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Characterization of the Binding of a Potent, Selective, Radioiodinated Antagonist to the Human Neurokinin-1 Receptor

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SUMMARY

We have synthesized a potent, selective, radioiodinated antagonist of the human neurokinin-1 (NK1) receptor and have characterized its binding to the cloned receptor expressed in Chinese hamster ovary cells. (*cis*)-2-(Diphenylmethyl)-*N*-[(2-iodophenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine (L-703606) inhibits binding of ^{125}I-Tyr^{8}-substance P to the human NK1 receptor with an IC_{50} of 2 nM. This compound is a competitive antagonist of substance P-induced inositol phosphate generation, with a K_b of 29 nM. [^{125}I]L-703606 binds to a single class of high affinity binding sites in human NK1/Chinese hamster ovary cell membranes (K_d = 0.3 nM). Substance P inhibits the binding of [^{125}I]L-703606 to 65% of the NK1 receptor sites with a K_d of 0.04 ± 0.03 nM and to the remaining 35% of the sites with a K_d of 1.5 ± 0.7 nM. Addition of the nonhydrolyzable GTP analog guanylyl-5′-(β,γ-imido)diphosphate [Gpp(NH)p] shifts >90% of the binding sites to the lower affinity state. In addition, Gpp(NH)p markedly alters the dissociation of substance P from the NK1 receptor by increasing the number of sites in the low affinity, rapidly dissociating state. However, Gpp(NH)p does not affect the rate of dissociation of [^{125}I]L-703606. These data suggest that the pharmacological properties of [^{125}I]L-703606 binding to the human NK1 receptor are similar to those of antagonists of nonpeptide guanine nucleotide-binding protein-coupled receptors and that this ligand will be useful for the biochemical and pharmacological characterization of the human NK1 receptor.

The tachykinins are a family of peptides that share a common carboxyl-terminal sequence (Phe-X-Gly-Leu-Met-NH_2) and that exert their physiological activities through activation of three subtypes of NK receptors. The mammalian tachykinins SP, NKA, and NKB[1] preferentially bind to the NK1, NK2, and NK3 receptors, respectively. The sequences for these three receptor subtypes from the rat have been determined by molecular cloning (1–4). More recently, several groups have cloned and characterized the human NK receptors (5–11).

The pharmacology of the NK receptors has been characterized extensively by using radiolabeled agonists that are selective for one or more of the receptor subtypes (12–16). However, pharmacological and biochemical analyses of these receptors have been hampered by the lack of selective, high affinity, high specific activity, radiolabeled antagonists. Recently, two nonpeptide antagonists that are selective for the NK1 receptor have been described (17, 18). The first of these, CP-96,345, has been radiolabeled with tritium and used to identify a binding site in guinea pig striatal membranes and to localize [^{3}H]CP-96,345 binding sites in guinea pig brain by autoradiography (19).

In the present report, we have synthesized a radioiodinated analog of CP-96,345 that is a potent selective antagonist at the human NK1 receptor. We have characterized the binding of this analog, [^{125}I]L-703606, to the cloned human NK1 receptor, heterologously expressed in CHO cells, and we have compared the properties of its binding site with those of the binding site for ^{125}I-Tyr^{8}-SP. The results indicate that the pharmacology and G protein coupling of the NK1 receptor, which is a peptide-binding G protein-coupled receptor, are similar to those of the receptors for small biogenic amines, such as the adrenergic and muscarinic receptors. This radioligand will be useful in the biochemical characterization of the NK1 receptor and its interaction with G proteins.

[1] The sequences for the mammalian tachykinins are as follows: SP, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2; NKA, His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2; NKB, Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2.

