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Deletion Mutation in the Putative Third Intracellular Loop of the Rat Neurotensin Receptor Abolishes Polyphosphoinositide Hydrolysis but Not Cyclic AMP Formation in CHO-K1 Cells

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SUMMARY

The tridecapeptide neurotensin is a putative neurotransmitter in the central nervous system. Previously, we showed that the rat neurotensin receptor expressed in CHO-K1 cells mediates polyphosphoinositide hydrolysis and cAMP formation. To further investigate these neurotensin receptor-mediated signal transduction systems, we constructed three deletion mutations in the putative third intracellular loop of this receptor and transfected these mutated genes into CHO-K1 cells. The equilibrium dissociation constants for specific [^{3}H]neurotensin binding to these mutants were not different from that for the wild-type receptor. However, one mutant, which lacked 27 amino acids (amino acids 270–296), did not stimulate polyphosphoinositide hydrolysis, whereas it retained its ability to stimulate cAMP formation. In addition, as found for the wild-type receptor, sequestration occurred with this mutant. We demonstrated here that the putative third intracellular loop of this receptor plays a role in coupling to certain G proteins that induce polyphosphoinositide hydrolysis but not in coupling to cAMP formation or in ligand binding. The two different signal transduction systems may be induced by different G proteins that couple at different sites of the neurotensin receptor protein in CHO-K1 cells. Furthermore, our data show that neurotensin receptor sequestration is independent of agonist-induced polyphosphoinositide hydrolysis.

The tridecapeptide neurotensin, initially isolated from bovine hypothalamus by Carraway and Leeman in 1973 (1), is a putative neurotransmitter in the central nervous system. Neurotensin, through its endogenous receptors, mediates many biological activities, including PI hydrolysis and synthesis of cGMP (2, 3). In addition, in MIA PaCa-2 human pancreatic cancer cells neurotensin stimulates production of cAMP in a dose-dependent manner (4). These latter results support some indirect evidence suggesting that the neurotensin receptor stimulates the formation of cAMP in rat midbrain dopaminergic neurons (5).

Neurotensin receptors are sequestered by cells after prolonged exposure to high concentrations of agonist. This agonist-induced loss of cell surface neurotensin receptors occurs in murine neuroblastoma cells (6–9), primary cultures of rat (10) and mouse (11) neurons, and human colonic adenocarcinoma HT-29 cells (12). The internalization of neostriatal neurotensin receptors, followed by retrograde axonal transport to the substantia nigra pars compacta, occurs in rat brain (13, 14). The mechanisms involved in neurotensin receptor internalization *in vivo* may be similar to those involved in receptor sequestration *in vitro*. We suggested that G proteins play a role in the mechanism of agonist-induced sequestration of neurotensin receptors in murine neuroblastoma clone N1E-115 cells (6, 9).

The rat neurotensin receptor has been molecularly cloned (15). It belongs to the superfamily of G protein-coupled receptors consisting of seven putative transmembrane regions and contains a large putative i-3 loop. Studies of other receptors from this family show that the i-3 loop is important for signal transduction and contains sites for G protein binding (16).

We showed that the cloned rat neurotensin receptor expressed in CHO-K1 cells mediates PI hydrolysis (17) and the increased synthesis of cAMP (18). Both responses require receptor and G proteins. We also showed that neurotensin receptor-mediated cAMP formation is observed under Ca^{2+}-free conditions using membrane preparations and is independent of PI hydrolysis in these cells (18).

CHO-K1 cells, commonly used as a host for stably transfected genes, have been used for similar studies with other receptors because they express multiple G proteins that couple

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ABBREVIATIONS: PI, polyphosphoinositide; CHO, Chinese hamster ovary; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; i-3 loop, third intracellular loop; IBMX, 3-isobutyl-1-methylxanthine; PBS-GS, phosphate-buffered saline solution with glucose and sucrose; PCR, polymerase chain reaction; PKC, protein kinase C.

to PI hydrolysis (19). To identify the regions of the neurotensin receptor that are important for different signal transduction systems and coupling to G proteins, we constructed three i-3 loop deletion mutants of this receptor and expressed each in CHO-K1 cells. Mutants I, II, and III lacked 31 (amino acids 270–300), 27 (amino acids 270–296), and 14 amino acids (amino acids 283–296), respectively (Table 1). In addition, to further characterize neurotensin receptor sequestration, we also examined the effect of i-3 loop deletion mutations on agonist-induced neurotensin receptor sequestration using these cell lines.

