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Characterization of the binding and activity of a high affinity, pseudoirreversible morpholino tachykinin NK_1 receptor antagonist

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Abstract

2(*S*)-((3,5-Bis(trifluoromethyl)benzyl)-oxy)-3(*S*)-phenyl-4-((3-oxo-1,2,4-triazol-5-yl)methyl)morpholine (L-742,694) is a selective morpholino tachykinin NK_1 receptor antagonist that inhibits the binding of ^{125}I-substance P to the human tachykinin NK_1 receptor with a $K_d = 37$ pM. Increasing concentrations of L-742,694 added to cells 15 min prior to agonist progressively increase the apparent EC_{50} of substance P for inducing the synthesis of inositol phosphate in Chinese hamster ovary (CHO) cells expressing human tachykinin NK_1 receptor and decrease the maximal level of stimulation observed. In contrast, addition of substance P and L-742,694 to the cells at the same time results in an increase in the EC_{50} for substance P with no decrease in the maximal level of stimulation. The compound also decreases the apparent number of binding sites for ^{125}I-substance P observed by Scatchard analysis. Analysis of the binding of [^{3}H]L-742,694 to the tachykinin NK_1 receptor shows that it associates with the receptor with $k_a = 3.98 \times 10^8$ M^{-1} min^{-1}, and dissociates with $k_d = 0.026$ min^{-1} and $t_{1/2} = 27$ min at 22°C. The slow rate of dissociation of L-742,694 from the tachykinin NK_1 receptor and the observation that altering the order of addition of antagonist and substance P attenuates the effect of the antagonist on the maximal activation suggest that L-742,694 is a competitive antagonist that can behave as a pseudoirreversible antagonist under some experimental conditions. L-742,694 has reduced affinity for tachykinin NK_1 receptors in which alanine has been substituted for Gln^{165}, His^{197} or His^{265} in transmembrane helices 4, 5 and 6, respectively. These three residues have previously been shown to be present in the binding site of tachykinin NK_1 receptor antagonists of several structural classes. In addition, L-742,694 inhibits binding of the quinuclidine antagonist (2*S*,3*S*)-*cis*-2-(diphenyl methyl)-*N*-[(2-iodophenyl)-methyl]-1-azabicyclo[2.2.2]octane 3-amine ([^{125}I]L-703,606) with the same affinity as it inhibits binding of ^{125}I-substance P. These data indicate that L-742,694 binds to the same site within the transmembrane domain of the receptor as previously described competitive antagonists. © 1997 Elsevier Science B.V.

Keywords: Tachykinin NK_1 receptor antagonist; Antagonism, pseudo-irreversible

1. Introduction

The endogenous ligands for the neurokinin receptors are peptides termed tachykinins that are widely distributed in the central and peripheral nervous systems, and whose C-terminal sequence is Phe-X-Gly-Leu-Met-NH_2. These peptides interact with three related receptor subtypes, with substance P, neurokinin A and neurokinin B having highest affinity for the tachykinin neurokinin-1 (NK_1) receptor, the NK_2 receptor and the NK_3 receptor, respectively. All three receptor subtypes are functionally coupled to G proteins and possess the seven hydrophobic transmembrane domains that are characteristic of receptors of this class. Antagonists of the tachykinin NK_1 receptor have potential clinical utility in the treatment of chronic pain, migraine, neurogenic inflammation and emesis (Eglezos et al., 1991; Nagahisa et al., 1992; Laird et al., 1993; Bountra et al., 1993; Tattersall et al., 1994).

The first non-peptidal tachykinin NK_1 receptor antagonist, (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxy

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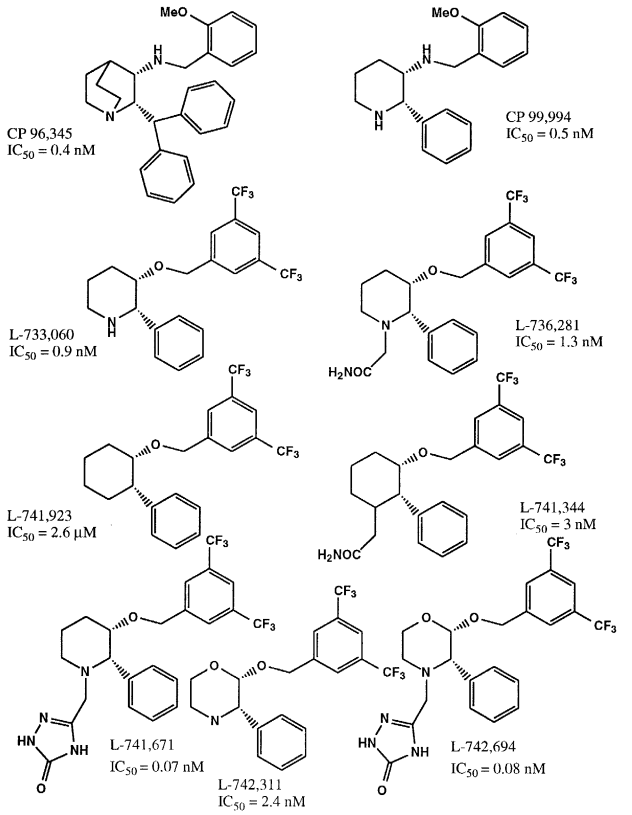

Fig. 1. Structure of L-742,694 and other structurally diverse tachykinin NK_1 receptor antagonists.

phenyl)-methyl]-1-azabicyclo[2.2.2]octane-3-amine (CP 96,345), was reported by Snider et al. (1991), and was a quinuclidine amine with subnanomolar affinity for the receptor and a competitive mechanism of action (See Fig. 1 for structures). Structure-activity studies of CP 96,345 analogs combined with receptor mutagenesis experiments have shown that this and related compounds bind within the transmembrane domain of the receptor, and interact with Gln^{165}, His^{197} and His^{265} of the human tachykinin NK_1 receptor via a hydrogen bonding interaction with the benzylic amine, an amino-aromatic interaction with the benzhydryl moiety and an aromatic interaction with the substituted benzyl moiety, respectively (Fong et al., 1993, 1994a,b). These three mutant receptors have normal affinity for either substance P or other non-peptidyl ligands suggesting that the mutations do not produce gross alterations in the structure of the receptor. Subsequent studies have shown that several other structurally diverse competitive antagonists also interact within this binding site (Cascieri et al., 1994, 1995a,b).

