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Interaction of the COOH-Terminal Domain of the Neurotensin Receptor with a G Protein Does Not Control the Phospholipase C Activation but Is Involved in the Agonist-Induced Internalization

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SUMMARY

The agonist-induced internalization of the neurotensin receptor was studied in transfected Chinese hamster ovary cells expressing either the wild-type or a truncated rat neurotensin receptor, lacking the complete intracellular COOH-terminal end. Incubation of cells expressing the wild-type neurotensin receptor in the presence of the peptide resulted in a dramatic decrease in the [^{3}H]neurotensin binding at the cell surface. This disappearance of cell surface binding sites resulted from the internalization of the receptor after the binding of the peptide. The receptor/peptide complexes were internalized in an intracellular compartment resistant to acid washes. The truncated receptor displayed high affinity binding properties for neurotensin in cell homogenates and activated phospholipase C as did the wild-type receptor. However, in cells expressing the truncated receptor, incubation with neurotensin only induced a partial decrease in cell surface binding, and internalization of the bound peptide was also impaired. On cell homogenates, the GTP analogue Gpp(NH)p was found to decrease the affinity of [^{3}H]neurotensin for the wild-type receptor, whereas no similar effect was observed with the truncated receptor. These results show that the intracellular COOH-terminal region of the rat neurotensin receptor is not required for its functional coupling with intracellular G protein but is involved in the shift of the affinity of the receptor for the agonist, which occurs as a consequence of receptor activation and coupling. Because the truncated receptor was shown to internalize poorly, it may be proposed that internalization is not directly related to the activation of G protein but rather is a consequence of modification of receptor affinity, after activation by the agonist.

Agonist-induced desensitization of cell surface receptors is a common feature of almost all GPCRs. This regulation has been reported to take place at different molecular and cellular levels, including molecular desensitization, internalization, and down-regulation of the receptor. Mutational analysis of many members of the GPCR family has provided evidence that the third intracellular loop and the COOH-terminal end of the receptors play a critical role in the coupling of the membrane receptor with intracellular G proteins (1). The same regions were also shown to be involved in the control of the receptor regulation processes (2). At the present time, no consensus sequences for G protein coupling or for receptor internalization have been clearly identified among the cloned GPCRs. However, it is now well established that the membrane proximal regions of the third intracellular loop of these receptors are required (but not always sufficient) for coupling (1). The desensitization at the molecular level appears to be related to the phosphorylation of amino acids located within both the third intracellular loop and the COOH-terminal end of the receptors (2). For the receptor internalization, multiple critical regions of the receptor have been identified (3–8). On the basis of these recent data, the relationship between the activation of a G protein and the internalization of the receptor remains controversial (see Discussion).

In the present study, we investigated the agonist-induced internalization of the NT receptor in transfected CHO cells expressing either the wild-type or a truncated NTR, lacking the complete intracellular COOH-terminal end. NT is a tridecapeptide distributed in both the central nervous system and the peripheral tissues of various mammalian species (9). This peptide displays a wide variety of biological activities as a central neurotransmitter or neuromodulator and as a periph-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; NT, neurotensin; CHO, Chinese hamster ovary; NTR, rat neurotensin receptor; IP, inositol phosphate; PBS, phosphate-buffered saline.

eral hormone. NT evokes its biological effects after specific binding to high affinity NTRs, which have been characterized in the rat and in the human brain, as well as in different cultured cell lines. Hydrolysis of the membrane phosphoinositides plays a major role in the transduction signal (10, 11), and the resulting accumulation of cytoplasmic IPs has been correlated with a transient increase in the intracellular calcium concentration (12, 13).

The NTR cDNA has been cloned from the rat brain (14) and functionally expressed in cell lines (15, 16). The NTR has been characterized as a member of the GPCRs family with seven transmembrane spanning domains separated by intracellular and extracellular loops. The regulation of the NTR at the second messenger level has been described in cultured neurons (12) and in HT29 (13) and N1E-115 (17) cell lines, whereas its desensitization by an internalization process was reported in primary cultures of rat (18) and murine (19) neurons and in the N1E-115 cell line (20). In cultured neurons, it was shown that after binding to cell surface receptors, NT undergoes rapid endocytosis through a mechanism that is receptor mediated. In previous studies, we characterized the internalization of the NTR in transfected CHO cells (21). At the molecular level, mutations in the third intracellular loop of the NTR indicated that this region was required for activation of phospholipase C but not for internalization (22). Recently, mutations and deletions within the COOH-terminal end of the NTR have been reported to impair the internalization of receptor-bound NT in transient transfected COS cells (23). According to this study, maximal loss of peptide internalization was observed after deletion of almost all of the COOH-terminal end of the receptor. Although this experimental model was proved to be useful for the study of NTR internalization, activation of phospholipase C was not observed after transient expression of both the full-length and the truncated receptors in COS cells. Therefore, in the present study, stable transfected CHO cells were used not only to study the consequence of the deletion of the COOH-terminal end of the NTR in the internalization of the NTR but also in the binding of NT, the coupling of the receptor with a G protein, and the activation of phospholipase C.

