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Agonism, Inverse Agonism, and Neutral Antagonism at the Constitutively Active Human Neurotensin Receptor 2

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ABSTRACT

Two G protein-coupled neurotensin (NT) receptors, termed NTR1 and NTR2, have been identified so far. In contrast to the NTR1, which has been extensively studied, little is known about the pharmacological and biological properties of the NTR2. In the course of characterizing NT analogs that exhibited binding selectivity for the NTR2, we discovered that this receptor constitutively activated inositol phosphate (IP) production. Here, we report on the constitutive activity of the human NTR2 (hNTR2) transfected in COS cells and on compounds that exhibit agonism, inverse agonism, and neutral antagonism at this receptor. IP levels increased linearly with time, whereas they remained constant in mock-transfected cells. Furthermore, IP production was proportional to the amount of hNTR2 present at the cell membrane. SR 48692, a nonpeptide antagonist of the NTR1, stimulated IP production, whereas levocabastine, a nonpeptide histamine H1 antagonist that binds the NTR2 but not the NTR1, behaved as a weak partial inverse agonist. NT analogs modified at position 11 of the NT molecule, in particular by the introduction of bulky aromatic D amino acids, exhibited binding selectivity at the hNTR2 and also behaved as partial inverse agonists, reversing constitutive IP production up to 50%. Finally, NT barely affected constitutive IP production but antagonized the effects of both agonist and inverse agonist compounds, thus behaving as a neutral antagonist. The unique pharmacological profile of the hNTR2 is discussed in the light of its sequence similarity with the NTR1 and the known binding site topology of NT and SR 48692 in the NTR1.

Neurotensin (NT) is a 13-amino acid peptide that exerts neuromodulatory functions in the central nervous system and endocrine/paracrine actions in the periphery (Vincent, 1995; Rostene et al., 1997). Three NT receptors, termed NTR1, NTR2 and NTR3 according to the order in which they were cloned, have been identified so far (Tanaka et al., 1990; Vita et al., 1993, 1998; Chalon et al., 1996; Mazella et al., 1996, 1998; Vincent et al., 1999). The NTR1 and NTR2 are G protein-coupled receptors and share 60% homology, whereas the NTR3 belongs to an entirely different family of proteins. In the past 25 years, a wealth of information has accumulated regarding the pharmacological and biological properties of the NTR1 (Vincent et al., 1999). Our understanding of the physiology of the NTR1 has been facilitated in the last 10 years by the development of SR 48692, a nonpeptide antagonist that binds preferentially to the NTR1 (Gully et al., 1993; Rostene et al., 1997). Much less is known about the

biological role of the NTR2, and the functions associated with NT binding to the NTR3 remain unknown.

The NTR2 is identical to the low-affinity NT binding sites (K_d in the nanomolar range) first described in rat brain (Mazella et al., 1983) and has been shown to be selectively recognized by levocabastine, a nonpeptide H1 histamine antagonist, in contrast to the high-affinity NT binding sites (K_d in the 100 pM range) that are totally insensitive to levocabastine and have been identified as the NTR1 (Schotte et al., 1986; Kitabgi et al., 1987). It was subsequently reported that the ontogeny and localization of the NTR1 and NTR2 in rat brain differ markedly and that although the NTR1 is almost exclusively neuronal, the NTR2 seems to be associated both with neurons and glial cells (Schotte and Laduron, 1987; Schotte et al., 1988; Mazella et al., 1996; Nouel et al., 1997, 1999). Recently, using antisense strategy in vivo to decrease the expression of the NTR2 in the mouse brain, we presented evidence that this receptor is responsible for the SR 48692-insensitive analgesic effect of centrally administered NT (Dubuc et al., 1994; Dubuc et al., 1999).

The NTR2 has been cloned from the rat, mouse, and hu-

ABBREVIATIONS: NT, neurotensin; NTR, neurotensin receptor; CHO, Chinese hamster ovary; IP, inositol phosphate; TM, transmembrane domain; E_{max}, maximal efficiency; GFP, green fluorescent protein; E, extracellular loop; I, intracellular loop; hNTR, human neurotensin receptor; mNTR, mouse neurotensin receptor; rNTR, rat neurotensin receptor.

man (Chalon et al., 1996; Mazella et al., 1996; Vita et al., 1998). When expressed in mammalian cell lines or in *Xenopus laevis* oocytes, this receptor exhibits a complex pharmacological behavior that seems to be species-dependent. Thus, in *X. laevis* oocytes that expressed the mNTR2, NT and levocabastine acted as agonists on chloride conductance and SR 48692 was without agonist or antagonist effects in this system (Mazella et al., 1996; Botto et al., 1997). In rNTR2-expressing Chinese hamster ovary (CHO) cells, SR 48692 and levocabastine efficiently activated Ca^{2+} mobilization, whereas NT had little effect on the Ca^{2+} response (Yamada et al., 1998). Finally, in hNTR2-expressing CHO cells, SR 48692 activated Ca^{2+} mobilization whereas both NT and levocabastine antagonized this response (Vita et al., 1998). These findings suggested that as-yet-unidentified endogenous ligands of the NTR2 may exist (Vita et al., 1998).

