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# Septide: an Agonist for the NK1 Receptor Acting at a Site Distinct from Substance P

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**SUMMARY**

The hexapeptide [*p*Glu$^6$,Pro$^9$]substance P $(SP)_{6-11}$, septide, has been shown to be an agonist as potent as SP in eliciting smooth muscle contraction in several *in vitro* preparations, while being a poor competitor of labeled SP binding. These results, as well as other pharmacological data, have suggested the existence of either a specific septide receptor or a septide site on the neurokinin (NK)1 receptor distinct from that for SP. We have used rat recombinant NK1 receptor expressed in COS-1 cells to address this issue. Both functional (agonist-induced inositol phosphate accumulation) and radioligand binding studies were conducted on transiently transfected cells. SP and septide elicited similar maximal increases (4–6-fold) in inositol phosphate levels in transfected cells, with $EC_{50}$ values of 0.05 ± 0.02 nM for SP and 5 ± 2 nM for septide. No additivity of the maximal responses to the two agonists was observed, and neither agonist evoked any response in sham-transfected cells. RP 67580 was a competitive inhibitor of SP responses, with an inhibition constant ($K_B$) of 13 ± 2 nM, in agreement with displacement studies of [$^3$H]SP binding to membranes and intact transfected cells ($K_i$ values of 10 ± 4 nM, and 1.16 ± 0.06 nM, respectively). In comparison, septide responses were inhibited by RP 67580 in an uncompetitive fashion, with an apparent $K_B$* value of 1.5 ± 0.2 nM. Septide was a weak competitor of [$^3$H]SP binding, with dissociation constants ($K_i$) of 2.9 ± 0.6 μM and 3.7 ± 0.9 μM for membranes and intact transfected cells, respectively. Similarly, septide at concentrations up to 10 μM did not affect [$^3$H]RP 67580 binding. In conclusion, we have demonstrated that septide is a potent functional agonist of the NK1 receptor but it seems to act at a specific subsite different from that for SP. Although not ruling out the existence of selective septide receptors in some tissues, these results could explain some of the discrepancies with regard to the pharmacological properties of septide. Furthermore, a specific septide site on the NK1 receptor could represent an original pharmacological target.

The three mammalian tachykinins, SP, NKA, and NKB, mediate their biological effects by interaction with three different receptors, $NK_1$, $NK_2$, and $NK_3$, which have been recently cloned and characterized (for review, see Ref. 1). Numerous tachykinin analogs have been synthesized in the search for ligands more selective than the endogenous peptides for each of the receptors. Thus, the short carboxyl-terminal SP analog septide ([*p*Glu$^6$,Pro$^9$]$SP_{6-11}$) has been described as a potent agonist of the NK1 receptor in several isolated organ preparations, as well as *in vivo*. In the guinea pig ileum, the initial system used to characterize its properties, septide is as potent as SP in eliciting smooth muscle contraction, with an $EC_{50}$ value of 2 nM (2). Similarly, septide and SP are equipotent in inhibiting $K^+$ release from rat parotid gland slices (3) and in inducing plasma extravasation (4) or muscle contraction (5) in rat urinary bladder. *In vivo*, septide is approximately 10 times more potent than SP in stimulating cutaneous plasma extravasation in the rat (6), stimulating bronchoconstriction in the guinea pig (7), and eliciting scratching/grooming behavior in the mouse after intracerebroventricular injection (8). In these latter studies, different degradation properties *in vivo* could account for the higher activity of septide.

This high potency of septide contrasts sharply with its lack of affinity for the NK1 receptor in binding studies. Even if large differences were observed between central and peripheral organs, as well as between rat and guinea pig tissues, in an increasing order of affinity (9–11), septide always displayed a 400 (guinea pig ileum) to >1000 (rat cortex) times lesser affinity than SP for the $NK_1$ receptor. The atypical properties of septide were further underlined when it was shown to be 50 times less active than SP in eliciting *myo*-[$^3$H]inositol uptake in the rat parotid gland (12) (although it should be noted that, in the same preparation, septide was almost as potent as SP in eliciting $K^+$ release, see above). Such findings have led some authors to postulate the existence of a new septide-selective tachykinin receptor subtype in the guinea pig ileum (12), although inhibition of both septide and SP responses by nonpeptide $NK_1$ antagonists was later demonstrated in this prepara-

**ABBREVIATIONS:** SP, substance P; NK, neurokinin; IP, inositol phosphate; CAT, chloramphenicol acetyl transferase; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tion (13, 14). The proposal of the existence of a new septide-selective tachykinin receptor is in keeping with similar demonstrations of $NK_2$ receptor subtypes, $NK_{2A}$ and $NK_{2B}$, based on pharmacological evidence (15). However, in this latter case, species differences might actually account for the pharmacological diversity, as has also been recently demonstrated for the nonpeptide $NK_1$ antagonists (16–18).

