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# [$^3$H]SR 48692, the First Nonpeptide Neurotensin Antagonist Radioligand: Characterization of Binding Properties and Evidence for Distinct Agonist and Antagonist Binding Domains on the Rat Neurotensin Receptor

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## SUMMARY

The binding of [$^3$H]SR 48692, a new potent and specific nonpeptide neurotensin (NT) receptor antagonist, was characterized in membranes from mouse fibroblast LTK$^-$ cells stably transfected with the G protein-coupled rat NT receptor. The binding of [$^3$H]SR 48692 was specific, time dependent, reversible, and saturable. Scatchard analysis of saturation experiments indicated that [$^3$H]SR 48692 bound to a single population of sites, with a $K_d$ of 3.4 nM and a $B_{max}$ value that was 30–40% greater than that observed in saturation experiments with [$^{125}$I]NT. Two SR 48692-related enantiomers, SR 48527 and SR 49711, were 10 and 1000 times less potent, respectively, than unlabeled SR 48692 in inhibiting [$^3$H]SR 48692. Unlabeled NT inhibited [$^3$H]SR 48692 binding in a complex manner that was best analyzed with a three-site model, with high ($K_i$ = 0.22 nM) and low ($K_i$ = 57 nM) affinity NT binding sites and a site insensitive to unlabeled NT (up to 10 $\mu$M), which represented 60, 20, and 20%, respectively, of the total number of [$^3$H]SR 48692 binding sites. Digitonin (10 $\mu$g/ml) markedly reduced the proportion of NT-insensitive sites without affecting [$^3$H]SR 48692 binding. $Na^+$ and guanosine-5′-(γ-thio)triphosphate differentially modulated [$^3$H]SR 48692 and [$^{125}$I]NT binding and inverted the proportions of the high and low affinity NT binding sites. A mutant rat NT receptor that contained a deletion in a region (amino acids 45–60) of the amino-terminal extracellular domain near the first transmembrane helix and was expressed in COS M6 cells retained the same affinity for [$^3$H]SR 48692 and the same stereoselectivity for SR 48527 and SR 49711 as the wild-type receptor. In contrast, it bound NT with 3000-fold lower potency. In conclusion, the data indicate that [$^3$H]SR 48692 represents a new, potent, nonpeptide antagonist radioligand of the NT receptor that differentiates between agonist- and antagonist-receptor interactions. Furthermore, the data demonstrate that the peptide agonist and the nonpeptide antagonist bind to distinct regions of the NT receptor.

Recently, we reported on the development and characterization of the first nonpeptide NT receptor antagonist, SR 48692 (1). The compound inhibits [$^{125}$I]NT binding to mammalian peripheral and central nervous system NT receptors with $K_i$ values ranging from 1 to 20 nM (1). In the periphery, SR 48692 antagonizes the cardiovascular and gastrointestinal effects of NT (2, 3). In the central nervous system, *in vivo* it blocks (after oral administration) the turning behavior and hyperlocomotor effects elicited in rats and mice by centrally injected NT (1, 4, 5), whereas *in vitro* it inhibits the NT potentiation of $K^+$-evoked dopamine release from cultured rat mesencephalic neurons and from rat and guinea pig striatal slices (1, 6). However, it does not antagonize centrally mediated, NT-induced hypothermia and analgesia (7), and it fails to inhibit NT responses in rat substantia nigra neurons *in vitro* (8), suggesting that these effects are initiated through a subtype of SR 48692-insensitive NT receptors.

So far, only one type of NT receptor has been cloned from rat brain and from the human adenocarcinoma HT-29 cell line (9, 10). This receptor belongs to the family of G protein-coupled receptors with seven putative transmembrane segments. Its activation leads to phospholipase C stimulation and consequently to an increased production of inositol phosphates and to intracellular $Ca^{2+}$ mobilization. We have shown that SR 48692 inhibits NT binding to cloned rat and human NT receptors transiently expressed in COS-7 cells (1, 10). Furthermore, we and others have recently reported that the antagonist blocks NT-induced $Ca^{2+}$ mobilization in

**ABBREVIATIONS:** NT, neurotensin; [$^{125}$I]NT, monoiodo-[$^{125}$I-Tyr$^3$]-neurotensin; GTPγS, guanosine-5′-(γ-thio)triphosphate; SR 48692, 2-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino]tricyclo(3.3.1.1.$^{3,7}$)decan-2-carboxylic acid; SR 48527, (*S*)-(+)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino]cyclohexylacetic acid; SR 49711, (*R*)-(−)-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino]cyclohexyl acetic acid.

mouse LTK$^-$ fibroblast and rat pheochromocytoma PC-12 cell lines stably transfected with the cloned rat brain NT receptor (11, 12). In these transfected systems, the $K_i$ values for the inhibitory effects of SR 48692 are in the same nanomolar range as those found in systems that normally express the NT receptor and are sensitive to the antagonist action of SR 48692. Thus, SR 48692 represents the prototype of a new series of high affinity ligands for the cloned NT receptor.