Recent studies have shown that potent tachykinin NK_1 receptor antagonists, possessing dramatically improved oral activity, could be obtained after substitution of the benzylic amine in CP 96,345 and its phenyl piperidine analog CP 99,994 with an ether linkage, and after further modification of the aryl substituents. 2(*S*)-Phenyl-3(*S*)-(3,5-bis(trifluoromethyl) benzyl-oxy) piperidine (L-733,060) (Fig. 1) inhibits binding with an $IC_{50} = 0.87$ nM (Harrison et al., 1995). Substitution of the piperidine nitrogen with a methyl carboxamide moiety (L-736,281, Fig. 1) did not alter affinity for the receptor, suggesting that a basic nitrogen in this position was not required for high affinity (Harrison et al., 1995). The cyclohexyl analog of L-733,060 (L-741,923, Fig. 1) has 2000-fold reduced affinity for the receptor, while the methyl carboxamide-substituted cyclohexyl analog (L-741,344, Fig. 1) has the same affinity as L-736,281 (Mills et al., 1995). These data suggest that the ring nitrogen is normally involved in a hydrogen bonding interaction with the receptor, and that this interaction can be replaced with an interaction with the methyl carboxamide side chain with no loss in affinity.

These data suggested that substitution at the ring nitrogen might be productively utilized to introduce potentially potency-enhancing substituents onto the molecule. Such enhancements were achieved with the introduction of triazole or triazolinone substituents onto the phenylpiperidine or morpholine scaffolds (L-741,671, L-742,694, Fig. 1) (Ladduwahetty et al., 1996; Hale et al., 1996). These compounds have high affinity for the human tachykinin NK_1 receptor with $K_d = 45$ pM and 37 pM, respectively. The data in the present report indicate that 2(*S*)-(3,5-bis(trifluoromethyl)benzyl)-oxy)-3(*S*)-phenyl-4-((3-oxo-1,2,4-triazol-5-yl)methyl)morpholine (L-742,694) is a high affinity, selective and pseudoirreversible antagonist of the human tachykinin NK_1 receptor.

2. Materials and methods

2.1. Synthesis of [^{3}H]L-742,694 (45 Ci / mmol)

The synthesis is described schematically in Fig. 2. 2-(3,5-Bis(trifluoromethyl)benzyloxy)-3-(*S*)-(3,5-dibromophenyl-4-benzylmorpholine (**1**) was prepared from (*S*)-(3,5-dibromo)-phenylglycine with methods analogous to those described for the preparation of L-742,694 (Hale et al., 1996) and was obtained as a 2:1 mixture of 2-(*S*) and 2-(*R*) diastereomers. The mixture was combined with an equal amount of 10% palladium on carbon in isopropanol and stirred under 0.3 atm of tritium gas for 2 h. The tritiated intermediate was further reduced with hydrogen gas and the resulting 2-(*S*) and 2-(*R*) diastereomers were separated by high pressure liquid chromatography (HPLC) on a Zorbax Rx-C_8 column, 50:50 to 85:15 (v/v) $MeCN/H_2O + 0.1\%$ trifluoroacetic acid gradient over 25 min, 1.0 ml/min) to afford 2-(*S*)-3,5-bis(trifluoromethyl)benzyloxy-3-(*S*)-[3,5-^{3}H]phenylmorpholine (**2**). This material was converted to [^{3}H]L-742,694 using the methods previously described (Hale et al., 1996) and was purified by HPLC (Zorbax Rx-C_8 column, 20:80 to 65:35 (v/v) $MeCN/H_2O + 0.1\%$ trifluoroacetic acid gradient over 25 min, 1.0 ml/min). The specific activity was calculated to be 45.47 Ci/mmol. Non-radioactive L-

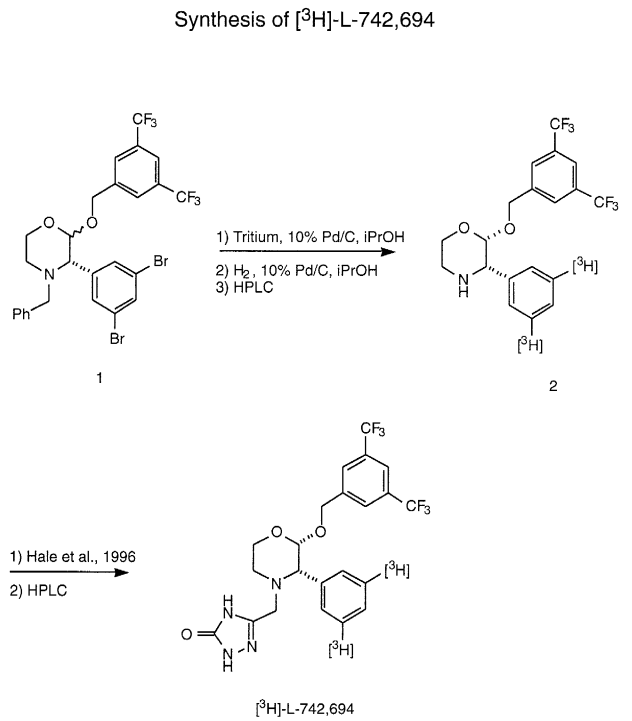

Fig. 2. Synthesis of [^{3}H]L-742,694.

742,694 was synthesized as previously described (Hale et al., 1996).

2.2. Cells and reagents

Receptor assays and functional assays were performed using stable Chinese hamster ovary (CHO) cell lines expressing 1×10^5 human tachykinin NK_1 receptors/cell that were selected and maintained as described previously (Cascieri et al., 1992). Mutants of the tachykinin NK_1 receptor were prepared as described previously and were assayed after transient expression in SV-40 transformed African green monkey kidney (COS) cells (Fong et al., 1993, 1994a,b). The human tachykinin NK_1 receptor expressed in baculovirus-infected Sf9 cells (Mazina et al., 1994) was utilized for binding experiments with [^{3}H]L-742,694.