Experimental Procedures

Materials. [^{3}H]Neurotensin was obtained from DuPont-NEN (Boston, MA). Neurotensin was synthesized at Mayo Clinic by solid-phase methods. IBMX, phenylmethylsulfonyl fluoride, creatine phosphate, creatine phosphokinase, forskolin, ATP, GTP, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). D-*myo*-[^{3}H]Inositol (18.3 Ci/mmol), [^{3}H]adenine (25 Ci/mmol), and cAMP assay kits were from Amersham Life Science (Arlington Heights, IL). Polypropylene or polyethylene plasticware was used for all experiments with neurotensin.

Construction of deletion mutants of the rat neurotensin receptor. We constructed three i-3 loop deletion mutants of the rat neurotensin receptor. Mutant I lacked 31 amino acids (amino acids 270–300), mutant II lacked 27 amino acids (amino acids 270–296), and mutant III lacked 14 amino acids (amino acids 283–296) (Table 1). All of the cDNA fragments encoding from the 5′ start codon to the i-3 loop domain and from different sites of the i-3 loop to the carboxyl end of the protein were generated by PCR amplification. First-strand rat neurotensin receptor cDNA was used as a PCR template for 35 cycles of denaturation at 95° for 1.5 min, annealing at 60° for 1.5 min, and synthesis at 72° for 2.0 min. The total volume of the PCR assays was 50 μl, containing 30 pmol of primer and 2.5 units of *Thermus aquaticus* polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, CT). Appropriate fragments were combined and the constructed mutant receptor cDNAs were subcloned into pBluescript SK+ vector. The sequences were analyzed by dideoxy sequencing. The deduced amino acid sequences of these mutants and that of the wild-type neurotensin receptor are presented in Table 1.

Transfection and cell culture. The mutant neurotensin receptor cDNA inserts were excised from the pBluescript SK+ vector and then directionally ligated into the pcDNA1neo expression vector (Invitrogen, San Diego, CA), which had been previously digested with *Bam*HI and *Xho*I restriction enzymes. These constructs of mutant neurotensin receptors were transfected into different groups of CHO-K1 cells (American Type Culture Collection, Rockville, MD) by the calcium phosphate method, as described by our group (17) for the wild-type receptor. The cells were selected by G418 (Geneticin) resistance and screened by [^{3}H]neurotensin binding assays using crude membrane preparations. The cells were then cultured as described before (17).

Neurotensin receptor binding assay. For neurotensin receptor binding assays using membrane preparations, we homogenized cells with a Brinkmann Polytron homogenizer in cold 50 mM Tris·HCl, pH 7.4, containing 1 mM EDTA. After centrifugation of the preparation at 40,000 × *g* for 10 min, the pellet was resuspended in buffer and the centrifugation was repeated. The final pellet was resuspended in 50 mM Tris·HCl, pH 7.4, containing 1 mM EDTA, 0.1% bovine serum albumin, and 0.2 mM bacitracin. The protein concentration of the membrane preparation was estimated by the method of Lowry *et al.* (20), using bovine serum albumin as the standard. The competition binding assays were carried out using different concentrations of unlabeled neurotensin with 2 nM [^{3}H]neurotensin, thus allowing determination of the B_{max} value (maximal number of binding sites) and the equilibrium dissociation constant (K_d) for the neurotensin receptors. The binding assays were performed at 20° for 30 min. The data were analyzed with the LIGAND program (21). Nonspecific binding was the amount bound in the presence of 1 μM unlabeled neurotensin. The assays were routinely terminated by simultaneous filtration of 48 samples on a cell harvester filtering unit (Brandel, Gaithersburg, MD) equipped with GF/B filter strips (Whatman, Clifton, NJ) that had been pretreated with 0.1% polyethylenimine for 60 min just before use. For neurotensin receptor binding assays using intact cells, we suspended cells (1 × 10^6 cells/tube) in PBS-GS (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2PO_4, 25 mM glucose, 70 mM sucrose, pH 7.35; 340 mOsmol). The binding assays were performed at 0° for 30 min with 2 nM [^{3}H]neurotensin in the presence of increasing concentrations of unlabeled neurotensin. Nonspecific binding was also defined as described for the membrane assays.

