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High Affinity Binding of [^{3}H]Propionyl-[Met(O$_2$)^{11}]Substance P(7–11), a Tritiated Septide-Like Peptide, in Chinese Hamster Ovary Cells Expressing Human Neurokinin-1 Receptors and in Rat Submandibular Glands

SANDRINE SAGAN, JEAN-CLAUDE BEAUJOUAN, YVETTE TORRENS, MONIQUE SAFFROY, GÉRARD CHASSAING, JACQUES GLOWINSKI, and SOLANGE LAVIELLE

Laboratoire de Chimie Organique Biologique, URA CNRS 493, Université Pierre et Marie Curie, 75252 Paris Cedex 05, France (S.S., G.C., S.L.), and Chaire de Neuropharmacologie, Institut National de la Santé et de la Recherche Médicale U114, Collège de France, 75005 Paris, France (J.-C.B., Y.T., M.S., J.G.)

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SUMMARY

Propionyl-[Met(O$_2$)^{11}]substance P(7–11) [ALIE-124 or propionyl-[Met(O$_2$)^{11}]SP(7–11)] has been designed as a septide-like ligand adequate for tritiation and, therefore, adequate for binding studies. In Chinese hamster ovary (CHO) cells expressing human tachykinin neurokinin (NK)-1 receptors, ALIE-124 displaced [^{3}H][Pro^9]substance P (SP) from its binding site at micromolar concentrations. However, ALIE-124 stimulated phosphatidylinositol hydrolysis, as previously shown for septide-like peptides. With [^{3}H]ALIE-124 (95 Ci/mmol), we have been able to reveal a high affinity binding site in CHO cells (K_d = 6.6 ± 1.0 nM), with a low maximal binding capacity. [^{3}H]ALIE-124 specific maximal binding represented only 15–20% of that observed with [^{3}H][Pro^9]SP in CHO cells. Septide-like peptides, including septide and NKA, were potent competitors (in the nanomolar range) of [^{3}H]ALIE-124 specific binding site. Interestingly, SP and [Pro^9]SP were also potent competitors, with 10-fold greater potency for sites labeled with [^{3}H]ALIE-124 than for sites labeled with [^{3}H][Pro^9]SP. The NK-1 antagonist RP 67580 also showed a higher potency for [^{3}H]ALIE-124 than for [^{3}H][Pro^9]SP-specific binding sites. NKB and [Lys^5,methyl-Leu^9,Nle^{10}]NKA(4–10) displaced [^{3}H]ALIE-124 binding but with lower potency, whereas senktide had no affinity. The existence of [^{3}H]ALIE-124 specific binding sites was also demonstrated in rat submandibular gland. In this tissue, [^{3}H]ALIE-124 specific maximal binding was higher, reaching 40–50% of that achieved with [^{3}H][Pro^9]SP.

[Pro^9]SP (1) and septide [pGlu^6,Pro^9]SP(6–11) (2) were originally described in 1986 as selective agonists of the tachykinin NK-1 receptor. However, the molecular basis for the action of septide on tachykinin receptors has been a paradox for the past 10 years (2–4). This synthetic carboxyl-terminal analogue of SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$) has a very low binding affinity for any of the described tachykinin NK-1, NK-2, or NK-3 receptors (IC$_{50}$ values in the micromolar range) (5, 6). Nevertheless, in numerous, but not all, *in vitro* preparations or *in vivo* experiments, septide is as active as SP (EC$_{50}$ values in the nanomolar range). Furthermore, specific NK-1 antagonists inhibit the action of septide; most of them are significantly more potent against septide-evoked than on substance P-evoked responses (3, 7–9). All these observations led us to postulate the existence of "septide-sensitive" tachykinin receptors in the guinea pig ileum (3, 7). This proposal was then supported by other groups who provided further pharmacological evidence for this paradoxical mode of action in other tissues (5). However, this hypothesis has proved controversial, and other groups have also suggested that septide behaves as an agonist for the tachykinin NK-1 receptor, acting at a site distinct from SP (10). Huang *et al.* (11) speculated that septide may occupy only part of the binding site devoted to the undecapeptide SP. Both conclusions arose from experiments performed with CHO or COS cells transfected with the tachykinin NK-1 receptor (for a review, see Ref. 6).

In CHO cells transfected with the tachykinin hNK-1 receptor cDNA, PI metabolism was similarly activated by both NK-1 agonists and previously classified "septide-like" molecules (i.e., peptides with low or micromolar affinity for specific NK-1 binding sites, respectively) (12). Furthermore, an excellent correlation was found between the EC$_{50}$ values of several tachykinin analogues on IP production in CHO cells

ABBREVIATIONS: SP, substance P; NK, neurokinin; ALIE-124, propionyl-[Met(O$_2$)^{11}]substance P(7–11); CHO, Chinese hamster ovary; PI, phosphatidylinositol; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethylsulfoxide; PLC, phospholipase C; hNK, human neurokinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

and their EC_{50} values determined in the guinea pig ileum bioassay (13). In contrast to PI turnover, cAMP production in CHO cells was stimulated only by NK-1 agonists, which present high affinity for [^{3}H][Pro^9]SP specific binding sites, with septide-like molecules being only weakly active (12). Moreover, the activities (EC_{50}) of tachykinin analogues on cAMP pathway correlated with their affinities (IC_{50}) for specific tachykinin NK-1 binding sites (13).

All attempts to identify a specific binding site for septide on the NK-1 receptor protein with iodinated septide-like peptides have so far failed. In the present study, with the use of a new tritiated septide-like molecule, [^{3}H]ALIE-124 ([2,3-^{3}H]propionyl-Phe-Phe-Gly-Leu-Met(O_2)NH_2), we have been able to detect a high affinity specific binding site for this radiolabeled peptide on membranes from CHO cells transfected with the tachykinin hNK-1 receptor. This high affinity specific binding site was also found on membrane preparations from rat submandibular glands. Interestingly, in both preparations, the maximal binding capacity (B_{max}) of [^{3}H]ALIE-124 was always lower than that found with [^{3}H][Pro^9]SP. Furthermore, the ratios of [^{3}H]ALIE-124 specific binding sites to those of [^{3}H] [Pro^9]SP were different in membranes from CHO cells transfected with the hNK-1 receptors and those prepared from submandibular glands.