2.3. Receptor binding assays

CHO cells (5×10^4) or membranes (1–2 μg) expressing the human tachykinin NK_1 receptor were incubated with ^{125}I-Tyr^8-substance P (0.1 nM, 2200 Ci/mmol; New England Nuclear) at room temperature for 45 min and then filtered over GF/C filters that had been presoaked in 0.1% polyethylenimine using a Tomtec 96-well harvester (Cascieri et al., 1992). Experiments with mutant receptors were carried out under the same conditions after transient expression of the receptors in COS cells. Assays using [^{125}I]L-703,606 were performed in a similar fashion as described in detail previously (Cascieri et al., 1992). The free energy of binding and the change in the free energy of binding were calculated using the formulas $\Delta G = -RT \ln K_d$ and $\Delta(\Delta G) = -RT \ln(K_{d,wt}/K_{d,mut})$.

Although specific binding of [^{3}H]L-742,694 is observed using membranes prepared from CHO cells expressing the human tachykinin NK_1 receptor, membranes prepared from baculovirus-infected Sf9 cells expressing the human tachykinin NK_1 receptor at higher density were utilized in these experiments in order to increase the signal to noise ratio. Scatchard analysis was performed using various concentrations of [^{3}H]L-742,694 (0.01–2 nM) and 7 μg of membrane protein in 0.5 ml under the conditions described above. The data were analyzed using the LIGAND program as purchased from Biosoft (Munson and Rodbard, 1980). In order to determine the rate of dissociation (k_{-1}) of [^{3}H]L-742,694 from the receptor, ligand (1.2 nM) was incubated with receptor (7 μg) for 45 min at 22°C, then dissociation was initiated by addition of 100-fold excess of unlabeled L-742,694. The amount of [^{3}H]L-742,694 remaining bound to the receptor was measured at 22°C at times from 0.5 to 120 min after the addition of unlabeled L-742,694. The dissociation was monophasic and the data were analyzed using the first-order rate equation, $\log[^3\text{H}]\text{L-742,694} * R/[^3\text{H}]\text{L-742,694} * R_o \times 100) = -k_{-1}t/2.3) + 2$.

The rate of association (k_1) of [^{3}H]L-742,694 with the receptor was determined by measuring the kinetics of association at five ligand concentrations (0.3 nM, 1.2 nM, 3 nM, 7.1 nM and 15 nM) at 22°C. The data were analyzed using the equation describing a one-phase exponential association, [^{3}H]L-742,694 Bound = [^{3}H]L-742,694 Bound_{max} $(1 - e^{-k_{obs}t})$, and the rate of association was calculated using the equation, $k_1 = (k_{obs} - k_{-1})/[[^3\text{H}]\text{L-742,694}]$.

2.4. Substance P-induced inositol phosphate production

The assay was performed as described by Berridge et al. (1982), using cells grown to confluence in 12-well tissue culture dishes. Cells were prelabeled with myo-[2-^{3}H]inositol for 24 h, washed, and incubated with LiCl in the presence or absence of L-742,694 for 15 min at 37°C. Substance P was added for 20 min, and inositol monophosphate was isolated after extraction and ion exchange chromatography (Cascieri et al., 1992).

3. Results

2(*S*)-(3,5-bis(trifluoromethyl)benzyl)-oxy)-3(*S*)-phenyl-morpholine (L-742,311), a morpholino tachykinin NK_1 receptor antagonist with no substitution on the ring nitrogen, inhibits ^{125}I-[Tyr^8]substance P binding to the human tachykinin NK_1 receptor with an $IC_{50} = 2.4 \pm 1.8$

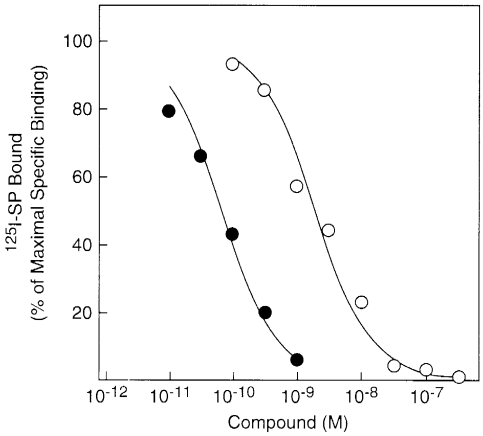

Fig. 3. Inhibition of ^{125}I-[Tyr^8]substance P binding to the human tachykinin NK_1 receptor by L-742,694 (closed circles) and L-742,311 (open circles). Ligand (0.1 nM) and CHO cells expressing the human tachykinin NK_1 receptor were incubated in the presence or absence of competitor as described in Section 2. Data shown are % of maximal specific binding observed without antagonists, and are the average of $n = 5$ or $n = 3$ experiments for L-742,694 and L-742,311, respectively.

nM ($n = 3$, Fig. 3). Introduction of the triazolinone substitution increases affinity 30-fold ($IC_{50} = 0.08 \pm 0.02$ nM, $n = 5$). The K_d calculated from these data is 37 pM, with a Hill coefficient of 0.86. L-742,694 inhibits the binding of the competitive quinuclidine benzylamine antagonist ((2*S*, 3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-iodophenyl)-methyl]-1-azabicyclo[2.2.2]octane 3-amine ([^{125}I]L-703,606) (Cascieri et al., 1992) to the tachykinin NK_1 receptor with an $IC_{50} = 0.1$ nM (data not shown). In contrast, L-742,694 has 80 000-fold and 1800-fold lower affinity for the human tachykinin NK_2 receptor ($IC_{50} = 7$ μM) and the human tachykinin NK_3 receptor ($IC_{50} = 150$ nM).

Preincubation (15 min at 37°C) of CHO cells expressing the human tachykinin NK_1 receptor with increasing concentrations of L-742,694 increases the apparent EC_{50} for substance P stimulation of inositol phosphate synthesis and decreases the maximal stimulation observed (Fig. 4A). These data suggest that L-742,694 is a non-competitive or irreversible antagonist of the tachykinin NK_1 receptor. In contrast, the unsubstituted L-742,311 increases the apparent EC_{50} for substance P but does not change the maximal stimulation observed under these conditions (Fig. 4B). Schild analysis of the data in Fig. 4B gives a $K_b = 3$ nM and a slope of 0.92, indicating that L-742,311 functions as a competitive antagonist.