Because the aforementioned studies were carried out on isolated organs in which several tachykinin receptor types were present, we decided to study the properties of septide on transfected cells expressing only the NK1 receptor. Septide was recently shown to be a potent agonist of the recombinant rat NK1 receptor, while displaying little affinity for either SP or NK1 antagonist RP 67580 binding sites (19). We have extended these previous studies in a different expression system and confirmed that septide displays a high functional activity (as measured by stimulation of IP accumulation) but little affinity for SP and RP 67580 binding sites under conditions closely matching the functional studies. Furthermore, septide responses were inhibited uncompetitively by RP 67580, which, however, behaved as a true competitive inhibitor of SP. Additionally, RP 67580 was approximately 8-fold more potent toward septide than toward SP.

## Materials and Methods

[prolyl$^{2,4}$-3,4($n$)-$^3$H]SP and *myo*-[2-$^3$H]inositol were obtained from Amersham (Buckinghamshire, UK). Cell culture media were from GIBCO-BRL (Saint-Ouen L'Aumone, France). SP and septide were purchased from Bachem (Switzerland). Septide was dissolved in 10% dimethylsulfoxide at a concentration of 600 μM and aliquots were stored at −80°C. RP 67580 and [$^3$H]RP 67580 (specific activity, 48 Ci/mmol in ethanol) were synthesized in the Rhône-Poulenc Rorer Chemistry Department.

**Cell culture and transfection.** COS-1 cells were cultured in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 3% glutamine. A pSV2 construct harboring the rat brain NK1 receptor cDNA sequence[1] (pSVNK1) was transfected into COS-1 cells in 10-cm plates using the Transfectam methodology (Sepracor). Control sham transfections were performed with the same expression vector containing the cDNA sequence for CAT (pSVCAT). Twenty-four hours after transfection, cells were trypsinized and plated onto six-well (150,000 cells/well) or 24-well (40,000 cells/well) culture dishes. Cells were used 2–3 days later for binding or IP experiments. Crude membrane fractions were prepared from cells in 10-cm plates 3 days after transfection.

**IP measurements.** IP formation was measured in cells plated onto six-well dishes using a modification of a previously described method (20). Cells were equilibrated for 18 hr in culture medium (1% fetal calf serum) containing 1 μCi of *myo*-[$^3$H]inositol and were then rinsed in Krebs-HEPES buffer (136 mM NaCl, 1.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 1.2 mM $CaCl_2$, 5.5 mM glucose, 5 mM $NaHCO_3$, 0.2 mM bacitracin, 20 mM HEPES, pH 7.4, 1 mg/ml BSA). Cells were then incubated for 1 hr in 0.5 ml of fresh buffer containing 10 mM LiCl and the agonists. Antagonists were preincubated with cells for 15 min before addition of agonist. All incubations were performed at 37° and triplicate determinations were performed for each treatment condition. At the end of the incubation period, cells were lysed with 50 μl of 1% Triton X-100 and the samples were transferred to separate test tubes containing 1.5 ml chloroform/methanol (1:2, v/v). An additional 0.3 ml of both chloroform and water was then added to separate the organic and aqueous phases. An aliquot of the aqueous phase was removed to determine the total radioactivity, whereas 1 ml of the remaining solution was applied to an anion exchange column (Dowex AG1x8, 200–400 mesh, formate form). Columns were eluted serially with increasing concentrations of formate, as described by Berridge *et al.* (21) and modified by Brown *et al.* (22). Free inositol was eluted with distilled water (10 ml), glycerophosphoinositides with 60 mM sodium formate/5 mM sodium tetraborate (20 ml), and finally IP with 1 M ammonium formate (2 ml). Preliminary experiments showed that 95% of the radioactivity in the last fraction coeluted with inositol monophosphate. Radioactivity was determined by liquid scintillation counting in Hionic fluor scintillant (Packard). To compensate for variations in cell number from well to well, individual data were expressed as the ratio of [$^3$H]IP levels to the total radioactivity in the aqueous phase (inositol uptake).

For antagonists, inhibtion constant ($K_B$) values were obtained from the equation $K_B$ = [RP 67580]/[($EC_{50}'/EC_{50}$) − 1], where $EC_{50}'$ and $EC_{50}$ are the agonist concentrations for half-maximal response in the presence or absence of the antagonist RP 67580, respectively, and [RP 67580] is the antagonist concentration. For septide, for which insurmountable antagonism occurred, apparent $K_B$* values were derived from the same equation, with the $EC_{50}'$ concentration corresponding to half the new maximal response in the presence of RP 67580.

