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Impaired G protein coupling of the neurotensin receptor 1 by mutations in extracellular loop 3

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Abstract

The neurotensin receptor 1, NTS1, is a G protein-coupled receptor. We have shown previously that the NTS1 receptor-binding site of the peptide agonist involved residues in extracellular loop 3 and at the extracellular junction of transmembrane domains 4 and 6. Here, we investigated by site-directed mutagenesis residues in extracellular loop 3 that might be involved in agonist-induced activation of the rat NTS1 (rNTS1) receptor. Wild type and mutated receptors were expressed in COS (African green monkey kidney fibroblasts) cells. Labeled agonist and antagonist binding as well as inositol phosphate and cAMP productions were studied. Compared to the wild type NTS1 receptor, the W339A, F344A, H348A and Y349A mutant receptors exhibited (i) decreased proportion of high over low affinity agonist binding sites, (ii) increased sensitivity of high affinity agonist binding to GTPγS, and (iii) impaired G protein coupling of high affinity agonist-receptor complexes. The data are consistent with the C-terminal part of extracellular loop 3 being essential for allowing high affinity agonist-NTS1 receptor complexes to couple to G proteins.

Keywords: G protein-coupled receptor; Neurotensin receptor 1; Mutagenesis; Inositol phosphate; cAMP; Transduction, impaired

1. Introduction

Neurotensin is a 13 amino acid peptide that exerts neuromodulatory functions in the central nervous system including the modulation of dopaminergic transmission, and endocrine/paracrine actions in the periphery (Kitabgi, 1989; Rostène and Alexander, 1997; Kinkead et al., 1999). Three neurotensin receptor subtypes, termed NTS1, NTS2 and NTS3 according to the order in which they were cloned, have been identified so far (Vincent et al., 1999). The NTS1 and NTS2 receptors are G protein-coupled receptors and share 60% homology while the NTS3 receptor belongs to an entirely different family of proteins. The NTS1 receptor has high affinity for neurotensin while the NTS2 receptor has lower affinity for the peptide and is selectively recognized by the antihistamine H_1 receptor antagonist levocabastine (Chalon et al., 1996; Mazella et al., 1996). The non-peptide neurotensin antagonist SR 48692, 2-([1-{7-chloro-4-quinolinyl}-5-{2,6-dimethoxy-phenyl}pyrazol-3-yl]carboxylamino)tricyclo(3.3.1.1.[3.7])decan-2-carboxylic acid, preferentially binds to the NTS1 receptor. Many of the known central and peripheral effects of neurotensin are blocked by SR 48692 and can therefore be attributed to the NTS1 receptor (Gully et al., 1993). In particular, SR 48692 antagonizes a number of effects that involve neurotensin interaction with central dopaminergic systems (Rostène et al., 1997; Kinkead et al., 1999). This has led to the suggestion that non-peptide agonist mimetics of neurotensin might be useful in the treatment of brain disorders such as Parkinson's disease and schizophrenia (Kitabgi, 1989; Kinkead et al., 1999). Knowledge of the structural determinants in the NTS1 receptor that participates to neurotensin binding might help for the design of such mimetics.

In a recent study, using mutagenesis approaches combined with structure–activity studies and computer-assisted molecular modeling, we established tridimensional models of the SR 48692 and neurotensin binding sites in the rat

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NTS1 (rNTS1) receptor (Labbe-Jullie et al., 1998; Barroso et al., 2000). As schematically depicted in Fig. 1, the residues that play a role in agonist and antagonist binding can be grouped in three categories. The first category comprises residues (Met^{208}, Phe^{331} and Arg^{327}) at or near the extracellular junction of transmembrane domains 4 and 6 that interact with both neurotensin and SR 48692. The second category comprises residues, most of them within transmembrane domain 7, that are involved in antagonist binding only. The third category comprises residues, Trp^{339}, Phe^{344} and Tyr^{347}, that interact with neurotensin only. They are all found in extracellular loop 3 and appear to contact Tyr^{11} in the neurotensin molecule with Tyr^{347} being essential in this regard as its mutation into alanine decreases neurotensin affinity by four orders of magnitude. These data point to extracellular loop 3 as playing an essential role for agonist binding to the NTS1 receptor.