Incubation of CHO cells expressing the human tachykinin NK_1 receptor with increasing concentrations of ^{125}I-[Tyr^8]substance P results in saturable binding with a $K_d = 0.1$ nM and a B_{max} of 3×10^4 receptors/cell (Fig. 5). L-742,694 (30 pM or 100 pM) decreases the apparent receptor number by 52% or 65%, respectively, without altering the apparent affinity for ^{125}I-[Tyr^8]substance P (Fig. 5). These data are also consistent with L-742,694 acting as a non-competitive or irreversible antagonist.

The dissociation of ^{125}I-[Tyr^8]substance P from the tachykinin NK_1 receptor is biphasic, with dissociation rates at 15°C of 0.012 ± 0.002 min^{-1} and 0.2 ± 0.2 min^{-1} for the high and low affinity states, respectively. In contrast to the observations above, excess L-742,694 has no affect on the rate of dissociation of ^{125}I-[Tyr^8]substance P or [^{125}I]L-703,606 ($k_{-1} = 0.085$ min^{-1} at 15°C) from the human tachykinin NK_1 receptor, consistent with a competitive mechanism (data not shown).

In order to assess if L-742,694 interacts with the same transmembrane domain binding site as other structurally diverse tachykinin NK_1 receptor antagonists, the affinity of L-742,694 and L-742,311 for receptors in which alanine was substituted for Gln^{165}, His^{197} and His^{265} was determined. Both compounds have reduced affinity for all three of these mutant receptors, indicating that they bind within the previously characterized binding site (Table 1). In

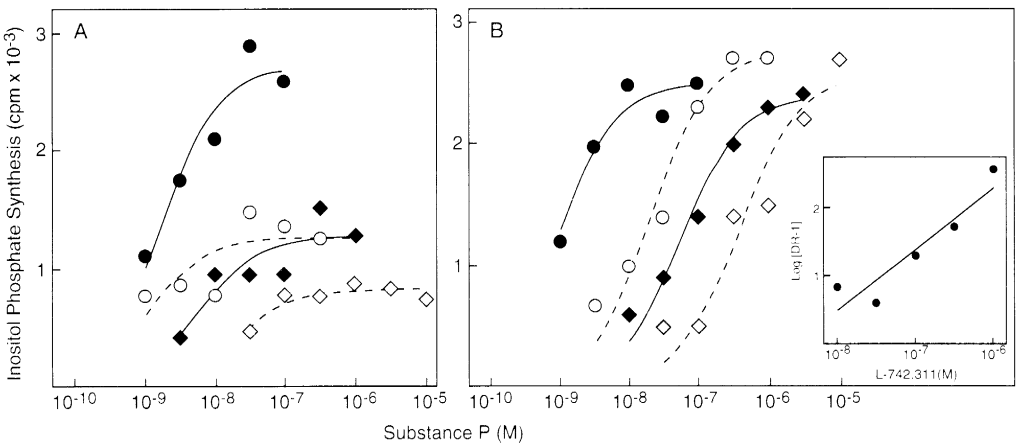

Fig. 4. Stimulation of inositol phosphate synthesis in human CHO cells expressing the human tachykinin NK_1 receptor by substance P in the presence of increasing concentrations of L-742,692 (A) or L-742,311 (B). Cells were incubated with LiCl and compound for 15 min before the addition of substance P. The reaction was terminated 30 min after addition of substance P. (A) Cells were incubated in the absence (closed circles) or presence of 1 nM (open circles), 3 nM (closed diamonds) or 30 nM (open diamonds) L-742,694. (B) Cells were incubated in the absence (closed circles) or presence of 100 nM (open circles), 300 nM (closed diamonds) or 1 μM (open diamonds) L-742,311. Inset: Schild analysis of the data for experiment shown. Data shown are representative of at least 2 experiments.

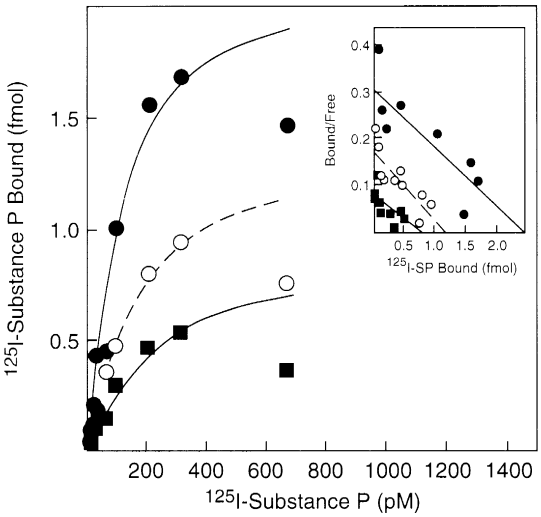

Fig. 5. Scatchard analysis of the binding of ^{125}I-[Tyr^8]substance P to the human tachykinin NK_1 receptor in the absence (closed circles) or presence of 30 pM (open circles) or 100 pM (closed squares) L-742,694. Scatchard analysis was performed using freshly prepared ligand and CHO cells expressing the human tachykinin NK_1 receptor as described in Section 2. Data shown are representative of 3 experiments.

addition, the loss in free energy of binding ($\Delta(\Delta G)$) of L-742,311 and L-742,694 for each of these mutants in comparison with the wild-type receptor is comparable (Table 1). These data suggest that addition of the triazolinone moiety in L-742,694 does not alter the way in which this compound interacts with the binding site in comparison with the competitive antagonist L-742,311.