**Binding.** Binding to membrane preparations was performed essentially as described previously (10), except than the total reaction volume was scaled down to 300 μl.

Binding to intact cells was carried out directly in 24-well culture dishes. In wells, cells were rinsed twice in cold Krebs-HEPES buffer and incubated with the appropriate ligand, [$^3$H]SP or [$^3$H]RP 67580, in the same buffer supplemented with bestatin and leupeptin (5 μg/ml each), at 4° for 3 hr. Cells were rinsed three times with 1 ml of cold Krebs buffer and lysed with 200 μl of 1 M NaOH. Lysates were transferred to scintillation vials, wells were rinsed with 200 μl of 1 M HCl and pooled with lysates, and Ready Gel (Beckman) scintillation fluid was added. For displacement studies, the radioligand concentration was approximately 1 nM. Nonspecific binding was determined in the presence of a 10 μM concentration of the respective unlabeled ligand. Specific binding amounted to >95% of total binding for [$^3$H]SP and >85% for [$^3$H]RP 67580. Results were treated by nonlinear least-squares curve-fitting procedures, using the EBDA/LIGAND program.

## Results

The rat $NK_1$ receptor was expressed in COS-1 cells by transient transfection of an expression vector containing the rat $NK_1$ cDNA sequence under the control of the early SV40 promoter.

### Agonist-Stimulated IP Accumulation

In transfected cells, SP applications elicited a rise in intracellular IP levels, with a maximum value of 5 ± 1.5 times basal IP levels (eight experiments) (Table 1). From concentration-response curves, an $EC_{50}$ (half-maximal response concentration) value of 0.05 ± 0.02 nM was obtained for SP, confirming the high potency of this NK1 agonist (Fig. 1A). Similarly, septide was a potent agonist of the IP response, with an $EC_{50}$ value of 5 ± 2 nM (Fig. 1B). Both agonists elicited the same maximal rise in IP levels and at saturating concentrations of each agonist these responses were not additive (Table 1). Neither agonist stimulated IP accumulation in nontransfected COS-1 cells or in COS-1 cells transfected with the pSVCAT plasmid (Table 1), demonstrating that both SP and septide responses were mediated by the transfected NK1 receptor.

These functional responses were further characterized by studying the effects of the NK1 antagonist RP 67580 (13). In the presence of the antagonist, the concentration-response curves for SP were shifted to the right (Fig. 1A). A Schild transformation of these results (Fig. 1A, *inset*) demonstrated

[1] L. Pradier, E. Habert-Ortoli, L. Emile, J. LeGuern, I. Loquet, M. D. Bock, J. Clot, V. Moras, L. Mercken, V. Fardin, C. Garret, and J. F. Mayaux. Molecular determinants of the species selectivity of NK1 receptor antagonists. Manuscript in preparation.

TABLE 1

**Agonist-induced IP accumulation in COS-1 cells transfected with the rat NK1 receptor and in sham-transfected cells**

COS-1 cells were transfected with either the pSVNK1 or pSVCAT construct and IP accumulation was measured as described in Materials and Methods. Results for comparison of maximal stimulation by the two agonists were obtained during simultaneous experiments, and values represent the mean ± standard error, with the number of separate experiments indicated in parentheses.

| | Basal IP formation | Maximal stimulated IP formation | | |
|---|---|---|---|---|
| | | SP ($10^{-7}$ M) | Septide ($10^{-6}$ M) | SP + septide |
| | *dpm* | *% of basal* | | |
| pSVNK1 | 484 ± 155 (8) | 500 ± 133 (12) | 486 ± 108 (12) | 504 ± 74 (4) |
| pSVCAT (sham) | 526 ± 179 (4) | 110 ± 12 (4) | 115 ± 13 (4) | ND[a] |

[a] ND, not determined.