Experimental Procedures

Materials

[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 (22.3 Ci/mmol) and [2,8-^{3}H]adenine (26.8 Ci/mmol) were from DuPont-New England Nuclear (Les Ulis, France). *N*-Succinimidyl-[2,3-^{3}H]propionate (95 Ci/mmol) was from Amersham (Les Ulis, France). SP, [Pro^9]SP, septide, ALIE-124, NKA, and [Lys^5, methyl-Leu^9,Nle^{10}]NKA(4–10) were synthesized in the Laboratoire de Chimie Organique Biologique (Paris, France). NKB and senktide were from Bachem Biochimie (Voisins-le-Bretonneux, France). RP 67580 was a generous gift from Dr. C. Garret (Rhône-Poulenc Rorer, Paris, France). BSA was from Calbiochem (Meudon, France). Ham's F-12 cell culture medium, fetal calf serum, HEPES, streptomycin, penicillin, and geneticin were from GIBCO (Eragny, France). 3-Isobutyl-1-methylxanthine, bacitracin, leupeptin, chymostatin, amastatin, phosphoramidon, polyethyleneimine, imidazole, LiCl, cAMP, ATP, EDTA, PMSF, leupeptin, soybean trypsin inhibitor, cholera toxin, and tunicamycin were from Sigma (Saint Quentin Fallavier, France). Captopril was a generous gift from Squibb Labs (Nevilly sur Seine, France). AG 50W-X4 Dowex resin, in the hydrogen form (200–400 mesh) and AG 1-X8 Dowex resin in the formate form (200–400 mesh) were from BioRad (Ivry-sur-Seine, France). Alumina *N*-Super I was from ICN Biomedicals (Orsay, France).

Materials and Methods

Peptide solubility. All peptides were dissolved in water and stored at a concentration of 1 mM at −20°, except NKB (final concentration, 0.1 mM), which was dissolved first with DMSO (final concentration, 4%) and then diluted with 0.1 N NaOH (final concentration, 2%) and water. ALIE-124 stock solutions (1 mM) were prepared in DMSO.

Cell culture. CHO cells expressing hNK-1 receptors were cultured in Ham's F-12 medium supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin, and 10% fetal calf serum. Cultures were kept at 37° in a humidified atmosphere of 5% CO_2. Stable transfectants were maintained by geneticin periodic selection. Deglycosylated cells were obtained after treatment every 24 hr for 40 hr with tunicamycin (5 μg/ml) as previously described (15). For experiments carried out with cholera toxin, the G_s activator was added (1 μg/ml) to the culture medium 20 hr before the experiments.

Membrane preparation from CHO cells. CHO cells were grown to confluence in 100-mm culture dishes. Membranes were then prepared as described with slight modifications (15). Briefly, cells were first washed with 150 mM NaCl in 50 mM Tris-Cl buffer, pH 7.4 (8 ml), and detached with the same buffer containing 2 mM EDTA. Collected cells were centrifuged at 700 × *g* for 15 min. The pellet was then resuspended in (0.5 ml/dish) 10 mM Tris-Cl buffer, pH 7.4, 1 mM EDTA, 0.5 mM PMSF, 40 μg/ml bacitracin, 5 μg/ml leupeptin, and 5 μg/ml soybean trypsin inhibitor and further left on ice for 30–60 min. Cells were homogenized (10–15 times) and centrifuged at 900 × *g* and then twice at 1000 × *g* to remove debris. The resulting supernatant was centrifuged at 48,000 × *g* for 30 min at 4°. The resulting membrane pellet was resuspended in 50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml soybean trypsin inhibitor at a protein concentration of 0.3–0.5 μg/μl, as determined according to Bradford (16).

Membrane preparations from rat submandibular glands. Male Sprague-Dawley rats (200–250 g; Charles River, Cléon, France) were used. Rat submandibular glands were rapidly removed and homogenized with a Kinematica apparatus for 15 sec in ice-cold HEPES buffer (20 mM, pH 7.4). After centrifugation at 30,000 × *g* for 30 min at 4°, the pellet was resuspended at 4° for 5 sec in the same buffer containing 300 mM KCl and 10 mM EDTA. This suspension was incubated for 30 min at 4° with intermittent mixing and then centrifuged at 30,000 × *g* for 30 min at 4°. The final membrane pellet was resuspended in Tris-Cl buffer (50 mM, pH 7.4) containing 3 mM $MnCl_2$, 0.1% BSA, 200 μg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin, 1 μM amastatin, 5 μM captopril, and 1 μM phosphoramidon. Protein concentrations were determined according to Bradford (16).

Synthesis of tritiated ALIE-124. [Met(O_2)^{11}]SP(7–11) (21 nmol) in DMSO (3.3 μl) was reacted with *N*-succinimidyl-[2,3-^{3}H]propionate (1 mCi, 95 Ci/mmol, 10.5 nmol) in 1.8 μl of DMSO in the presence of 0.05 M borate buffer, pH 8.5 (2.2 μl), for 2 hr at room temperature. After the addition of 50 μl of DMSO, the crude reaction mixture was purified by high performance liquid chromatography (Lichrospher RP8*e*, 4 × 250 mm, 10 μm) using a gradient of CH_3CN in 0.1% (v) trifluoroacetic acid from 29% to 51% CH_3CN in 30 min. The radiolabeled peptide [^{3}H]ALIE-124, which eluted with a retention time identical to that of the nonlabeled peptide (15.5 min), was collected. After the removal of CH_3CN (at reduced pressure) and lyophilization, [^{3}H]ALIE-124 was aliquoted in DMSO and stored in liquid nitrogen (specific activity, 95 Ci/mmol; 1 Ci = 37 GBq). For comparison, the retention time of [Met(O_2)^{11}]SP(7–11) with this gradient system was 7.3 min.

