Biochemical Pharmacology, Vol. 51, pp. 1243–1246, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00031-7

SHORT COMMUNICATION

Pharmacological and Molecular Characterization of the Neurotensin Receptor Expressed in Sf9 Cells

Hélène Boudin, Jean Labrecque,†*
Anne-Marie Lhiaubet, Michael Dennis,† William Rostène* and Didier Pélaprat*‡*

*INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE, UNITÉ 339, HÔPITAL ST ANTOINE, 184 RUE DU FAUBOURG ST ANTOINE, 75571 PARIS CEDEX 12, FRANCE, AND †BIOSIGNAL INC., 1744 RUE WILLIAM, MONTRÉAL, QUÉBEC, H3J 1R4 CANADA

ABSTRACT. The rat neurotensin receptor was expressed in *Spodoptera frugiperda* insect (Sf9) cells using infection with a recombinant baculovirus. Immunoblot experiments performed with an antibody raised against the C-terminus of the receptor showed major bands at 47 (corresponding to the unglycosylated receptor protein) and 50 kDa, and minor bands at 65 and 36 kDa. The expressed receptor bound ^{125}I-neurotensin with high affinity, was coupled to endogenous G-proteins, and agonist-induced inositol phosphate production was observed at early times after infection. These results show that the rat neurotensin receptor retains functional properties when expressed in the heterologous insect cell system. BIOCHEM PHARMACOL 51;9:1243–1246, 1996.

KEY WORDS. neurotensin receptor; baculovirus expression; antipeptide antibody; phospholipase C

NT§ is a tridecapeptide with various central and peripheral actions mediated through specific receptors, which have been characterized in a variety of tissue preparations and cell lines [1]. A cDNA encoding a rat NTR has been cloned, showing that this receptor belongs to the family of G-protein-coupled receptors possessing seven transmembrane-spanning helices [2]. Depending on the cell type, NT stimulates the production of IPs and/or modulates cAMP and cGMP levels [1, 3].

The baculovirus-infected insect cell system has been used to express a variety of G protein-coupled receptors, including catecholamine receptors, such as β2-adrenergic [4] or dopaminergic [5, 6] receptors, and some peptide receptors, such as neurokinin receptors [7]. Levels of receptor expression are generally much higher than in mammalian cells, providing convenient models for studying the properties of these receptors; some of them were shown to be coupled to endogenous effector pathways [4, 5]. However, this information was not provided for all the receptors expressed in this system, and it is not certain that this coupling ability will be retained by all receptors from mammalian sources when expressed in the insect cells.

In the present work, we report the properties of the rat NTR expressed in the baculovirus Sf9 cell system, and show that the NTR expressed in this system is functionally linked to phosphatidylinositol hydrolysis at early times after infection. We have also developed an antibody against the C-terminal part of the NTR to characterize the expressed receptor.

MATERIALS AND METHODS

The expression of the NTR in Sf9 insect cells was achieved using a recombinant baculovirus, as previously described for the β2-adrenergic receptor [4]. Briefly, the recombinant baculovirus was obtained by subcloning the StuI-BamHI restriction fragment containing the entire coding sequence of the NTR (kindly provided by Dr S. Nakanishi) into the baculovirus transfer vector pJVETLZ/NheI; the NTR-plasmid construct was then cotransfected into Sf9 cells with wild-type AcNPV viral DNA, using the calcium phosphate method, and recombinant baculoviruses were purified and amplified.

For membrane preparation, Sf9 cells (2×10^6 cells/mL, grown at 27°C in Grace's supplemented medium containing 10% fetal bovine serum, 50 μg/mL of gentamycin sulfate and 2.5 μg/mL of fungizone) were infected with virus stock at an infection multiplicity of 2. Cells were harvested at 24, 48, or 72 hr postinfection, washed in cold PBS, and lysed in ice-cold 10 mM Tris-HCl (pH 7.4), 2 mM EDTA. The lysates were centrifuged at 500 × g for 5 min and the supernatants centrifuged at 45,000 × g for 30 min at 4°C. The pelleted membranes were resuspended in 50 mM Tris-HCl, 2 mM EDTA, and stored at −80°C.