that RP 67580 was a competitive inhibitor of SP responses, with a $pA_2$ value of 7.86 ($K_B$ = 13 ± 2 nM, four experiments) and a slope not significantly different from unity (1.2 ± 0.2), in agreement with the known affinity of this antagonist of the NK1 receptor in rat brain tissues (13) (see also below). At the lowest concentration tested (10 nM), RP 67580 also induced a rightward shift of the concentration-response curves for septide, but at higher concentrations the progressive shift was accompanied by an insurmountable block of the response, i.e., even high concentrations of septide could not overcome the blocking effect of RP 67580 (Fig. 1B; note that 10-fold lower concentrations of RP 67580 were used, compared with Fig. 1A). In the presence of increasing concentrations of the antagonist 10, 30, and 60 nM, the maximal septide responses decreased to 94 ± 4% (five experiments), 65 ± 5% (four experiments), and 60 ± 6% (four experiments), respectively, of control responses in the absence of antagonist. Thus, RP 67580 was an uncompetitive antagonist of the septide response, whereas it was competitive with regard to SP. Taking into account the apparent decline in septide efficacy, apparent $EC_{50}$* values could be determined. At all concentrations, the apparent $K_B$* values derived for RP 67580 were similar, i.e., 1.3 ± 0.5 nM (five experiments), 1.3 ± 0.5 nM (four experiments), and 2.0 ± 0.6 nM (three experiments) at 10, 30, and 60 nM, respectively, with a mean $K_B$* value of 1.5 ± 0.2 nM. Therefore, RP 67580 was apparently 8–9 times more potent at antagonizing septide versus SP responses at the NK1 receptor and behaved uncompetitively toward septide.

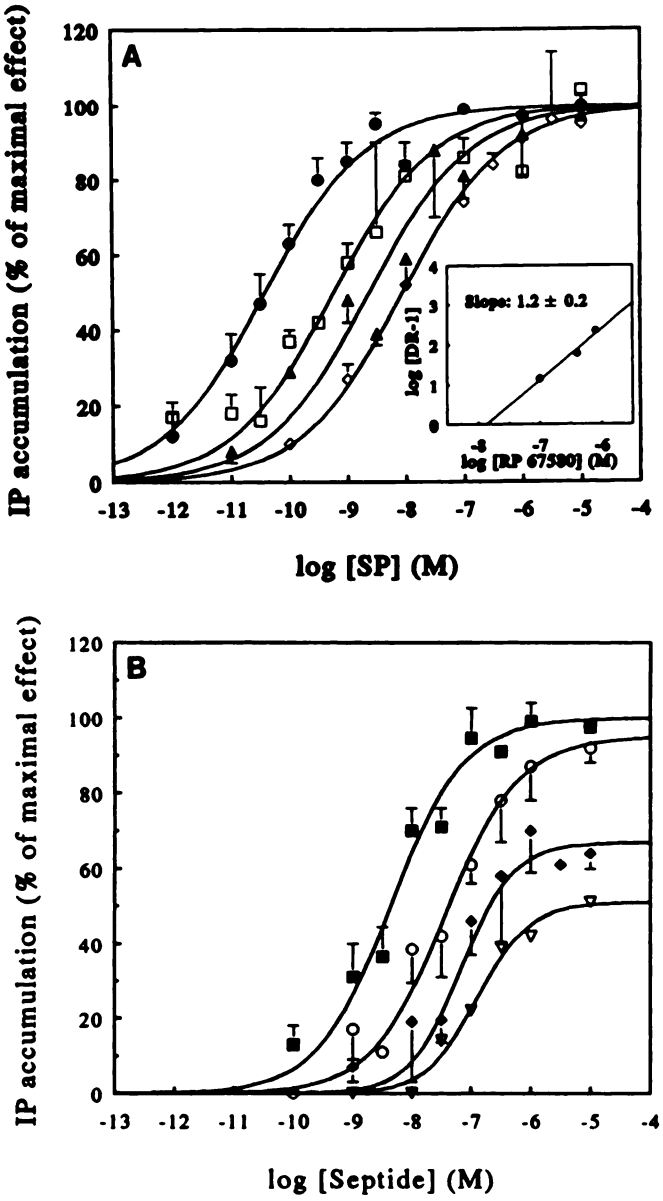


**Fig. 1.** SP- and septide-induced IP accumulation in rat NK1 receptor-transfected COS-1 cells and inhibition by RP 67580. COS-1 cells were transiently transfected and plated 2 days before measurement of agonist-induced IP accumulation, as described in Materials and Methods. Total IPs were measured by chromatography. Data represent the mean ± standard error of four to 10 experiments, each performed in triplicate. A, Competitive inhibition by RP 67580 of SP-induced IP accumulation. Concentration-response curves of SP-induced IP accumulation were obtained in the presence of the NK1 antagonist RP 67580 at the concentrations of 0 (●), 100 (□), 400 (▲), and 800 (◇) nM. *Inset*, Schild transformation of these data, which yielded a $pA_2$ value of 7.86 and a slope of 1.2 ± 0.2. *DR*, dose ratio of the agonist half-maximal concentrations in the presence and absence of RP 67580. B, Uncompetitive inhibition by RP 67580 of septide-induced IP accumulation. Concentration-response curves for septide-induced IP accumulation were obtained in the presence of 0 (■), 10 (○), 30 (♦), or 60 (▽) nM RP 67580. Note that the antagonist concentrations are at least 1 order of magnitude lower than in A.