Binding assays on CHO cells. Binding assays were carried out both on whole cells and membranes. For binding assays using whole cells, different densities of cells were seeded onto 24-well plates 24 hr before experiments (i.e., 5 × 10^3/and 10^5 cells/well for [^{3}H][Pro^9]SP and [^{3}H]ALIE-124 binding, respectively). With these conditions, <10% of radioligand was bound. Krebs-phosphate buffer consisted of 120 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 15.6 mM NaH_2PO_4, pH 7.2, containing 0.04% BSA (w/v), 0.03 mg/ml bacitracin, and 0.6% glucose (w/v). Cells were first washed three times with 0.5 ml of the buffer and then incubated in 200 μl of Krebs-phosphate buffer at room temperature (22°) with [^{3}H][Pro^9]SP (0.3–0.7 nM, 65 Ci/mmol) for 100 min or with [^{3}H]ALIE-124 (2–5 nM, 95 Ci/mmol) for 70 min. These incubation times correspond to the binding equilibrium as determined from kinetics experiments performed under the same conditions. The incubation was stopped by aspiration of the supernatant and washing the cells three times with 0.5 ml of cold (4°) buffer. Cells were then lysed with 0.1% Triton X-100 (0.5 ml), and the radioactivity in the lysates was determined after the addition of Aquasol-2.

Membranes were also used and prepared as described above. In Eppendorf tubes, membrane suspension containing 5–10 μg of protein ([^{3}H][Pro^9]SP, 0.3–0.7 nM) or 50–60 μg of protein ([^{3}H]ALIE-124, 2–5 nM) was incubated in 50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.04% BSA (w/v), 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml soybean trypsin inhibitor in a total volume of 200 μl. Membranes were incubated at room temperature (22°) for 10 min with [^{3}H][Pro^9]SP and for 70 min with [^{3}H]ALIE-124 as determined from kinetic studies. Incubation was stopped by centrifugation of the samples for 2 min at 13,000 × g, washing the pellet with 1 ml of cold (4°) buffer, and centrifugation again for 1 min at 13,000 × g. Radioactivity in the pellets was counted after the addition of Aquasol-2.

All determinations were carried out at least three times in duplicate. Nonspecific binding was estimated in the presence of 1 μM concentration of the corresponding unlabeled peptide.

Binding assays on membrane preparations from rat submandibular glands. Rat submandibular gland membranes (60 μl, ~150 μg of protein/assay) were incubated at 20° (final volume, 200 μl) for 15 or 90 min with [^{3}H][Pro^9]SP (65 Ci/mmol, 0.5–1 nM) and [^{3}H]ALIE-124 (95 Ci/mmol, 1–2 nM), respectively. Incubations were stopped by filtration with a J.S.I. Multividor apparatus through Whatman GF/C filters pretreated at 4° for 3–4 hr with 0.1% polyethyleneimine. Filters were then washed three times with 3 ml of Tris-Cl buffer 50 mM, pH 7.4, containing 3 mM $MnCl_2$ and 0.1% BSA at 4°. Radioactivity bound to membranes was counted after the addition of Aquasol-2

Measurements of inositol phosphate and cAMP formations in CHO cells. PI hydrolysis was determined as previously described (12, 17). CHO cells were seeded onto 24-well plates (10^5 cells/well) 48 hr before the assay. [^{3}H]Inositol (0.5 μCi/well) was added to the culture medium for 24 hr.

cAMP levels were estimated as previously reported (12, 18). CHO cells were seeded onto 24-well plates (10^5 cells/well) 24 hr before the assay. [^{3}H]Adenine (0.2 μCi/well) was added to the culture medium for 2 hr.

Analysis of data. All binding studies (kinetics, saturation, competition) were analyzed with the program LIGAND (19). The curves presented have been fitted using SIGMA PLOT software (Jandel Scientific, Erkrath, Germany).

Results

Design of a tritiated septide-like radioligand. As recently reported, acetyl-Arg-septide (CH_3CO-Arg-Phe-Phe-Gly-Leu-Met-NH_2), the water-soluble septide, was shown to behave as an NK-1 agonist in CHO cells transfected with the hNK-1 tachykinin receptor (12). In contrast to septide, acetyl-Arg-septide presented an affinity in the nanomolar range for the specific binding site labeled with [^{3}H][Pro^9]SP, and acetyl-Arg-septide stimulated both PI and cAMP pathways; the production of cAMP is a specific signal for tachykinin NK-1 agonists (12, 13). In the same study, [pGlu^6]SP(6–11) behaved as a septide-like peptide (i.e., with an affinity for specific NK-1 binding sites in the micromolar range), being active only on IP production. These observations led us to search for the shortest carboxyl-terminal analogue of SP(6–11) that could be easily tritiated and still act as a septide-like peptide: a peptide with a micromolar affinity for specific tachykinin NK-1 receptor labeled with [^{3}H][Pro^9]SP but highly potent on IP formation in CHO cells transfected with the tachykinin hNK-1 receptor. Propionyl-SP(7–11) became the lead compound.

Preliminary results with [2,3-^{3}H]propionyl-SP(7–11) showed a gradual decrease in specific binding, suggesting that storage of this peptide in DMSO led to oxidation into the sulfoxides. This hypothesis was confirmed by the pharmacological properties of the corresponding synthetic sulfoxides and sulfone:propionyl-[Met(O)^{11}]SP(7–11) and propionyl-[Met(O_2)^{11}]SP(7–11) (i.e., ALIE-124). Indeed, on CHO cells transfected with the tachykinin hNK-1 receptor, the diastereoisomeric sulfoxides weakly stimulated PI turnover (EC_{50} = 650 nM), whereas the sulfone, ALIE-124, was as potent as propionyl-SP(7–11) on IP formation (EC_{50} = 37 and 12.5 nM, respectively) (Table 1). Incidentally, similar unexplained behaviors have also been obtained with SP and its corresponding sulfoxides and sulfone (Table 1) (20). Therefore, the tritiated sulfone analogue propionyl-[Met(O)^{11}]SP(7–11), [^{3}H]ALIE-124, was prepared and used as the septide-like radioligand.