The binding assay was performed at 22°C for 30 min using 2 μg (for 48 and 72 hr postinfection) or 10 μg (for 24 hr) of membranes and 50 pM [^{125}I]-Tyr^{3}NT (^{125}I-NT, 2000

‡ Corresponding author. Tel. (33-1) 49-28-46-92; FAX (33-1) 43-40-82-70.

§ *Abbreviations:* Gpp[NH]p, 5′-guanylylimidodiphosphate; IPs, total inositol phosphates; NT, neurotensin; NTR, neurotensin receptor; Sf9 cells, *Spodoptera frugiperda* cells.

Received 27 June 1995; accepted 11 December 1995.

Ci/mmol) prepared as previously described [8] in 250 μL of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.2% BSA, 0.8 mM 1,10-*o*-phenanthroline. Nonspecific binding was measured in the presence of 10^{-6} M NT. The assay was terminated by centrifugation at 12,000 × *g* for 4 min at 4°C. NT, Acetyl-NT_{8-13} ($AcNT_{8-13}$), NT_{1-11}, and neuromedin N (NN) were purchased from Neosystem (Strasbourg, France), and Gpp[NH]p from Sigma (St Louis, MO, U.S.A.). SR 48692 was kindly provided by Dr D. Gully (Sanofi, Toulouse, France). Data were analyzed using the LIGAND program [9].

For measurement of inositol phosphate production, growing cell cultures were prelabeled for 24 hr with 1 μCi/mL [^{3}H]myo-inositol (NEN-Dupont) prior to infection. Cell suspension assays were started by addition of labeled cells to a 96-well deepwell from Beckman containing drugs to be tested. The cells were then incubated for 20 min at 27°C, and the incubations stopped by addition of perchloric acid. Total ^{3}H-labeled IPs were measured by scintillation counting following isolation by anion exchange chromatography on AG 1 X-8 resin (BioRad) [10].

For antireceptor antibody production, a peptide (i4) corresponding to the C-terminal portion of the NTR (amino acids 405–422) with an additional tyrosine at the amino terminus was coupled to BSA using ϵ-maleimido-caproïc acid-N-hydroxysuccinimide ester. Ten prebled balb c mice were immunized with the conjugate (10 μg peptide equivalent) in Freund's complete adjuvant, boosted every 3 weeks with 10 μg of peptide in incomplete adjuvant and bled 2 weeks after each boost. Serum obtained after the fourth boost was used for immunoblotting analysis.

For immunoblotting analysis, 5 μg of Sf9 membranes solubilized in 60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol were subjected to SDS/10% PAGE and proteins were transferred to nitrocellulose membrane. The blots were incubated overnight at 4°C with anti-i4 antiserum or preimmune serum (1/500), and then incubated for 2 hr with alkaline phosphatase-conjugated goat antimouse IgG (1/300). Visualization was obtained using Nitroblue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate as substrates.

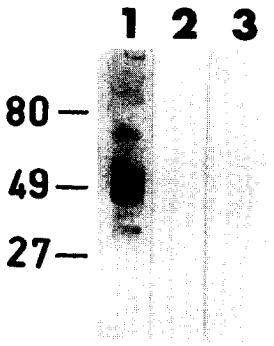

FIG. 1. Western blot analysis of the NTR expressed in Sf9 cells. Membranes (5 μg protein) were prepared at 48 hr postinfection from cells expressing NTR (lanes 1 and 3) or from cells infected with the wild-type baculovirus (lane 2). The anti-i4 antibody (lanes 1 and 2) and the preimmune serum (lane 3) were used at a 1/500 dilution. Migration of molecular mass markers in kDa is indicated on the left.

RESULTS AND DISCUSSION

Specific binding of ^{125}I-NT to membranes of infected Sf9 cells increased with time after infection, leading to binding site densities (B_{max}) of 0.5 and 6.4 pmol/mg protein at 24 and 48 hr postinfection, respectively, and reaching 10 pmol/mg protein at 72 hr. This level of expression is in the range seen with other G protein-coupled receptors expressed in Sf9 cells [4, 6] and is approximately 500 times greater than that observed in rat brain membranes [11], and 4 to 10 times greater than that observed in transfected CHO cells [3].