Since L-742,694 has 30-fold higher affinity for the receptor than L-742,311, it is possible that L-742,694 interacts with the receptor in an irreversible or pseudoirreversible manner to produce its effects on the functional response to substance P. Therefore, we sought to characterize the interaction of L-742,694 with tachykinin NK_1 receptors that have reduced affinity for this compound. The receptor in which His^{197} is replaced with serine has 20-fold reduced affinity for L-742,694 (IC_{50} = 1.6 nM). Scatchard analysis of ^{125}I-[Tyr^8]substance P binding to the H197S tachykinin NK_1 receptor expressed in COS cell membranes shows that the apparent K_d for ^{125}I-[Tyr^8]substance P is increased 10-fold by inclusion of 10 nM

Table 1
Interaction of L-742,311 and L-742,694 with tachykinin NK_1 receptors with mutations in the non-peptidyl antagonist binding site

Receptor	L-742,311		L-742,694	
	IC_{50} (n) (nM)	$\Delta(\Delta G)$ (kcal/mol)	IC_{50} (n) (nM)	$\Delta(\Delta G)$ (kcal/mol)
NK-1R (WT)	1.8 ± 0.8 (3)		0.18 ± 0.09 (8)	
Q165A	38 ± 8 (2)	1.8	1.9 ± 0.4 (2)	1.4
H197A	9 ± 1 (2)	1	2.4 ± 0.2 (2)	1.5
H265A	73 ± 27 (2)	2.2	4.6 ± 0.4 (2)	2

Table 2
Scatchard analysis of ^{125}I-[Tyr^8]substance P binding to wild-type and H197S human tachykinin NK_1 receptors in the presence and absence of L-742,694

Receptor	K_d (pM)		[Receptor] (pM)	
	Control	+L-742,694[a]	Control	+L-742,694[a]
NK_1R (WT)	19 ± 4	80 ± 10	7.2 ± 0.7	3.2 ± 0.3
H197S	27 ± 3	300 ± 30	9.3 ± 0.3	8.5 ± 0.7

[a][L-742,694] = 0.5 nM for NK_1R, 10 nM for H197S.

L-742,694, while there is no change in the apparent number of receptor binding sites (Table 2). In contrast, the apparent number of binding sites observed for the wild-type receptor under these conditions is reduced 56% by inclusion of 0.5 nM L-742,694.

L-742,694 inhibits the binding of ^{125}I-[Tyr^8]substance P to the rat tachykinin NK_1 receptor with 1000-fold lower affinity than the human receptor (IC_{50} = 80 nM). Preincubation (15 min at 37°C) of CHO cells expressing the rat tachykinin NK_1 receptor with up to 10 μM L-742,694 increases the apparent EC_{50} for substance P stimulation of inositol phosphate synthesis without altering the maximal response (Fig. 6). Schild analysis of these data gives a K_b = 790 nM and a slope = 0.97, indicating that L-742,694 acts as a competitive antagonist at the rat tachykinin NK_1 receptor. Although the 10-fold difference between the affinity determined in receptor binding and the K_b calculated from the Schild analysis suggests that equilibrium between antagonist and agonist had not been achieved, these data still suggest that the interaction of L-742,694 with tachykinin NK_1 receptors for which it has lower

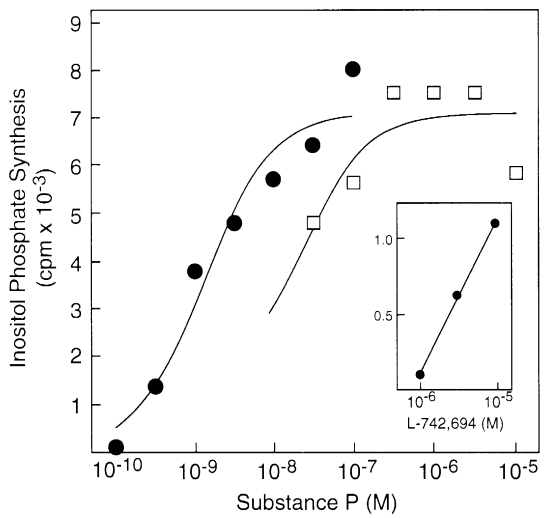

Fig. 6. Stimulation of inositol phosphate synthesis in CHO cells expressing the rat tachykinin NK_1 receptor by substance P in the absence (closed circles) or presence (open squares) of 10 μM L-742,694. Cells were incubated with LiCl and L-742,694 for 15 min before addition of substance P. The reaction was terminated after 30 min as described in Section 2. Inset: Schild analysis of the data from this experiment.

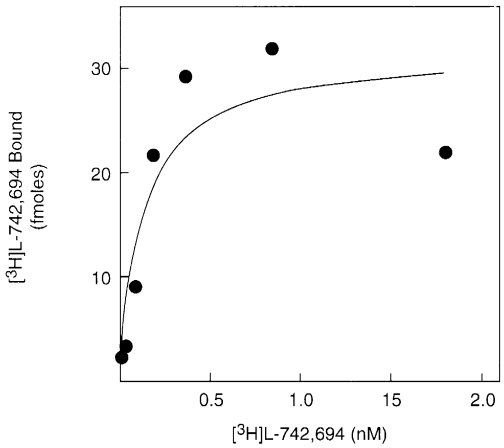

Fig. 7. Scatchard analysis of the binding of [^{3}H]L-742,694 to the human tachykinin NK_1 receptor. Ligand and receptor as expressed in baculovirus-infected Sf9 cells were incubated as described in Section 2. Experiment shown is representative of 2 replicate experiments.

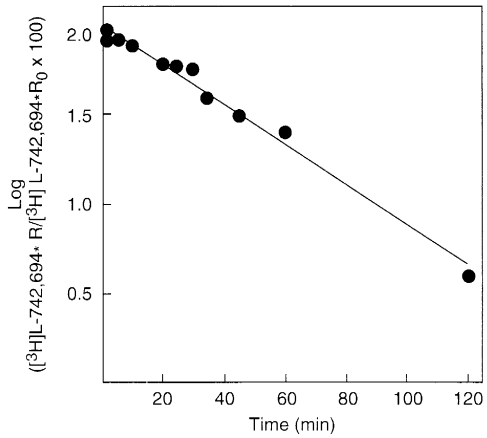

Fig. 8. Dissociation of [^{3}H]L-742,694 from the human tachykinin NK_1 receptor. Ligand (1.2 nM) was incubated with receptor as expressed in baculovirus-infected Sf9 cells until equilibrium, and dissociation was initiated with addition of 100-fold excess of unlabeled ligand. Data shown are the average of 2 experiments.

affinity than the human tachykinin NK_1 receptor does not produce the apparent irreversible antagonism demonstrated with the human receptor.