Binding of [^{3}H]ALIE-124 and [^{3}H][Pro^9]SP on CHO cells transfected with hNK-1 receptors: Pharmacological properties of tachykinin analogues. Association kinetics of [^{3}H]ALIE-124 (2 nM) indicated that this peptide binds slowly to tachykinin NK-1 receptors. Equilibrium was reached only after 60-min incubation with membranes and intact CHO cells expressing tachykinin hNK-1 receptors, with both curves being almost identical (Fig. 1A, binding shown for membranes). Specific binding accounted for 70–80% and >90% of the total binding in membranes and intact CHO cells, respectively. From the association curve, k_{obs} = 0.0480 ± 0.005 min^{-1} and k_{-1} = 2.67 ± 0.17 M/min were determined. Dissociation curve of [^{3}H]ALIE-124 (obtained after the addition of 1 μM ALIE-124 at the plateau) on both membranes and intact CHO cells was best fitted with a biexponential decay model with $k_{-1}{}^1$ = 0.35 ± 0.09 min^{-1} and $k_{-1}{}^2$ = 0.0436 ± 0.005 min^{-1} (data shown for membranes in Fig. 1A). From these data, two affinity states were deduced: K_{d1} = 1.6 ± 0.2 nM (45% of the receptors) and K_{d2} = 13 ± 3 nM (55% of the receptors). No binding of [^{3}H]ALIE-124 (2 nM) could be detected on nontransfected CHO cells.

In contrast to [^{3}H]ALIE-124, the binding of [^{3}H][Pro^9]SP to membrane-bound NK-1 receptors was very fast, with a plateau reached after 5 min (Fig. 1B). A very fast dissociation rate was also observed from membrane-bound receptors, using [^{3}H][Pro^9]SP. After a 1-min incubation, 75% of the [^{3}H][Pro^9]SP specific binding was already displaced by an excess (1 μM) of [Pro^9]SP, and after 6 min, 95% of the labeled peptide was replaced (Fig. 1B). These association/dissociation rates to and from membranes were too fast to allow accurate determination of the kinetic parameters. The situation was different in intact CHO cells because equilibrium was achieved only after 90 min; dissociation kinetics of [^{3}H][Pro^9]SP also were much slower (12). The specific binding of [^{3}H][Pro^9]SP accounted for >90% of the total binding in both membrane preparations and intact CHO cells.

TABLE 1

Comparison of the potencies of ALIE-124, SP, and their oxidized analogues

Comparison was made of the ability to compete with [^{3}H][Pro^9]SP and stimulate both inositol phosphates and cAMP second messenger pathways in intact CHO cells expressing human NK-1 receptors, as described in Materials and Methods.

Peptide	[^{3}H][Pro^9]SP (K_i)	PLC (EC_{50})	cAMP (EC_{50})
		nM	
Propionyl-SP(7–11)	2000 ± 530	12.5 ± 2.5	2600 ± 560
Propionyl-[Met(O)^{11}]SP(7–11)	>5000	650 ± 20	>5000
ALIE-124	4200 ± 300	37 ± 4	>5000
SP	1.6 ± 0.4	1.0 ± 0.6	8 ± 2
[Met(O)^{11}]SP	40 ± 10	3.5 ± 0.5	>5000
[Met(O_2)^{11}]SP	0.75 ± 0.35	1.0 ± 0.2	14 ± 2

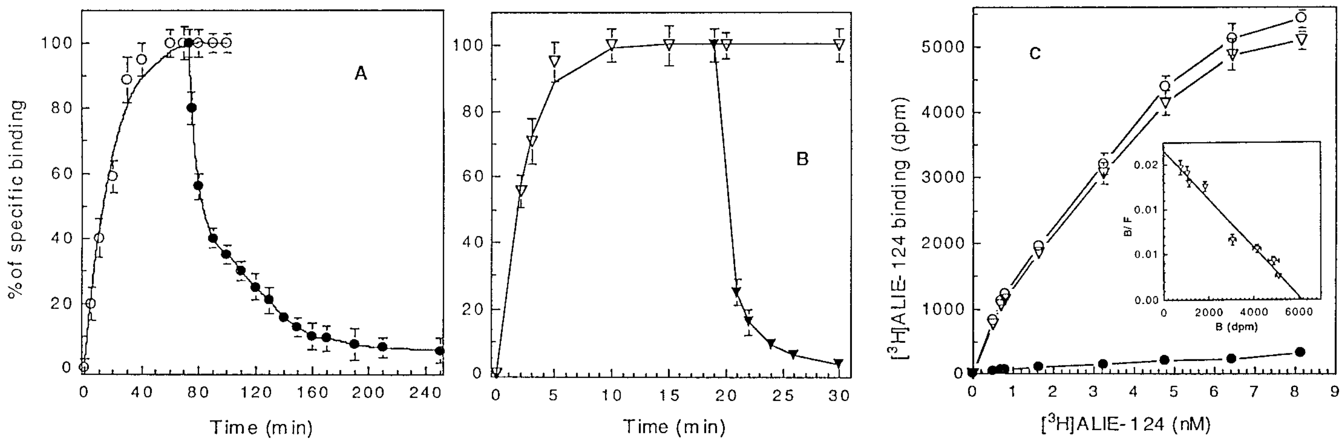

Fig. 1. Association (○ and ▽) and dissociation (● and ▼) binding kinetics of [^{3}H]ALIE-124 (2 nM, 95 Ci/mmol, ○ and ●) (A) and [^{3}H][Pro^9]SP (0.5 nM, 65 Ci/mmol, ▽ and ▼) (B) with membranes (50 and 5 μg of proteins, respectively) prepared from CHO cells expressing hNK-1 tachykinin receptors. At the steady state for association kinetics, 1 μM concentration of the corresponding unlabeled peptide was added to initiate dissociation. *Curve*, mean ± standard error of three independent experiments performed in triplicate. C, Saturation binding analysis of [^{3}H]ALIE-124 to intact CHO cells (50 μg of proteins, 10^5 cells) as described in Materials and Methods. ○, Total binding; ●, nonspecific binding; ▽, specific binding. *Inset*, Scatchard analysis of these data performed with use of the program LIGAND (9).