Experimental Procedures

Materials. Reagents and materials were obtained from the following sources: [^{3}H]NT (specific activity, 107 Ci/mmol in ethanol) and *myo*-[2-^{3}H]inositol (specific activity, 12.3 Ci/mmol in ethanol/water 9:1) were from New England Nuclear (Boston, MA); NT, NT1–8, NT8–13, neuromedin N, bacitracin, bovine serum albumin, G418, and phenylarsine oxide were from Sigma Chemical Co. (St. Louis, MO); AG 1-X8 resin (in the formate form, 100–200 mesh) was from Bio-Rad Laboratories (Richmond, CA); cell culture media and fetal calf serum were from GIBCO (Paisley, UK), cell culture plasticware was from Nunc (Roskilde, Denmark), and Aqua luma was from Lumac (Schaesberg, The Netherlands). SR48692 (batch number 9200VI) was kindly provided by Dr. D. Gully (Sanofi Recherches, Toulouse, France).

Cell culture. CHO cells were cultured in Ham-F12 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cultures were maintained at 37° in a humidified atmosphere of 5% CO_2/95% air. For binding experiments with intact cells, cells were grown in 24-well plates, and for IP measurements, cells were cultured in 6-well plates.

Cloning and transfection procedure. The molecular cloning of the cDNA sequence encoding the rat NTR in the plasmid pSVK3 has been previously described (15). The cDNA encoding the truncated receptor was obtained through polymerase chain reaction performed on the full-length rat NTR cDNA. The 5′oligonucleotide (5′ GAA TTC GAA TTC CCG GCA CCC ACC ATG CAC CTC AA 3′) corresponded to the 5′ end sequence of the NTR cDNA and contained an *Eco*RI restriction site; the 3′oligonucleotide (5′ T CTA GAT CTA GAC CAG GTT GTA GAG GAT GGG ATT 3′) was complementary to nucleotides 1092–1126 of the sequence of the receptor and contained a *Xba*I site and an in-frame TAG stop codon at position 1116. The polymerase chain reaction product (corresponding to amino acids 1–372) was cloned in the plasmid pSVK3.

The pSVK3 constructs were transfected together with the plasmid pSV2neo into CHO cells using the calcium phosphate precipitation method as follows: a coprecipitate containing 1 μg of pSV2neo and 9 μg of pSVK3 was added to the culture medium of 800,000 CHO cells. After overnight incubation, the cells were washed extensively with PBS to remove the precipitate and further incubated with fresh culture medium. Three days after transfection, the cells were selected for their resistance to aminoglycoside by the addition of increasing concentrations (150–600 μg/ml) of G418 to the culture medium. Resistant cell populations were submitted to limited dilutions to isolate cellular clones.

Binding assays on homogenates. Cell homogenates were prepared as previously described (18). An aliquot of cell homogenates (50–100 μg of protein) was incubated in the presence of increasing concentrations of [^{3}H]NT in 0.6 ml of homogenate binding buffer (Tris·HCl 50 mM, pH 7.4, containing 0.1% bovine serum albumin, 0.2 mM bacitracin, and 0.6 mM $MgCl_2$) for 20 min at 20° in plastic tubes. The nonspecific binding of [^{3}H]NT was measured in the presence of 1 μM of unlabeled peptide. The incubation was terminated by the addition of 3 ml of ice-cold homogenate binding buffer, and the suspension was rapidly filtered with GF/C Whatman glass fiber filters that had been previously soaked in 0.5% polyethylenimine for 1 hr. Each filter was washed twice with 3 ml of ice-cold buffer. The radioactivity retained by the filter was counted in a Beckman liquid scintillation counter using 7 ml of Aqua luma.

Binding on intact cells. Binding of [^{3}H]NT on intact cells was measured on cells grown in 24-well plates for 4–5 days. After being washed twice with ice-cold PBS (1 ml) and once with the intact cell binding buffer (F12 medium containing 0.1% bovine serum albumin and 0.2 mM bacitracin) and after equilibration at the binding temperature, the cells were incubated with [^{3}H]NT. The nonspecific binding was determined in the presence of 1 μM unlabeled NT and subtracted from the total binding to calculate the specific binding. After binding, the cells were rapidly washed three times with 1 ml of ice-cold PBS and dissolved in 500 μl deoxycholic acid 1%, pH 11.3. The radioactivity contained in 200 μl was counted by liquid scintillation, and the protein concentration was determined using bovine serum albumin as a standard. For the internalization studies, the amount of internalized ligand was measured after two additional acid washes (2 min each) using ice-cold PBS at pH 2.5.