Recently, we described NT analogs modified on the tyrosyl residue at position 11 of the NT molecule that exhibited 10- to 100-fold higher affinity for the mNTR2 than for the rNTR1 (Dubuc et al., 1999). While comparing the binding and biological potencies of these compounds at the hNTR2 versus the hNTR1, we discovered that the hNTR2 exhibited spontaneous phosphoinositide hydrolyzing activity when transfected in COS cells. Here we describe the constitutive activity of the hNTR2 transfected in COS cells on inositol phosphate (IP) production. Furthermore, we show that SR 48692 acts as an agonist at the hNTR2 whereas the above-mentioned NT analogs and other analogs also modified on Tyr11 exhibit partial inverse agonist activity. Finally, we show that NT behaves as a neutral antagonist, reversing the effects of either agonist or inverse agonists and having no effect by itself.

Materials and Methods

Drugs. Neurotensin was from Neosystem (Strasbourg, France) and SR 48692 was from Sanofi Recherche (Montpellier, France). Monoiodo-$[^{125}I$-Tyr$^3]$neurotensin (2000 Ci/mmol) (^{125}I-NT) was prepared as described previously (Bidard et al., 1993). Levocabastine was from Janssen Pharmaceutica (Beerse, Belgium). NT analogs were synthesized according to the method of Doulut et al. (1992). Table 1 lists the structures and abbreviations of the pseudopeptide and peptide NT analogs used in the present study.

Cell Culture and Transfection. COS M6 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Cergy-Pontoise, France) containing 10% fetal bovine serum (Dutcher, Brumath, France) and 50 μg/ml gentamicin (Sigma, St Quentin Fallavier, France). For transient transfection, 100-mm cell culture dishes,

seeded with 10^6 cells the day before, were washed twice with Tris-buffered saline (25 mM Tris, 137 mM NaCl, 2.3 mM CaCl$_2$, 0.5 mM MgCl$_2$, 0.4 mM Na$_2$HPO$_4$, pH 7.4) and incubated for 30 min with 1 μg of recombinant hNTR2-pcDNA3 or hNTR1-pcDNA3 plasmid in the presence of DEAE Dextran (0.5 mg/ml; Sigma) at room temperature. After 3 h in culture medium supplemented with 100 μM chloroquine (Sigma), cells were washed twice with Tris-buffered saline, and cultured for 48 to 72 h. For inositol phosphate measurement as a function of hNTR2 expression, several transient transfections were performed with increasing recombinant plasmid amounts ranging from 10 ng to 5 μg per 100-mm cell culture dish.

Cell Membranes Preparation. 48 h after transfection, cells were washed twice with phosphate-buffered saline and collected in ice-cold 5 mM Tris/HCl, pH 8. After homogenization by repeated passages through a syringe needle and centrifugation at 4°C for 30 min at 100,000g, cell membranes were resuspended in 300 μl per dish of 5 mM Tris/HCl, pH 7.5, and stored at -20°C. Membrane protein concentration was determined with the use of the Bio-Rad Protein Assay (Bio-Rad, Ivry sur Seine, France).

Binding Experiments. Binding experiments with ^{125}I-NT were carried out with 20 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-orthophenantroline (Sigma), for 30 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, Bohemia, NY), followed by two washes of the tube and filter with 2 ml of the same buffer. Nonspecific binding was determined in the presence of 1 μM unlabeled ligand. Saturation and competition experiments with membranes from hNT2-expressing cells were performed essentially as described previously (Martin et al., 1999). Briefly, competition experiments were carried out with 0.1 nM ^{125}I-NT and increasing concentrations of unlabeled compounds. Saturation experiments were performed by incubating increasing concentrations of ^{125}I-NT ranging from 0.05 nM to 0.8 nM and then by adding increasing concentrations of unlabeled NT (0.5 to 20 nM) to a fixed concentration of 0.4 nM ^{125}I-NT. We have previously shown that iodination of NT on the Tyr^3 position yields a peptide with the same binding affinity as unlabeled NT (Sadoul et al., 1984). Competition experiments with the hNTR1 were performed similarly with 0.05 nM ^{125}I-NT and 2 μg of membrane protein (Labbé-Jullié et al., 1998). Data were analyzed with the use of the LIGAND software (Munson and Rodbard, 1980).