Binding experiments for [^{3}H]ALIE-124 and [^{3}H][Pro^9]SP were performed in side-by-side experiments with the same membrane preparations; only difference was the amounts of proteins and the concentrations of radioligands used in the assays.

Saturation experiments performed with [^{3}H][Pro^9]SP and [^{3}H]ALIE-124 indicated that both radioligands bound to a single high affinity binding site in membrane preparation of CHO cells expressing tachykinin hNK-1 receptor (Fig. 1C and Table 2). For [^{3}H][Pro^9]SP, the K_d value (0.35 nM) was similar to that previously observed (12), whereas that of [^{3}H]ALIE-124 was 6.0 nM. The major finding was that the maximal binding capacities for [^{3}H][Pro^9]SP and [^{3}H]ALIE-124 were very different (B_{max} = 2570 and 570 fmol/mg of proteins, respectively). With a CHO clone expressing a lower level of hNK-1 receptors, similar results were obtained for intact CHO cells with [^{3}H][Pro^9]SP and [^{3}H]ALIE-124 (i.e., $K_d = 0.6 \pm 0.1$ nM, $B_{max} = 1000 \pm 240$ fmol/mg of protein, and $K_d = 22 \pm 5$ nM, $B_{max} = 160 \pm 35$ fmol/mg of protein, respectively). The difference in K_d values with [^{3}H]ALIE-124 between the two clones is significant ($p = 0.02$) but could result from the low amount of radioactivity bound to the cells, with the clone expressing lower level of receptors and the subsequent calculations of K_d and B_{max} values with the program LIGAND.

These K_d and B_{max} values were unaltered by pretreatment of the transfected CHO cells with either tunicamycin, which was shown to inhibit *N*-glycosylation (15), or cholera toxin (Table 2). After incubation with cholera toxin, the tachykinin hNK-1 receptor was uncoupled to G_s, as demonstrated by subsequent experiments on cAMP production performed with [Pro^9]SP and ALIE-124 on these pretreated cells (data not shown).

Competition studies were performed with [^{3}H]ALIE-124 (2–5 nM) and different molecules on intact CHO cells expressing tachykinin hNK-1 receptors (Table 3). For comparison, binding potencies previously obtained with [^{3}H][Pro^9]SP are also shown in Table 3. The potency of ALIE-124 to displace [^{3}H]ALIE-124 was similar to that found in saturation experiments. Tachykinin NK-1 ligands such as SP and [Pro^9]SP were 10-fold more potent competitors for the site labeled with [^{3}H]ALIE-124. Septide and NKA also inhibited [^{3}H]ALIE-124 binding at nanomolar concentration, whereas higher concentrations were needed to inhibit [^{3}H][Pro^9]SP specific binding (Table 3). The selective tachykinin NK-2 agonist [Lys^5,methyl-Leu^9,Nle^{10}]NKA(4–10) and the endogenous NK-3 ligand NKB were weak competitors of [^{3}H]ALIE-124 specific binding. All these competitors inhibited [^{3}H]ALIE-124 specific binding to the same maximal extent, except the selective tachykinin NK-3 agonist senktide, which inhibited only marginally [^{3}H]ALIE-124 specific binding (40% inhibition at 10^{-5} M). Finally, the selective nonpeptidic tachykinin

TABLE 2

Scatchard analysis of [^{3}H]ALIE-124 and [^{3}H][Pro^9]SP saturation binding experiments

Analyses were performed in untreated or tunicamycin- or cholera toxin-pretreated CHO cells transfected with the human NK-1 receptors, membranes prepared from CHO cells expressing human NK-1 receptors, and membranes prepared from rat submandibular glands, as described in Materials and Methods. Data were analyzed with the programm LIGAND and are the mean ± standard error of at least three independent experiments performed in triplicate.

		CHO cells			CHO cell membranes	Rat submandibular gland membranes
		− Tunicamycin	+ Tunicamycin	+ Cholera toxin (1 μg/ml)		
[^{3}H]ALIE-124[a]	K_d (nM)	6.6 ± 1.0	7.7 ± 1.5	10 ± 2	6.0 ± 1.2	11.4 ± 0.4
	B_{max}(fmol/mg of protein)	750 ± 130	800 ± 170	800 ± 150	570 ± 70	160 ± 15
[^{3}H][Pro^9]SP[b]	K_d (nM)	0.3 ± 0.1	0.35 ± 0.02	0.5 ± 0.15	0.35 ± 0.15	0.7 ± 0.1
	B_{max}(fmol/mg of protein)	6500 ± 550	6000 ± 350	6400 ± 800	2570 ± 540	370 ± 80

[a] Incubations performed at 22° for 70 min on whole cells and membrane-bound preparations and at 20° for 90 min in membranes from rat submandibular glands.
[b] Incubations performed at 22° for 100 min on whole cells and 10 min for CHO membranes and rat submandibular gland preparations.

TABLE 3
Comparison of the potencies of different tachykinins
Comparison was made of the ability to compete with [^{3}H]ALIE-124 or [^{3}H][Pro^9]SP and to stimulate both inositol phosphates and cAMP second messenger pathways in intact CHO cells expressing human NK-1 receptors, as described in the text.