PI hydrolysis assay. The assay used for measurement of D-*myo*-inositol-1-phosphate accumulation was essentially as described before (17). D-*myo*-[^{3}H]Inositol-1-phosphate produced in the cells was isolated chromatographically from cells that had been labeled with the radioactive precursor D-*myo*-[^{3}H]inositol before stimulation by the agonist. In brief, the cells were harvested for use as described for the binding assays. The washed cells were collected by centrifugation. The cellular pellet was resuspended in 4 ml of CO_2-saturated Kreb's solution (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.18 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, pH 7.4) and transferred to a 25-ml Erlenmeyer flask. Twenty microliters of the suspension were removed for enumeration of cells. After a 60-min incubation at 37° with 60 μCi of D-*myo*-[^{3}H]inositol, the cells were resuspended in a volume of PBS-GS (containing 10 mM LiCl). Cells were then allowed to equilibrate for 10 min at 37°. The final assay volume used in each tube was 300 μl. Each tube of cells, oscillating at 80 times/min, was then stimulated by the addition of PBS-GS (to obtain basal D-*myo*-[^{3}H]inositol-1-phosphate values) or agonist. Each reaction was routinely terminated after 30 min by the addition of 750 μl of ice-cold chloroform/methanol (1:2). D-*myo*-[^{3}H]Inositol-1-phosphate levels were then determined as described (17).

Assay for cAMP synthesis using membrane preparations. We removed cells from culture plates with modified Puck's D1 solution containing 2 mM EGTA. Cells were shaken loose by gentle tapping and were collected by low speed centrifugation (200 × *g* for 5 min). The cells were resuspended in 5 mM Tris·HCl buffer, pH 7.4, containing 2 mM EDTA and 50 μM phenylmethylsulfonyl fluoride. Cells were allowed to swell and lyse on ice for 10 min. The cellular lysate was then centrifuged at 48,000 × *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 50 mM Tris·HCl buffer, pH 7.4,

TABLE 1

Deduced amino acid sequences of the putative i-3 loop of rat wild-type and mutant neurotensin receptors

The putative i-3 loop of rat wild-type and mutant neurotensin receptors is contained between predicted transmembrane regions 5 and 6. The cDNAs encoding wild-type and mutant neurotensin receptors were constructed, sequenced, subcloned into an expression vector, and transfected into CHO-K1 cells as described in Experimental Procedures. CHO-K1 cell lines stably expressing these receptors were used for the present study.

	Deduced amino acid sequence
Wild type	5th←/ANKLTVMVH/Q^{270}AAEQGRVCTVGT/H^{283}NGLEHSTFNMTIE^{296}/PGRV^{300}/QALRHGVL/→6th
Mutant I	5th←/ANKLTVMVH/----------/QALRHGVL/→6th
Mutant II	5th←/ANKLTVMVH/---------- /PGRV^{300}/QALRHGVL/→6th
Mutant III	5th←/ANKLTVMVH/Q^{270}AAEQGRVCTVGT^{282}/----------/PGRV^{300}/QALRHGVL/→6th