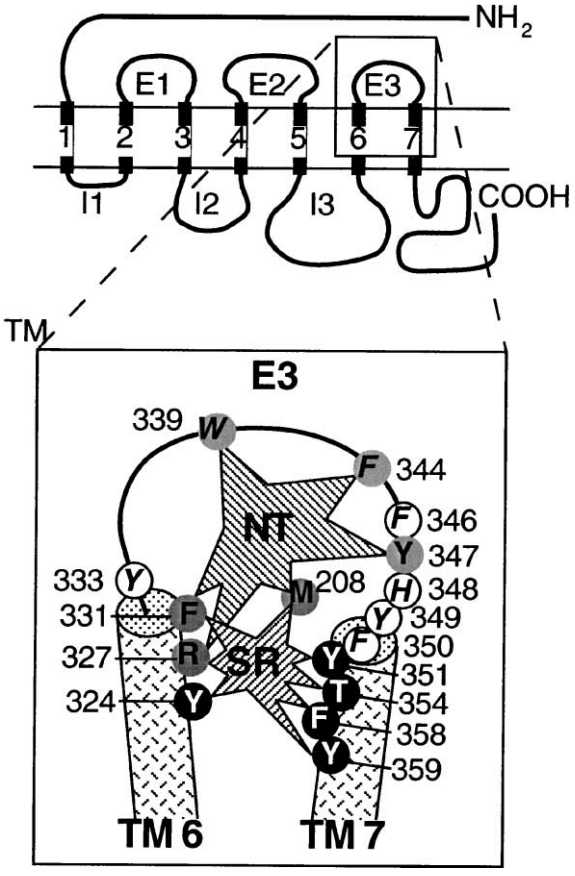

Fig. 1. At the top is shown a serpentine drawing of the rNTS1 receptor with the three extracellular (E) and the three intracellular (I) loops and the seven transmembrane (TM) domains. At the bottom, the transmembrane domains 6 and 7 and the connecting extracellular loop 3 have been enlarged. The agonist and antagonist binding sites (Labbe-Jullie et al., 1998; Barroso et al., 2000) are schematically represented. The receptor residues involved solely in neurotensin binding are shown in light grey, those involved only in SR 48692 binding in black and those involved in both agonist and antagonist binding in grey. The residues whose mutation was studied in the present work are labeled in italic.

Previous studies with stably or transiently rNTS1 receptor-transfected cells have shown that neurotensin could discriminate three binding components with high, low and very low agonist affinity, the latter component representing vesicular receptors that were inaccessible to the peptide agonist (Labbe-Jullie et al., 1995). In contrast, SR 48692 displayed the same affinity for the three components. In the course of characterizing the binding properties of rNTS1 receptors mutated in extracellular loop 3, we obtained preliminary evidence that the W339A and F344A mutations changed the proportion of high and low agonist affinity binding sites and the potency of agonist-induced second messenger production (Barroso et al., 2000). In the present work, we examined the effect of a number of mutations in extracellular loop 3 on agonist and antagonist equilibrium binding properties and on receptor-G protein coupling. In addition to the W339A and F344A mutations, two other mutations, H348A and Y349A, markedly decreased the proportion of high affinity neurotensin binding sites and impaired coupling of the rNTS1 receptor to G proteins. Paradoxically, sensitivity to GTPγS of the high affinity binding neurotensin site was increased in the mutant receptors. The data show that extracellular loop 3 in the rNTS1 receptor is not only essential for agonist binding but also for agonist-induced activation of the receptor. They are discussed with regard to our model of the NT/NTS1 receptor complex and to the existence of the high and low affinity agonist binding components of the NTS1 receptor.

2. Materials and methods

2.1. Materials

Neurotensin was from Neosystem and SR 48692 from Sanofi Recherche. Monoiodo-[^{125}I-Tyr^{3}]-neurotensin (^{125}I-neurotensin) was prepared as described (Bidard et al., 1993). [^{3}H]SR 48692 was from Amersham. Mutant receptors were obtained in the pcDNA3 plasmid as previously described (Barroso et al., 2000).

2.2. Cell culture and transfection

COS M6 cells (African green monkey kidney fibroblasts) were grown in Dulbecco's modified Eagle's medium (Gibco) containing 8% fetal bovine serum (Dutcher) and 50 μg/ml gentamicine (Sigma). For transient transfection, 100 mm cell culture dishes seeded with 10^6 cells the day before were washed twice with Tris-buffered saline (Tris 25 mM, NaCl 137 mM, $CaCl_2$ 2.3 mM, $MgCl_2$ 0.5 mM, Na_2HPO_4 0.4 mM, pH 7.4) and incubated for 30 min with 1 μg recombinant pcDNA3 plasmid in the presence of DEAE Dextran (0.5 mg/ml) at room temperature. After 3 h in culture medium supplemented with 100 μM chloroquine, cells were washed twice with Tris-buffered saline, and cultured for 48 to 72 h. For the wild type receptor,

several transient transfections were realized with increasing recombinant pcDNA3 plasmid ranging from 1 ng to 1 μg per 100 mm cell culture dish.

2.3. Cell membranes preparation

Transfected cells were washed twice with phosphate-buffered saline and collected in ice-cold Tris/HCl 5 mM, pH 8. After homogenization by repeated passages through a syringe needle and centrifugation at 4 °C for 30 min at 100,000 × *g*, cell membranes were resuspended in 300 μl per dish of Tris/HCl 5 mM, pH 7.5, and stored at −20°C. Membrane protein concentration was determined by the Bio Rad Protein Assay.

2.4. Inositol phosphate determination

Twenty-four hours after transfection with the wild type rNTS1 receptor or mutant receptor, cells were trypsinized and grown for 18 h in 12 well plates in culture medium in the presence of 0.5 μCi [^{3}H]myo-inositol (ICN). After two washes with Earle buffer (HEPES 25 mM, Tris 25 mM, NaCl 140 mM, KCl 5 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.9 mM, glucose 5 mM containing 0.1% bovine serum albumin), cells were incubated for 15 min at 37 °C in 500 μl of 20 mM LiCl in Earle buffer. Then, neurotensin was added at the indicated concentrations in 5 μl of Earle buffer for 15 min. The reaction was stopped by 750 μl of ice cold 10 mM HCOOH. After 1 h at 4 °C, the supernatant was collected and neutralized by 3 ml of 5 mM NH_4OH. Total [^{3}H]-inositol phosphates were separated from free [^{3}H]-inositol on Dowex AG1-X8 (Bio Rad) chromatography by eluting successively with 5 ml of water and 4 ml of 40 mM and 1 M ammonium formate buffer, pH 5.5. The radioactivity contained in the 1 M fraction was counted after addition of 5 ml Ecolume (ICN).