Peptide	K_i		Ratio of [^{3}H][Pro^9]SP to [^{3}H]ALIE-124	EC_{50}	
	[^{3}H][Pro^9]SP	[^{3}H]ALIE-124		Inositol phosphates	cAMP
	nM			*nM*	
SP	1.6 ± 0.4[a]	0.22 ± 0.02	7	1.0 ± 0.6[a]	8 ± 2[a]
NK-A	4700 ± 200[a]	4.9 ± 1.1	960	6 ± 2	1240 ± 60[a]
NK-B	>5000[a]	27 ± 5		300 ± 50	>5000[a]
[Pro^9]SP	1.1 ± 0.1[a]	0.15 ± 0.04	7	0.8 ± 0.2	10 ± 2
	1.20 ± 0.04[b]	0.56 ± 0.07[b]	2		
Septide	490 ± 10[a]	2.4 ± 0.5	204	2.7 ± 0.5[a]	5200 ± 200[a]
ALIE-124	4200 ± 300	8.0 ± 0.7	525	37 ± 4	>5000
	250 ± 50[b]	17 ± 4[b]	15		
[Lys^5,methyl-Leu^9,Nle^{10}]NKA(4–10)	5000 ± 1000[a]	68 ± 10	73	170 ± 30	>5000[a]
Senktide	>5000[a]	>5000		>5000[a]	>5000[a]
RP 67580	92 ± 11[a]	15 ± 1	6		

[a] Data from Ref. 12.
[b] Affinity (K_i) measured in membrane preparations from rat submandibular glands.

NK-1 antagonist RP 67580 was a better competitor of [^{3}H]ALIE-124 than of [^{3}H][Pro^9]SP specific binding (Table 3).

As previously found with septide (12), kinetic studies of PLC activation indicated that ALIE-124 (1 μM) and [Pro^9]SP (1 μM) stimulate the hydrolysis of PI with the same rate and that both peptides elicit the same maximal response after a 30-min stimulation. When ALIE-124 and [Pro^9]SP were simultaneously added (each at 1 μM), no additivity was observed between both responses (data not shown). Concentration-response curves of IP formation determined by incubation of the peptides with transfected CHO cells for 8 min showed that ALIE-124 is a weaker agonist (EC_{50} = 37 nM) compared with septide (2.4 nM) and [Pro^9]SP (0.8 nM) (Table 3 and Fig. 2A). However, [Pro^9]SP and ALIE-124 showed different rates and different maximal responses in studies on cAMP accumulation (Fig. 2, B and C). Kinetic analysis of cAMP formation indicated that ALIE-124 responses (10 μM) and [Pro^9]SP responses (1 μM) were partially additive (Fig. 2B). Dose-response studies after 8 min of stimulation with both peptides showed that [Pro^9]SP was highly potent to evoke the formation of cAMP (EC_{50} = 10 nM), whereas ALIE-124, as septide (12), was only a weak stimulator of the cAMP pathway (EC_{50} > 5000 nM) (Fig. 2C and Table 3). Nevertheless, ALIE-124 was not a partial agonist, as demonstrated by additivity experiments, because ALIE-124 (≤100 μM) was unable to antagonize responses to [Pro^9]SP.

In the clone expressing lower amounts of receptor, [Pro^9]SP, septide, and ALIE-124 were unable to stimulate cAMP accumulation, both at concentrations ranging from 1 nM to 1 μM for 10 min and at 10 μM for 1 hr. Nevertheless, in this clone, these three agonists activated IP formation to the same extent. At maximal concentrations (1 μM for [Pro^9]SP and septide and 10 μM for ALIE-124), [Pro^9]SP, septide, and ALIE-124 elicit at the same rate the same maximal response after a 40-min stimulation. The relative potency of these peptides to stimulate IP formation were similar with both clones after 10 min of stimulation. All peptides were slightly less potent (ratio, 2–4), with the clone expressing a lower amount of tachykinin hNK-1 receptor (EC_{50} = 1.55 ± 0.05, 10 ± 3, and 90 ± 10 nM for [Pro^9]SP, septide, and ALIE-124, respectively). With this clone, the responses elicited by septide and ALIE-124 seemed to be biphasic, with flat concentration-response curves (slope values = 0.64 ± 0.04 and

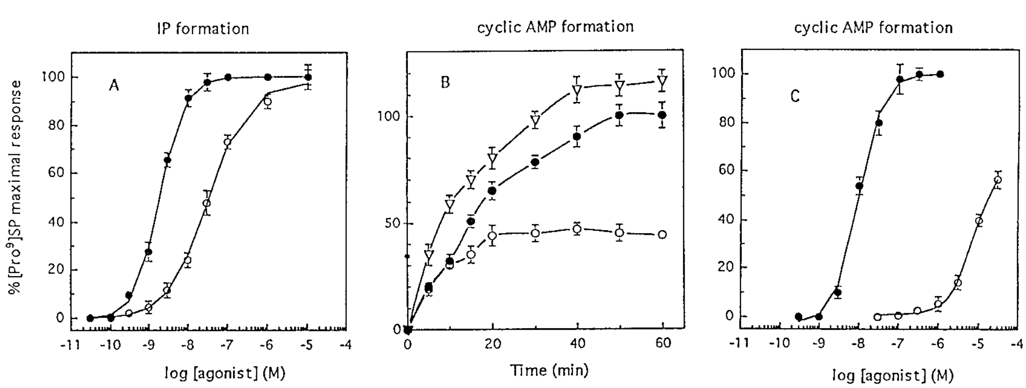

Fig. 2. A, Concentration-response curves of inositol phosphate formation carried out with ALIE-124 (○) or [Pro^9]SP (●) on intact CHO cells (10^5/well), as described in the text. *Curves*, best fit to the mean ± standard error of five different experiments performed in triplicate. B, Time course of cAMP accumulation stimulated by 10 μM ALIE-124 (○) or 1 μM [Pro^9]SP (●) and by 10 μM ALIE-124 and 1 μM [Pro^9]SP added simultaneously (▽). Intact CHO cells (10^5/well) were incubated at 37° for the indicated times. CAMP accumulation was evaluated as mentioned in the text. *Curves*, mean ± standard error of four independent experiments performed in triplicate. C, Concentration-response curves of cAMP formation were carried out on intact CHO cells at 37° for 8 min with either ALIE-124 (○) or [Pro^9]SP (●). *Curves*, best fit to the mean ± standard error of five different experiments performed in triplicate.

0.63 ± 0.05 for septide and ALIE-124, respectively) in contrast to that obtained with [Pro9]SP (slope = 1.30 ± 0.01).