containing 2 mM EDTA. We homogenized the lysate with a Polytron PT3000 homogenizer (5000 rpm for 10 sec) and then centrifuged the preparation (48,000 × *g* for 10 min). The final pellets were resuspended in 50 mM Tris·HCl buffer, pH 7.4, for assay. Protein was estimated as described above. For measurement of cAMP formation, membrane protein was incubated with each concentration of neurotensin, in a total volume of 150 μl of 50 mM Tris·HCl buffer, pH 7.4, containing 1.6 mM ATP, 5 mM $MgCl_2$, 1.0 mM EGTA, 1.5 mM IBMX, 0.1% (v/v) bovine serum albumin, 0.5 mM GTP, 67 units/ml creatine phosphokinase, and 2.5 mM creatine phosphate, for 5 min at 32°. At the end of the incubation, the tubes were placed in a boiling water bath for 5 min. We collected the supernatant after the solution was centrifuged (9000 × *g* for 1 min). cAMP content in the supernatant was measured with a cAMP assay kit (Amersham Life Science) as described by our group previously (18).

Assay for cAMP synthesis using intact cells. The assay used for measurement of cAMP formation in intact cells was that described before (3, 18), in which [^{3}H]cAMP produced in the cells was isolated chromatographically from cells that had been labeled with radioactive precursor before stimulation. Briefly, the cells were suspended in PBS-GS and prelabeled with 20 μCi of [^{3}H]adenine at 37° for 45 min. The cells were resuspended in a volume of PBS-GS containing 1.5 mM IBMX, which provided 2 × 10^5 cells/well of a Linbro 24-well plate (Flow Laboratories, McLean, VA). The cells were then stimulated with several concentrations of neurotensin at 37° for 5 min. Each reaction was terminated by the addition of 30 μl of 50% (w/v) trichloroacetic acid solution. [^{3}H]cAMP formed in the cells was isolated by chromatography and then determined as described previously (3, 18).

Sequestration of wild-type and mutant rat neurotensin receptors. For sequestration studies, the cells were exposed to 100 nM neurotensin or PBS-GS (as a control) at 37° for 15 min, without peptidase inhibitors, as described before (6, 8, 9). After five washes with ice-cold PBS-GS, intact cells were examined for changes in neurotensin receptor binding as described above. The receptor sequestration was also examined after exposure at 0°. Additionally, the recovery of binding sites was studied after washed cells were incubated for 30 min at 20° (the temperature of our binding assays with membrane preparations).

Results

Neurotensin receptor binding. Stably transfected clonal cell lines were selected by resistance to G418 and screened for agonist binding using [^{3}H]neurotensin. Binding of [^{3}H]neurotensin to i-3 loop mutants of the rat neurotensin receptor was specific and saturable, with K_d values not significantly different from that found for the wild-type rat neurotensin receptor (Table 2). All data fit well to a model for a single population of high affinity agonist binding sites. Calculated B_{max} values (using membrane preparations) were found to be high for the wild-type receptor, intermediate for mutant I (lacking 31 amino acids), and low for mutant II (lacking 27 amino acids) and mutant III (lacking 14 amino acids). However, mutant I receptors were not detected using intact cells (data not shown), whereas binding was found using membrane preparations.

Neurotensin-induced PI hydrolysis. We previously showed that wild-type rat neurotensin receptors expressed in CHO-K1 cells mediate PI hydrolysis (17). The stimulation of PI hydrolysis was not detected using CHO-K1 cells before transfection (17). The i-3 loop deletion mutants of the rat neurotensin receptor were tested for their ability to stimulate PII•hydrolysisin response to the agonist neurotensin. Receptor-mediated PI hydrolysis, in a dose-dependent manner, was observed with mutant III (lacking 14 amino acids) (Fig. 1). The EC_{50} value for this mutant receptor was 61 nM, significantly different from the value found for the wild-type receptor (EC_{50} = 3 nM for the wild-type receptor) (Table 2). On the other hand, mutant II (lacking 27 amino acids) lost the ability to stimulate PI hydrolysis in response to neurotensin. We could not detect the stimulation of PI hydrolysis using the cells expressing mutant I (lacking 31 amino acids) receptors.