Immunoblot analysis (Fig. 1), carried out with the antibody raised against the carboxyl-terminal part of the NTR, revealed multiple bands of immunoreactivity with major bands at 47 (corresponding to the molecular mass of the unglycosylated receptor [2]), and 50 kDa, and minor bands at 65 and 36 kDa. Therefore, a large portion of the NTR expressed in Sf9 cells presents molecular masses lower than that (55 kDa) previously reported for the glycosylated NTR purified from rat cerebral cortex [12], probably due to the fact that Sf9 cells produce smaller N-linked oligosaccharides than mammalian cells [13]. No signal was obtained in cells infected with wild-type baculovirus, with the preim-

TABLE 1. Effect of Gpp[NH]p (60 μM) on ^{125}I-NT binding in membranes from Sf9 cells

		High-affinity site		Low-affinity site	
		K_d, nM	% B_{max}	K_d, nM	% B_{max}
24 hr	control	0.22 ± 0.02	100	N.D.	N.D.
	+Gpp[NH]p	0.17 ± 0.01	30.7 ± 1.3*	1.73 ± 0.16	69.3 ± 1.3
48 hr	control	0.10 ± 0.02	22.9 ± 5.5	1.25 ± 0.17	77.1 ± 5.6
	+Gpp[NH]p	0.09 ± 0.02	7.5 ± 1.6*	1.78 ± 0.23	92.5 ± 1.6*

Membranes were prepared from Sf9 cells at 24 or 48 hr postinfection. Values are the means ± SEM from 3 (24 hr) or 4 (48 hr) independent experiments. *$P < 0.05$ vs respective control value. N.D., not detected. The B_{max} values (high + low affinity = 100%) were 0.57 ± 0.06 (24 hr, control), 0.52 ± 0.09 (24 hr, + Gpp[NH]p), 6.4 ± 0.8 (48 hr, control) and 5.7 ± 0.8 pmol/mg protein (48 hr, + Gpp[NH]p), respectively.