### Binding to Cell Membranes

[$^3$H]SP binding studies were performed with membrane preparations from rat NK1 receptor-transfected COS-1 cells. From saturation experiments, an equilibrium dissociation constant ($K_d$) of 0.56 ± 0.18 nM (four experiments) was determined for SP, with a binding density ($B_{max}$) of 1.2 ± 0.2 pmol/mg of protein.[1]

In displacement studies, SP and RP 67580 were potent competitors of [$^3$H]SP, with binding constant ($K_i$) values of 5.4 ± 0.9 nM (three experiments) and 10 ± 4 nM (10 experiments), respectively (Fig. 2). The equilibrium binding constant for SP was 10 times higher than its $EC_{50}$ value in functional measurements (Table 2), indicating that a large NK1 receptor reserve pool was present in our expression system. The antagonist RP 67580, however, displayed a $K_i$ value very similar to its inhibition constant in the functional test ($K_B$ = 13 ± 2 nM). In comparison, in the same displacement studies septide was shown to be a poor competitor of [$^3$]SP labeling, with a $K_i$ value of 2.9 ± 0.6 μM (four experiments).

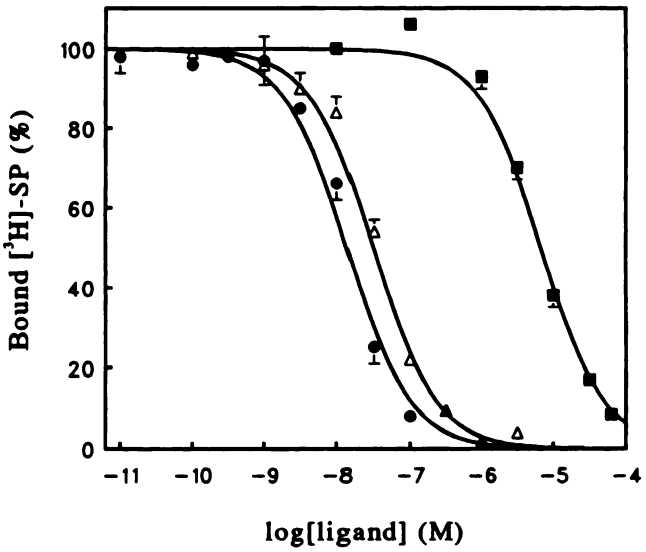


**Fig. 2.** [³H]SP displacement studies with SP, RP 67580, and septide on membranes of rat NK1 receptor-transfected cells. [³H]SP binding to crude membranes from rat NK1 receptor-transfected COS-1 cells was performed as described in Materials and Methods. Displacement curves correspond to SP (●), RP 67580 (△), and septide (■). Data represent the mean ± standard error of four experiments, each performed in duplicate.

### Binding to Intact Cells

To further characterize the interaction between septide and the NK1 receptor, binding studies were performed on intact adherent NK1-transfected cells under the same ionic conditions (Krebs-HEPES buffer) as those used in the functional IP experiments. In some cases, the same aliquots of septide stock solutions were used in both types of experiments. Both [³H]SP and [³H]RP 67580 were used as labeled ligands and for both equilibrium binding was reached in 2 hr at 4° (data not shown).

**[³H]SP.** From saturation experiments, an equilibrium dissociation constant ($K_d$) of 0.53 ± 0.03 (two experiments) was determined for SP, with a binding density ($B_{max}$) of 230,000 ± 30,000 receptors/cell (data not shown). SP and RP 67580 were potent competitors of [³H]SP labeling on intact cells (Fig. 3A; Table 2), with $K_i$ values of 0.47 ± 0.17 nM (three experiments) and 1.16 ± 0.06 nM (three experiments), respectively, in good agreement with values obtained previously with membranes.

Under these conditions, septide was again a poor competitor of [³H]SP binding, with a $K_i$ value of 3.7 ± 0.9 μM (three experiments). Preincubation of cells with septide (15 min) did not improve its inhibition properties (data not shown), suggesting that the lack of competition does not arise from differences in the association kinetics of the two peptides. [³H]SP binding was also performed under alternate ionic conditions (Dulbecco's modified Eagle's medium/BSA or Tris/NaCl/BSA) and comparable results were obtained for the displacement properties of septide (data not shown).