2.5. cAMP determination

Twenty-four hours after transfection with the wild type rNTS1 receptor or mutant receptor, cells were trypsinized and grown for 18 h in six well plates in culture medium in the presence of 1 μCi [2,8-^{3}H]adenine (ICN). After two washes with Earle buffer (HEPES 25 mM, Tris 25 mM, NaCl 140 mM, KCI 5 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.9 mM, glucose 5 mM containing 0.1% bovine serum albumin), cells were incubated for 10 min at 37 °C in 800 μl of 1 mM isobutylmethylxanthine in Earle buffer. Then, cells were incubated for 20 min with varying concentration of neurotensin in 1 mM isobutylmethylxanthine–Earle buffer. The incubation medium was removed and the reaction was stopped by 800 μl of ice cold 5% Trichloroacetic acid containing 2 mM of unlabeled cAMP and 2 mM ATP as carriers. After 30 min at 4 °C, the supernatant was collected and each well was washed with 500 μl of water and the wash was combined with the supernatant fraction (final sample volume: 1 ml). Samples were loaded on 1.2 ml Dowex 50W-X4 columns (200–400 mesh, hydrogen form Fluka), pre-equilibrated in water. First, the columns were rinsed with 3 ml of H_2O which eluted most of the [^{3}H]-ATP. Then, the Dowex columns were eluted with 10 ml of H_20 that were directly loaded on 0.8 ml alumina (Sigma) columns equilibrated in 100 mM imidazole–HCl buffer pH 7.5. Each alumina column was subsequently eluted with 6 ml of imidazole buffer. Eluates, containing [^{3}H]-cAMP, were mixed with 7 ml Ecolume (ICN) and counted for radioactivity.

2.6. Binding experiments

Binding experiments with ^{125}I-neurotensin and [^{3}H]SR 48692 were performed as previously described (Labbe-Jullie et al., 1998; Barroso et al., 2000). Briefly, experiments with both radioligands were carried out with 1 to 10 μg of cell membrane proteins in a final volume of 250 μl of 50 mM Tris/HCl, pH 7.5, containing 0.1% bovine serum albumin and 0.8 mM 1,10-phenantroline, for 20 min at room temperature. The reaction was stopped by addition of 2 ml of ice cold buffer and filtration on cellulose acetate filter (0.2 μm, Sartorius) followed by two washes of the tube and filter with 2 ml of the same buffer. Non-specific binding was determined in the presence of 1 μM unlabeled ligand. For saturation experiments, concentrations of radioligand ranging from 0.01 to 2 nM for ^{125}I-neurotensin or from 0.1 to 10 nM for [^{3}H]SR 48692 were tested. For competitive inhibition experiments, increasing concentrations of unlabeled ligands were incubated with 0.05 nM ^{125}I-neurotensin or 2 nM [^{3}H]SR 48692. Saturation and competition data were analyzed by the LIGAND software (Munson and Rodbard, 1980). Competition data were fitted according to a three-site model (Labbe-Jullie et al., 1995). In order to facilitate the determination of low affinity neurotensin binding parameters from competition experiments of [^{3}H]SR 48692 binding by unlabeled NT, high affinity neurotensin binding parameters (K_d and B_{max}) determined in parallel from saturation experiments with ^{125}I-neurotensin using the same membrane preparations were entered as fixed parameters in LIGAND analysis of competition curves. Best fit to a three-site model was significantly better than to one- or two-site models ($P < 0.01$).

2.7. Statistics

All comparisons were made using the Student's *t*-test.

3. Results

3.1. Effect of mutations in extracellular loop 3 of the rNTS1 receptor on agonist and antagonist equilibrium binding parameters

A number of residues in extracellular loop 3 of the rNTS1 receptor, essentially aromatic (Fig. 1), were mutated into alanine, and the wild type and mutant receptors were

transiently transfected in COS cells. As recalled in the Introduction, previous studies with the rNTS1 receptor have shown that neurotensin could discriminate three receptor populations, two of them having, respectively, high and low affinity for neurotensin and the third one being inaccessible to the peptide, whereas SR 48692 displayed the same affinity for the three components (Labbe-Jullie et al., 1995). In order to assess the effect of the mutations on the high affinity binding component, saturation experiments with ^{125}I-neurotensin were performed with membranes prepared from the transfected cells (in the concentration range used in these experiments, ^{125}I-neurotensin binding occurs chiefly to the high affinity binding component). Linear Scatchard plots were obtained for all receptors (not shown). The data in Table 1 show that, when compared to the wild type rNTS1 receptor, none of the mutations markedly affected the K_d values of neurotensin, except the W339A and F334A mutations which, as previously reported (Barroso et al., 2000), increased 6- to 10-fold the agonist K_d value, and the Y349A mutation which increased 4-fold the neurotensin K_d value. Furthermore, the Y333A, F346A and F350A mutations yielded neurotensin B_{max} values that were comparable to that obtained with the wild type rNTS1 receptor. In contrast, the W339A, F344A, H348A and Y349A mutations resulted in B_{max} values that were 15 to 50 times lower than that of the wild type receptor (Table 1).