Materials and Methods

Synthesis of [^{125}I]L-703606. The trimethylsilyl precursor (*cis*)-2-(diphenylmethyl)-3-[2-(trimethylsilyl)benzylamino]-1-azabicyclo-

ABBREVIATIONS: NK, neurokinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-703606, (*cis*)-2-(diphenylmethyl)-*N*-[(2-iodophenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine; Gpp(NH)p, guanylyl-5′-(β,γ-imido)diphosphate; SP, substance P; CHO, Chinese hamster ovary; G protein, guanine nucleotide-binding protein; ^{125}I-SP, ^{125}I-Tyr^{8}-substance P; PMSF, phenylmethylsulfonyl fluoride.

[2.2.2]octane was prepared by reacting 7.2 mmol of (*cis*)-3-amino-2-(diphenylmethyl)-1-azabicyclo[2.2.2]octane (20) and 7.1 mmol of 2-(trimethylsilyl)benzaldehyde (21) in 30 ml of methanol containing 500 mg of powdered molecular seives. Citric acid (3.4 mmol) and sodium borohydride (7 mmol) were added. After 1 hr, the mixture was filtered, the methanol was evaporated, and the residue was extracted with dichloromethane/sodium bicarbonate. The organic layer was washed with brine, dried, and evaporated. After purification over alumina, the trimethylsilyl precursor was characterized by NMR and elemental analysis. This precursor (0.5 mg) was reacted with 5 mCi of Na^{125}I (IMS 30; Amersham), in the presence of an Iodobead (Pierce), in 50 μl of trifluoroacetic acid. After 45 min at room temperature, the reaction was neutralized with ammonium hydroxide and the products were purified using a C18 Vydac column, with elution with acetonitrile/0.1 M ammonium formate, pH 3 (1:1). The [^{125}I]L-703606 product peak was collected, the solvent was removed by Speed Vac evaporation, and the dried product was dissolved in ethanol. The radiochemical purity was >95%, and the specific activity of several syntheses was at least 200–400 Ci/mmol.

Expression of human NK1 receptors in CHO cells. A stable CHO cell line expressing 1 × 10^5 human NK1 receptors/cell was selected from neomycin-resistant clones by ^{125}I-SP binding, after transfection with a vector containing a human NK1 receptor expression cassette (5). Cell lines were selected and maintained in Iscove's modified Dulbecco's medium containing 25 mM HEPES, 2 mM L-glutamine, penicillin-streptomycin (1:100; GIBCO), 10% fetal bovine serum (heat-inactivated), hypoxanthine-thymidine (1:500; American Type Culture Collection), and 1 mg/ml G418.

Binding of ^{125}I-Tyr^8-SP to human NK1 receptors. Human NK1/CHO cells (4 × 10^6 cells/ml) were lysed and homogenized in 1 mM Tris·HCl, pH 7.5, containing 0.1 mM PMSF, 2 μg/ml pepstatin, 40 μg/ml bacitracin, 10 μM phosphoramidon, and 2 μg/ml leupeptin. The homogenate was centrifuged at 1000 × *g* for 10 min, and the resulting supernatant was centrifuged at 38,000 × *g* for 1 hr. The pellet was suspended at 2–10 mg/ml and stored at −70° before use. Ligand binding was performed in 0.25 ml of 50 mM Tris·HCl, pH 7.5, containing 5 mM $MnCl_2$, 0.02% bovine serum albumin (Sigma), 50 μg/ml chymostatin (Peninsula), 0.1 mM PMSF, 2 μg/ml pepstatin, and 2 μg/ml leupeptin. ^{125}I-Tyr^8-SP (0.1 nM, 2200 Ci/mmol; New England Nuclear)[2] was incubated with membranes (1–2 μg) for 40 min at room temperature and then filtered over GF/C filters that had been presoaked in 0.1% polyethylenimine, using a Tomtec 96-well harvester. The steady state plateau of ^{125}I-SP binding was reached after 30 min and was stable for at least 2 hr. Nonspecific binding was determined using excess SP (1 μM) and was <10% of total ligand binding to membranes.