**[³H]RP 67580.** Binding on intact cells was saturable, with an equilibrium dissociation constant ($K_d$) of 1.8 ± 0.7 nM (three experiments) (data not shown) and a binding density ($B_{max}$ = 270,000 ± 35,000 receptors/cell) similar to that obtained for SP. Saturation experiments in the presence of SP (0.5 and 5 nM) demonstrated that RP 67580 and SP were interacting competitively (data not shown). From displacement studies

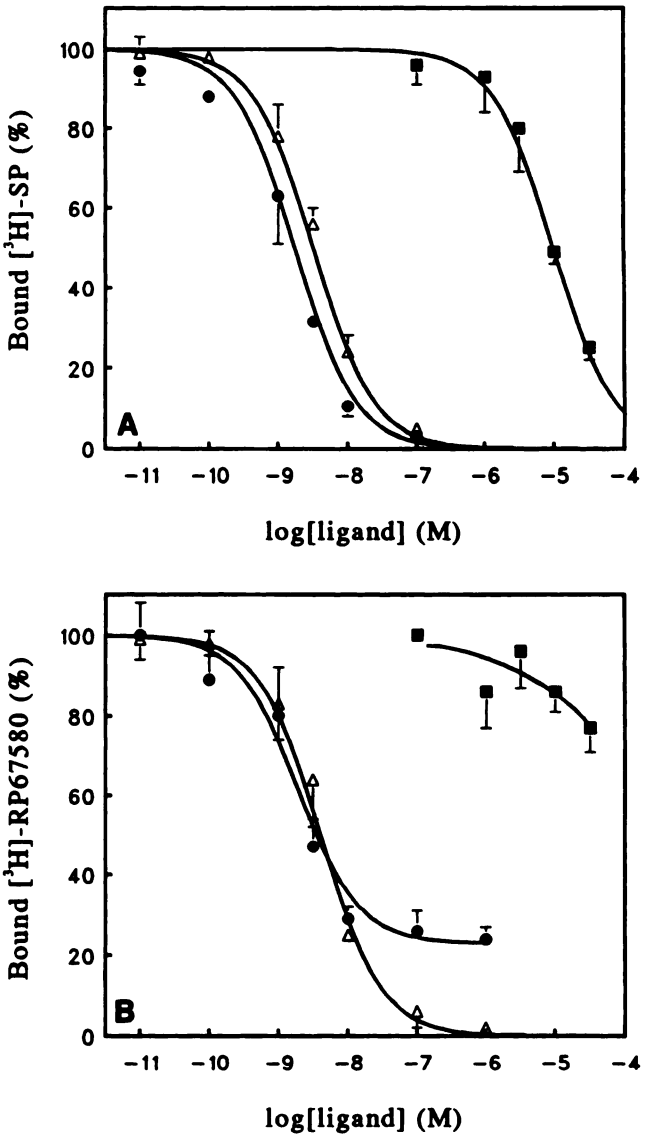


**Fig. 3.** [³H]SP and [³H]RP 67580 displacement studies with SP, RP 67580, and septide on intact rat NK1 receptor-transfected cells. Binding to intact cells was performed as described in Materials and Methods. Displacement studies were performed with SP (●), RP 67580 (△), and septide (■). Data are given as the mean ± standard error of three experiments, each performed in duplicate. A, Displacement of [³H]SP binding, with calculated $IC_{50}$ values of 1.1 ± 0.2, 3.3 ± 0.4, and 9900 ± 1800 nM for SP, RP 67580, and septide, respectively. B, Displacement of [³H]RP 67580 binding, with calculated $IC_{50}$ values of 2.0 ± 0.3 and 4.2 ± 1.2 nM for SP and RP 67580, respectively. Septide was inactive at concentrations up to 10 μM.

with SP and RP 67580, $K_i$ values could be determined as 1.0 ± 0.1 nM (three experiments) and 2.1 ± 0.5 nM (three experiments), respectively (Fig. 3B; Table 2), in agreement with data obtained using [³H]SP as a ligand (see above). SP did not fully displace [³H]RP 67580 binding (25 ± 3% bound [³H]RP 67580 in the presence of 1 μM SP, three experiments) (Fig. 3B). Similar low [³H]RP 67580 binding, insensitive to SP but displaceable by RP 67580, was also detected in sham-transfected cells and represents an additional non-NK1-related binding site for this ligand. It was not further characterized, and no such binding was found with the [³H]SP ligand. In comparison, septide was totally inactive in displacing [³H]RP 67580 at