TABLE 2

Pharmacological properties of wild-type and mutant rat neurotensin receptors expressed in CHO-K1 cells

Binding of [^{3}H]neurotensin to wild-type and mutant rat neurotensin receptors was specific and saturable. All data fit well to a model for a single population of high affinity agonist binding sites. The receptor-mediated PI hydrolysis was studied as described in Experimental Procedures. Neurotensin receptor-mediated cAMP formation under Ca^{2+}-free conditions, using membrane preparations, was examined as described in Experimental Procedures. Data are expressed as geometric means ± standard errors of three or four independent experiments, each determined in triplicate.

Receptor type	Receptor binding, K_d	PI hydrolysis, EC_{50}	cAMP formation, EC_{50}
	nM	nM	nM
Wild-type	2.2 ± 0.2	3.0 ± 0.2	4.7 ± 0.1
Mutant I	2.0 ± 0.3[a]	ND[b]	6 ± 1[a]
Mutant II	3.8 ± 0.4[a]	ND[b]	3.7 ± 0.8[a]
Mutant III	4.0 ± 0.7[a]	61 ± 1[c]	3.1 ± 0.6[a]

[a] Not significantly different from the value for the wild-type receptor (Student's *t* test).
[b] ND, PI hydrolysis was not observed at detectable levels.
[c] Significantly different from the value for the wild-type receptor ($p < 0.05$, Student's *t* test).

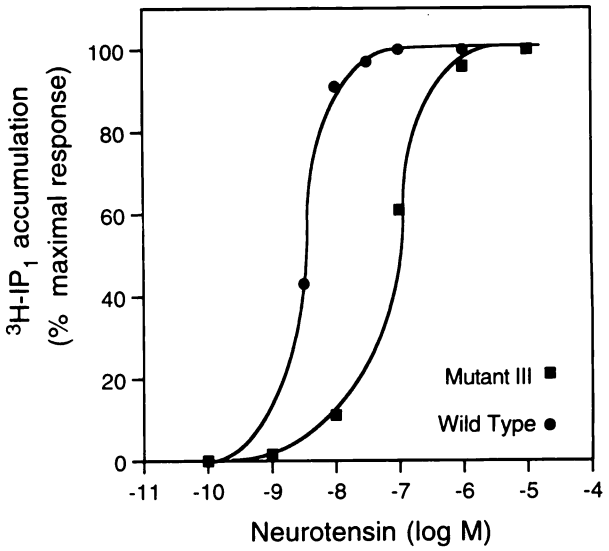

Fig. 1. Neurotensin-induced PI hydrolysis with wild-type and mutant III (lacking 14 amino acids, amino acids 283–296) rat neurotensin receptors expressed in CHO-K1 cells. The receptor-mediated [^{3}H]inositol trisphosphate (^{3}*H-IP*$_1$) accumulation was studied as described in Experimental Procedures. Data are representative results from one of seven and one of four independent experiments, respectively, with each *point* determined in triplicate. Data are expressed as a percentage of the maximal response for each receptor. On average, the basal level of [^{3}H]inositol-1-phosphate was 2000 dpm/10^6 cells and the maximal response was 60-fold and 20-fold over basal levels for the wild-type and mutant III receptors, respectively.

Neurotensin-induced cAMP formation. More recently, we showed, using membrane preparations, that the cloned, wild-type, rat neurotensin receptor mediates cAMP formation in CHO-K1 cells under Ca^{2+}-free conditions (18). cAMP formation was not detected in CHO-K1 cells using either intact cells or membrane preparations before transfection (18). Therefore, we tested neurotensin receptor mutants for their abilities

to stimulate cAMP formation. Neurotensin also stimulated cAMP formation with each of the mutant neurotensin receptors, in a dose-dependent manner, in membrane preparations (Fig. 2). EC_{50} values were not significantly different from the value found for the wild-type receptor (Table 2). Additionally, CHO-K1 cells expressing wild-type and mutant receptors (except mutant I) showed a positive correlation between the level of cAMP formation and the number of binding sites (correlation coefficient, 0.94). However, despite its B_{max} value, mutant I, with the largest deletion mutation (lacking 31 amino acids), caused a small cAMP response, compared with the wild-type receptor (Table 3). On the other hand, cAMP formation was not detected using intact cells expressing mutant I receptors. This result is consistent with that of PI hydrolysis studies using the same cells.