Binding of [^{125}I]L-703606 to human NK1 receptors. Ligand (75–300 pM, >200–400 Ci/mmol) was incubated with 1–2 μg of human NK1/CHO cell membranes in 50 mM Tris·HCl, pH 7.5, 1 mM $MnCl_2$, 0.02% bovine serum albumin, 40 μg/ml bacitracin, 4 μg/ml leupeptin, 10 μM phosphoramidon, for 20 min at room temperature. The steady state plateau of [^{125}I]L-703606 binding was reached after 20 min and remained stable for at least 2 hr. Peptidase inhibitors had minimal effects on the specific binding of [^{125}I]L-703606 but were included to prevent the degradation of SP and other peptides during competition experiments. The reaction was terminated by filtration over GF/D filters, as described above. Approximately 2% and 5–10% of the ligand bound to the filters in the absence and presence of human NK1/CHO membranes, respectively. Nonspecific binding was determined in the presence of excess (1 μM) L-703606 and was <10% of the total ligand binding to the membranes.

Dissociation of ^{125}I-SP from the human NK1 receptor. Membranes were incubated with ^{125}I-SP for 40 min at room temperature. Dissociation was initiated by the addition of excess unlabeled SP (100 nM), in the absence or presence of antagonist or nucleotide as indicated. Dissociation was assayed at 15° at times from 10 sec to 2 hr after the addition of SP. The data are expressed as the logarithm of the ratio of bound ^{125}I-SP at a given time after SP addition to bound ^{125}I-SP at equilibrium. The amount of ^{125}I-SP binding in the absence of excess unlabeled SP remained constant over the course of the dissociation phase of the experiment.

Dissociation of [^{125}I]703606 from the human NK1 receptor. Membranes were incubated with [^{125}I]L-703606 for 20 min at room temperature. Dissociation was initiated by the addition of excess unlabeled L-703606 (100 nM), in the absence or presence of SP (100 nM) or Gpp(NH)p (100 μM). Dissociation was assayed at 15° at times from 10 sec to 2 hr after the addition of unlabeled L-703606.

SP-induced inositol phosphate production. The assay was performed as described by Berridge *et al.* (22). Cells (2.5 × 10^5) were plated in 12-well tissue culture dishes and grown to confluence. Cells were prelabeled with *myo*-[2-^{3}H]inositol (19 Ci/mmol, 10 μCi/2 ml; Amersham) for 24 hr. The cells were washed twice and then incubated with 10 mM LiCl, in the presence or absence of L-703606, for 15 min at 37°, in 50 mM HEPES containing 138 mM NaCl, 5.4 mM KCl, 4 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 0.02% bovine serum albumin, 0.1 mM PMSF, 50 μg/ml chymostatin, and 10 μM phosphoramidon. SP was added and the incubation was continued for another 30 min. The reaction was terminated by removal of the medium and addition of 0.1 N HCl. The cells were sonicated and the homogenate was extracted with chloroform/methanol (1:1). The upper phase was loaded onto Dowex AG 1-X8, which was washed sequentially with 0.1 N formic acid and 0.025 M ammonium formate/0.1 N formic acid. Inositol monophosphate was eluted with 0.2 M ammonium formate/0.1 N formic acid and counted.

Results

^{125}I-Tyr^8-SP binds with high affinity to the human NK1 receptor expressed in CHO cells. Incubation of membranes prepared from these cells with increasing concentrations of ^{125}I-SP results in saturable binding, with a K_d of 0.125 ± 0.006 nM (mean ± standard deviation, three experiments), and Scatchard analysis of these data gives a linear fit. A representative experiment is shown in Fig. 1. Scatchard analysis of the binding of ^{125}I-SP to intact cells is also linear, with a K_d of 0.35 nM and a B_{max} of 1 × 10^5 receptors/cell (data not shown).