Inositol Phosphate Determination. Twenty-four hours after transfection, cells were trypsinized and grown for 18 h in 12-well plates in culture medium in the presence of 0.5 μCi of myo-$[^3H]$inositol (ICN Biomedicals, Orsay, France). After 2 washes with Earle's buffer (25 mM HEPES, 25 mM Tris, 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 0.9 mM MgCl$_2$, and 5 mM glucose containing 0.1% bovine serum albumin), cells were incubated for 30 min at 37°C in 500 μl of Earle's buffer containing 20 mM LiCl in the absence or presence of the different drugs used in the present study at the indicated con-

TABLE 1

Comparison of binding potencies and selectivity at the hNTR2 and hNTR1 of NT, SR 48692, levocabastine, and NT(8–13) analogs

K_i values were derived from competitive inhibition curves of ^{125}I-NT specific binding to membrane homogenates of COS cells expressing hNTR1 or hNTR2. Binding assays were performed as described under *Materials and Methods*. Specific binding in the absence of competitor was 17.0 $\pm$ 2.5 and 770 $\pm$ 85 fmol/mg for the hNTR2 and hNTR1, respectively. B_{max} values were 290 $\pm$ 34 and 3900 $\pm$ 410 fmol/mg for the hNTR2 and hNTR1, respectively. Selectivity ratio (1/2) is the ratio of K_i value for hNTR1 to that for hNTR2. Values are the means $\pm$ S.E. from three independent experiments.

Compound	Structure	Ki hNTR1, nM	Ki hNTR2, nM	Selectivity ratio (1/2)
NT	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH	0.22 $\pm$ 0.04	1.44 $\pm$ 0.17	0.15
SR 48692		4.3 $\pm$ 2.1	418 $\pm$ 82.2	0.01
Levocabastine		no affinity	166 $\pm$ 15.5	—
JMV 431	Boc-Arg-Arg-Pro-Tyr-ψ(CH$_2$-NH)-Ile-Leu-OH	5178 $\pm$ 1468	97 $\pm$ 9	53
JMV 457	Boc-Arg-Arg-Pro-D-Trp-Ile-Leu-OH	1475 $\pm$ 113	168 $\pm$ 27	9
JMV 509	Boc-Lys-Lys-Pro-D-Nal-Ile-Leu-OH	13490 $\pm$ 6222	521 $\pm$ 10	26
JMV 2004	H-Lys-Lys-Pro-ψ(CH$_2$-NH)-D-Trp-Ile-Leu-OH	2088 $\pm$ 480	62 $\pm$ 12	34
JMV 458	Boc-Arg-Arg-Pro-L-Trp-Ile-Leu-OH	1.55 $\pm$ 0.37	2.12 $\pm$ 0.46	0.73
JMV 510	Boc-Lys-Lys-Pro-L-Nal-Ile-Leu-OH	152.5 $\pm$ 29.4	176 $\pm$ 48	0.86

Boc, *tert*-butoxy-carbonyl; ψ, pseudopeptide bond; Nal, naphtylalanine.

centrations. For IP measurement as a function of time, cells were incubated in LiCl-Earle buffer for varying times ranging from 0 to 30 min. The reaction was stopped by 800 μl of ice-cold 10 mM HCOOH. After 1 h at 4°C, the supernatant was collected and neutralized by 2.5 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 of Ecolume (ICN Biomedicals). In all experiments, control IP measurements were performed after a 30 min incubation in Earle buffer without LiCl. Except in Fig. 1, where total IP production is represented, IP production in the absence of LiCl was subtracted from total IP levels measured in the presence of LiCl and the data are expressed as the percentage of basal (constitutive) IP production in the absence of added drugs. In experiments designed to measure IP production as a function of hNTR2 expression levels, 24 h after transfection with varying amounts of plasmid, cells in 100 mm culture dishes were either left in culture medium for another 24 h and harvested for binding assay (see preceding section) or treated as described above for IP measurement.

Transfection Efficiency. A green fluorescent protein (GFP)-pcDNA3 plasmid was constructed and varying amounts of plasmid ranging from 10 ng to 3 μg were transfected in COS cells using the same procedure as that used for transfecting the hNTR2-pcDNA3 plasmid. Forty-eight hours after transfection, cells were trypsinized, centrifuged, and resuspended in phosphate-buffered saline containing 0.5% bovine serum albumin. Transfection efficiency was analyzed by flow cytometry on a FACScan flow cytometer (BD Biosciences SA, Pont de Claix, France). The data show that the percentage of transfected cells increased from 30 to 70% between 10

and 300 ng of plasmid and reached a plateau thereafter. Fluorescence intensity distribution in the transfected cell population showed a single mode with a peak whose intensity increased approximately 3-fold between 10 ng and 3 μg of plasmid. The fluorescence distribution was nearly symmetrical, declining sharply and then trailing somewhat on the low fluorescence intensity side and declining sharply on the high intensity side. Whatever the amount of plasmid used, no cell had a fluorescence signal that exceeded four times mean fluorescence intensity (data not shown).