Receptor internalization experiments. After being washed, transfected CHO cells were incubated in the presence of various concentrations of NT in intact cell binding buffer. Thereafter, the plates were chilled on ice, and the cells were washed four times with neutral PBS and incubated in the binding buffer for 2 hr at 0–4° before the binding experiments. At the end of this washing period, phenylarsine oxide (10 μM) was added, and the cells were further incubated for 15 min at 0–4°. Then, the number of NT binding sites present on the cell surface was determined in binding experiments conducted with 1 nM [^{3}H]NT for 30 min at 37° in the presence of phenylarsine oxide. Radioactivity associated with the cells was measured as described above.

Production of IPs. Cells were grown in six-well plates and equilibrated for 18 hr in F12 medium without serum and containing 1 μCi/ml *myo*-[2-^{3}H]inositol. After being washed with PBS, the cells were incubated for 30 min at 37° in 1 ml intact cell binding buffer containing 30 mM LiCl. The incubation medium was then replaced by

fresh buffer supplemented with various concentrations of NT and further incubated at 37° for 30 min. The reaction was terminated by aspiration of the medium and the addition of 500 μl of deoxycholic acid 1%, pH 11.3. The accumulation of [^{3}H]IPs was determined as previously reported (15)

Results

Molecular cloning. Amplification of the rat NTR cDNA by polymerase chain reaction was performed as previously described using cDNA obtained after reverse transcription of total RNA purified from primary cultures of rat neurons (15). In the present study, this cDNA was used as a template in the amplification of the cDNA sequence encoding a truncated form of the NTR receptor. In this receptor, designated NTRdel372, the 52 amino acids that constitute the COOH-terminal intracellular tail of the transmembrane protein were eliminated. The constructs encoding either the full-length or the truncated NTR were used for stable transfection of CHO cells. Crude membrane preparations from individual clones of transfected cells were isolated and tested for their ability to bind [^{3}H]NT. Cellular clones expressing similar [^{3}H]NT binding levels were selected for further experiments. CHO cells bearing the full-length or the truncated NTRs are designated CHONTRwt and CHONTRdel372, respectively.

Binding on cell homogenates. Homogenates from transfected cells were incubated in the presence of increasing concentrations of [^{3}H]NT (0.03–8 nM) for 20 min at 20°. The specific binding of [^{3}H]NT to NTRwt and NTRdel372 was saturable. In both cell lines, the Scatchard analysis of the [^{3}H]NT binding revealed the presence of a single population of high affinity binding sites (Fig. 1A). The K_D value measured on the truncated receptor was not significantly different from that measured on the wild-type NTR (Table 1). In addition, the ability of the nonpeptide antagonist SR48692 to displace the specific binding of [^{3}H]NT was found to be the same for both receptors (Fig. 1B and Table 1).

Effects of Gpp(NH)p. To investigate the interaction of the NTR with an intracellular G protein, we measured the affinity of [^{3}H]NT in the presence of Gpp(NH)p, a nonhydrolyzable analogue of GTP, which is known to interfere with the formation of the high affinity agonist/receptor/G protein ternary complex. As shown in Fig. 2A, an important decrease in the binding of [^{3}H]NT was measured in the presence of increasing concentrations of Gpp(NH)p (EC_{50} = 0.54 ± 0.17 μM). A maximal decrease (55%) was observed at a concentration of 0.1 mM Gpp(NH)p. The binding of [^{3}H]NT to the truncated receptor was, on the contrary, completely unaffected by the presence of Gpp(NH)p. Saturation experiments conducted in the presence of 0.1 mM Gpp(NH)p indicated that the decrease in [^{3}H]NT binding observed for the wild-type receptor resulted from a 3-fold decrease in the affinity for the peptide (Fig. 2B and Table 1), whereas no significant modification of the affinity of [^{3}H]NT for the truncated receptor was found in the presence of Gpp(NH)p.

Binding of [^{3}H]NT on intact cells. The affinity of [^{3}H]NT for the receptors expressed on intact cells was measured after analysis of the saturation of [^{3}H]NT specific binding. These experiments were performed at 0–4° to avoid ligand internalization after binding to the receptor. As shown in Fig. 3, saturation curves of the binding of [^{3}H]NT to