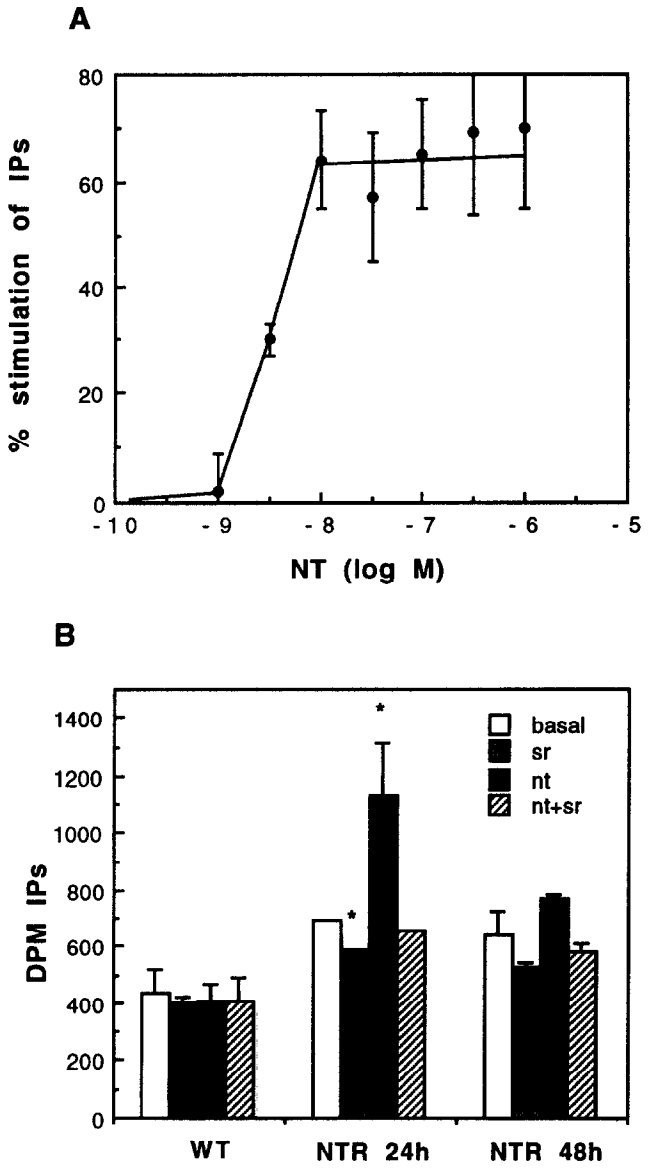

FIG. 2. Functional coupling of the rat NTR to endogenous phospholipase C activity in Sf9 cells. Following a 24-hr preincubation with [^{3}H]myo-inositol (1 μCi/mL), cells were incubated 20 min with or without addition of drugs and total [^{3}H]-labeled inositol phosphates (IPs) were measured on whole cells. (A) Effect of NT on IPs production in Sf9 cells expressing NTR at 24 hr postinfection. Results represent the % stimulation of IPs production by NT over basal level. Data are presented as the mean ± SEM for at least 3 different determinations performed in triplicate. (B) Cells were incubated without the addition of drugs (open bars), with 10^{-5} M SR48692 (grey bars), with 10^{-7} M NT (black bars), or with both 10^{-7} M NT and 10^{-5} M SR48692 (hatched bars). DPM values are normalized for 2.5 × 10^5 cells to correct for variations in cell densities between conditions. Each value represents the mean ± SEM of triplicate determinations and similar results were obtained in 3 independent experiments. *$P < 0.05$ vs control without drug (ANOVA followed by Dunnett's test).

mune serum in NTR-expressing cells (Fig. 1), nor when the antipeptide antibody was incubated in the presence of free immunogenic peptide (not shown).

At 24 hr postinfection, the data obtained in competition experiments with unlabeled NT fitted with a one-site model, suggesting the presence of a single class of high-affinity binding sites with an equilibrium dissociation constant (K_d) of 0.22 ± 0.02 10^{-9} M (mean ± SEM, n = 3) (Table 1). The selectivity pattern of these sites indicated that NT (IC_{50} = 0.25 ± 0.03nM) and $AcNT_{8-13}$ (0.24 ± 0.04) were 10 times more potent than neuromedin N (1.71 ± 0.29), and NT_{1-11} was inactive. The nonpeptide antagonist SR48692 [14] (IC_{50} = 5.02 ± 0.06nM) was 20 times less potent than NT. This selectivity was similar to that previously described for the high-affinity ^{125}I-NT binding sites in rat brain [14]. In the presence of Gpp[NH]p, a fraction of the high affinity site was converted to a ten-fold lower affinity site (Table 1), suggesting that the NTR expressed in Sf9 cells was coupled to endogenous G-proteins. Interestingly, not all the receptors expressed in this system are sensitive to guanine nucleotides. For instance, a sensitivity of this type was observed for the D1 dopamine receptor [5], but not for the D2 dopamine receptor [6]. In contrast to what was observed at 24 hr postinfection, two sites were already present even in the absence of Gpp[NH]p at 48 hr (Table 1), the high affinity site representing only 22.9% of the total sites. The presence of Gpp[NH]p decreased the proportion of high-affinity sites. Thus, the large increase in expression of the NTR (0.5 vs 6 pmol/mg protein) between 24 and 48 hr was not only associated with an increase in the amount of high affinity sites, but also with the appearance of a large fraction of uncoupled receptors.

At 24 hr postinfection, NT stimulated IPs production (Fig. 2A) with an EC50 of 5.08 ± 1.0 nM (mean ± SEM, n = 3), and this effect was inhibited by SR 48692 (Fig. 2B). However, at 48 hr (and 72 hr, not shown), no significant effect of NT was observed, in spite of the presence of greater receptor levels. These results indicate that the NTR is coupled to phosphatidylinositol hydrolysis in Sf9 cells, and that this coupling is maximal at early times after infection. It was observed that the maximal response to agonist (60% increase at 24 hr) was lower than that previously observed when this receptor was expressed in CHO cells (600% increase) [3], perhaps reflecting partial incompatibility between the mammalian receptor and the insect G protein-effector relay.

Interestingly, basal IPs levels in the NTR-expressing cells were higher than in the wild-type cells (Fig. 2B), and were slightly decreased by SR 48692. These results could be due to the release of endogenous NT-like material by the insect cells. However, they could also suggest that a fraction of the baculovirus-expressed NTR is spontaneously active. Such an agonist-independent activation of second messenger systems was previously evidenced for some other receptors such as the β-adrenergic receptor [15].