Binding experiments of [3H]ALIE-124 and [3H][Pro9]SP on membrane preparation of submandibular glands. Kage *et al*. (21) have shown by photolabeling that two forms of tachykinin NK-1 receptors were present in membranes from submandibular glands. Therefore, binding experiments were carried out with [3H]ALIE-124 and [3H][Pro9]SP in this tissue. In rat submandibular gland, specific binding accounted for 68% of total binding. Kinetic parameters of [3H]ALIE-124 specific binding in membranes from submandibular glands were also similar to those obtained with membrane preparation of CHO cells transfected with tachykinin hNK-1 receptor. With membranes from rat submandibular glands, high concentrations of peptide inhibitors were used to prevent enzymatic degradation of [3H]ALIE-124. With these conditions, degradation of the radioligand was limited to 15% during the 90-min incubation. The binding of [3H][Pro9]SP was faster than that of [3H]ALIE-124 to membranes of submandibular glands, as for membranes from CHO cells transfected with the tachykinin hNK-1 receptor. Saturation experiments with [3H][Pro9]SP and [3H]ALIE-124 showed that both radioligands bound to a single high affinity binding site (Table 2). K_d values for both [3H][Pro9]SP and [3H]ALIE-124 were similar to those obtained with membranes of transfected CHO cells (K_d = 0.7 and 11 nM, respectively).

Competition studies showed that the potency of ALIE-124 for [3H]ALIE-124 specific binding sites (K_d = 17 nM) was similar to that observed on CHO cells transfected with tachykinin hNK-1 receptor. When [3H][Pro9]SP was the radioligand, the potency of ALIE-124 was higher than that seen in CHO cells (K_d = 250 and 4200 nM, respectively) (Table 3). In this tissue, the maximal binding capacities for both radioligands were also different (B_{max} = 370 and 160 fmol/mg of proteins, for [3H][Pro9]SP and [3H]ALIE-124 specific binding sites, respectively) (Table 2). In membranes from submandibular glands, the proportion of [3H]ALIE-124 specific binding sites was higher compared with that found in membranes from CHO cells transfected with the tachykinin hNK-1 receptor (Table 2).

Discussion

We developed a new radioligand, [3H]ALIE-124, which allowed the demonstration, for the first time, of the presence of a specific binding site for septide-like peptides. This site was found in membranes prepared from both CHO cells transfected with the tachykinin hNK-1 receptor and rat submandibular glands. This specific binding site for [3H]ALIE-124 was also detected on intact CHO cells transfected with the tachykinin hNK-1 receptor, proving that it was not an artefact originating from membrane preparation. In membrane preparations from both tissues, kinetics of association/dissociation were very fast for [3H][Pro9]SP and rather slow for [3H]ALIE-124. This difference in behavior was not observed with intact CHO cells because both ligands reached their respective plateaus with similar slow kinetics regardless of whether the cells were untreated or treated with tunicamycin to prevent glycosylation (15).

Although the pharmacological analysis remains preliminary, septide-like molecules showed high affinity for the specific binding site labeled with [3H]ALIE-124 (i.e., septide, ALIE-124, and NKA). This result raises the question as to whether, under physiological conditions, NKA can interact on NK-1 receptors, because NKA is coreleased with SP. The potency of the nonpeptide antagonist RP 67580 was 6-fold higher for [3H]ALIE-124 than for [3H][Pro9]SP specific binding site. Previously, RP 67580 was shown to be 6-fold more potent in inhibiting septide than [Pro9]SP stimulation of the PLC pathway (12). However, the higher potency of [Pro9]SP and SP for [3H]ALIE-124 specific binding site compared with that labeled with [3H][Pro9]SP was unexpected. This finding may, however, explain some of fluctuations in IC_{50} values obtained, in various tissues, with tachykinins and carboxyl-terminal fragments of SP (22, 23). Indeed, the high affinity binding site for SP (i.e., [3H]ALIE-124 site) might be preferentially labeled with the lowest concentrations of ^{125}I-labeled SP that could be used. In contrast, affinities obtained with tritiated SP analogues might correspond to the labeling of both specific binding sites, and differences in affinities should be expected, depending on the ratio of these sites. For instance, in competition experiments with [3H][Pro9]SP, ALIE-124 had a higher potency in membrane preparations from rat submandibular glands (B_{max} ratio between [3H]ALIE-124 and [3H][Pro9]SP = close to 1:2) than in CHO cells (ratio between both sites = 1:7).

Noteworthy, with the seven active agonists used with CHO cells transfected with the tachykinin hNK-1 receptor, an excellent correlation was found between K_d values for the [3H]ALIE-124 specific binding site and EC_{50} values on IPs production (r = 0.97) (Fig. 3). In contrast, as previously established, the K_d values of these analogues for the [3H][Pro9]SP specific binding sites in these CHO cells correlate with their EC_{50} values for cAMP formation (13).

Saturation studies showed different binding capacities for both radioligands. The undecapeptide [3H][Pro9]SP always has higher binding capacity compared with [3H]ALIE-124. According to respective B_{max} values, specific sites labeled by [3H]ALIE-124 in comparison with [3H][Pro9]SP varied from 11% (intact transfected CHO cells) to 22% (CHO membranes) to 43% (membranes from rat submandibular glands). In in-