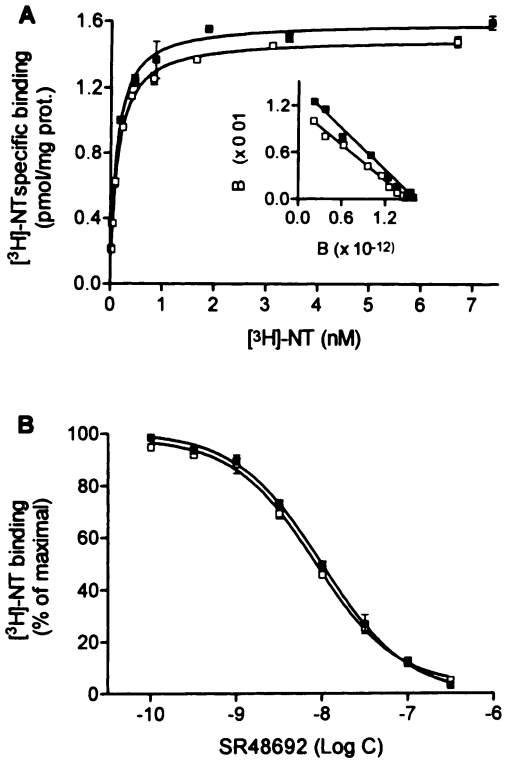

Fig. 1. Analysis of the specific binding of [^{3}H]NT on cell homogenates of CHONTRwt (■) and CHONTRdel372 cells (□). A, Saturation curves of the specific binding of [^{3}H]NT. *Inset*, Scatchard plot of the specific binding. Data correspond to mean ± standard deviation values of typical experiments performed five times and three times, respectively. B, Displacement of [^{3}H]NT (0.5 nM) specific binding by compound SR48692. Results are expressed in percentage of maximal specific binding, and data represent to mean ± standard deviation values of three different experiments performed in triplicate.

NTRwt or NTRdel372 were very different in these experimental conditions. As a consequence, the accurate calculation of the binding parameters of the NTRwt required [^{3}H]NT concentrations up to 60 nM, whereas saturation of the specific binding of [^{3}H]NT to the truncated receptor was obtained with concentrations of ~10 nM. Taken together, these experiments show that at low temperature, the affinity of [^{3}H]NT for the NTRwt was lower on intact cells than on cell homogenates (Table 1). Such a difference was not observed when the binding of [^{3}H]NT was measured on cells expressing the truncated receptor. The low affinity of [^{3}H]NT on CHONTRwt cells at 0–4° leads to very low binding of the labeled ligand when used at a concentration of 1 nM. This was not observed when the binding of [^{3}H]NT on intact cells was measured at 15° and 37°. For this reason, when binding experiments were carried out at 0–4° on CHONTRwt, they were conducted with a 20 nM [^{3}H]NT concentration (see Receptor internalization studies).

Phospholipase C activation. Despite the absence of effect of Gpp(NH)p on the high affinity binding of [^{3}H]NT to the truncated receptor in cell homogenates, we investigated the ability of this receptor to activate phospholipase C. Therefore, the accumulation of IPs was measured after stimulation of the cells with NT in the presence of lithium. As shown in Fig. 4, a similar accumulation of IPs was observed in the transfected cells expressing either NTRwt or NTRdel372. After 30 min, the maximal increase in IPs in both transfected

TABLE 1

Ligand binding characteristics

Results of binding experiments using [^{3}H]NT on CHONTRwt and CHONTRdel372 cells. The values presented are the results of experiments illustrated in Figs. 1–3.

Cell line	K_D	B_{max}	IC_{50} of SR48692
	nM	pmol/mg protein	nM
Homogenates, 20°			
CHONTRwt	0.14 ± 0.02 (5)	1.57 ± 0.20 (5)	10.10 ± 1.69 (3)
CHONTRdel372	0.16 ± 0.02 (3)	1.48 ± 0.15 (3)	8.52 ± 0.73 (3)[a]
CHONTRwt + 100 μM Gpp(NH)p	0.45 ± 0.09 (5)[b]	1.26 ± 0.34 (5)	n.d.
CHONTRdel372 + 100 μM Gpp(NH)p	0.15 ± 0.02 (3)[c]	1.44 ± 0.14 (3)	n.d.
Intact cells, 0–4°			
CHONTRwt	21.42 ± 2.76 (3)	0.46 ± 0.08 (3)	n.d.
CHONTRdel372	2.07 ± 0.26 (3)	0.53 ± 0.09 (3)	n.d.

[a] No significant difference compared with CHONTRwt.
[b] Significant difference ($p < 0.01$ Student's t test) compared with CHONTRwt in the absence of Gpp(NH)p.
[c] No significant difference compared with CHONTRdel372 in the absence of Gpp(NH)p.
n.d. indicates not determined.