SP inhibits the binding of ^{125}I-SP to its receptor with an IC_{50} of 1 nM. NKA and NKB also inhibit this binding, with 15-fold and 50-fold lower affinities, respectively (Fig. 2), consistent with the expression of an NK1 receptor subtype. The antagonist CP-96,345 inhibits the binding of ^{125}I-SP to the human NK1 receptor with an IC_{50} of 0.5 nM. Replacement of the *ortho*-methoxy substituent of CP-96,345 with ^{127}I results in a compound (L-703606) that inhibits ^{125}I-SP binding with an IC_{50} of 2 nM (Fig. 2). In contrast, L-703606 (1 μM) does not inhibit the binding of ^{125}I-Bolton Hunter-labeled NKA or ^{125}I-Bolton Hunter-labeled eledoisin to the cloned human NK2 and NK3 receptors, respectively (data not shown), consistent with the NK1 specificity previously demonstrated for CP-96,345 (17). In addition, L-703606 has a markedly reduced affinity for the cloned rat NK1 receptor expressed in CHO cells (K_d = 0.3 μM). A similar species selectivity has been observed for CP-96,345, using pharmacological analysis (23).

In human NK1/CHO cells, increasing concentrations of L-703606 shift the dose-response for SP-induced inositol phosphate production progressively to the right, without altering

[2] Although earlier experiments, including those done in our own laboratory, showed that ^{125}I-Tyr^8-SP Bolton-Hunter-SP was the preferred ligand for SP (NK1) receptors, the ^{125}I-Tyr^8-SP currently commercially available binds NK1 receptors with high affinity and low nonspecific binding. In our laboratories, both ligands are used interchangeably and with equally good results on the cloned receptors and in rat brain cortex membranes.

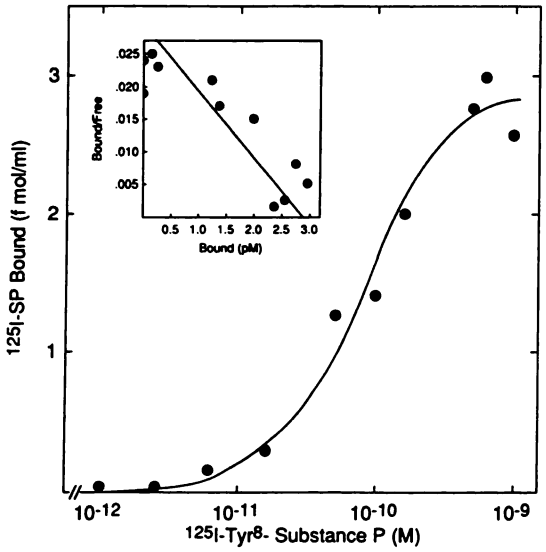

Fig. 1. Saturation binding analysis of ^{125}I-SP binding to the human NK1 receptor. Membranes (2 μg) were incubated with various concentrations of ^{125}I-SP (1 pM to 1.5 nM), as described in Materials and Methods, except that 10 μM SP was used to determine the amount of nonspecific binding. The data were analyzed using LIGAND (26), as purchased from Biosoft. *Inset*, Scatchard analysis of these data.

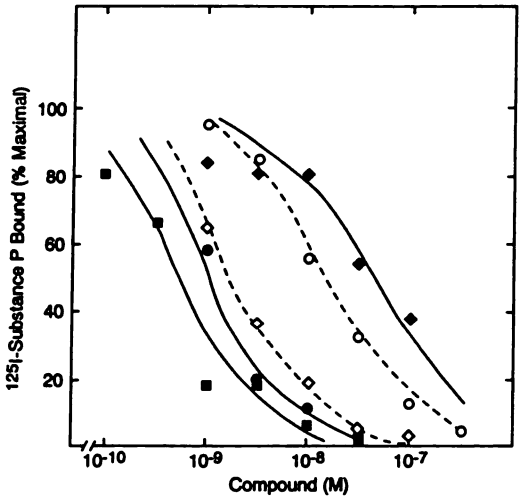

Fig. 2. Inhibition of ^{125}I-SP binding to intact human NK1/CHO cells by SP (●), NKA (○), NKB (♦), L-703606 (◇), and CP-96,345 (■). ^{125}I-SP (0.1 nM) and cells were incubated in the presence or absence of competing peptides or antagonists, as described in Materials and Methods. Data points are the average of two experiments, with each point done in duplicate.