Results

The hNTR2 Exhibits Constitutive Activity. COS cells were either mock-transfected or transfected with the hNTR2 and IP production was measured as a function of time in the presence of Li^+ (Fig. 1A). IP production did not vary significantly over a 30-min interval in mock-transfected cells and remained similar to IP levels measured in the absence of Li^+ (Fig. 1A). In contrast, IP production rose linearly with time in cells expressing the hNTR2, increasing 5-fold over that measured in mock-transfected cells after a 30-min incubation. In a second series of experiments, COS cells were transfected with varying amounts of hNTR2-encoding plasmid. The amount of expressed hNTR2 was measured in binding experiments with ^{125}I-NT and membranes prepared from each batch of cells, and IP production was determined after a 30-min incubation in intact cells. In these experiments, the relationship between receptor expression and plasmid amount was not linear. There was an increase of approximately 10-fold in hNTR2 expression (as determined in binding experiments) when plasmid concentration rose from 30 ng to 3 μg per dish. Above 3 μg, receptor expression reached a plateau. Furthermore, B_{max} values varied somewhat between sets of transfection experiments performed with different plasmid preparations. For these reasons, experiments were grouped according to B_{max} values rather than to the amount of plasmid used to transfect the cells. This explains the horizontal standard deviations in Fig. 1B. IP production was proportional to the amount of expressed hNTR2 in COS cells and was increased nearly 10-fold at the highest level of receptor expression tested in these experiments (Fig. 1B). Furthermore, IP levels measured after a 30-min incubation in the absence of Li^+ remained low and barely varied as receptor expression increased. Finally, for comparison, IP production was measured in the presence or absence of Li^+ in COS cells transfected with the hNTR1. The cells expressed 3.7 ± 0.3 pmol of hNTR1/mg of membrane protein as determined in binding experiments (i.e., a receptor concentration that was four times higher than the highest hNTR2 concentration tested). Figure 1B shows that there was no measurable increase in IP levels in hNTR1-transfected cells. Altogether, these data clearly demonstrate the constitutive activity of the hNTR2.

Effects of Neurotensin, Levocabastine, and SR 48692 on the Constitutively Active hNTR2. SR 48692 stimulated IP formation above the spontaneous activity of the hNTR2 (Fig. 2) in a concentration-dependent manner, with an EC_{50} value of 50 ± 6.8 nM ($n = 4$). Maximal stimulation obtained with 10 μM SR 48692 was greater than 200% of constitutive activity. In contrast, NT at concentrations up to 10 μM did not affect the spontaneous activity of the hNTR2 (Fig. 2). Levocabastine decreased IP production in a concentration-dependent manner below the level of spontaneous
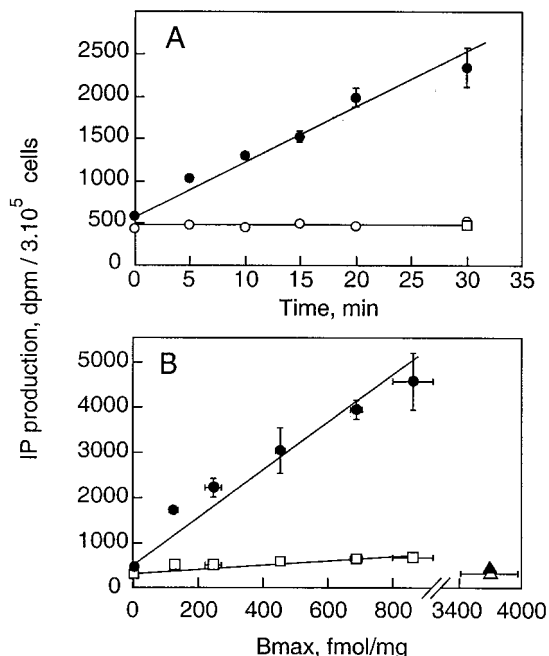

Fig. 1. Constitutive inositol phosphate production in COS cells expressing the hNTR2. A, Basal IP production as a function of time in hNTR2-transfected cells (●), or in mock-tranfected cells (○). Also shown are IP levels measured after 30 min in the absence of Li^+ in hNTR2-transfected cells (□). The B_{max} value for NT in these experiments was 240 ± 24 fmol/mg as determined in binding experiments with cell membranes. B, Basal IP production as a function of the amount of hNTR2 expressed at the cell membrane (B_{max}) after a 30-min incubation in the presence (●) or absence (□) of Li^+. Also shown are the levels of IP production in hNTR1-expressing COS cells in the presence (▲) or absence (△) of Li^+. Values are the means ± S.E. from three independent experiments. S.E. values smaller than symbol size are not represented.

activity (Fig. 2). IC_{50} and E_{max} values for the levocabastine effect are shown in Table 2. Maximal inhibition of basal IP levels reached 30%. Hence, levocabastine behaved as a partial inverse agonist of the hNTR2.