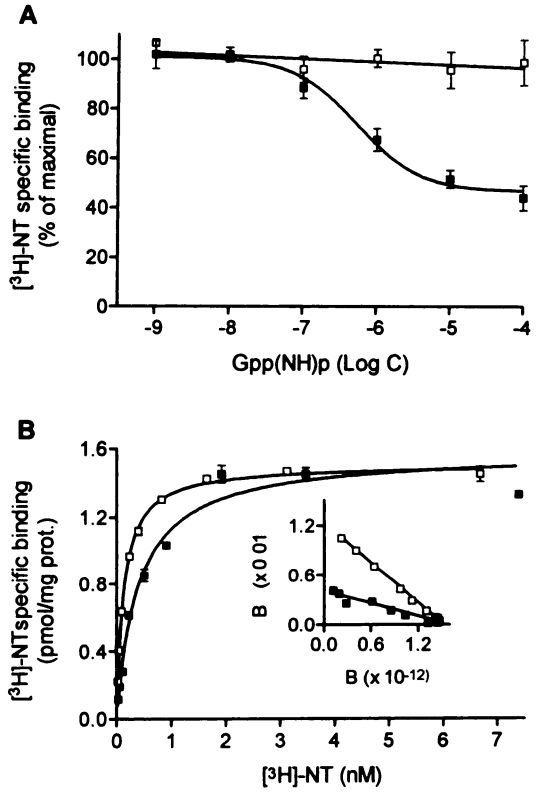

Fig. 2. Effects of Gpp(NH)p on the specific binding of [^{3}H]NT on homogenates of CHONTRwt (■) and CHONTRdel372 (□) cells. A, Effect of increasing concentrations of Gpp(NH)p on the specific binding of [^{3}H]NT (0.25 nM). Results are expressed in percentage of total specific binding, and data represent mean ± standard deviation values of four and three different experiments performed in triplicate, respectively. B, Saturation curves of the specific binding of [^{3}H]NT in the presence of Gpp(NH)p (0.1 mM). *Inset*, Scatchard plot of the specific binding. Data represent mean ± standard deviation values of typical experiments performed five times and three times, respectively.

cell lines was ~30-fold above the baseline. The EC_{50} values resulting from the activation by NT of NTRwt or NTRdel372 were 1.71 ± 0.67 nM (four experiments) and 1.08 ± 0.45 nM (four experiments), respectively (p = NS, Student's t test).

Internalization studies. The internalization of [^{3}H]NT in transfected CHO cells was studied by measuring the amount of peptide that remained associated to intact cells after an acid washing step. The efficacy of acidic PBS to remove cell surface-associated NT was first investigated by incubating the CHONTRwt cells with 20 nM [^{3}H]NT for 2 hr at 0–4° and washing the cells twice for 2 min with acidic PBS. An almost complete release of the bound radioligand was observed in these experimental conditions (>95%, not shown). After binding at 15° and 37°, [^{3}H]NT (1 nM) was rapidly internalized in intact CHONTRwt cells, as revealed by the increasing amount of radioactivity resistant to acid washing. The maximal internalization was observed after 10 min at 37° and represented ~80% of total associated ligand (Fig. 5A). Interestingly, a similar internalization of the peptide was observed at 15°. In CHONTRdel372, internalization of [^{3}H]NT was lower: after a 1-hr incubation at 37°, the internalization of [^{3}H]NT reached ~40% of the total associated ligand (Fig. 5B). At 15°, a slow internalization was observed, reaching only ~20% of the total associated ligand after 1-hr incubation.

The internalization of [^{3}H]NT in both cell lines was completely prevented in the presence of a large excess (1 μM) of either unlabeled NT, the active fragment of NT (NT8–13), neuromedin N (specific ligand of the NTR), or the specific NT antagonist SR48692. On the contrary, this internalization was not affected by the inactive fragment of NT (NT1–8) (Fig. 6). In addition, internalization was inhibited in the presence of phenylarsine oxide. Finally, nontransfected CHO cells did not show any ability to internalize [^{3}H]NT (not shown). All of these results indicate that the transfected CHO cells internalize the peptide through receptor-mediated endocytosis. Having established that the COOH-terminal end of the receptor plays a key role in the internalization of [^{3}H]NT by transfected CHO cells, we investigated internalization of the receptor. We have previously shown that incubation of CHONTRwt cells with NT induces a dramatic reduction in the cell surface NT binding (24). This reduction was time and concentration dependent. A maximal decrease was observed after ~30 min and involved 71.2% of cell surface binding. In the same conditions, incubation of CHONTRdel372 with NT was found to result in only a 27.8% decrease in cell surface binding (Fig. 7A). The EC_{50} value for NT in promoting the internalization of cell surface receptors was similar for both cell lines (0.92 ± 0.08 nM and 1.23 ± 0.14 nM in CHONTRwt and CHONTRdel372, respectively) (Fig. 7B).

Discussion

We previously reported the stable expression of the rat NTR in cultured CHO cells (15). In the present study, trans-