The above data could mean either that the mutations decreased the proportion of high affinity neurotensin binding sites or that they resulted in lower global expression of the rNTS1 receptor. In order to address this point, we performed saturation experiments with [^{3}H]SR 48692 that binds to all populations of NTS1 receptor binding sites. Linear Scatchard plots were obtained for the wild type and the mutant receptors (not shown). Table 1 shows that the mutations did not significantly affect the K_d value of SR 48692. There were indeed variations in B_{max} values for the radiolabeled antagonist that ranged between 2.9 pmol/mg for the F344A mutant and 17.2 pmol/mg for the Y349A mutant (Table 1). However, the ratio of the B_{max} value for [^{3}H]SR 48692 to that for ^{125}I-neurotensin was similar for the Y333A, F346A and F350A mutant receptors, whereas it was 5 to 25 times higher for the W339A, F344A, H348A and Y349A mutant receptors, as compared to that for the wild type rNTS1 receptor (Table 1). In particular, the Y349A mutant that exhibited the same SR 48692 B_{max} value as the wild type rNTS1 receptor displayed approximately 25 times less high affinity neurotensin binding sites than the wild type receptor. These data suggest that the W339A, F344A, H348A and Y349A mutations decreased the proportion of high affinity neurotensin binding sites independently of global receptor expression levels.

Table 1
K_d and B_{max} values for ^{125}I-neurotensin and [^{3}H]SR 48692 binding to wild type and mutated NTS1 receptors were derived from Scatchard analysis of saturation experiments as described under Materials and methods

Receptor	^{125}I-neurotensin		[^{3}H]SR 48692		B_{max} SR/NT
	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	
WT	0.12 ± 0.02	7.5 ± 2.1	2.6 ± 0.2	16 ± 5	2.2
Y333A	0.20 ± 0.05[a]	5.8 ± 1.3	2.9 ± 0.6[b]	14 ± 2	2.4
W339A	1.1 ± 0.2[c]	0.40 ± 0.10	2.6 ± 0.1[b]	5.1 ± 0.9	12
F344A	0.69 ± 0.18[a]	0.16 ± 0.06	2.5 ± 0.4[b]	2.9 ± 0.3	18
F346A	0.22 ± 0.02[a]	4.0 ± 0.6	3.1 ± 0.4[b]	9.8 ± 1.9	2.5
H348A	0.22 ± 0.02[a]	0.45 ± 0.16	1.7 ± 0.3[b]	12 ± 2	27
Y349A	0.45 ± 0.10[a]	0.33 ± 0.12	3.0 ± 0.8[b]	17 ± 7	52
F350A	0.23 ± 0.03[a]	4.3 ± 0.9	5.4 ± 1.2[b]	9.3 ± 1.9	2.1

B_{max} SR/NT: ratio of the B_{max} value for SR 48692 to that for neurotensin. The data for the F344A and W339A mutants are taken from Barroso et al. (2000). Values are the means ± S.E.M. from three independent determinations.

[a] $P < 0.05$ when compared to wild type.

[b] Not significant when compared to wild type.

[c] $P < 0.01$ when compared to wild type.

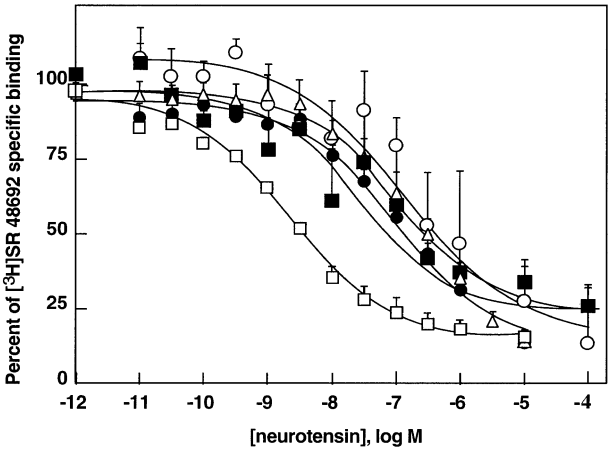

Fig. 2. Competitive inhibition of [^{3}H]SR 48692 specific binding by neurotensin to wild type and mutated rNTS1 receptors. Competition binding experiments were performed with membrane homogenates of COS M6 cells transfected with the wild type rNTS1 receptor (open squares) and the W339A (closed squares), F344A (open circles), H348A (closed circles) or Y349A (open triangles) mutant receptors. The data for the F344A and W339A mutants are taken from Barroso et al. (2000). The results are expressed as the percent of [^{3}H]SR 48692 specific binding in the absence of competitor. The values are the means ± S.E.M. from three independent experiments.

In order to determine the proportion of low affinity neurotensin binding sites and of sites that are not accessible to the agonist, competition experiments of [^{3}H]SR 48692 binding by unlabeled neurotensin were performed with the wild type rNTS1 receptor and the W339A, F334A, H348A and Y349A mutant receptors. Fig. 2 shows that the competition curves were largely shifted to the right for the four mutant receptors as compared to the wild type rNTS1 receptor. The curves were fitted to a three-site model using the LIGAND software as explained in Materials and methods. The data in Table 2 show that low affinity K_d values (site 2) were higher for the mutants than for the wild type receptor and that the proportion of low over high affinity neurotensin binding sites varied from 0.8 for the wild type

Table 2
Determination of high, low and very low affinity equilibrium binding parameters for neurotensin with the wild type and extracellular loop 3 mutant receptors