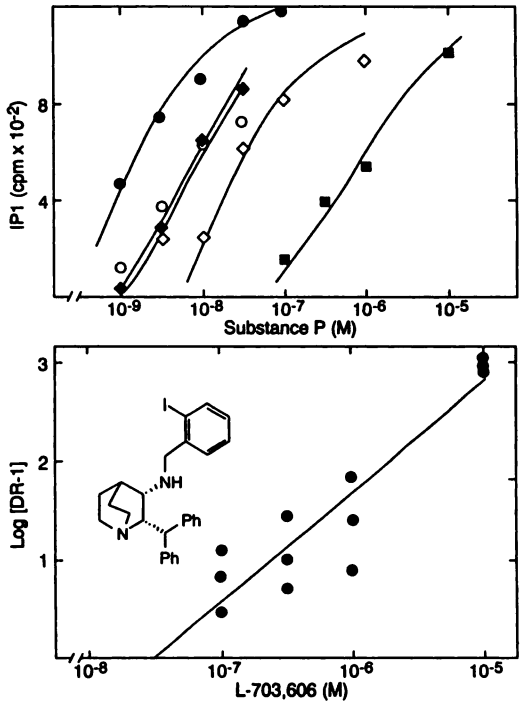

Fig. 3. Inhibition of SP-induced inositol monophosphate (*IP1*) generation in human NK1/CHO cells by L-703606. *Top*, dose-response curves for SP in the absence (●) or presence of 0.1 μM (○), 0.3 μM (♦), 1 μM (◇), or 10 μM (■) L-703606. *Bottom*, Schild analysis of the data at three response levels. *Inset*: Structure of L-703606.

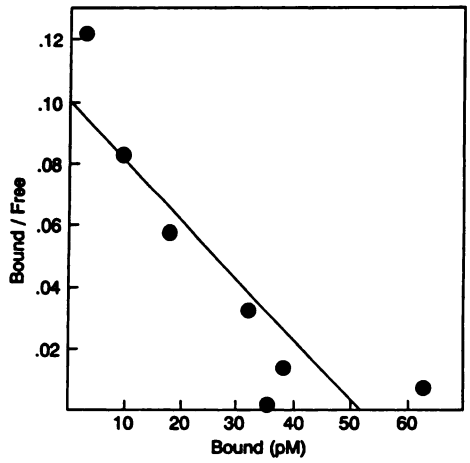

Fig. 4. Scatchard analysis of the binding of L-703606 to the human NK1 receptor. Membranes (1.6 μg) prepared from human NK1/CHO cells were incubated with [^{125}I]L-703606 (300 pM) in the presence or absence of unlabeled L-703606 (0.1–100 nM), as described in Materials and Methods. The data were analyzed using LIGAND (26), as purchased from Biosoft.

the maximum SP response attained (Fig. 3). Schild analysis of these data gives a straight line, with a slope of 1.1 and a K_b of 29 nM, consistent with competitive antagonism of the NK1 receptor by L-703606.

L-703606 was synthesized with Na^{125}I, to a specific activity of >200–400 Ci/mmol, and this ligand was used to further characterize agonist and antagonist binding to the human NK1 receptor. [^{125}I]L-703606 shows specific saturable binding to membranes from human NK1/CHO cells. Scatchard analysis of the data gives a linear curve, consistent with a single class of binding sites, with a K_d of 0.5 nM and a B_{max} of 2–10 pmol/mg of protein in several membrane preparations (Fig. 4). SP and CP-96,345 inhibit binding of [^{125}I]L-703606 to the human NK1 receptor with IC_{50} values of 0.3 nM and 0.2 nM, respectively (Table 1). The relative potencies of agonists and antagonists for inhibiting ^{125}I-SP and [^{125}I]L-703606 binding to human NK1 receptors are summarized in Table 1.