In the present study we report on the use of [$^3$H]SR 48692 as a novel radioligand for studying NT receptor-antagonist interactions at the molecular level. The binding of [$^3$H]SR 48692 to membranes prepared from mouse LTK$^-$ fibroblasts stably transfected with the cloned rat brain NT receptor was characterized with respect to kinetic and equilibrium properties. The stereoselectivity of the binding was assessed using two enantiomers, SR 48527 and SR 49711, that are structurally related to SR 48692 (Fig. 1). [$^3$H]SR 48692 and [$^{125}$I]NT binding characteristics were compared in saturation experiments, in inhibition experiments with unlabeled NT and antagonists, and in assays of their sensitivity to $Na^+$ and guanyl nucleotides. It was found that, unlike NT, SR 48692 does not discriminate between different affinity states of the NT receptor and that SR 48692 and NT bindings are differentially modulated by $Na^+$ and GTPγS. Finally, [$^3$H]SR 48692 binding experiments were performed with a mutant rat NT receptor that contained a deletion in a region of the amino-terminal domain near the first transmembrane segment and was transiently expressed in COS M6 cells. These studies demonstrated that SR 48692 and NT recognize distinct epitopes on the NT receptor.

## Materials and Methods

**Drugs.** SR 48692, SR 48527, and SR 49711 (Fig. 1) were synthesized at Sanofi Recherche (Montpellier, France). [*methoxy*-$^3$H]SR 48692 was prepared by Amersham (Buckinghamshire, UK), by methylation of a phenolic intermediate with [$^3$H]methyl iodide. The product was purified by high performance liquid chromatography on a 5-μm Ultrasphere ODS column, using a linear gradient of water/methanol (1:1) to methanol over 30 min, at a flow rate of 1 ml/min. The product was 98.5% pure and had a specific radioactivity of 86 Ci/mmol (3.18 TBq/mmol). The location of the tritium atoms in SR 48692 is shown in Fig. 1. [$^{125}$I]NT was prepared and purified as described previously (13). All synthetic peptides were from Neosystem (Strasbourg, France).

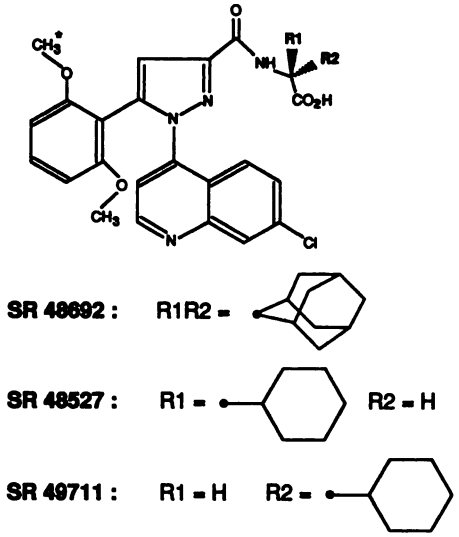


**Fig. 1.** Chemical structures of SR 48692, SR 48527, and SR 49711. *, Location of the three tritium atoms in SR 48692.

**Cell culture.** All media and reagents were from GIBCO-BRL (Eragny, France) and Sigma Chemical Co. (St. Louis, MO). The mouse fibroblast LTK$^-$ and monkey kidney epithelial COS M6 cell lines were cultured in 100-mm Falcon Petri dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin. Stable transfection of the rat NT receptor into LTK$^-$ cells, clone selection and propagation, and characterization of NT binding and signal transduction have been described previously (11).

**Site-directed mutagenesis and transfection in COS M6 cells.** The cDNA encoding the rat NT receptor (kindly provided by Dr. S. Nakanishi, Kyoto University, Kyoto, Japan), minus its 3′ untranslated region, was subcloned into pBluescript II vector (Stratagene, La Jolla, CA) for single-strand production. The deletion of nucleotides 133–180 in the open reading frame, to obtain mutant Δ45–60 (deletion of amino acids 45–60), was performed according to the method of Kunkel (14). A mutant clone was identified after restriction analysis and verified by dye dideoxy terminator sequencing (Applied Biosystems, Foster City, CA). The wild-type and mutant receptors subcloned into CDM8 (Invitrogen, NV Leek, The Netherlands) were transfected into COS M6 cells by the DEAE-dextran precipitation method (1).

**Preparation of cell homogenates.** Transfected LTK$^-$ cells at 70% confluency or COS M6 cells obtained 48 hr after transfection were washed twice with phosphate-buffered saline and lysed in 5 mM Tris·HCl, pH 8, at 4° for 30 min. The cells were scraped and homogenized by repeated passages through a syringe needle. The homogenates were centrifuged at 125,000 × *g* for 1 hr. The pellet was resuspended in 5 mM Tris·HCl, pH 7.5 (4 ml/10 dishes). Protein concentration was determined using the Bio-Rad protein assay reagent.