Receptor	Neurotensin binding parameters						B_{max} ratio
	Site 1		Site 2		Site 3		Site 2/ Site 1
	K_d (nM)	B_{max} (%)	K_i (nM)	B_{max} (%)	K_i (nM)	B_{max} (%)	
WT	0.12	48±4	19±4.5	37±3	>10,000	15±1	0.8
W339A	1.13	8.3±0.1[a]	40±16	72±3[a]	>10,000	20±3[b]	9
F344A	0.69	6.3±0.8[a]	160±30	75±3[a]	>10,000	19±2[b]	12
H348A	0.22	3.7±0.1[a]	110±30	80±3[a]	>10,000	16±3[b]	22
Y349A	0.45	1.9±0.1[a]	170±20	84±2[a]	>10,000	14±2[b]	44

Site 1 (high affinity) binding parameters were derived from Scatchard analysis of ^{125}I-neurotensin saturation experiments (Table 1), and site 2 (low affinity) and site 3 (very low affinity) binding parameters were derived from competition of [^{3}H]SR 48692 binding by unlabeled neurotensin (Fig. 2) using LIGAND software analysis, as described in Materials and methods. B_{max} values are given as the percent of the total SR 48692 binding capacity. Values are the means±S.E.M. from three independent experiments.

[a] $P<0.01$ when compared to wild type.

[b] Not significant when compared to wild type.

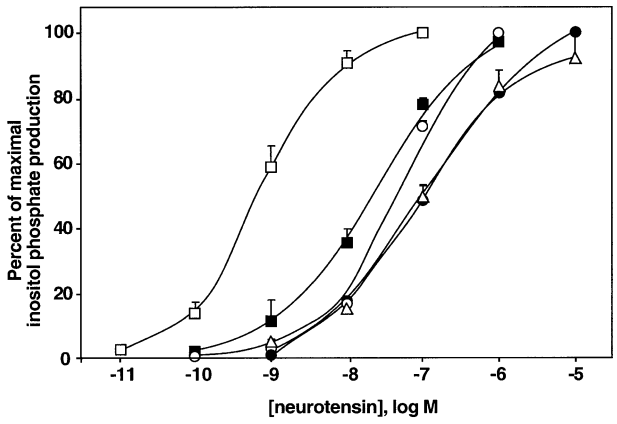

Fig. 4. Effect of neurotensin on inositol phosphate production in COS M6 cells transfected with wild type and mutant receptors. Concentration–response curves for NT-stimulated inositol phosphate production were performed with the wild type receptor (open squares) and W339A (closed squares), F344A (open circles), H348A (closed circles) or Y349A (open triangles) mutant receptors. The data for the F344A and W339A mutants are taken from Barroso et al. (2000). The results are expressed as the percent of the maximal NT-stimulated inositol phosphate production obtained with each receptor (see E_{max} values in Table 3). The values are the means±S.E.M. from three independent experiments.

rNTS1 receptor to 10–40 for the four mutant receptors. Furthermore, the portion of [^{3}H]SR 48692 binding that could not be competed for by unlabeled neurotensin, which represents sites that are inaccessible to the peptide agonist, was similar for all receptors (15–20%). These data clearly establish that the four mutations in extracellular loop 3 markedly decreased the proportion of high affinity neurotensin binding sites.

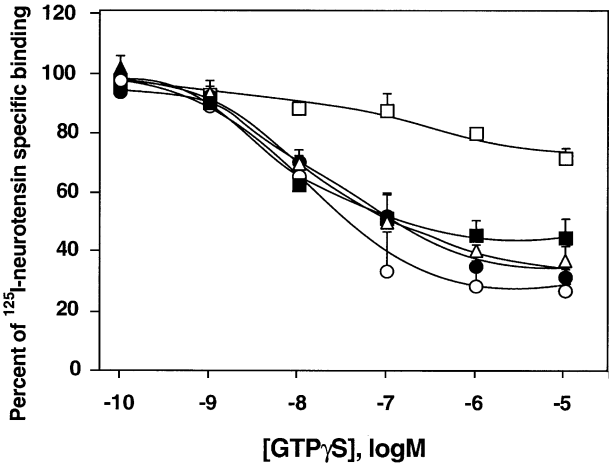

Fig. 3. Effect of GTPγS on ^{125}I-neurotensin specific binding to wild type and mutant rNTS1 receptor. Binding experiments were performed with membrane homogenates from COS M6 cells transfected with wild type (open squares) and W339A (closed squares), F344A (open circles), H348A (closed circles) or Y349A (open triangles) mutant receptors. The data are expressed as the percent of ^{125}I-neurotensin specific binding in the absence of nucleotide. The values are the means±S.E.M. from three independent experiments.