Agonist-induced sequestration of the neurotensin receptor. Our previous studies showed that the neurotensin receptor is rapidly sequestered from the cell surface after exposure of N1E-115 murine neuroblastoma cells to agonist (6–9). In this study, neurotensin (100 nM) caused a loss of 60% of neurotensin binding sites after 15 min of exposure at 37° in cells expressing wild-type rat neurotensin receptors. The loss of binding was not recovered after incubation at 20° for 30 min (conditions of the binding assay used in this study). No sequestration was observed at 0° (data not shown). Neurotensin caused the loss of binding to the same degree for the mutant neurotensin receptors as for the wild-type receptor (Table 4).

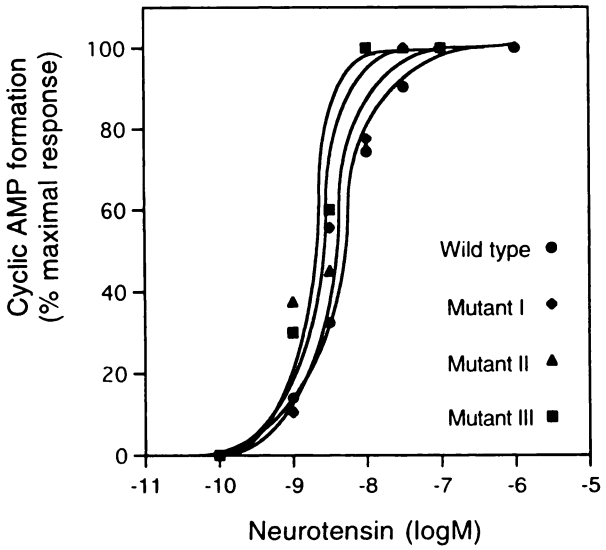

Fig. 2. Neurotensin receptor-mediated cAMP formation under Ca^{2+}-free conditions with wild-type and mutant rat neurotensin receptors, examined as described in Experimental Procedures, using membrane preparations. Data are representative results from one of three independent experiments, with each *point* determined in triplicate. Data are expressed as a percentage of the maximal response for each receptor. The maximal response observed for each receptor is summarized in Table 3.

TABLE 3

Correlation between the magnitude of cAMP formation and the number of binding sites for wild-type and mutant rat neurotensin receptors

Binding of [^{3}H]neurotensin to wild-type and mutant rat neurotensin receptors was specific and saturable. All data fit well to a model for a single population of high affinity agonist binding sites. Neurotensin receptor-mediated cAMP formation under Ca^{2+}-free condition, using membrane preparations, was examined as described in Experimental Procedures. Data are expressed as geometric means ± standard errors of three or four independent experiments determined in triplicate. A linear regression of B_{max} versus maximal cAMP formation for CHO-K1 cells expressing wild-type and mutant receptors (except mutant I) showed a positive correlation ($r = 0.94$).

Receptor type	B_{max}	Maximal cAMP formation	cAMP formation/ B_{max}
	fmol/mg of protein	*pmol/mg of protein*	
Wild-type	2200 ± 200	180 ± 10	0.08
Mutant I	854 ± 6	17 ± 1[a]	0.02[a]
Mutant II	160 ± 10	20.1 ± 0.8	0.12
Mutant III	200 ± 20	16.8 ± 0.7	0.08

[a] Despite its B_{max} value, maximal cAMP formation obtained for mutant I was extremely low, compared with the wild-type receptor.

Discussion

The rat neurotensin receptor belongs to the superfamily of G protein-coupled receptors that consist of seven putative transmembrane regions and a putative large i-3 loop (15). From studies of receptors from this family it is thought that the i-3 loop and surrounding structural regions of these receptors are important for G protein coupling (16). For example, the i-3 loop of the human muscarinic acetylcholine (Hm1) receptor contains sites for G protein binding, which mediates PI hydrolysis (22). The i-3 loop of the β-adrenergic receptor is important for coupling to a G protein (e.g., G_s) that stimulates adenylate cyclase (23, 24). Chimeric receptors generated from several different receptors of this family also support this hypothesis. In those chimeric mutants, second messenger systems of the receptors could be changed by simply substituting the i-3 loop and surrounding domains of one receptor for another (25–28). However, other researchers reported that the important domain for coupling to G proteins that mediate PI hydrolysis is encoded not in the i-3 loop but elsewhere in the rat tachykinin (neurokinin type 2) receptor (29) and Hm1 muscarinic receptor (30).