**Binding experiments.** SR 48692, SR 48527, SR 49711, and [$^3$H]SR 48692 were solubilized and diluted in dimethylsulfoxide. The final concentration of dimethylsulfoxide in the binding assays did not exceed 1%. [$^{125}$I]NT binding was performed as described previously (1). Time course studies of [$^{125}$I]NT binding to membranes from transfected LTK$^-$ cells indicated that the specific binding increased with time and reached a plateau by 15 min (data not shown). Binding experiments with [$^3$H]SR 48692 were conducted at 20° for 20 min (or for various time periods in association kinetic experiments), with 10 μg of transfected LTK$^-$ or 3 μg of transfected COS M6 cell membrane proteins in 0.25 ml of 50 mM Tris·HCl, pH 7.4, 0.1% bovine serum albumin, 0.8 mM *o*-phenanthroline. In saturation experiments, [$^3$H]SR 48692 concentrations ranged from 10 pM to 10 nM. Competition experiments were performed with 2 nM [$^3$H]SR 48692 and unlabeled compounds at the indicated concentrations. For dissociation kinetic experiments, 2 nM [$^3$H]SR 48692 was incubated for 20 min with cell membrane fractions. Unlabeled SR 48692 was then added at a final concentration of 1 μM, and incubations were stopped at the indicated times. Bound ligand was separated from free ligand by dilution of the incubation medium with 2 ml of ice-cold 50 mM Tris·HCl, pH 7.4, 0.1% bovine serum albumin, and vacuum filtration through 0.2-μm cellulose acetate filters (Sartorius, Goettingen, Germany), followed by two washes of the tube and filter with 2 ml of the same buffer. The radioactivity trapped on the filters was counted by liquid scintillation. Nonspecific binding was determined by incubation with 1 μM SR 48692. All experiments were performed at least three times in duplicate. Competition binding data were analyzed using LIGAND software (15).

It was verified that untransfected LTK$^-$ and COS M6 cells were devoid of specific [$^3$H]SR 48692 binding. With the amounts of membrane protein (3–10 μg) used in the binding assays, nonspecific [$^3$H]SR 48692 binding consisted mostly (>80%) of radioactivity bound to the filters. The absence or presence of bovine serum albu-

min in the binding assay buffer did not affect [$^3$H]SR 48692 binding (either total or nonspecific). Albumin was used in the buffer because it is known to prevent NT at low concentrations (such as those used in competition experiments or in [$^{125}$I]NT binding studies) from adhering to the glassware.

## Results

**Kinetic experiments.** Fig. 2A shows the specific binding of 2 nM [$^3$H]SR 48692 to homogenates of transfected LTK$^-$ cells as a function of time. The binding increased with time and reached a plateau by 20 min. Because bound ligand represented <5% of total ligand, the data can be analyzed as a pseudo-first-order reaction according to the equation

$$\ln[B_{eq}/(B_{eq} - B)] = (k_1[L] + k_{-1})t,$$

in which $B_{eq}$ and $B$ are the concentrations of bound ligand at equilibrium and at time $t$, respectively, [L] is the concentration of free labeled ligand, and $k_1$ and $k_{-1}$ are the rate constants of association and dissociation, respectively. The plot of $\ln[B_{eq}/(B_{eq} - B)]$ versus time yielded a straight line (Fig. 2A, *inset*) with a slope value equal to $k_1[L] + k_{-1}$ (Table 1).

Fig. 2B depicts the time course of dissociation of specifically bound [$^3$H]SR 48692 from homogenates of LTK$^-$ cells. When the data were plotted according to the equation $\ln(B/B_0) = -k_{-1}t$, in which $B_0$ is the concentration of bound ligand at time 0 of dissociation, a straight line was obtained (Fig. 2B, *inset*), indicating that dissociation was a first-order process. The slope of the straight line yielded $k_{-1}$, which could then be used to calculate $k_1$ from association kinetics (Table 1). From the ratio $k_{-1}/k_1$, an estimate of the dissociation constant ($K_d$) for [$^3$H]SR 48692 was obtained (Table 1).

**Saturation experiments.** Fig. 3A presents the total, nonspecific, and specific binding of [$^3$H]SR 48692 to LTK$^-$ cell homogenates as a function of labeled ligand concentration. The nonspecific binding increased linearly with increasing ligand concentrations and represented a low proportion of total binding even at high [$^3$H]SR 48692 concentrations. The specific binding obtained by subtracting nonspecific binding from total binding was saturable. Scatchard analysis of the data (Fig. 3A, *inset*) yielded a linear plot, from which $K_d$ and $B_{max}$ (maximal binding capacity) values were derived (Table 1). Note that the $K_d$ value obtained from equilibrium experiments agrees quite well with that calculated from kinetic studies. Saturation binding experiments were also performed, with the same membrane preparations, using [$^{125}$I]NT as the labeled ligand (Fig. 3B). A linear Scatchard plot was obtained (Fig. 3B, *inset*). $K_d$ and $B_{max}$ values for [$^{125}$I]NT are given in Table 1. It is noteworthy that the $B_{max}$ value obtained with [$^{125}$I]NT was significantly lower (32%) than that obtained with [$^3$H]SR 48692.