3.2. The W339A, F344A, H348A and Y349A mutations increase neurotensin binding sensitivity to GTPγS

^{125}I-neurotensin binding to the high affinity binding component of the wild type rNTS1 receptor is guanyl-nucleotide sensitive, as previously reported (Labbe-Jullie et al., 1995). Here, the effect of increasing concentrations of GTPγS on the binding of 0.05 nM ^{125}I-neurotensin was compared for the wild type rNTS1 receptor and the four mutant receptors (Fig. 3). At this concentration of labeled agonist, most of the binding (>90%) occurs to the high affinity component. GTPγS concentration-dependently inhibited neurotensin binding to the wild type receptor with a maximal inhibitory

Table 3
EC_{50} and E_{max} values for neurotensin-stimulated inositol phosphate and cAMP productions in wild type and mutated rNTS1 expressing cells were derived from concentration–response experiments as represented in Fig. 4 (inositol phosphate production) and Fig. 5 (cAMP production)

Receptor	Inositol phosphate		cAMP	
	Neurotensin EC_{50} (nM)	E_{max} (dpm/well)	Neurotensin EC_{50} (nM)	E_{max} (dpm/well)
WT	0.77±0.18	1740±480	5.5±1.8	1400±380
W339A	28±5[a]	940±360	9100±6500	1250±420
F344A	47±4[a]	630±200	7800±1900	2300±570
H348A	110±20[a]	3200±380	4200±1500	560±90
Y349A	140±30[a]	3900±1600	14100±1200	850±170

EC_{50}: neurotensin concentration eliciting half-maximal effect. E_{max}: maximal neurotensin-stimulated inositol phosphate or cAMP production minus basal levels in the absence of agonist. Values are the means±S.E.M. from three independent determinations.

[a] $P<0.01$ when compared to wild type.

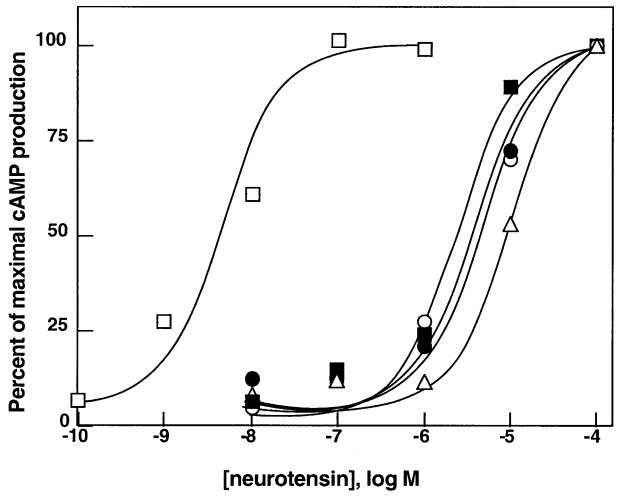

Fig. 5. Effect of neurotensin on cAMP production in COS M6 cells transfected with wild type and mutant receptors. Concentration–response curves for NT-stimulated cAMP production were performed with the wild type receptor (open squares) and W339A (closed squares), F344A (open circles), H348A (closed circles) or Y349A (open triangles) mutant receptors. The data are expressed as the percent of the maximal NT-stimulated cAMP production obtained with each receptor (see E_{max} values in Table 3). The values are from a typical experiment.

effect of 30% (Fig. 3). Interestingly, the inhibitory effect of the nucleotide on neurotensin binding was much more pronounced for the receptors mutated in extracellular loop 3, reaching 50% with the W339A mutant and 70% with the three other mutants (Fig. 3).

3.3. The W339A, F344A, H348A and Y349A mutations affect G protein coupling of the rNTS1 receptor

The above data suggest that mutations in extracellular loop 3 of the rNTS1 receptor affect the coupling of the receptor to G proteins. Therefore, concentration–response curves were established for the ability of neurotensin to stimulate phosphoinositide hydrolysis in COS cells expressing the wild type and the four mutant receptors. As seen in Fig. 4, the mutant receptors were functionally coupled to inositol phosphate production. However, EC_{50} values were 40 to 200 times higher for the mutants than for the wild type receptor (Table 3). Thus, EC_{50} values for the mutant receptors were closer to low than to high affinity K_d values, in contrast to the wild type receptor for which the reverse was true. It can further be noted by comparing Table 3 and Table 1 that for the four mutant receptors E_{max} values for neurotensin-stimulated inositol phosphate production correlated with B_{max} values for the low affinity neurotensin binding sites (Y349A>H348A>W339A>F344A) whereas they showed no correlation with high affinity B_{max} values.

Previous studies have shown that high expression of the NTS1 receptor in transfected cell systems led to its coupling to G_s, though the potency of neurotensin for stimulating cAMP production was lower than that for inducing inositol phosphate production (Yamada et al., 1994). In agreement with these data, Fig. 5 shows that neurotensin stimulated cAMP production in wild type rNTS1 receptor-expressing COS cells with an EC_{50} that was 5- to 10-fold higher than that obtained for inositol phosphate production (Table 3). Neurotensin also stimulated cAMP production in COS cells expressing the four extracellular loop 3 mutant receptors, but in this case the dose–response curves were largely shifted to the right (Fig. 5). The EC_{50} values obtained in this test with the four extracellular loop 3 mutant receptors were two to three orders of magnitude higher than corresponding EC_{50} values for inositol phosphate production (Table 3).