Neurotensin Analogs That Behave as Inverse Agonists at the hNTR2. We have noted previously that some NT analogs modified on position 11 of the NT sequence showed selectivity for the mNTR2 compared with the rNTR1 (Dubuc et al., 1999). Here we show that four such analogs also presented selectivity for the hNTR2 versus the hNTR1. Thus, the compounds competed for ^{125}I-NT binding with potencies that were 10 to 50 times greater at the hNTR2 than at the hNTR1 in contrast to NT, which showed higher binding potency for the hNTR1 than for the hNTR2 (Fig. 3A, Table 1). Interestingly, when tested for their ability to affect IP production in intact hNTR2-expressing COS cells, all the selective NT analogs inhibited constitutive IP production in a concentration dependent manner (Fig. 3 B). IC_{50} and E_{max} values for IP production inhibition are given in Table 2. In general, IC_{50} values for inhibiting IP production matched K_i values for competing with ^{125}I-NT binding (less than a 4-fold difference at the most), except for JMV 2004, the IC_{50} value of which was 15-fold higher than its K_i value (Tables 1 and 2). JMV 2004 is the only analog in this series that is not protected at its N terminus (Table 1). We have previously shown that such NT analogs are degraded by aminopeptidases when exposed to cells or tissues at 37°C (Lugrin et al., 1991). IP measurements were performed at 37°C, whereas binding assays were carried out at room temperature in the presence of the metallopeptidase inhibitor phenanthroline. These different conditions might have led to greater degradation of JMV 2004 in the bioassay than in the binding assay. This hypothesis was tested directly by comparing the effects of JMV 2004 on IP production in the absence or presence of 10 μM bestatin, an aminopeptidase inhibitor. In the presence of bestatin, there was a 4- to 5-fold reduction in IC_{50} values for the ability of the analog to inhibit IP production (790 ± 160 nM and 170 ± 50 nM, $n = 3$, in the absence and presence of bestatin, respectively), with no change in E_{max} value (presented as the percentage of inhibition of constitutive activity: 39 ± 3.7 and 37 ± 4.2, $n = 3$, in the absence and presence of bestatin, respectively). Maximal inhibition reached 30 to 50%
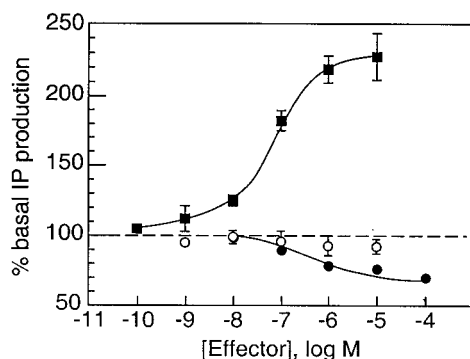
for all analogs. Thus, the selective analogs can be defined as partial inverse agonists at the hNTR2. Three of the four analogs had bulky D-amino acid residues (D-Trp or D-Nal) in position 11 of the NT molecule. For comparison, two isomer analogs of JMV 457 and JMV 509 with L-Trp and L-Nal in position 11 (JMV 458 and JMV 510, respectively) were tested in binding and biological assays. They showed no selectivity for the hNTR2 over the hNTR1 (Table 1) and did not affect constitutive IP production at concentrations as high as 10 μM (not shown), behaving in this regard like NT.

Neurotensin Is a Neutral Antagonist at the hNTR2. It has been reported previously that NT antagonized the SR 48692 response in hNTR2-expressing CHO cells (Vita et al., 1998). Here we show in COS cells transfected with the

TABLE 2
Inhibition of the constitutive IP production in hNTR2-expressing cells by NT(8–13) analogs and levocabastine.

IC_{50} and E_{max} values were derived from concentration-response curves for levocabastine and NT(8–13) analogs as represented in Figs. 2 and 3B, respectively. IC_{50}, concentration of the compound corresponding to half-maximal inhibition of basal IP production. E_{max}, maximal drug efficiency to inhibit basal IP production. Values are the means ± S.E. from three independent experiments.

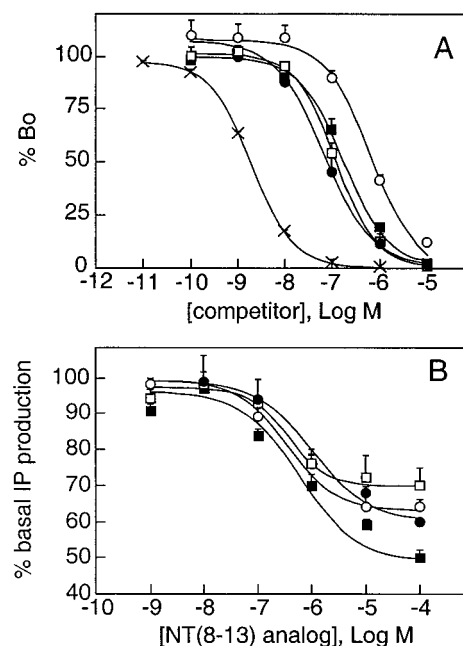| Compound	IC_{50}	E_{max}, (inhibition of IP production)
	nM	*%*
JMV 431	350 ± 54	30 ± 4.8
JMV 457	444 ± 68	49.7 ± 2.3
JMV 509	459 ± 69	36.3 ± 2.2
JMV 2004	889 ± 147	39.6 ± 4.5
Levocabastine	451 ± 205	30 ± 1.4