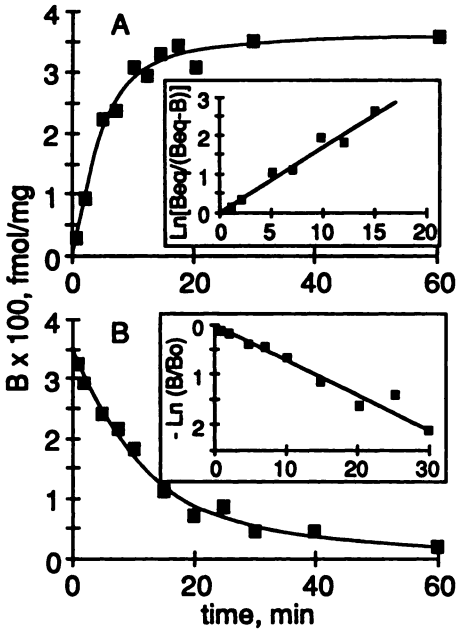


Fig. 2. Association (A) and dissociation (B) kinetics of [$^3$H]SR 48692 binding to homogenates of transfected LTK$^-$ cells. The values are from a typical experiment. *Insets*, pseudo-first-order representation of the association kinetics (A) and first-order representation of the dissociation kinetics (B), according to the equations given in the text.

**Competition experiments.** The inhibition of [$^3$H]SR 48692 and [$^{125}$I]NT binding by increasing concentrations of unlabeled SR 48692, SR 48527, SR 49711, and NT in LTK$^-$ cell homogenates is shown in Fig. 4. The $K_i$ values for the nonpeptide antagonists were slightly (1.5–2.5-fold) lower in competition experiments with [$^3$H]SR 48692, compared with [$^{125}$I]NT (Table 2). In both assays the potencies of the enantiomers, relative to that of SR 48692, were similar, with SR 48527 and SR 49711 being 5–7 and 500–700 times less potent, respectively, than SR 48692; Hill coefficients were close to unity for all three antagonists (Table 2). Note that the $K_i$ value of unlabeled SR 48692 in competing with [$^3$H]SR 48692 binding (5.6 nM) agrees reasonably well with the $K_d$ value of [$^3$H]SR 48692 as determined in saturation experiments (3.4 nM). Inhibition of [$^{125}$I]NT and [$^3$H]SR 48692 binding by unlabeled NT yielded markedly different curves (Fig. 4). NT inhibited [$^{125}$I]NT binding with a Hill coefficient close to unity (1.02 ± 0.12, three experiments) and a $K_i$ value of 0.17 ± 0.05 nM (three experiments), quite similar to the $K_d$ value determined for [$^{125}$I]NT from saturation experiments. In contrast, NT inhibition of [$^3$H]SR 48692 binding produced a shallow curve (Hill coefficient of 0.55) with a portion (~20%) of specific [$^3$H]SR 48692 binding that was not displaceable by up to 10 μM NT. Computer analysis of the data indicated that they fit best to a three-site model with high and low affinity NT binding sites, which represented approximately 60% and 20%, respectively, of the total population of [$^3$H]SR 48692 binding sites, and a third site with no or very low affinity for NT, which accounted for the remaining 20% (Table 3). Interestingly, the low and very low affinity NT binding sites, which could not have been detected in saturation experiments with [$^{125}$I]NT, amounted to 40% of total [$^3$H]SR 48692 binding, a value close to the difference between the $B_{max}$ values obtained in saturation experiments with [$^3$H]SR 48692 and [$^{125}$I]NT (Fig. 3; Table 1).

**Effect of digitonin.** The portion of [$^3$H]SR 48692 binding that was not inhibited by NT might represent a fraction of vesiculated NT receptors that were accessible to the hydrophobic nonpeptide antagonist but not the more hydrophilic peptide agonist. To test this hypothesis, the binding of [$^3$H]SR 48692 and [$^{125}$I]NT was measured in the presence of increasing concentrations of digitonin. Fig. 5 shows that low concentrations of detergent (3 or 10 μg/ml) increased [$^{125}$I]NT binding, whereas they did not affect [$^3$H]SR 48692 binding. Higher concentrations of digitonin resulted in a loss

TABLE 1

**Binding parameters for [$^3$H]SR 48692 and [$^{125}$I]NT binding to homogenates of transfected LTK$^-$ cells**

The values are the means ± standard errors of three experiments.

| | $k_1$[L] + $k_{-1}$[a] | $k_{-1}$[b] | $k_1$[c] | $K_d$[d] | $K_d$[e] | $B_{max}$[e] |
|---|---|---|---|---|---|---|
| | *min*$^{-1}$ | *min*$^{-1}$ | *nM*$^{-1}$ *min*$^{-1}$ | *nM* | *nM* | *pmol/mg* |
| [$^3$H]SR 48692 | 0.12 ± 0.04 | 0.062 ± 0.002 | 0.028 ± 0.008 | 2.2 | 3.4 ± 1.0 | 3.8 ± 0.3 |
| [$^{125}$I]NT | | | | | 0.22 ± 0.04 | 2.6 ± 0.3[f] |

[a] Derived from association kinetics (Fig. 2A).
[b] Derived from dissociation kinetics (Fig. 2B).
[c] Calculated from ($k_1$[L] + $k_{-1}$) and $k_{-1}$ values.
[d] Calculated as the ratio $k_{-1}/k_1$.
[e] Derived from Scatchard analysis (Fig. 3).
[f] $p < 0.5$, compared with the $B_{max}$ value obtained with [$^3$H]SR 48692 (Student's *t* test).