3.4. Effect of NTS1 receptor expression levels on agonist and antagonist binding and on G protein coupling

The above data suggest that even though the mutant receptors exist in a high agonist affinity, GTP-sensitive state, this state is not coupled to inositol phosphate production. However, the amount of high affinity binding sites expressed in cells transfected with the mutant receptors was much lower than that obtained with the wild type rNTS1 receptor (Table 1). In order to check whether expression levels of the high affinity binding site could influence its coupling to inositol phosphate production, varying amounts of wild type rNTS1 receptor were transfected in COS cells

Table 4
Neurotensin-stimulated inositol phosphate production as a function of wild type rNTS1 receptor expression

Binding			Inositol phosphate production		
^{125}I-neurotensin K_d (nM)	^{125}I-neurotensin B_{max} (pmol/mg)	B_{max} SR/NT	Basal (dpm/well)	E_{max} (dpm/well)	EC_{50} (nM)
0.19 ± 0.08	0.53 ± 0.23	4.0 ± 0.8	150 ± 4	480 ± 95	2.2 ± 1.1
0.21 ± 0.03	1.6 ± 0.4	3.9 ± 0.8[a]	210 ± 10	1300 ± 150	3.2 ± 0.9[a]
0.19 ± 0.04	7.7 ± 3.0	3.1 ± 1.1[a]	240 ± 30	2300 ± 450	3.2 ± 0.7[a]

COS cells transfected with varying amounts of wild type rNTS1 receptor/pcDNA3 plasmid were divided in 24 well plates for inositol phosphate measurement and 100 mm culture dishes for cell membrane preparation. K_d and B_{max} values for radioligand binding were determined from saturation experiments with cell membranes. B_{max} SR/NT: ratio of the B_{max} value for SR 48692 to that for neurotensin. Basal: inositol phosphate production in absence of agonist. E_{max}: maximal neurotensin-stimulated inositol phosphate production. EC_{50}: neurotensin concentration eliciting half-maximal inositol phosphate production. Values are the means ± S.E.M. from three independent experiments.

[a] Not significant when compared to values obtained at lowest receptor expression level.

and saturation binding experiments with ^{125}I-neurotensin and [^{3}H]SR 48692 as well as concentration–response curves for NT-stimulated inositol phosphate production were performed. The data in Table 4 show that while high affinity neurotensin binding capacities varied more than 10-fold there was no significant difference in high affinity K_d values. Similarly, maximal neurotensin-stimulated inositol phosphate production (E_{max} values) rose with increasing receptor expression levels but EC_{50} values did not significantly vary (Table 4). Note that the lowest high affinity B_{max} value obtained for the wild type rNTS1 receptor (0.5 pmol/mg) in these experiments was within the range of high affinity B_{max} values observed for the mutant receptors (Table 1). Therefore, it is unlikely that the high neurotensin EC_{50} values for inositol phosphate production observed with the mutant receptors resulted from low expression levels of the high affinity neurotensin binding site. It can further be noted in Table 4 that antagonist over agonist B_{max} ratio values showed little variation as a function of receptor expression. Finally, Table 4 shows that basal inositol phosphate production was virtually independent of the level of NTS1 receptor expression in transfected COS cells.

4. Discussion

In the present work, we show that the W339A, F344A, H348A and Y349A mutations in extracellular loop 3 of the rNTS1 receptor exhibited a strikingly similar phenotype characterized by (i) markedly decreased proportion of receptors having high agonist affinity, (ii) increased sensitivity of high affinity agonist binding to GTPγS, and (iii) strongly decreased potency of neurotensin to stimulate inositol phosphate production as compared to the wild type NST1 receptor. Furthermore, EC_{50} values for stimulating inositol phosphate production were close to low affinity K_d values and E_{max} values paralleled low affinity neurotensin B_{max} values. These data suggest that neurotensin activated the mutant receptors through a low affinity agonist/receptor complex. It may therefore seem paradoxical that high affinity agonist binding was more sensitive to GTPγS with the mutant receptors than with the wild type rNTS1 receptor. This might suggest that the high affinity agonist binding component of the mutant receptors could efficiently couple to G proteins, in contradiction with the observation that the mutations markedly decreased neurotensin potency to promote coupling of the NTS1 receptor to its preferred G protein, G_q. It might be hypothesized that the mutations directed the preference of the NTS1 receptor to other G proteins. However, the data presented here exclude that the high affinity mutant receptors coupled to G_s as the potency of neurotensin to stimulate cAMP formation was even more decreased by the mutations than the potency to activate inositol phosphate production. As no inhibitory effect of neurotensin on cAMP production could be observed in COS cells transfected with the wild type and mutant receptors (data not shown), a coupling of the NTS1 receptor to the family of G_i proteins in this system can also be excluded. It remains possible that the mutant receptors retained or acquired the capability to couple to an as yet unidentified G protein. Similar observation was recently reported for a chimeric mutant of the angiotensin AT_1 receptor in which transmembrane domain 4 was replaced by the corresponding domain of the AT_2 receptor (Feng and Karnik, 1999). The chimeric receptor was unresponsive to the agonist and, nonetheless, exhibited high affinity agonist binding that was sensitive to guanyl nucleotides. The preferred coupling of the AT_1 receptor to G_q was abolished by the mutation and no other coupling could be detected (G_s, G_i/G_o). The authors concluded that the mutant AT_1 receptor coupled to an unidentified G protein.