The nonhydrolyzable GTP analog Gpp(NH)p inhibits 60% of the binding of the agonist ^{125}I-SP to the human NK1 receptor (IC_{50} = 500 nM) but does not inhibit the binding of the antagonist [^{125}I]L-703606 (Fig. 5).

The inhibition of [^{125}I]L-703606 binding to the human NK1 receptor by SP is best described by a two-site fit, in which 65% of the sites have high affinity for SP (Fig. 6). In the presence

TABLE 1

Comparison of agonist and antagonist potencies for inhibition of ^{125}I-SP and [^{125}I]L-703606 binding to the human NK1 receptor

	IC_{50}	
	^{125}I-SP[a]	[^{125}I]L-703606
	nM	
SP	1	0.3
NKA	15	>100
NKB	50	>100
CP-96,345	0.5	0.2
L-703606	2	0.3

[a] Data derived from Fig. 2.

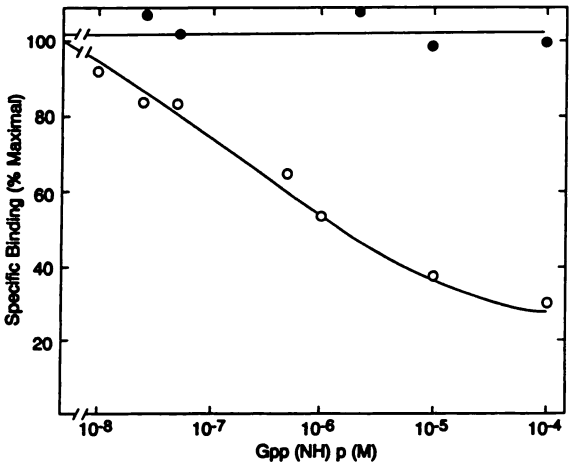

Fig. 5. Inhibition by Gpp(NH)p of [^{125}I]L-703606 binding (●) and ^{125}I-SP binding (○) to the human NK1 receptor. Membranes (1.6 μg) were incubated with ligands, as described in Materials and Methods, in the presence or absence of the indicated concentrations of Gpp(NH)p.

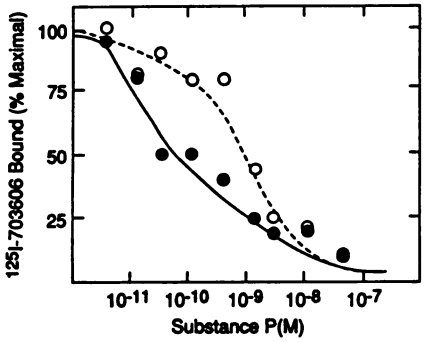

Fig. 6. Inhibition of the binding of [^{125}I]L-703606 to the human NK1 receptor by SP, in the presence (○) or absence (●) of Gpp(NH)p (100 μM). [^{125}I]L-703606 (300 pM) and membranes (1.6 μg) were incubated as described in Materials and Methods, in the presence or absence of SP and Gpp(NH)p. Data were analyzed using LIGAND (26), as purchased from Biosoft.

of 100 μM Gpp(NH)p, >90% of the receptors are shifted to the state having low affinity for SP. In four experiments of this type, SP has a K_d of 0.04 ± 0.03 nM (mean ± standard deviation) for the high affinity site and a K_d of 1.5 ± 0.7 nM for the low affinity site observed in the presence of Gpp(NH)p.

