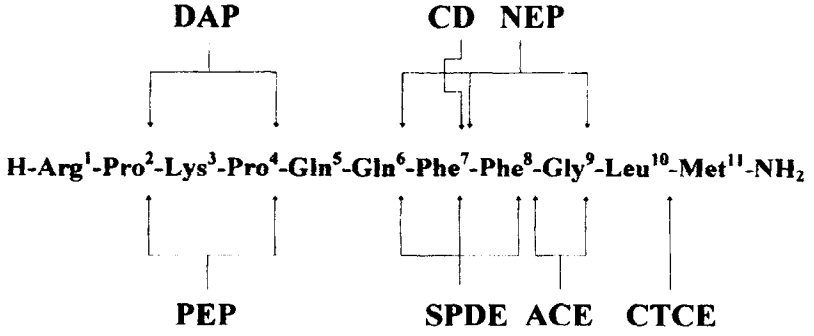

Fig. 5

Sites of action of substance P degrading peptidases. ACE = angiotensin converting enzyme; CD = cathepsin D; CTCE = C-terminal cleaving enzyme; DAP = dipeptidyl aminopeptidase; NEP = neutral endopeptidase; PEP = propyl endopeptidase; SPDE = substance P degrading enzyme (modified from Mussap et al., 1993).

The first hypothesis is supported by previous observation (8) indicating that when the gamma carboxamide group of the glutamine^5 residue of SP was covalently linked to the primary amino group of the polyamine Spm various biological activities of the neuropeptide *in vitro* (contractile effect on isolated guinea pig ileum and rat stomach strips and duodenum) and *in vivo* (edema induction in the rat hind limb) dramatically change. Moreover, it has been shown that TGase-catalyzed structural modification of SP prevents the binding of the neuropeptide to its receptors in the tested systems since Spm-SP was also unable to antagonize both spasmogenic and inflammatory effects of the naturally occurring peptide (8). The second hypothesis is based on the knowledge that SP is a substrate of different proteolytic enzymes *in vitro* (Fig. 5) (22). However, the cleavage reported patterns do not necessarily reflect the situation *in vivo*, because not only the substrate specificity but also the tissue distribution of the single protease should be taken into account. Since neurokinins are charged hydrophilic peptides, confined to the extracellular space, it is conceivable that they might be target of some specific membrane-bound proteases (i.e. the neutral endopeptidase, or the angiotensin converting enzyme) either in the isolated organs assayed *in vitro* or in the *in vivo* biological systems (23). In fact, thiorphan, a well known inhibitor of neutral endopeptidases, resulted able to potentiate and to prolong SP effects. In contrast Spm-SP and [Sar^9 Met $(O_2)^{11}$]SP potency resulted completely unmodified by thiorphan. On the other hand, the rapid degradation of SP has been reported (24) and, the biological effects of the neuropeptide is known to be persistent suggesting that its activity may be due, at least in part, to some bioactive metabolites (25). Therefore, possible explanations for our results could assume either that the decay of Spm-SP develops more slowly or the degradation products of Spm-SP possess a different capability to link NK-3 receptors than the SP metabolites.

In conclusion, the present findings suggest that the introduction of Spm mojety at the glutamine^5 level of SP gives rise to an analogue of SP which a) possess a longer biological life, b) displays a selectivity for NK receptors different from that of the parent peptide and c) originates bioactive metabolites having receptorial activity distinct from that produced from the native SP.

Acknowledgments

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