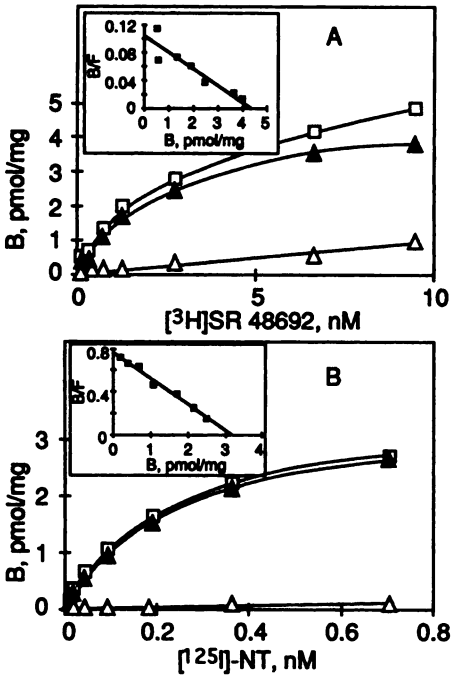


**Fig. 3.** Total (□), specific (▲), and nonspecific (△) [$^3$H]SR 48692 (A) and [$^{125}$I]NT (B) binding to homogenates of transfected LTK$^-$ cells as a function of radioligand concentration. The values are from a typical experiment. *Insets*, Scatchard transformation of specific binding data. *B*, bound; *B/F*, bound/free.

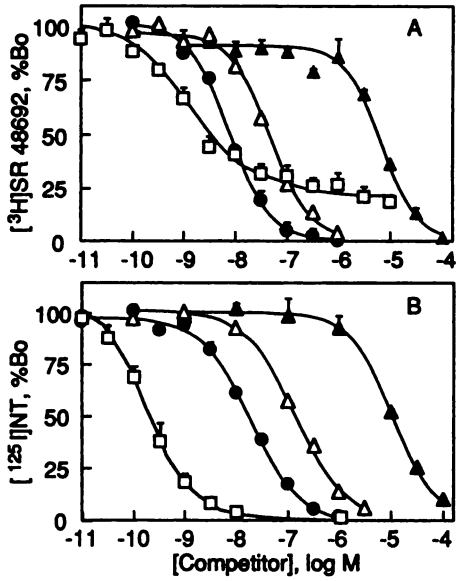


**Fig. 4.** Competitive inhibition of [$^3$H]SR 48692 (A) and [$^{125}$I]NT (B) specific binding to homogenates of transfected LTK$^-$ cells by unlabeled NT (□), SR 48692 (●), SR 48527 (△), and SR 49711 (▲). The values are the means ± standard errors of three independent experiments.

of both [$^{125}$I]NT and [$^3$H]SR 48692 binding. The inhibition of [$^3$H]SR 48692 binding by unlabeled NT and SR 48692 in the absence and presence of 10 μg/ml digitonin is shown in Fig. 6. The detergent had no effect on the SR 48692 inhibition curve (see $K_i$ values for SR 48692 in Table 3). In contrast, it increased the proportions of high and low affinity NT binding sites and markedly reduced the NT-insensitive fraction of binding (Table 3).

**Effects of sodium and guanyl nucleotide.** The inhibition of [$^3$H]SR 48692 binding by increasing concentrations of unlabeled SR 48692 and NT was studied in the absence or presence of 140 mM $Na^+$, 10 μM GTPγS, or a combination of both agents. Fig. 6 shows that the SR 48692 inhibition curve was slightly shifted to the left by the combination $Na^+$ plus GTPγS and that, in contrast, the NT inhibition curve was markedly shifted to the right. Analysis of the data (Table 3) indicated that $Na^+$ and GTPγS had similar effects, i.e., they concomitantly decreased and increased the proportions of high and low affinity NT binding sites, respectively. Tested in combination, they inverted the proportions of high and low affinity NT binding sites, compared with the control (Table 3). In contrast, the $K_i$ value for unlabeled SR 48692 was unaffected by GTPγS and slightly (2-fold) decreased by $Na^+$ (Table 3).

TABLE 2

**Binding parameters for the inhibition of [$^{125}$I]NT and [$^3$H]SR 48692 binding by unlabeled nonpeptide antagonists in homogenates of transfected LTK$^-$ cells**

The values are the means ± standard errors of three experiments.

| | [$^{125}$I]NT | | [$^3$H]SR 48692 | |
|---|---|---|---|---|
| | $K_i$ | Slope | $K_i$ | Slope |
| | *nM* | | *nM* | |
| SR 48692 | 11.2 ± 1.4 | 0.95 ± 0.05 | 5.6 ± 0.4 | 0.98 ± 0.05 |
| SR 48527 | 85 ± 3 | 0.97 ± 0.05 | 31 ± 3 | 0.99 ± 0.06 |
| SR 49711 | 6200 ± 220 | 1.02 ± 0.01 | 4200 ± 970 | 1.03 ± 0.12 |