TABLE 2

**Comparison of functional and binding properties of SP and septide on the rat NK1 receptor**

IP measurements on intact cells were performed as described in Materials and Methods. $K_B^*$ (apparent inhibition constant of RP 67580 versus septide) and $K_B$ were calculated from the equation $K_B$ = [RP 67580]/(DR − 1) where DR is the dose ratio of the agonist half-maximal concentrations in the presence and in the absence of RP 67580. Inhibition constants ($K_i$) for SP, RP 67580, and septide in displacement studies of [$^3$H]SP and [$^3$H]RP 67580 are given for intact transfected cells as the mean ± standard error, with the number of separate experiments indicated in parentheses. $K_i$ values were calculated from $IC_{50}$ values using the Cheng-Prusoff equation.

| IP stimulation | | | |
|---|---|---|---|
| SP | | Septide | |
| $EC_{50}$ | RP 67580 ($K_B$) | $EC_{50}$ | RP 67580 ($K_B^*$) |
| nM | | | |
| 0.05 ± 0.02 (7) | 13 ± 2 (4) | 5 ± 2 (8) | 1.5 ± 0.2 (5) |

| Ligand | Binding, $K_i$ | | |
|---|---|---|---|
| | SP | RP 67580 | Septide |
| | nM | | |
| [$^3$H]SP | 0.47 ± 0.17 (3) | 1.16 ± 0.06 (3) | 3,700 ± 900 (3) |
| [$^3$H]RP 67580 | 1.0 ± 0.1 (2) | 2.1 ± 0.5 (3) | Inactive, 10,000 (3) |

concentrations up to 10 μM. Even with a 1-hr preincubation period, septide (1 and 10 μM) did not affect [$^3$H]RP 67580 binding in saturation experiments (concentrations of labeled ligand ranging from 0.1 to 12 nM) (data not shown).

## Discussion

The hexapeptide septide has been shown to be a NK1 agonist as potent as SP in several preparations *in vitro* and *in vivo* (1–8). Using rat recombinant NK1 receptor reexpressed in a cell line, we demonstrate here that indeed septide can activate the NK1 receptor (as measured by IP stimulation) with an $EC_{50}$ value of 5 nM, confirming recent data obtained with a Chinese hamster ovary cell line stably expressing the same receptor (19). SP is, however, 100 times more potent than septide in this system. The absence of SP degradation under the present defined conditions could account for its high activity ($EC_{50}$ value of 0.05 nM), compared with isolated organs, whereas the shorter peptide septide, which is fairly stable in most tissues (23), would be unaffected. Alternatively, different septide receptors could be present in certain tissues or different post-translational modifications of the NK1 receptor could modify septide potency. The lack of response to either peptide in nontransfected or sham-transfected cells indicates that the responses in transfected cells are mediated by the NK1 receptor and do not result, for example, from a direct activation of G proteins (24).

RP 67580, a nonpeptide NK1 antagonist, inhibited SP-induced IP stimulation competitively, with a $K_B$ value of 13 nM ($pA_2$ = 7.86), in general agreement with its binding affinity for the rat NK1 receptor. In comparison, RP 67580 is an uncompetitive inhibitor of septide-induced IP stimulation with an apparent inhibition constant ($K_B^*$) 8–9 times lower than that obtained against SP. The higher potency of NK1 antagonists, both peptide and nonpeptide, versus septide rather than SP (or other NK1-selective agonists) responses has already been demonstrated (a) in the guinea pig ileum for GR 71251 ($pK_B$ values of 7.9 and 6.5, respectively) (12), [D-Pro$^9$,Pro$^{10}$,Trp$^{11}$]SP ($pA_2$ values of 6.5 versus septide and 5 versus [Pro$^9$]SP) (24), RP 67580 ($pA_2$ values of 7.6 and 7.2, respectively) (13), and (±)-CP96,345 ($pA_2$ values of 9.24 versus septide and 8.17 versus [Sar$^9$]SP sulfone) (14) and (b) in the rat urinary bladder for RP 67580 ($pK_B$ values of 7.5 and 6.7, respectively) and CP 96,345 ($pK_B$ values of 6.5 and 5.7, respectively) (5). Similarly, *in vivo* in the guinea pig bronchoconstriction model RP 67580 ($IC_{50}$ value of 0.65 mg/kg versus septide and inactive versus SP) and CP 96,345 ($IC_{50}$ values of 0.03 mg/kg and 1 mg/kg, respectively) are more potent toward septide than SP stimulation (7). Interestingly, this higher sensitivity of septide responses to antagonists has in part led to the suggestion of the existence of a specific septide receptor distinct from the NK1 receptor (12, 25). Although not ruling out such an hypothesis, the present data demonstrate, however, that the instrinsic properties of the NK1 receptor suffice to explain the high potency of septide as well as the higher potency of RP 67580 toward septide than toward SP. A major difference in the present data, compared with results obtained in the guinea pig ileum preparation, is that the inhibitions of both SP and septide responses were described as competitive in the latter (13, 14). In isolated organ preparations, cumulative concentration-response curves for agonists are usually generated and applications are short-lasting. Furthermore, access to the adequate receptors could be somewhat restricted in tissues. These methodological differences as well as possible differences in receptor post-translational modification could account for this major discrepancy. The study of additional agonists and nonpeptide antagonists in this recombinant system, as well as variations of NK1 receptor expression levels, will be needed to help explain this difference.