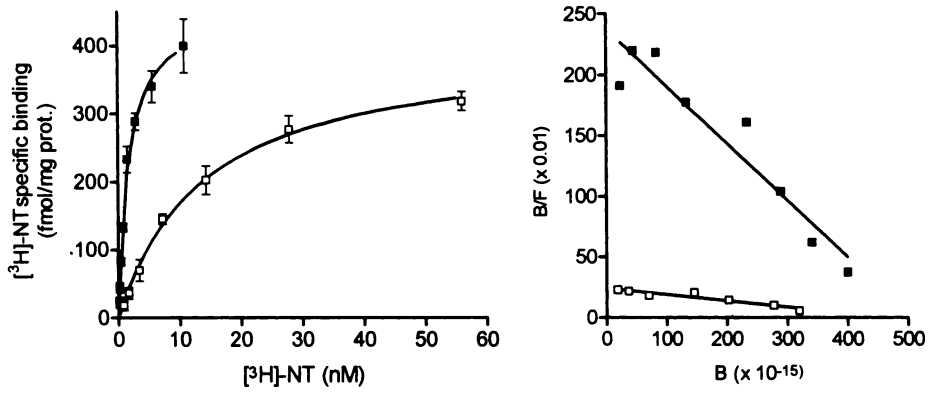

Fig. 3. Binding of [^{3}H]NT on intact CHONTRwt (■) and CHONTRdel372 (□) cells at 0–4°. Saturation curves of the specific binding of [^{3}H]NT on intact cells in monolayer cultures. *Inset*, Scatchard plot of the specific binding. Data represent to mean ± standard deviation values of typical experiments performed three times in quadruplicate.

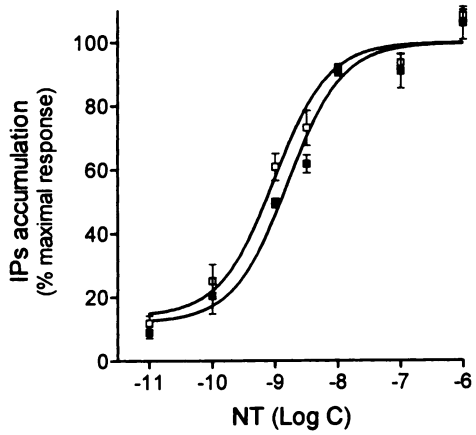

Fig. 4. Dose-response curves for the effects of NT on the accumulation of IPs on CHONTRwt (■) and CHONTRdel372 (□) cells. Total IPs were measured from intact cells stimulated with increasing concentrations of NT. Results are expressed in percentage of maximal IP level and correspond to ~30-fold basal level in both cell lines. Data represent mean ± standard deviation values of five different experiments performed in triplicate.

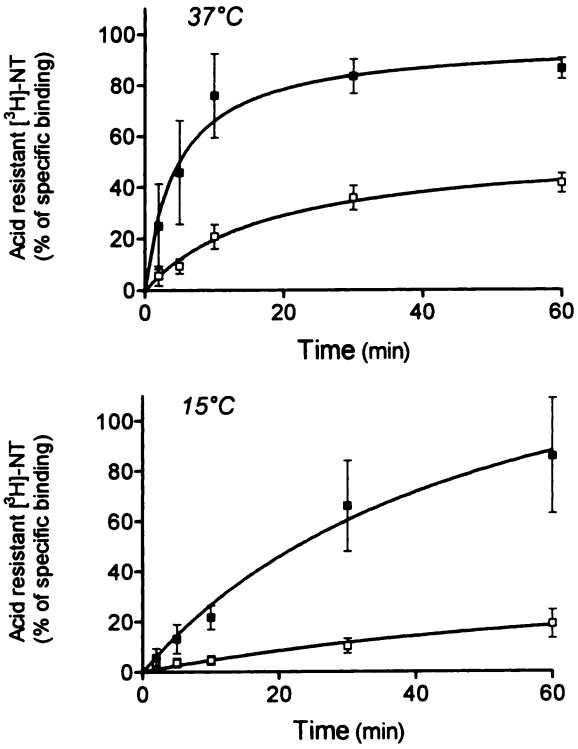

Fig. 5. Time course of the internalization of [^{3}H]NT (1 nM) in intact CHONTRwt (■) and CHONTRdel372 (□) cells at 15° and 37°. After binding of [^{3}H]NT, intact cells were washed with neutral PBS, pH 7.4, or acidic PBS, pH 2.5. The percentage of internalization was obtained by dividing acid-resistant binding by total specific binding. Data represent mean ± standard deviation values of four different experiments performed in quadruplicate.

fected cells expressing the full-length NTR or a receptor deleted from the COOH-terminal intracytoplasmic domain were used to evaluate the importance of this region of the receptor in the binding of NT, the coupling of the receptor with a G protein, the activation of phospholipase C, and the agonist-induced internalization of the receptor.

In CHO cells expressing the wild-type receptor, NT was found to bind with high affinity to cell homogenates and to promote the functional activation of a G protein, leading to activation of phospholipase C. The coupling of the receptor with a G protein was further confirmed by a decrease of the affinity in the presence of a nonhydrolysable GTP analogue. According to previous studies (25, 26), such a shift in agonist affinity results from the uncoupling of the receptor from the G protein. Usually, uncoupling of the receptor by the addition of GTP analogues gives rise to more dramatic changes in affinity for agonists, especially for adrenergic receptors (27, 28). However, according to multiple studies conducted with different GPCRs, the shift in agonist affinity is more important for receptors coupled to G_s or G_i than for those coupled to G_p.