**Mutagenesis studies.** Amino acids 45–60 of the rat NT receptor, in the amino-terminal extracellular region near the first transmembrane segment, were deleted (Fig. 7). The wild-type and mutant (Δ45–60) receptors were transiently transfected into COS M6 cells and the binding of [$^{125}$I]NT and [$^3$H]SR 48692 was tested in homogenates of transfected cells. No [$^{125}$I]NT binding could be detected with the mutant

TABLE 3

**Binding parameters for NT and SR 48692 inhibition of [$^3$H]SR 48692 binding to homogenates of transfected LTK$^-$ cells and effects of digitonin (10 μg/ml), $Na^+$ (140 mM), GTPγS (10 μM), and a combination of $Na^+$ (140 mM) and GTPγS (10 μM)**

The values are the means ± standard errors of three experiments.

| | NT | | | | | | SR 48692, $K_i$ |
|---|---|---|---|---|---|---|---|
| | Site 1 | | Site 2 | | Site 3 | | |
| | $K_i$ | $B_{max}$ | $K_i$ | $B_{max}$ | $K_i$ | $B_{max}$ | |
| | nM | % | nM | % | nM | % | nM |
| Control | 0.22 ± 0.08 | 59 ± 6 | 57 ± 16 | 21 ± 3 | >$10^5$ | 20 ± 5 | 5.6 ± 0.4 |
| Digitonin | 0.28 ± 0.12 | 71 ± 8 | 215 ± 62 | 26 ± 6 | >$10^5$ | 3 ± 2 | 3.9 ± 0.5 |
| $Na^+$ | 1.1 ± 0.7 | 43 ± 10 | 78 ± 52 | 37 ± 8 | >$10^5$ | 20 ± 2 | 2.8 ± 0.3 |
| GTPγS | 0.10 ± 0.05 | 49 ± 12 | 20 ± 15 | 36 ± 7 | >$10^5$ | 14 ± 6 | 4.3 ± 0.5 |
| $Na^+$ + GTPγS | 1.4 ± 0.8 | 27 ± 1 | 68 ± 24 | 59 ± 4 | >$10^5$ | 14 ± 5 | 2.2 ± 0.2 |

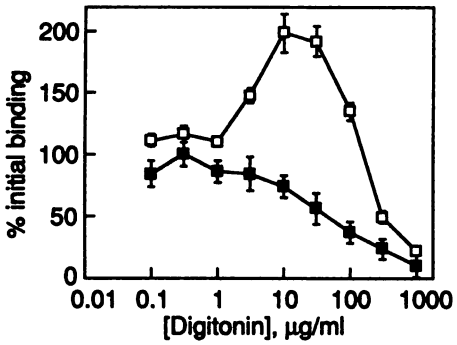


**Fig. 5.** Effect of increasing digitonin concentrations on the specific binding of 2 nM [$^3$H]SR 48692 (■) and 0.05 nM [$^{125}$I]NT (□) to homogenates of transfected LTK$^-$ cells. The values are the means ± standard errors of three independent experiments.

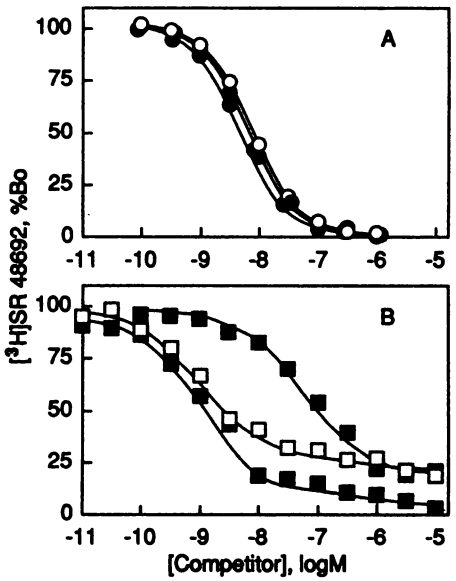


**Fig. 6.** Competitive inhibition of [$^3$H]SR 48692 specific binding to homogenates of transfected LTK$^-$ cells by unlabeled SR 48692 (A) and NT (B) in the absence (*open symbols*) or presence of 10 μg/ml digitonin (*closed symbols*) or 140 mM NaCl plus 10 μM GTPγS (*hatched symbols*). The data are expressed as the percentage of initial [$^3$H]SR 48692 binding (*Bo*). The values are the means of three independent experiments. Standard errors did not exceed 10% of the means and were omitted for clarity. The $K_i$ values for NT and SR 48692 are given in Table 3.

receptor (data not shown). In contrast, saturation experiments with [$^3$H]SR 48692 showed that the wild-type and mutant receptors exhibited the same $K_d$ (wild-type, 2.6 ± 0.2 nM; Δ45–60, 3.0 ± 0.3 nM; three experiments) and $B_{max}$ (wild-type, 21 ± 4 pmol/mg; Δ45–60, 23 ± 4 pmol/mg; three experiments) values for the labeled antagonist. Inhibition of [$^3$H]SR 48692 binding by increasing concentrations of SR 48692 and the enantiomers SR 48527 and SR 49711 yielded virtually superimposable curves for each compound with the wild-type receptor, compared with the mutant NT receptor (Fig. 7A). The $K_i$ values for the three unlabeled antagonists were quite similar to those given in Table 2 for the wild-type NT receptor stably transfected into LTK$^-$ cells. Unlabeled NT inhibited [$^3$H]SR 48692 binding to the wild-type NT receptor transfected into COS M6 cells (Fig. 7B) in a fashion similar to that observed with stably transfected LTK$^-$ cells (Fig. 4A). In marked contrast, the NT inhibition curve obtained with the mutant receptor was shifted to the right by >3 orders of magnitude (see $K_i$ values for NT in the legend to Fig. 7). Otherwise, the curve was parallel to that for the wild-type NT receptor. In particular, it exhibited the same shallow slope, with approximately 20% of specific [$^3$H]SR 48692 binding not being displaceable by NT (Fig. 7B).