To analyze further the effects of guanine nucleotides on the NK1 receptor, the dissociation kinetics of agonists and antagonists were examined in the presence and absence of Gpp(NH)p. Gpp(NH)p (10 μM) increases the rate of dissociation of ^{125}I-SP from the human NK1 receptor (Fig. 7). In the

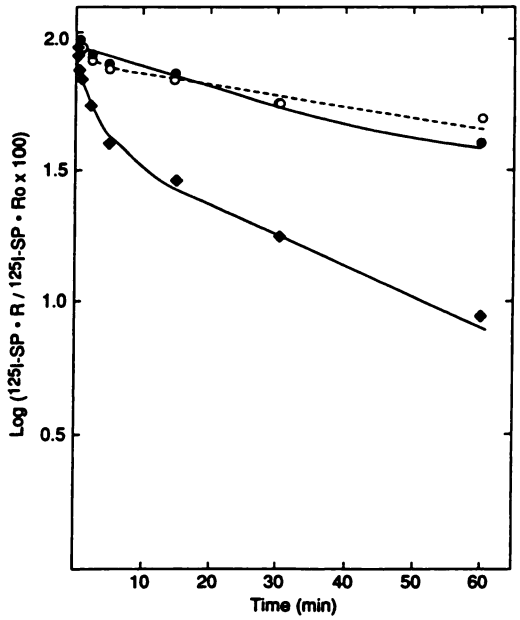

Fig. 7. Dissociation of ^{125}I-SP from the human NK1 receptor in the absence or presence of L-703606 or Gpp(NH)p. Dissociation was initiated by addition of 100 nM SP in the absence (●) or presence of 100 nM L-703606 (○) or 10 μM Gpp(NH)p (♦). Data were analyzed as described by Duggleby (27), and theoretical curves were generated using the formula $Y = R_1e^{-(X \cdot k-1)} + R_2e^{-(X \cdot k-2)}$, where Y is the ratio ^{125}I-SP·R/^{125}I-SP·R_o, X is time, R_1 and R_2 are the percentages of receptors in the low and high affinity states, and k_{-1} and k_{-2} are the dissociation rates from the low and high affinity sites, respectively. Data points are the average of two separate experiments.

absence of guanine nucleotide, ^{125}I-SP has a biphasic dissociation curve, with dissociation rates of 0.015 ± 0.001 min^{-1} and 4 ± 4 min^{-1} for the high and low affinity sites, respectively. In the membrane preparation shown in Fig. 7, 90% of the sites are in the high affinity, slowly dissociating state, although this parameter varies from 50 to 90% among different membrane preparations. The major effect of Gpp(NH)p is to decrease the percentage of sites in the slowly dissociating state to 45%, although there is also a small effect on the dissociation rates. L-703606 does not significantly affect the rate of dissociation of ^{125}I-SP from its receptor, consistent with a competitive mechanism of antagonism.

In contrast to the biphasic kinetics observed for the dissociation of ^{125}I-SP from the receptor, [^{125}I]L-703606 dissociates from the human NK1 receptor with a single exponential rate (k = 0.085 min^{-1}) (Fig. 8). The rate of dissociation of [^{125}I]L-703606 from the receptor is not affected by the addition of either Gpp(NH)p (k = 0.081 min^{-1}) or SP (k = 0.080 min^{-1}).

Discussion

In order to obtain a radiolabeled antagonist with higher specific activity than [^{3}H]CP-96,345, we have prepared an iodinated analog of this NK1 antagonist. The quinuclidine L-703606 is a high affinity, selective antagonist of the human NK1 receptor. It displays nanomolar affinity for the human NK1 receptor but >1000-fold lower affinity for the human NK2 and NK3 receptors. As observed *in vivo*, the NK1 receptor expressed in CHO cells is coupled to the phospholipase C second messenger pathway, which leads to inositol phosphate synthesis, and L-703606 functions as a competitive antagonist

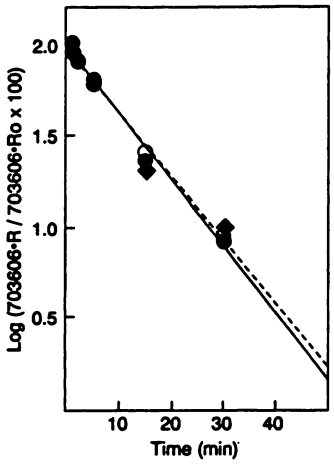

Fig. 8. Dissociation of [^{125}I]L-703606 from the human NK1 receptor. Dissociation was initiated by the addition of 100 nM L-703606 in the absence (●) or presence of 100 nM SP (♦) or 100 μM Gpp(NH)p (○). The data were analyzed using the first-order rate equation $\log([^{125}I]L\text{-}703606 \cdot R/[^{125}I]L\text{-}703606 \cdot R_o) = k/2.3t$. Data points are the average of at least two separate experiments.