In order to directly assess the kinetics of L-742,694 binding to the human tachykinin NK_1 receptor, [^{3}H]L-742,694 (45 Ci/mmol) was synthesized. The human tachykinin NK_1 receptor expressed in baculovirus-infected Sf9 cells was utilized for these experiments in order to increase the signal to noise ratio in the ligand binding assay. The binding of [^{3}H]L-742,694 to the tachykinin NK_1 receptor reached equilibrium at 30 min and was stable for at least 180 min at 22°C. Scatchard analysis of the binding of increasing concentrations of [^{3}H]L-742,694 gave a $K_d = 0.12 \pm 0.01$ nM and a maximal number of binding sites of 4.2 ± 0.3 pmol/mg protein ($n = 2$, Fig. 7). These data are consistent with the maximal number of receptor binding sites in this preparation determined using the quinuclidine tachykinin NK_1 receptor antagonist, [^{125}I]L-703,606 or [^{3}H]substance P (Mazina et al., 1994). The binding is inhibited by unlabeled L-742,694, CP 96,345 and the inactive enantiomer of CP 96,345 with $K_i = 0.12$ nM, 1.4 nM and > 100 nM, respectively. These data are consistent with binding to the tachykinin NK_1 receptor.

In order to measure the rate of dissociation of [^{3}H]L-742,694 from the tachykinin NK_1 receptor, membranes were incubated with radioactive ligand (1.2 nM) until equilibrium was achieved (45 min), and then 100-fold excess of unlabelled L-742,694 was added to the incubation. [^{3}H]L-742,694 dissociates from the receptor with $k_{-1} = 0.026 \pm 0.002$ min^{-1} and $t_{1/2} = 27 \pm 2$ min at 22°C (Fig. 8).

The rate of association (k_{obs}) measured for 0.3 nM, 1.17 nM, 3 nM, 7.1 nM and 15 nM [^{3}H]L-742,694 is 0.40 min^{-1}, 0.61 min^{-1}, 0.82 min^{-1}, 1.28 min^{-1} and 6.4

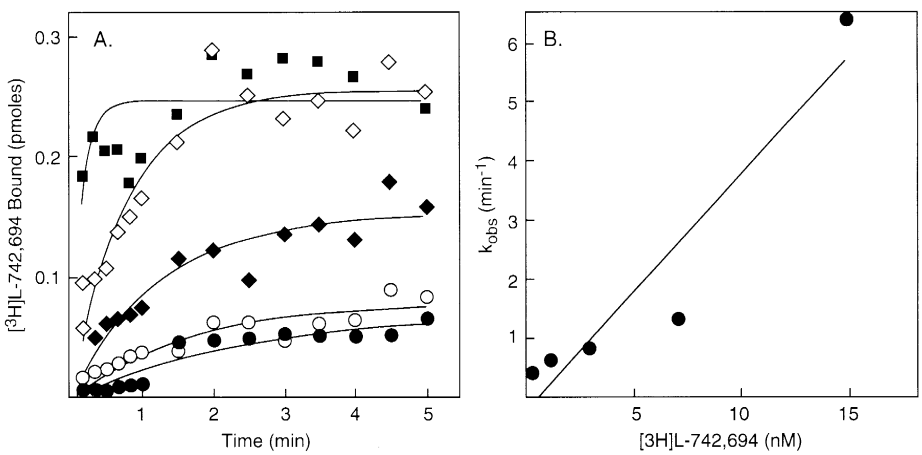

Fig. 9. Association of [^{3}H]L-742,694 with the human tachykinin NK_1 receptor. (A) The amount of specific binding of [^{3}H]L-742,694 vs. time at 0.3 nM (closed circles), 1.2 nM (open circles), 3 nM (closed diamonds), 7.1 nM (open diamonds) and 15 nM (closed squares) ligand was determined. The observed rate of association (k_{obs}) at each concentration was calculated as described in Section 2. (B) Correlation between k_{obs} and concentration of [^{3}H]L-742,694. The rate of association (k_1) was calculated as described in Section 2.

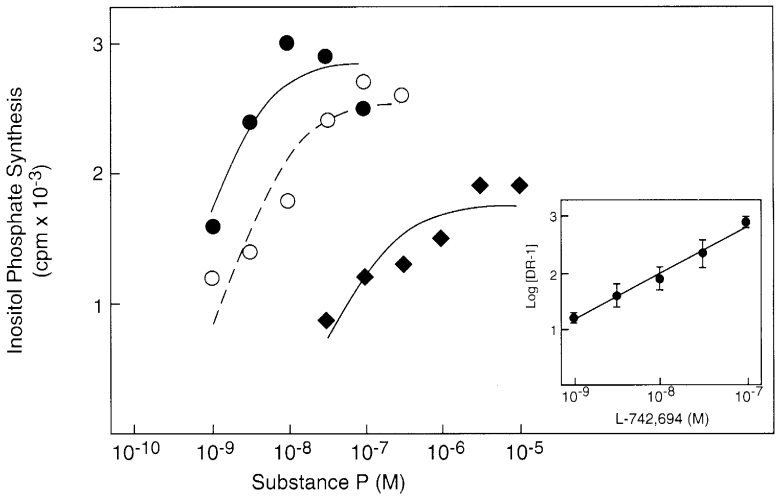

Fig. 10. Stimulation of inositol synthesis in CHO cells expressing the human tachykinin NK_1 receptor by substance P in the absence (closed circles) or presence of 1 nM (open circles) or 100 nM (closed diamonds) L-742,694. Cells were preincubated with LiCl, then substance P and L-742,694 were added simultaneously. The incubation was terminated after 30 min. Inset: Schild analysis of these data. Data shown are representative of 2 experiments.

min^{-1}, respectively (Fig. 9A). The relationship of k_{obs} to concentration of [^{3}H]L-742,694 is linear, and the association constant k_1 calculated from these data is 3.76×10^8 M^{-1} min^{-1} when the *Y*-intercept is constrained to the value for k_{-1} determined above (Fig. 9B). The K_d calculated from these kinetic measurements (i.e., k_{-1}/k_1) is 69 pM.

If the reduction in maximal responsiveness to substance P were produced by a pseudoirreversible interaction of L-742,694 with the tachykinin NK_1 receptor due to a slow rate of dissociation, alteration of the order of addition of antagonist and agonist might be expected to alter the effects of L-742,694 on the maximal responsiveness. In contrast to the data observed with preincubation of antagonist (Fig. 4), simultaneous addition of agonist and antagonist does not reduce the maximal responsiveness to substance P at 1 nM L-742,694, and the maximal responsiveness in the presence of 100 nM L-742,694 is reduced by only 50% (Fig. 10). Schild analysis of these data gives a $K_b = 40$ pM and a slope of 0.83.