## Discussion

This paper presents the first detailed biochemical and pharmacological characterization of the binding properties of a newly developed, radiolabeled, nonpeptide NT receptor antagonist, [$^3$H]SR 48692. The characterization was carried out using a mouse fibroblast LTK$^-$ cell line stably transfected with the rat NT receptor. The transfected cell line has been shown to express functional NT receptors that are antagonized by SR 48692 (11). [$^3$H]SR 48692 bound to the transfected rat NT receptor in a specific, time-dependent, reversible, and saturable manner. The nonspecific binding represented a trivial portion of the total binding over a wide range of [$^3$H]SR 48692 concentrations. The stereoselectivity of the binding was demonstrated with SR 48527 and SR 49711, two enantiomers that are structurally related to SR 48692 and that inhibited [$^3$H]SR 48692 binding with potencies that differed by 2 orders of magnitude. Similar differences in potencies have been reported for the inhibition by the enantiomers of [$^{125}$I]NT binding to guinea pig brain, ileal, and colonic membranes and for their ability to antagonize NT augmentation of $K^+$-evoked dopamine release in guinea pig striatal slices, NT-induced contraction in guinea pig ileum, and NT-induced relaxation in guinea pig colon (3, 6).

Scatchard analysis of saturation experiments indicated

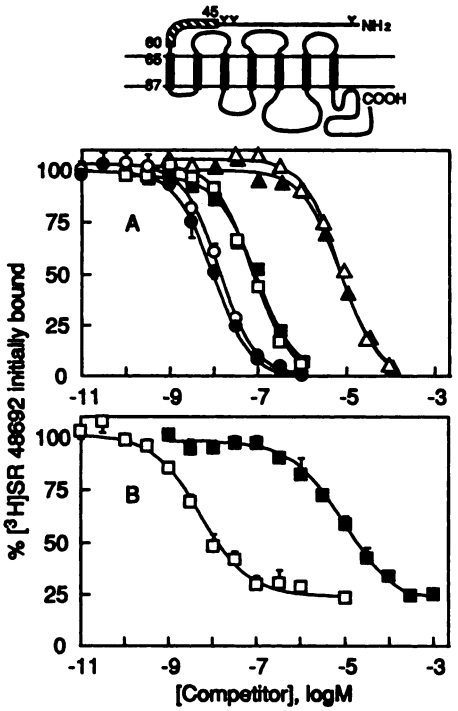


**Fig. 7.** Competitive inhibition of [$^3$H]SR 48692 specific binding to homogenates of COS M6 cells transfected with wild-type (*open symbols*) or Δ45–60 mutant (*closed symbols*) NT receptors by unlabeled SR 48692 (*circles*), SR 48527 (*squares*), and SR 49711 (*triangles*) (A) and unlabeled NT (*squares*) (B). *Drawing at the top*, NT receptor, showing the location of the deleted sequence (residues 45–60) (*hatched box*) relative to the first transmembrane segment (residues 65–87) and the putative glycosylation sites (*Y*). The NT binding data in B were best fitted to a three-site model. For the wild-type receptor, $K_i$ values were 0.22 ± 0.17, 14 ± 7, and >$10^5$ nM for sites 1, 2, and 3, respectively. Corresponding $B_{max}$ values were 34 ± 13%, 45 ± 11%, and 22 ± 3%. For the Δ45–60 mutant, $K_i$ values were 0.8 ± 0.5, 14 ± 10, and >100 μM. Corresponding $B_{max}$ values were 28 ± 8%, 57 ± 4%, and 15 ± 5%. The values are the means ± standard errors of three independent experiments.

that [$^3$H]SR 48692 apparently recognized a single class of binding sites. The labeled antagonist detected 30–40% more binding sites than did [$^{125}$I]NT in homogenates of transfected LTK$^-$ cells. Competition experiments with unlabeled SR 48692 yielded a Hill coefficient near unity, thus confirming that the antagonist bound to an apparently homogeneous receptor population. In contrast, competition experiments with unlabeled NT led to complex inhibition curves that were best fitted to a three-site model. The site with high affinity for NT (Table 3) matched, with respect to affinity and binding capacity, the single class of sites seen in saturation experiments with [$^{125}$I]NT (Table 1). In addition, two other sites, one with low affinity and the other with no affinity for NT, were detected. Each of these sites accounted for 20% of the total number of [$^3$H]SR 48692 binding sites.