In CHONTRwt, [^{3}H]NT bound to the cell surface was internalized into an intracellular compartment, where it was protected from an acid wash. According to previous studies conducted with the NTR (19, 23), this process was fully inhibited in the presence of phenylarsine oxide, a strong inhibitor of the endocytosis. The endocytosis of the labeled peptide was receptor mediated as it was not observed in nontransfected cells and was prevented by high concentrations of specific ligands of the NTR (NT, NT8–13, neuromedin N) and by the nonpeptide antagonist SR48692 (29). Moreover, the preincubation of the transfected cells with unlabeled NT resulted in a marked decrease in the number of cell surface binding sites, as measured with *in vitro* binding assays.

Binding experiments performed with homogenates of CHONTRdel372 indicate that the complete removal of the COOH-terminal domain of the NTR did not affect the ability of the receptor to bind [^{3}H]NT or the NTR antagonist SR48692. In accordance with other data obtained with truncated GPCR (3, 4, 6, 30), our data indicate that the COOH-

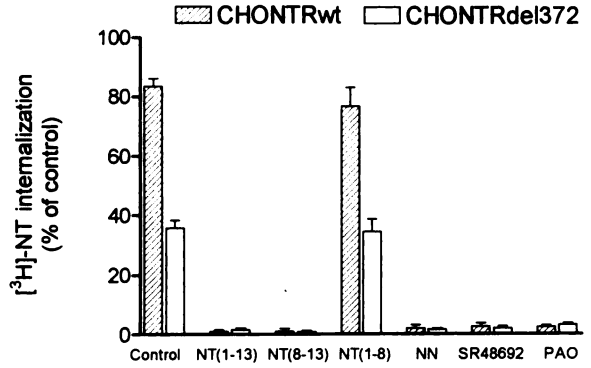

Fig. 6. Effect of different NT analogues SR48692 and phenylarsine oxide on [^{3}H]NT (1 nM) internalization in CHONTRwt and CHONTRdel cells. Intact CHONTRwt and CHONTRdel372 cells were incubated for 30 min with 1 nM [^{3}H]NT in the presence of 1 μM of either NT, NT(1–13), NT(1–8), NT(8–13), neuromedin N (*NN*), SR48692, or phenylarsine oxide (*PAO*). The internalized ligand was measured after washing the cells with acidic PBS, pH 2.5. Results are expressed as percentage of internalized ligand, and data represent mean ± standard deviation values of two different experiments performed in quadruplicate.

terminal end of the rat NTR is not required for the functional coupling to a G protein as complete removal of this region yielded a receptor that remains fully active in mediating phospholipid hydrolysis. It was recently demonstrated that the third intracellular loop of the NTR plays a key role in the agonist-induced activation of phospholipase C (22).

Interestingly, despite the ability of the truncated receptor to activate phospholipase C, the high affinity binding of [^{3}H]NT on CHONTRdel372 cell homogenates was not decreased in the presence of Gpp(NH)p. The first evidence for GTP-sensitive binding of agonist was provided in the early 1970s (28). Many different studies have shown a correlation between the ability of a GPCR to activate a second messenger cascade and a GTP-sensitive binding of the agonists (31–34). However, some studies have clearly indicated that these two properties have to be dissociated, in particular, with mutated receptors. In recent reports, some mutations have been shown to interfere with the activation of a G protein, preventing the production of second messengers, whereas the GTP sensitivity of high affinity agonist binding was unaffected (35, 36). On the contrary, an absence of GTP-sensitive binding of agonists does not necessarily indicate a failure in the coupling of a G protein (37, 38). Accordingly, limited mutations within intracellular regions of the α_2-adrenergic receptor have been demonstrated to eliminate the sensitivity to GTP analogues without eliminating the activation of intracellular effectors (39). Consequently, coupling to a G protein is different than the activation of a G protein (35).

Another difference between the full-length and the truncated receptor concerns the [^{3}H]NT binding measured on intact cells at low temperature. A similar modification of agonist binding at low temperature has been reported for other peptide receptors (40). The reduced binding of the peptide to the full-length receptor was not observed using cells expressing the truncated receptor. On the basis of the differences in the GTP sensitivity of the agonist binding, the low affinity of the NTRwt measured on intact cells at low temperature could result from the interaction of endogenous GTP with a G protein on binding of the agonist and the absence of hydrolysis of the GTP molecule at low temperature.