In conclusion, we have presented here the pharmacolog-

ical and molecular characterization of the rat NTR expressed in Sf9 cells. The expressed receptor bound ^{125}I-NT with high affinity, interacted with endogenous G-proteins and was coupled to inositol phosphate production, indicating that the rat NTR retains functional properties when expressed in heterologous insect cells.

We are grateful to Dr S. Nakanishi (Institute for Immunology, Kyoto, Japan) for his generous gift of the rat NTR cDNA and Dr D. Gully (Sanofi Recherche, Toulouse, France) for kindly providing us with SR48692. This work was supported by Grant CRG 940325 from NATO and by the French Ministère de l'Enseignement Supérieur et de la Recherche (fellowship to H.B.).

References

1. Kitabgi P and Nemeroff CB, The neurobiology of neurotensin. *Ann NY Acad Sci* **668:** 1–371, 1992.
2. Tanaka K, Masu M and Nakanishi S, Structure and functional expression of cloned rat neurotensin receptor. *Neuron* **4:** 847–854, 1990.
3. Watson MA, Yamada M, Yamada M, Cusack B, Veverka K, Bolden-Watson C and Richelson E, The rat neurotensin receptor expressed in chinese hamster ovary cells mediates the release of inositol phosphates. *J Neurochem* **59:** 1967–1970, 1992.
4. Mouillac B, Caron M, Bonin H, Dennis M and Bouvier M, Agonist-modulated palmitoylation of β_2-adrenergic receptor in Sf9 cells. *J Biol Chem* **267:** 21733–21737, 1992.
5. Ng GYK, Mouillac B, George SR, Caron M, Dennis M, Bouvier M and O'Dowd B, Desensitization, phosphorylation and palmitoylation of the human dopamine D1 receptor. *Eur J Pharmacol* **267:** 7–19, 1994.
6. Javitch JA, Kaback J, Li X and Karlin A, Expression and characterization of human dopamine D2 receptor in baculovirus-infected insect cells. *J Receptor Res* **14:** 99–117, 1994.
7. Aharony D, Little J, Powell S, Hopkins B, Bundell KR, McPheat WL, Gordon RD, Hassall G, Hockney R, Griffen R and Graham A, Pharmacological characterization of a cloned human NK-2 [neurokinin A] receptor expressed in a baculovirus/Sf21 insect cell system. *Mol Pharmacol* **44:** 356–363, 1993.
8. Sadoul JL, Mazella J, Amar S, Kitabgi P and Vincent JP, Preparation of neurotensin selectively iodinated on the tyrosine 3 residue. Biological activity and binding properties on mammalian neurotensin receptors. *Biochem Biophys Res Commun* **120:** 812–819, 1984.
9. Munson PJ and Rodbard D, Ligand: a versatile computerized approach for characterization of ligand binding systems. *Analytic Biochem* **107:** 220, 1980.
10. Fargin A, Raymond JR, Regan JW, Cotecchia S, Lefkowitz RJ and Caron MG, Effector coupling mechanism of the cloned HT-1A receptor. *J Biol Chem* **264:** 14848–14852, 1989.
11. Kitabgi P, Checler F, Mazella J and Vincent JP, Pharmacology and biochemistry of neurotensin receptors. *Rev Clin Basic Pharmacol* **5:** 397–486, 1985.
12. Miyamoto-Lee Y, Shiosaka S and Tohyama M, Purification and characterization of neurotensin receptor from rat brain with special reference to comparison between newborn and adult age rats. *Peptides* **12:** 1001–1006, 1991.
13. Luckow VA and Summers MD, Trends in the development of baculovirus expression vectors. *Bio/Technology* **6:** 47–55, 1988.
14. Gully D, Canton M, Boigegrain R, Jeanjean F, Molimard JC, Poncelet M, Gueudet C, Heaulme M, Leyris R, Brouard A, Pélaprat D, Labbé-Jullié C, Mazella J, Soubrié P, Maffrand JP, Rostène W, Kitabgi P and Le Fur G, Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of neurotensin receptor. *Proc Natl Acad Sci USA* **90:** 65–69, 1993.
15. Chidac P, Hebert TE, Valiquette M, Dennis M and Bouvier M, Inverse agonist activity of B-adrenergic antagonists. *Mol Pharmacol* **45:** 490–499, 1994.