4. Discussion

L-742,694 is a potent, selective tachykinin NK_1 receptor antagonist with increased receptor affinity and improved pharmacokinetic properties compared to previously described compounds (Hale et al., 1996). The K_d of L-742,694 for the tachykinin NK_1 receptor is 37 pM, 69 pM or 120 pM when determined by inhibition of ^{125}I-[Tyr^8]substance P binding, measurement of the kinetic constants for [^{3}H]L-742,694 binding, or Scatchard analysis of [^{3}H]L-742,694 binding, respectively. Although these experiments have been performed using the receptor expressed in CHO cells, COS cells and baculovirus- infected Sf9 cells, these comparisons are valid since ^{125}I-substance P and [^{3}H]L-742,694 have similar affinity in all three systems, and unlabeled L-742,694 inhibits this binding with similar affinity.

Characterization of the effects of L-742,694 on substance P stimulation of inositol phosphate synthesis and on ^{125}I-[Tyr^8]substance P binding isotherms indicates that the compound is an insurmountable antagonist. Thus, the maximal level of receptor activation and the apparent number of ^{125}I-substance P binding sites observed are decreased by addition of L-742,694. These data suggest that L-742,694 is either a non-competitive or irreversible antagonist. However, the compound has no effect on the rate of dissociation of ^{125}I-[Tyr^8]substance P or [^{125}I]L-703,606 from the tachykinin NK_1 receptor, consistent with competitive antagonism.

L-742,311, the *des*-triazolinone analog of L-742,694, is a competitive antagonist under these same experimental paradigms, and the reduction in the affinity of both these compounds for tachykinin NK_1 receptor mutants at Gln^{165}, His^{197} and His^{265} is similar. Since these residues have been demonstrated previously to be components of the binding site for several structural classes of competitive non-peptidal antagonists of this receptor (Fong et al., 1994a,b; Cascieri et al., 1994, 1995b), these data indicate that L-742,694 binds to the same site as competitive tachykinin NK_1 receptor antagonists, and that addition of the triazolinone moiety does not alter the nature of its interaction with these three residues.

Schambye et al. (1994a,b) conclude that competitive and insurmountable antagonists of the angiotensin II type 1 receptor bind to different, but overlapping sites within the transmembrane domain. Thus, alterations within transmembrane domains VI and VII result in more pronounced loss in affinity for competitive antagonists than for insur-

mountable, non-competitive antagonists. However, it is not clear from these experiments if the mutated amino acid residues interact directly with the angiotensin II antagonists, or if the mutations result in alterations in the size and shape of the antagonist binding pocket. Thus, it has not been demonstrated that the quantitative differences in affinity of competitive and insurmountable antagonists for the angiotensin II type 1 receptor mutants are due to differences in the molecular interactions shared by these antagonists.

Several additional lines of evidence suggest that the apparent non-competitive behavior of L-742,694 is due to pseudoirreversible antagonism resulting from a slow rate of dissociation from the human tachykinin NK_1 receptor. L-742,694 is a competitive antagonist at the rat tachykinin NK_1 receptor and at the human H197S mutant tachykinin NK_1 receptor, which have 1000-fold and 20-fold reduced affinity for the compound, respectively. In addition, altering the order of addition of agonist and antagonist in the inositol phosphate assay attenuates the effect of the antagonist on maximal substance P-mediated activation.

Lastly, direct measurement of the rate of dissociation of [^{3}H]L-742,694 from the tachykinin NK_1 receptor gives a $k_{-1} = 0.026$ min^{-1} and a $t_{1/2} = 27$ min at 22°C. The binding is fully reversible, and, thus, inconsistent with a covalent interaction between antagonist and receptor. Dissociation of both ^{125}I-[Tyr^8]substance P and the competitive quinuclidine antagonist [^{125}I]L-703,606 is too rapid to be accurately measured at 22°C. However, [^{125}I]L-703,606 dissociates with a $t_{1/2} = 8$ min at 15°C (Cascieri et al., 1992). ^{125}I-[Tyr^8]substance P dissociates with a $t_{1/2} = 46$ min and 0.2 min for the G-protein coupled and uncoupled receptor states, respectively, at 15°C (Cascieri et al., 1992). Thus, the dissociation of L-742,694 from the tachykinin NK_1 receptor is significantly slower than that of either substance P or L-703,606. These data are consistent with L-742,694 acting as a pseudoirreversible antagonist when added to the receptor 15 min prior to addition of substance P in the functional assay. The apparent lack of reversibility of L-742,694 results from its slow rate of dissociation from the receptor, and correlates with the extremely high affinity of this antagonist for the human tachykinin NK_1 receptor.

In summary, L-742,694 is a novel, high affinity tachykinin NK_1 receptor antagonist whose binding kinetics produce prolonged receptor antagonism. Thus, it is the first pseudoirreversible antagonist of the tachykinin NK_1 receptor described to date. In addition, we have demonstrated for the first time that such pseudoirreversible antagonists and competitive antagonists share qualitatively and quantitatively similar molecular interactions with the tachykinin NK_1 receptor. The addition of the triazolinone moiety to the competitive antagonist L-742,311 to give L-742,694 results in an additional molecular interaction with the receptor that confers higher affinity and a slower rate of dissociation.

References

Berridge, M.J., Downes, C.P., Hanley, M.R., 1982. Lithium amplifies agonist dependent phosphatidylinositol responses in brain and salivary glands. Biochem. J. 206, 587–596.

Bountra, C., Bunce, K., Dale, T., Gardner, C., Jordan, C., Twissell, D., Ward, P., 1993. Anti-emetic profile of a non-peptide neurokinin NK1 antagonist, CP 99,994 in ferrets. Eur. J. Pharmacol. 249, R3–R4.

Cascieri, M.A., Ber, E., Fong, T.M., Sadowski, S., Bansal, A., Swain, C., Seward, E., Frances, B., Burns, D., Strader, C.D., 1992. Characterization of the binding of a potent, selective, radioiodinated antagonist to the human neurokinin-1 receptor. Mol. Pharmacol. 42, 458–463.