Fig. 3. Binding and inverse agonist activity of NT(8–13) analogs. A, competitive inhibition of 0.1 nM ^{125}I-NT specific binding to membrane homogenates of COS cells expressing the hNTR2, with NT (×), JMV 431 (□), JMV 457 (■), JMV 509 (○), and JMV 2004 (●). The results are expressed as the percentage of specific binding in the absence of competitor (Bo). Mean Bo value in these experiments was 17.0 ± 2.5 fmol/mg. B, effect of NT(8–13) analogs on basal IP production in hNTR2-transfected COS cells; concentration-response curves with JMV 431 (□), JMV 457 (■), JMV 509 (○), and JMV 2004 (●). IP levels measured in the absence of Li$^+$ were subtracted from the data that are expressed as the percentage of IP production without added drugs (1670 ± 140 dpm/3.10^5 cells). Values are the means ± S.E. from three independent experiments. S.E. values smaller than symbol size are not represented.

Fig. 2. Effect of SR 48692, NT, and levocabastine on IP production. Concentration-response curves for the effect of SR 48692 (■), NT (○) and levocabastine (●) on basal IP production in hNTR2-transfected cells. IP levels measured in the absence of Li$^+$ were subtracted from the data that are expressed as the percentage of IP production without added drugs (1595 ± 85 dpm/3 × 10^5 cells). Values are the means ± S.E. from three independent experiments. S.E. values smaller than symbol size are not represented.

hNTR2 that NT concentration dependently reversed the effect of 1 μM SR 48692 on IP production back to the constitutive activity of unstimulated cells with an IC_{50} value of 130 $\pm$ 25 nM (Fig. 4 A). We also tested the effect of NT on the inhibitory response of inverse agonists. JMV 457 was chosen in these studies because it is the most efficient of the inverse agonists. Figure 4B shows that NT antagonized the effect of 10 μM JMV 457 in a concentration-dependent manner with an IC_{50} value of 1760 $\pm$ 150 nM, bringing IP production back to the levels of constitutive activity. Hence, although NT has no effect by itself on the hNTR2, it is able to antagonize agonist and inverse agonist effects and can therefore be defined as a neutral antagonist at this receptor. From these data, one can estimate K_i values for the ability of NT to reverse the effects of SR 48692 and JMV 457 by using the Cheng and Prusoff (1973) relationship (Craig, 1993), $K_i = IC_{50} / (1 + A/EC_{50})$, in which A represents the fixed concentration of SR 48692 (1 μM) or JMV 457 (10 μM) used in the experiments and EC_{50} the half-maximally effective concentration of SR 48692 (50 nM, Fig. 2) or JMV 457 (444 nM, Table 2) on IP production. Calculation yielded K_i values of 6.2 and 75 nM for the ability of NT to antagonize the effects of SR 48692 and JMV 457, respectively. Note that these values are 4- to 50-fold higher than the K_d value of NT for binding to the hNTR2.

Discussion

The major finding of the present study is that the hNTR2, when expressed transiently in COS cells, exhibits a robust constitutive activity on IP production. Spontaneous activity of native GPCRs in the absence of agonists with respect to G protein activation, either in membrane or in reconstituted systems, has long been known (Cerione et al., 1984; Costa and Herz, 1989; Freissmuth et al., 1991). The first described constitutively active G protein-coupled receptor obtained by mutation was the α1-adrenergic receptor (Cotecchia et al., 1990). Since then, a great number of constitutively active GPCRs for neurotransmitter, neuropeptides, and hormones have been engineered by mutagenesis (reviewed in Milligan et al., 1997; Leurs et al., 1998) and a growing number of native GPCRs with constitutive activity have been identified

(reviewed in de Ligt et al., 2000). The hNTR2 joins the latter group.