of SP-induced inositol phosphate production in human NK1/CHO cells. In kinetic experiments, L-703606 has no effect on the dissociation rate of SP from the human NK1 receptor and SP has no effect on the dissociation rate of L-703606. These data indicate that L-703606 is a competitive antagonist of the human NK1 receptor.

In intact cells, [^{125}I]L-703606 interacts with a low affinity, high capacity, uptake system that is not related to the NK1 receptor, because the binding is observed in nontransfected cells and is not inhibited by SP. Therefore, its interaction with the human NK1 receptor was analyzed in membrane preparations. [^{125}I]L-703606 binds with high affinity to membranes prepared from cells expressing the human NK1 receptor. This binding is inhibited by SP at 0.3 nM and by NKA and NKB with >100-fold lower affinity, consistent with the NK1 receptor subtype. The NK1 receptor antagonist CP-96,345, but not its inactive stereoenantiomer, is a high affinity inhibitor of [^{125}I]L-703606 binding. The relative potencies of these compounds are the same as their relative potencies in inhibiting ^{125}I-SP binding, indicating that the [^{125}I]L-703606 binding site is the human NK1 receptor.

The sequence of the human NK1 receptor predicts that it is a member of the family of G protein-coupled receptors (6–8). For G protein-coupled receptors that bind small molecules, notably the adrenergic and muscarinic receptors, high affinity competitive antagonists have long been available. Radiolabeled antagonists have been useful probes to characterize the ligand-binding parameters of these receptors. For the adrenergic and muscarinic receptors, these experiments have shown that association with agonist promotes the formation of a high affinity ternary complex of agonist, receptor, and G protein. The formation of this G protein-coupled receptor population is attenuated by addition of guanine nucleotides. Thus, agonists bind to the receptor with higher affinity in the absence than in the presence of GTP. In contrast, antagonists bind to the receptor with a single affinity that is independent of G protein coupling. These observations have not been extended to G protein-coupled receptors that bind peptides, because of the lack of radiolabeled antagonist probes. Thus, [^{125}I]L-703606 provides a new tool with which to probe the mechanism of G protein coupling to the NK receptor.

It was previously shown that GTP and its nonhydrolyzable analogs inhibited the binding of ^{125}I-SP to various tissues (24, 25). In the present study, we have found that Gpp(NH)p inhibits binding of the agonist ^{125}I-SP to the human NK1 receptor but does not inhibit binding of the antagonist [^{125}I]L-703606. As shown by the dissociation experiments in Fig. 7, the inhibition of SP binding by Gpp(NH)p is due to an increase in the percentage of the receptor population in the low affinity, rapidly dissociating state.

This is also observed in equilibrium binding experiments using [^{125}I]L-703606. SP binds to the receptor with two classes of sites. The K_d for the high affinity site (0.04 ± 0.03 nM) agrees with the K_d of ^{125}I-SP for the receptor (0.1 nM). As observed in the dissociation experiments, Gpp(NH)p increases the percentage of the receptor population in the low affinity, G protein-uncoupled state. The uncoupled receptor has 200-fold lower affinity for SP, and the dissociation rate of ^{125}I-SP is 100–200-fold greater for this form of the receptor, similar to the affinity shifts observed for other G protein-coupled receptors.

The use of [^{125}I]L-703606 as a probe for the human NK1 receptor has enabled us to characterize fully the effects of G protein coupling on the agonist-binding properties of this receptor. The availability of a high affinity, radioiodinated antagonist should permit more detailed biochemical and pharmacological characterization of the human NK1 receptor.

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