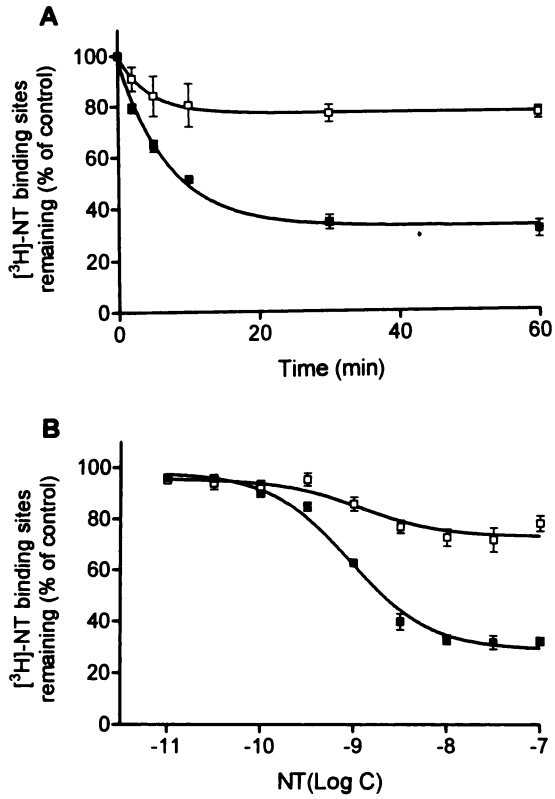

Fig. 7. NT-induced decrease in the cell surface [^{3}H]NT binding on the surface of CHONTRwt (■) and CHONTRdel372 (□) cells. A, Kinetics of internalization. The cells were incubated with NT 10 nM for different periods of time. Thereafter, the cells were extensively washed, and the relative amount of NTR remaining on the cell surface was estimated in binding experiments performed with [^{3}H]NT (1 nM) on intact cells in the presence of phenylarsine oxide. Results are expressed as percentage of control, and data present mean ± standard deviation values of five different experiments performed in quadruplicate. B, Decrease in cell surface [^{3}H]NT (1 nM) specific binding observed after incubation of the cells in the presence of increasing concentrations of NT for 30 min. Results are expressed as percentage of control, and data present mean ± standard deviation values of four different experiments performed in quadruplicate.

In agreement with the recent study of Chabry *et al.* (23) concerning the internalization of [^{3}H]NT, our present data indicate that the intracellular COOH-terminal tail of the NTR is involved in endocytosis of the ligand/receptor complex. Both endocytosis of the peptide and internalization of the receptor were dramatically decreased in the CHO cells expressing the NTRdel372. However, the endocytosis was not completely suppressed, indicating either that another component of the receptor might be involved in the internalization process or that the receptor could be partially internalized during constitutive endocytosis. Mutagenesis studies have demonstrated the role of the different intracellular domains of GPCRs in triggering internalization. Some segments of the COOH-terminal domain of the yeast pheromone α-factor receptor, thyrotropin receptor, gastrin receptor, parathyroid hormone receptor, and angiotensin AT_{1A} receptor were found to be specifically involved in the sequestration process (5, 6, 8, 41, 42), whereas truncated forms of the β-adrenergic and the muscarinic M1 receptors were internalized to a similar extent compared with the wild-type receptors (4, 30). No obvious explanation for such discrepancy has been reported, but it was proposed that the internalization

process might require the presence of sequences of critical length within the COOH-terminal end of the receptor (6).

The molecular mechanisms responsible for the receptor-mediated endocytosis of the NTR are still poorly understood. The observation that the truncated receptor is still able to mediate the activation of phospholipase C indicates that the coupling with the second messenger system is not sufficient to induce the internalization of the NT/NTR complex. The opposite is also true, as a mutated rat NTR, unable to activate phospholipase C, was recently found to be internalized like the wild-type receptor (22). However, these results contradict those of a previous study from the same group indicating that the inhibition of phospholipase C by U-73122 impaired the agonist-induced internalization of the NTR in N1E-115 neuroblastoma cells and in transfected CHO cells (17, 22). This discrepancy can, however, be explained because U73122 does not directly inhibit phospholipase C but is able to disrupt the function of the G protein that activates phospholipase C (43).

In multiple studies, large mutations of GPCRs have led to uncoupling of the receptors from G protein (1). Frequently, this loss of functional coupling is correlated with a decrease in receptor internalization (3, 31). However, some mutated receptors were found to internalize normally, whereas they did not activate intracellular effectors (35, 44); moreover, functional coupling with impaired receptor internalization has been demonstrated (4–6, 41). From these studies, the relationship between second messenger production and receptor internalization remains controversial. In the present study, we demonstrated that the receptor-mediated activation of a G protein, leading to production of second messengers, is neither sufficient nor required for the internalization of the NTR. However, the molecular coupling between the receptor and a G protein leading to a decrease in the affinity for agonists appears to be required for the NTR internalization. Accordingly, it was shown that different mutated β_2-adrenergic receptors that interact with a G protein but are unable to promote second messenger responses are internalized like the wild-type receptor (35, 45). In the same way, it was shown that a mutation of the internalization-defective avian β-adrenergic receptor restores both receptor internalization and GTP sensitivity of agonist binding (46).

In conclusion, we demonstrated that the intracellular COOH-terminal domain of the NTR is dispensable for activation of a G protein leading to hydrolysis of phospholipids and that this domain is required for a coupling step with a G protein leading to GTP-sensitive binding of agonists. Because the same COOH-terminal domain was also found to be of importance in the agonist-induced internalization of the NTR, we suggest that the receptor internalization and the GTP-sensitive binding of the agonists are related processes.

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