The pharmacological properties of the hNTR2 have been characterized by others in CHO cells stably transfected with this receptor (Vita et al., 1998). Coupling of the hNTR2 to IP formation was described and it was reported that SR 48692 behaved as an agonist at the hNTR2, whereas NT antagonized SR 48692 (Vita et al., 1998). Similar findings were made here for the hNTR2 transiently expressed in COS cells. However, contrary to our observation, the hNTR2 was not reported to be constitutively active in CHO cells (Vita et al., 1998). The reasons for this discrepancy are not clear. Coupling of the receptor in both systems activates the same transduction pathway (i.e., stimulation of phospholipase C). Furthermore, the present data show that constitutive activity of the hNTR2 in COS cells could be detected at low concentrations of receptor (100 fmol/mg of membrane protein) well below the amount of receptor (0.5–1 pmol/mg) expressed at the membrane of stably transfected CHO cells (Vita et al., 1998). It could be argued that transient transfection results in heterogeneous protein expression. This point was assessed by transient transfection of a GFP-pcDNA3 plasmid and fluorescence-activated cell-sorting analysis in COS cells (see *Materials and Methods*). If the data obtained with GFP-pcDNA3- can be extrapolated to hNTR2-pcDNA3-transfected cells, it can be estimated that when mean receptor expression level is 100 fmol/mg, no cell should express greater than 400 fmol/mg of receptor, a value that remains below hNTR2 concentrations in stably transfected CHO cells. Hence, hNTR2 expression in CHO cells would seem sufficient to detect constitutive activity in this system. Along the same lines, it might be interesting to compare NTR2 concentrations in our system with those found in normal tissues that express the receptor. NTR2 concentrations are 100 to 150 fmol/mg and 150 fmol/mg in membranes prepared from whole adult rat brain and primary cultures of rat cortical astrocytes, respectively (Kitabgi et al., 1987; Nouel et al., 1999). Furthermore, the NTR2 distribution in these systems is heterogeneous, being predominantly glial in the brain and concerning a subpopulation representing only 30 to 40% of primary cultured astrocytes (Nouel et al., 1999). Therefore, the concentrations of NTR2 in rat brain are clearly within the range of those achieved in COS cells and might therefore be compatible with the expression of constitutive activity in vivo. It will now be necessary to establish whether constitutive activation of the NTR2 is dependent on the cell system used to express the receptor and whether it is species-dependent and observable in cells and tissues that normally express the receptor.

We have reported previously that some NT analogs modified at position 11 of the NT molecule, in particular JMV 431 and JMV 509, had higher affinity for the mNTR2 than for the rNTR1 (Dubuc et al., 1999). Here, we show this to be true for the hNTR2 versus the hNTR1. In addition, two other analogs, JMV 457 and JMV 2004, were found to share the same property. Interestingly, when tested for their ability to affect IP production in hNTR2-expressing COS cells, all the compounds partially inhibited (30 to 50%) the spontaneous activity of the receptor. Hence, the analogs can be defined as partial inverse agonists at the hNTR2. Such a behavior seems to be related in part to the introduction of a bulky aromatic D-amino acid in position 11, because analogs that
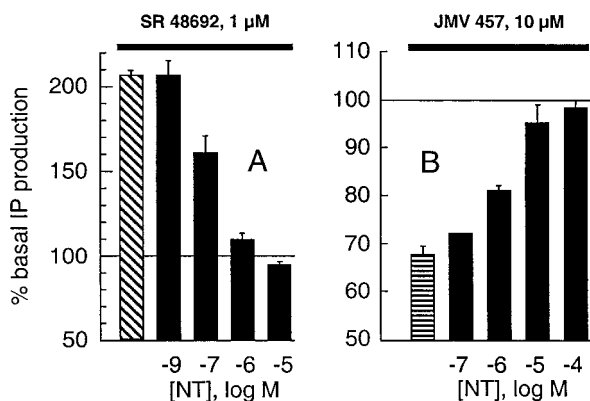

Fig. 4. Neutral antagonism of neurotensin on IP production. Effect of increasing concentrations of NT on IP production stimulated by 1 μM SR 48692 (A) or inhibited by 10 μM JMV 457 (B) in hNTR2-expressing cells. IP levels measured in the absence of Li$^+$ were subtracted from the data that are expressed as the percentage of IP production without added drugs (1170 $\pm$ 110 dpm/3 $\times$ 10^5 cells). Values are the means $\pm$ S.E. from three independent experiments.

have an L-Trp (JMV 458) or L-Nal (JMV 510) in this position did not affect constitutive IP production in hNTR2-expressing cells. The latter analogs, in contrast to the partial inverse agonists, lacked selectivity for the hNTR2 versus the hNTR1 (see Table 1). Interestingly, levocabastine, a totally selective NTR2 versus NTR1 ligand, exhibited partial inverse agonist activity in the present study. This raises the question of whether inverse agonism is linked to selectivity at the hNTR2. We are currently testing other NT analogs to answer this question.