Due to the present lack of labeled septide ligand, further characterization of the septide-NK1 interaction was performed by binding studies using labeled SP or RP 67580. In both cases, septide was a very poor competitor of ligand binding to the rat NK1 receptor. This lack of affinity for either binding site was observed with membranes (see also Ref. 19) and intact cells under conditions closely matching those of functional studies. Overall, the present data demonstrate that, acting on the NK1 receptor itself, septide is a potent agonist of the receptor but interacts only very weakly with the binding sites for SP or the antagonist RP 67580. Moreover, RP 67580, a competitive SP antagonist, is an uncompetitive septide inhibitor with a 8-fold higher potency toward septide than toward SP. The latter results strongly suggest that septide and SP could activate the NK1 receptor by different mechanisms.

Recently, a comparable unsurmountable antagonism of the rat brain 5-hydroxytryptamine type 2 receptor by lysergic acid diethylamide and bromo-lysergic acid diethylamide has been described (26). Extremely slow dissociation kinetics of the antagonist accounted for the unsurmountable block, inasmuch as "the agonist equilibrates with the available receptors and produces its measured response before any adjustment of antagonist occupancy has time to occur," according to the hemiequilibrium kinetic model of Paton and Rang (27) (for a review on insurmountable antagonism, see Ref. 28). However, in the case of the NK1 receptor (the present study), RP 67580 does not display slow dissociation kinetics, inasmuch as its blocking effect (after preincubation) can be reversed completely by increasing SP concentrations. Additionally, the time courses of IP stimulation by SP and septide were very similar (data not

shown), confirming that both peptides had comparable kinetic properties, as usually observed in most organ preparations. Such an uncompetitive antagonism could also occur with an irreversible antagonist if a large receptor reserve was present. However, RP 67580 has been shown in several systems to be rapidly and fully reversible (4, 13).

A very high intrinsic activity of septide could account for its high efficacy and poor affinity. Indeed, the large receptor reserve in our expression system, as observed in the 10-fold difference between SP potency ($EC_{50}$ in IP tests) and its affinity, would add support to this hypothesis, but similar results have been obtained in a Chinese hamster ovary cell line stably expressing the NK1 receptor, where little receptor reserve was present (19), as well as in the guinea pig ileum, where septide, which is equipotent with SP in eliciting contraction, has a 400 times lower affinity than SP for the NK1 receptor (10). Furthermore, the higher potency of RP 67580 toward septide (as well as the uncompetitive nature of its antagonism) is in contradiction to a high intrinsic activity of septide.

Therefore, our results tend to favor a model in which septide activates the NK1 receptor via a secondary binding site different from that of SP. RP 67580, a competitive antagonist of SP, would lock the receptor in an inactive state. Whereas a multiple-activation site model is well documented for multimeric ion channel-coupled receptors such as the nicotinic acetylcholine receptor (29) and the $\gamma$-aminobutyric acid type A receptor (30), such a model is very uncommon for G protein-coupled receptors. However, allosteric antagonists of the muscarinic acetylcholine receptor such as gallamine have been described (31), suggesting the existence of secondary binding sites on receptors of this type. Moreover, a recent report has suggested the existence of more than two binding sites in cloned D2 and D3 dopamine receptors (32), but data are also consistent with the high and low affinity state model for these receptors. Additional conformation states for $\alpha$- and $\beta$-adrenergic receptors have also been postulated (33) as an extension of the ternary complex model and could represent possible targets for septide. However, in the framework of that model, the present results are consistent with the existence of separate activation sites for septide and SP on the NK1 receptor. Partial deletions in the receptor amino-terminal region lead to a great loss of potency for SP, NKA, NKB, and septide upon activation of the receptor (34), which suggests that septide and SP binding might be sharing some structural determinants. This does not exclude the possibility that future mutants may distinguish between septide and SP binding sites. However, the final characterization of these sites might await the availability of labeled septide and the use of photoaffinity cross-linking studies on the NK1 receptor. In addition, a specific septide subsite on the NK1 receptor could represent an original pharmacological target for development of novel functional antagonists of that receptor.

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