Cascieri, M.A., Macleod, A.M., Underwood, D., Shiao, L.-L., Ber, E., Sadowski, S., Yu, H., Merchant, K.J., Swain, C.J., Strader, C.D., Fong, T.M., 1994. Characterization of the interaction of *N*-acyl-L-tryptophan benzyl ester neurokinin antagonists with the human neurokinin-1 receptor. J. Biol. Chem. 269, 2728–2732.

Cascieri, M.A., Fong, M.A., Strader, C.D., 1995a. Molecular characterization of a common binding site for small molecules within the transmembrane domain of G-protein coupled receptors. J. Pharmacol. Toxicol. Methods 33, 179–185.

Cascieri, M.A., Shiao, L.-L., Mills, S.G., MacCoss, M., Swain, C.J., Yu, H., Ber, E., Sadowski, S., Wu, M.T., Strader, C.D., Fong, T.M., 1995b. Characterization of the interaction of diacylpiperazine antagonists with the human neurokinin-1 receptor: identification of a common binding site for structurally dissimilar antagonists. Mol. Pharmacol. 47, 660–665.

Eglezos, A., Giuliani, S., Viti, G., Maggi, C.A., 1991. Direct evidence that capsaicin-induced plasma protein extravasation is mediated through tachykinin NK_1 receptors. Eur. J. Pharmacol. 209, 277–279.

Fong, T.M., Cascieri, M.A., Yu, H., Bansal, A., Swain, C., Strader, C.D., 1993. Amino-aromatic interaction between histidine 197 of the neurokinin-1 receptor and CP-96,345. Nature 362, 350–353.

Fong, T.M., Yu, H., Cascieri, M.A., Underwood, D., Swain, C.J., Strader, C.D., 1994a. The role of histidine 265 in antagonist binding to the neurokinin-1 receptor. J. Biol. Chem. 269, 2728–2732.

Fong, T.M., Yu, H., Cascieri, M.A., Underwood, D., Swain, C.J., Strader, C.D., 1994b. Interaction of glutamine 165 in the fourth transmembrane domain of the human neurokinin-1 receptor with quinuclidine antagonists. J. Biol. Chem. 269, 14957–14961.

Hale, J.J., Mills, S.G., MacCoss, M., Shah, S., Qi, H., Mathre, D.J., Cascieri, M.A., Sadowski, S., Strader, C.D., Pivnichny, J.V., MacIntyre, D.E., Metzger, J.M., 1996. 2(*S*)-((3,5-bis(trifluoromethyl)benzyl)oxy)-3(*S*)-phenyl-4-((3-oxo-1,2,4-triazol-5-yl)methyl)-morpholine (1): a potent, orally active, morpholine-based human NK-1 receptor antagonist. J. Med. Chem. 39, 1760–1762.

Harrison, T., Owens, A.P., Williams, B.J., Swain, C.J., Baker, R., Hutson, P.H., Sadowski, S., Cascieri, M.A., 1995. Piperidine-ether based NK-1 antagonists 2: investigation of the effect of N-substitution. Biorg. Med. Chem. Lett. 5, 209–212.

Ladduwahetty, T., Baker, R., Cascieri, M.A., Chambers, M.S., Haworth, K., Keown, L., MacIntyre, D.E., Metzger, J.M., Owen, S., Rycroft, W., Sadowski, S., Seward, E.M., Shepheard, S., Swain, C.J., Tattersall, F.D., Williamson, D., Hargreaves, R.J., 1996. Heteroaryl-2-phenyl-3-(benzyloxy) piperidines: a novel class of potent orally active human NK_1 antagonists. J. Med. Chem. 39, 2907–2914.

Laird, J.M.A., Hargreaves, R.J., Hill, R.G., 1993. Effect of RP 67,580, a non-peptide neurokinin 1 receptor antagonist, on facilitation of a nociceptive spinal flexion reflex in the rat. Br. J. Pharmacol. 109, 713–718.

Mazina, K.E., Strader, C.D., Fong, T.M., 1994. Expression and solubilization of a recombinant human neurokinin-1 receptor in insect cells. J. Recept. Res. 14, 63–73.

Mills, S.G., MacCoss, M., Underwood, D., Shah, S.K., Finke, P.E., Miller, D.J., Budha, R.J., Cascieri, M.A., Sadowski, S., Strader, C.D., 1995. 1,2,3-Trisubstituted cyclohexyl substance P antagonists: signifi-

cance of the ring nitrogen in piperidine-based NK-1 receptor antagonists. Biorg. Med. Chem. Lett. 5, 1345–1350.

Munson, P.J. and Rodbard, D., 1980. LIGAND: a versatile computerized approach for characterization of ligand binding systems. Anal. Biochem. 107, 220–239.

Nagahisa, A., Kanai, Y., Suga, O., Taniguchi, K., Tsuchiya, M., Lowe, J.A., Hess, H.-J., 1992. Antiinflammatory and analgesic activity of a non-peptide substance P receptor antagonist. Eur. J. Pharmacol. 217, 191–195.

Schambye, H.T., Hjorth, S.A., Bergsma, D.J., Sathe, G., Schwartz, T.W., 1994a. Differentiation between binding sites for angiotensin II and nonpeptide antagonists on the angiotensin II type 1 receptors. Proc. Natl. Acad. Sci. USA 91, 7046–7050.

Schambye, H.T., Wijk, B., Hjorth, S.A., Wienen, W., Entzeroth, M., Bergsma, D.J., Schwartz, T.W., 1994b. Mutations in transmembrane segment VII of the AT_1 receptor differentiate between closely related insurmountable and competitive angiotensin antagonists. Br. J. Pharmacol. 113, 331–333.

Snider, R.M., Constantine, J.W., Lowe, J.A., Longo, K.P., Lebel, W.S., Woody, H.A., Drozda, S.E., Desai, M.C., Vinick, F.J., Spencer, R.W., Hess, H.-J., 1991. A potent nonpeptide antagonist of the substance P (NK_1) receptor. Science 251, 435–437.

Tattersall, F.D., Rycroft, W., Hill, R.G., Hargreaves, R.J., 1994. Enantioselective inhibition of apomorphine-induced emesis in the ferret by the neurokinin-1 receptor antagonist CP 99,994. Neuropharmacology 33, 259–260.