One of the striking findings of this work is the neutral antagonist behavior of NT. The peptide had no effect by itself on IP production and reversed the effects of both SR 48692 (agonist) and JMV 457 (inverse agonist). However, its potency against SR 48692 and JMV 457 was noticeably lower than its binding potency determined in a homologous binding assay. It is generally assumed that neutral ligands have the same affinity for the R (inactive) and R* (active) states of a receptor as defined in the allosteric ternary complex model (Lefkowitz et al., 1993). However, a recent study with the constitutively active viral chemokine receptor ORF-74 pointed to a more complex mode of binding of neutral ligands (Rosenkilde and Schwartz, 2000). ORF-74 can bind a variety of structurally related chemokines that behave either as agonists, neutral ligands, or inverse agonists at the receptor. Rosenkilde and Schwartz (2000) showed that high-affinity neutral ligands (as determined in homologous binding assays) competed with low affinity for binding against either a radiolabeled agonist or a radiolabeled inverse agonist. Such a situation might apply here to NT and account for the discrepancy between binding affinity and biological potency against SR 48692 or JMV 457. This hypothesis, to be tested directly, will require the availability of radiolabeled agonist or inverse agonist ligands with sufficient affinity for the NTR2.

The NTR1 and NTR2 share 60% homology in their amino acid sequence and yet exhibit strikingly different pharmacological properties, such as constitutive activity for the hNTR2 and opposite pharmacological profiles for NT and SR 48692. Comparison of the sequences of both receptors might thus shed some light as to which structural domains may confer its "strange" behavior to the hNTR2. 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 NT binding sites in the rNTR1 (Labbé-Jullié et al., 1998; Barroso et al., 2000). The data showed that the SR 48692 binding site comprised residues in transmembrane domains (TM) 6 and 7 whereas that of NT involved residues in TM6 and in the third extracellular loop (E3) that connects TM6 and TM7 (Fig. 5). With very few exceptions, the residues involved in SR 48692

and NT binding are conserved in the hNTR1 and the hNTR2. Furthermore, the exceptions concern phenylalanyl residues in the rNTR1 that are substituted by tyrosyl residues in the hNTR1 or hNTR2. This suggests that the binding sites for SR 48692 and NT are similar in both the NTR1 and NTR2 and, therefore, that the opposite pharmacological behaviors of SR 48692 and NT on the two receptors are not the consequence of differences in ligand binding domains.

In general, NTR1 and NTR2 sequences are highly conserved in transmembrane domains and extracellular loops (Vincent et al., 1999). In contrast, they are poorly conserved in the N-terminal domain and intracellular loops. In particular, the third intracellular loop of both receptors shows virtually no similarity, being much longer in the NTR2 ($\sim$ 50 residues) than in the NTR1 ($\sim$ 30 residues) and sharing only five identical residues. This loop has been shown to be involved in coupling the NTR1 to Gq (Yamada et al., 1994). It is therefore quite possible that the constitutive activation of phospholipase C by the NTR2 might result from the divergent structure of the third intracellular loop in both receptors. The loop in the hNTR2 would activate the G protein in the absence of ligand. It might also modify the relative orientation of connecting TM6, E3, and TM7 in such a way that SR 48692 and NT binding results in the inverse pharmacological profiles of the ligands observed in the hNTR2 compared with the NTR1. These hypotheses could be tested directly by swapping the third intracellular loop of each NT receptor subtype and testing the pharmacological response of the chimeric receptors to SR 48692 and NT.

The previous observation by others that SR 48692 was agonist and antagonized by NT in hNTR2-expressing cells led to the interesting suggestion that there might be an undiscovered endogenous agonist of the NTR2 (Vita et al., 1998). The present finding that the hNTR2 exhibits constitutive activity and that NT analogs can behave as inverse agonists at this receptor raises the intriguing possibility that an endogenous inverse agonist of the hNTR2 might also exist. Complex modulation of the NTR2 would ensue from the interplay of agonist, inverse agonist, and neutral antagonist, and in such a system, NT might elicit a biological response by reversing the effect of either an agonist or an inverse agonist. As mentioned above, it will now be necessary to demonstrate that the NTR2 is constitutively active in cells or tissues that normally express the receptor. Primary culture of cortical glial cells might be a useful system in this regard as these cells that are devoid of NTR1 have been reported to express NT receptors with pharmacological properties similar to those of the NTR2 (Nouel et al., 1999). The NT analogs described here with selectivity for and inverse agonist activity at the NTR2 should provide tools for assessing the constitutive activity of the glial NT receptor. Finally, the ongoing search for more selective ligands of the NTR2 should hopefully help investigating the biological function of this interesting receptor.

Fig. 5. Sequence alignment of rNTR1, hNTR1, and hNTR2. Alignment of E3 and part of connecting TM6 and TM7 amino acid sequences of the rNTR1, hNTR1, and hNTR2. The residues involved in NT binding to the rNTR1 are indicated by ○, those in SR 48692 binding by ●, and those in the binding of both ligands in ◐.

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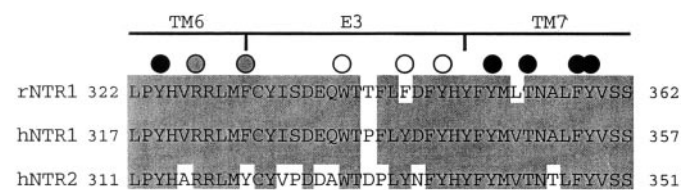
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