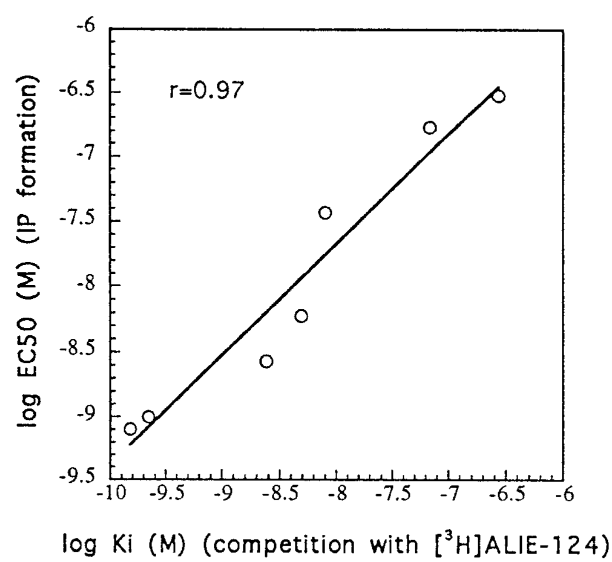

Fig. 3. Correlation between the binding affinities for [3H]ALIE-124 specific binding site (log K_d) on intact CHO cells transfected with hNK-1 receptors and log (EC_{50} values) for PI hydrolysis of SP, [Pro9]SP, NKA, NKB, septide, ALIE-124, and [Lys5,methyl-Leu9,Nle10]NKA(4–10).

tact CHO cells transfected with the tachykinin NK-1 receptor, the percentage of [^{3}H]ALIE-124 specific binding sites was not altered by the extent of glycosylation. Because [Pro^9]SP is ~10-fold more potent on [^{3}H]ALIE-124- than on [^{3}H][Pro^9]SP- specific binding, [^{3}H][Pro^9]SP must also label [^{3}H]ALIE-124 specific binding sites. Thus, the relative proportion of [^{3}H][ALIE-124 specific binding sites versus those labeled with [^{3}H][Pro^9]SP should be slightly higher in all tissues.

If different binding sites for tachykinin NK-1 agonists and septide-like molecules were located on the same NK-1 receptor protein, the maximal binding capacities for both radioligands should have been the same (24). At least two hypotheses can be proposed to explain these differences in B_{max} values: the presence of two pools or the presence of two forms of NK-1 receptor proteins.

One explanation for the different binding capacities for both radioligands could be the presence of two pools of NK-1 receptor protein that do not exchange; if they do, the equilibrium would be far beyond the 2-hr incubation time. Because, in CHO cells, tachykinin NK-1 agonists such as SP and [Pro^9]SP stimulate the cAMP pathway but septide-like molecules do not activate G_s (12), we speculated that high levels of G_s proteins tightly precoupled to tachykinin NK-1 receptors might have hampered this equilibrium. According to this hypothesis, part of the [^{3}H][Pro^9]SP specific binding sites would be "quenched" as a complex either by high concentrations of G_s and/or because of an unusual high affinity for G_s. [^{3}H]ALIE-124 specific binding sites would correspond to high affinity/low capacity NK-1 receptor precoupled to $G_{q/11}$ proteins. Conversely, [^{3}H][Pro^9]SP specific binding would represent a lower affinity/higher capacity NK-1 receptor precoupled to G_s proteins. This hypothesis may be ruled out for two reasons. First, B_{max} values for both [^{3}H][Pro^9]SP and [^{3}H]ALIE-124 remained unchanged after preincubation of CHO cells with cholera toxin (25), a treatment that uncoupled receptor and G_s proteins. Second, similar discrepancies in the B_{max} values were obtained with the clone expressing lower level of receptors, although the tachykinin hNK-1 receptor is no longer coupled to G_s, both [Pro^9]SP and septide being unable to stimulate cAMP accumulation.

A second explanation, which is in agreement with reports in the literature, could be the existence of two forms of NK-1 receptor protein. These forms may arise from either a defective palmitoylation or a carboxyl-terminal truncation of the NK-1 receptor protein. In CHO cells, palmitoylation of the transfected tachykinin NK-1 receptor could be incomplete, yielding two forms of NK-1 receptors that might preferentially activate different intracellular pathways. This selectivity has already been shown with mutated endothelin receptor A (26). The nonpalmitoylated receptor still activates cAMP turnover but is unable to stimulate PLC activation, unlike the wild-type endothelin receptor A, which activates both pathways (26). This hypothesis cannot be rejected. However, B_{max} values are also different for both radioligands in membrane from rat submandibular glands, and to our knowledge, defective palmitoylation has yet not been reported in mammalian tissues.

Kage *et al.* (21) previously demonstrated the existence of a long and a carboxyl-terminal truncated form of NK-1 receptor by photolabeling experiments in rat submandibular glands. In rat submandibular glands, Mantyh *et al.* (27) found only low levels of NK-1 receptor immunoreactivity in contrast to high levels of ^{125}I-SP specific binding sites. According to the specificity of the antibodies, this may indicate that the expression of a carboxyl-terminal truncated form of NK-1 receptor predominates in this tissue, corroborating the photolabeling experiment (21). Interestingly, we found high concentrations of [^{3}H]ALIE-124 labeling in rat submandibular glands, the ratio of [^{3}H]ALIE-124 versus [^{3}H][Pro^9]SP specific binding sites being close to 1:2. In CHO cells transfected with cDNA of tachykinin hNK-1 receptor, this ratio was much lower (1:9). Even with high levels of transfection, the B_{max} value of [^{3}H]ALIE-124 specific binding site was probably still too low to be detected by photolabeling with a tritiated photoreactive analogue of [Pro^9]SP, accounting for the results of our previous study (15).

However, the key point is that this new specific binding site for tachykinins is present in both mammalian tissue and intact cells transfected with the cDNA of tachykinin NK-1 receptors.

While this article was being reviewed for publication, Hastrup and Schwartz (28) reported binding data obtained with ^{125}I-NKA and [^{3}H]septide. They found that both radioligands bound to the tachykinin hNK-1 receptor expressed in COS-7 cells, also yielding B_{max} values different from those obtained with ^{125}I-Bolton Hunter SP. Binding data from homologous and heterologous competition studies with SP, NKA, and septide are provided. The authors concluded with the necessity to perform homologous binding assays to determine the "actual binding affinity" of a ligand for a receptor. However, no interpretation was given for the large discrepancies in B_{max} values.

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Send reprint requests to: Dr. Solange Lavielle, Laboratoire de Chimie Organique Biologique, URA CNRS 493, Université Paris 6, 4, place Jussieu, 75252 Paris Cedex 05, France. E-mail: lavielle@ccr.jussieu.fr