The site with no affinity for NT might represent either specific binding sites for SR 48692 that are unrelated to the NT receptors or a fraction of the NT receptors expressed in LTK$^-$ cells that are inaccessible to the peptide agonist. The first hypothesis can be ruled out because one might expect that SR 48692 binding sites molecularly distinct from the transfected NT receptors would also be present in untransfected LTK$^-$ cells and no [$^3$H]SR 48692 specific binding could be detected in homogenates of untransfected cells. Rather, the experiments with digitonin support the second hypothesis. Indeed, low concentrations of the detergent increased [$^{125}$I]NT binding without affecting [$^3$H]SR 48692 binding and markedly reduced the proportion of NT-insensitive [$^3$H]SR 48692 binding sites. These digitonin concentrations were much lower than those needed to solublize the transfected NT receptor in LTK$^-$ cells (11) and were in the range used to permeabilize cell membranes (16). This suggests that a fraction of NT receptors were vesicular and therefore rendered inaccessible to the hydrophilic peptide agonist, whereas they could be reached by the hydrophobic nonpeptide antagonist. This fraction of receptors (20% of $B_{max}$) could represent vesicular, newly synthesized, intracellular receptors. In keeping with this interpretation, it was shown by electron microscopic autoradiography of [$^{125}$I]NT binding to rat midbrain slices that a significant fraction (~20%) of specific binding was intraneuronal and frequently associated with rough endoplasmic reticulum and Golgi structures, suggesting that the intracellular binding sites corresponded in part to newly synthesized NT receptors (17).

[$^3$H]SR 48692 also labeled a site that exhibited low affinity for NT and accounted for 20% of maximal [$^3$H]SR 48692 binding. Interestingly, $Na^+$ and GTPγS markedly increased the proportion of the low affinity NT binding site, while diminishing that of the high affinity NT binding site. This is consistent with previous studies of [$^{125}$I]NT binding to membranes prepared from tissues and cell lines that functionally respond to NT, showing that the NT receptor exists in two states, i.e., a high affinity state that can be converted to a lower affinity state by $Na^+$ and GTP (3, 18, 19). It is well documented that antagonists of G protein-coupled receptors do not discriminate between receptor states with high and low agonist binding affinity and that $Na^+$ and guanyl nucleotides differentially modulate antagonist and agonist binding (20). The results obtained here conform to this general pattern. Thus, [$^3$H]SR 48692 bound with the same affinity to the high and low affinity NT binding sites. Furthermore, [$^3$H]SR 48692 binding, unlike [$^{125}$I]NT binding, was not reduced by $Na^+$ and GTPγS. Such a differential modulation of NT and SR 48692 binding by monovalent cations and guanyl nucleotides has recently been observed in studies of [$^{125}$I]NT binding to guinea pig intestinal membranes (3). This property could be useful to screen for compounds with agonist or antagonist activity at the NT receptor, using a binding assay.

A mutant rat NT receptor (Δ45–60) lacking residues 45–60 of the receptor amino acid sequence was constructed. The deleted sequence lies in the amino-terminal extracellular region of the receptor near the first transmembrane segment, which is presumed to comprise residues 65–87 (9). When the wild-type and mutant NT receptors were transiently expressed in COS M6 cells, it was observed in saturation experiments that Δ45–60 bound [$^3$H]SR 48692 with the same $K_d$ and $B_{max}$ values, compared with the wild-type receptor. This result indicated that the two receptors were expressed at similar levels in COS M6 cells. Furthermore, competition experiments with [$^3$H]SR 48692 and unlabeled SR 48692, SR 48527, and SR 49711 revealed identical pharmacological and stereoselectivity profiles for the wild-type and mutant receptors. In contrast, Δ45–60 was devoid of [$^{125}$I]NT binding. Competition experiments with unlabeled NT showed that the peptide bound to the mutant receptor but with a >1000-fold decrease in apparent affinity. These data demonstrate that

the deleted portion of the amino-terminal extracellular region (amino acids 45–60) of the rat NT receptor is crucial for agonist binding, whereas it plays no role in binding SR 48692 and related compounds. This, in turn, strongly suggests that the peptide agonist and the nonpeptide antagonist recognize, at least in part, distinct epitopes of the NT receptor. This further generalizes what has been observed with nonpeptide antagonists of other neuropeptide receptors, such as the cholecystokinin (21), tachykinin (22, 23), and angiotensin (24) receptors. The considerable loss in NT binding affinity observed with the mutant receptor implies a major perturbation of the agonist binding site. The deleted sequence could be an integral part of the peptide binding pocket and/or it could contribute indirectly to the proper conformation of the agonist binding site. This cannot be determined at present. It seems, however, that the portion of the amino-terminal extracellular segment of the NT receptor involved in agonist binding is restricted to a region near the first transmembrane segment, because a deletion mutant receptor lacking residues 14–28 was found to retain full affinity for both [$^{125}$I]NT and [$^{3}$H]SR 48692.[1] Further mutagenesis studies of the NT receptor are in progress, to map more precisely the agonist and antagonist binding domains. The availability of a potent and specific radiolabeled nonpeptide NT antagonist such as [$^{3}$H]SR 48692 should greatly facilitate these studies.

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[1] C. Labbé-Jullié and J.-M. Botto, unpublished observations.

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