The relationship between the high and low affinity agonist/NTS1 receptor complexes described here deserves some discussion. The current theory of G protein-coupled receptor/ligand interaction, i.e., the allosteric ternary complex model (Lefkowitz et al., 1993; Kenakin, 2001), postulates that, in the absence of ligand, the receptor exist in at least two states, R and R*, that are in conformational equilibrium. R and R* have low and high affinity for G protein(s), respectively, and their proportion (R/R*) is governed by an isomerization constant. Agonists would act by conformational selection, having greater affinity for R* than for R. If it is assumed that the G protein is in excess of the receptor and that all species in the ternary complex model equilibrate within the time period during which binding experiments are carried out, then simulation with the model shows that R and R* should exhibit similar agonist saturation curves with the same apparent K_d value (that falls between high and low affinity K_d values). This is not what was observed here where the high affinity neurotensin binding site was saturated independently of the low affinity site by the agonist. Independent saturation of high and low affinity sites by the agonist might arise if the receptor is in excess of the G protein. Then saturation of high affinity species corresponding to the formation of maximal concentrations of active ternary complexes could be reached before total saturation of uncoupled receptor species. However, in this case the apparent high affinity K_d value and the ratio of low affinity over high affinity B_{max} values should decrease as the receptor concentration is decreased relative to that of the G protein. Again, this is not what was observed here when receptor expression levels varied over a greater than tenfold range. It appears therefore that for the wild type and mutant rNTS1 receptors, the high and low affinity NT/receptor complexes are not in equilibrium, at least within the time required to perform binding experiments. They could represent either distinct receptor species (through post-translational modification), or receptor conformations that are in slow equilibrium. Further studies will be necessary to address this issue.

Whatever the relationship between high and low affinity neurotensin binding components, it is clear that the high

affinity neurotensin/receptor complex for the wild type rNTS1 receptor differs in its coupling properties from that observed with the mutant receptors. Whereas the present data and those of others (Yamada et al., 1994) are consistent with the high affinity neurotensin/wild type rNTS1 receptor complex being involved in mediating inositol phosphate production through activation of G_q, the data obtained here indicate that the high affinity agonist/receptor complexes formed with the mutant receptors are not coupled to G_q. This is not the result of the moderate expression of high affinity binding sites observed with the mutant receptors as transfection of COS cells with low amounts of wild type rNTS1 plasmid resulting in low levels of high affinity binding sites does not modify the coupling of the receptor to inositol phosphate production. Thus, mutations in the extracellular loop 3 of the rNTS1 receptor appear to reveal receptor conformation(s) that are able to form high affinity complexes with the agonist and yet are unable to couple to G_q. This raises the question as to how modifications in the extracellular loop 3 of the rNTS1 receptor can impair interactions of the receptor intracellular domains with G proteins.

It is generally thought that stimulus- or agonist-induced G protein-coupled receptor activation involves a rearrangement of transmembrane helices that in turn transforms intracellular domains in a way that permits their interaction with G proteins. This is particularly well documented in the case of the photoreceptor rhodopsin and adrenergic receptors (Robinson et al., 1992; Gether et al., 1997; Gether and Kobilka, 1998; Porter and Perez, 1999) for which the agonist binding site is localized in a pocket within the transmembrane helices (Wang et al., 1991; Han et al., 1997; Ji et al., 1998). However, for a number of other G protein-coupled receptors including in particular neuropeptide receptors, the agonist binding site is often localized either in extracellular domains or at the junction of extracellular and transmembrane domains. In these cases, transformation of the intracellular domains that interact with G proteins would have to occur through a rearrangement of transmembrane helices brought about by agonist binding to extracellular regions of the receptor. In the case of the rNTS1 receptor, we have previously shown that the peptide agonist binding domain comprises residues within extracellular loop 3 and transmembrane domains 6 and 7 (Barroso et al., 2000; see Fig. 1). Others have shown that the G_q-activating domain of the NTS1 receptor lies in intracellular loop 3 (Yamada et al., 1994). Interestingly, this domain connects to extracellular loop 3 through transmembrane domain 6. It is therefore tempting to speculate that formation of high affinity complexes between neurotensin and the NTS1 receptor modifies the conformation of extracellular loop 3 and that this conformational change results in movements of adjacent transmembrane domains, thereby allowing the receptor to couple to G proteins. The extracellular loop 3 mutations described here, although still allowing the formation of high affinity neurotensin/NTS1 receptor complexes, would markedly impair the conformational change that promotes G protein coupling.

It has long been known from structure–activity studies that Tyr^{11} in the neurotensin molecule is essential for binding to and for activating the NTS1 receptor (Kitabgi et al., 1985). Recent studies have shown that Tyr^{11} chiefly interacts with extracellular loop 3 in the NTS1 receptor (Pang et al., 1996; Barroso et al., 2000). In particular, Tyr^{347} in extracellular loop 3 plays an essential role in agonist binding by interacting strongly with Tyr^{11} (Barroso et al., 2000). It should be noted that the phenotype of the Y347A mutation differs from that of the nearby W339A, F344A, H348A and Y349A mutations, as unlike the latter mutants the Y347A mutant receptor is totally devoid of high affinity neurotensin binding. It may be suggested that Tyr^{347} is an absolute requirement for the correct positioning in extracellular loop 3 of neurotensin residue Tyr^{11} and that Trp^{339}, Phe^{344}, His^{348} and Tyr^{349} may be involved in stabilizing the Tyr^{347}–Tyr^{11} interaction and, more importantly, in participating to the conformational changes of extracellular loop 3 that lead to receptor activation. In this context, it is interesting to note that of all the peptide analogs of neurotensin ever synthesized, the only ones that displayed partial antagonist activity had bulky L or D aromatic residues in position 11 (Saint-Pierre et al., 1984), which presumably would affect their positioning in extracellular loop 3. As our knowledge of the topography of the neurotensin binding site in the NTS1 receptor gets more precise and as receptor modeling techniques improve, it may become feasible in a near future to rationally design new classes of selective non-peptide agonist compounds of the NTS1.

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