In the present study, to identify the regions of the rat neurotensin receptor that are important for different signal transduction systems we constructed three i-3 loop deletion mutants of this receptor and studied the pharmacological properties of these mutant receptors. The equilibrium dissociation constants (K_d values) for specific [^{3}H]neurotensin binding were identical among the mutant and wild-type receptors (Table 2). This result suggests that the i-3 loop is not important for ligand binding to the receptor protein. To examine the pharmacological properties of mutant receptors, it is ideal to use cells expressing comparable levels of each receptor. However, [^{3}H] neurotensin binding assays showed that the levels of expression of the mutant receptors were lower than that of the wild-type receptor (Table 3). One may speculate that differences in transfection efficiency and mRNA stability contributed to this

TABLE 4

Agonist-induced sequestration of neurotensin receptors

The loss of neurotensin binding sites after 15 min of exposure to neurotensin (100 nM) at 37° was studied in cells expressing wild-type and mutant rat neurotensin receptors, as described in Experimental Procedures. Data are expressed as means ± standard errors of three independent experiments, each determined in triplicate.

Receptor type	Sequestration of cell surface receptor
	% decrease of control binding sites
Wild-type	62.0 ± 0.7
Mutant I	NE[a]
Mutant II	61.7 ± 0.1[b]
Mutant III	55.9 ± 0.8[b]

[a] NE, not examined.

[b] Not significantly different from the value for the wild-type receptor (Student's *t* test).

phenomenon. Interestingly, the largest deletion mutant (mutant I, lacking amino acids 270–300) was not detected in binding assays using intact cells, whereas this mutant was found using membrane preparations. Additionally, neither PI hydrolysis nor cAMP formation was detected in this CHO-K1 cell line using intact cells. These results also support the hypothesis that the mutant I receptor is not expressed on the surface of the cell (or is expressed at undetectable levels). We have screened a number of cellular clones after transfection of this mutant receptor cDNA; all of the cellular clones analyzed gave similar results (data not shown), suggesting that the phenomena observed were not due to the chromosomal insertion site of the transfected DNA. A similar phenomenon was observed with the leutropin/β-adrenergic receptor chimeras (31). The reason for such complexity is still largely unknown. Changes in secondary and tertiary structure or post-translational modification of this receptor protein may be responsible for this phenomenon. Our results may suggest that a portion of i-3 loop is important for receptor expression on the cellular surface.

Previously we showed that the molecularly cloned rat neurotensin receptor expressed in CHO-K1 cells mediates the release of inositol phosphates (17). In this study, mutant III (lacking amino acids 283–296) retained the ability to stimulate neurotensin-mediated PI hydrolysis, whereas mutant II (lacking amino acids 270–296) did not stimulate PI hydrolysis. Our results suggest that the site encompassing amino acids 270–282 is important for coupling to the G protein that mediates PI hydrolysis. However, the EC_{50} value obtained for this mutant was significantly greater than that for the wild-type receptor. This was not due to a difference in the affinities of these receptors for their agonist neurotensin (Table 2). Thus, the data suggest that this mutant receptor couples less efficiently to G proteins, possibly due to conformational changes of the receptor protein.

Recently, we isolated a cDNA clone encoding the neurotensin receptor from a human substantia nigra cDNA library (32). As shown in Table 5, the amino acid sequence of this receptor from amino acid 270 to amino acid 282 is well conserved between human and rat. In the putative i-3 loop of the human receptor, four amino acids (amino acids 283–286, HNGL) found in the rat neurotensin receptor are missing. Nonetheless, this receptor, when expressed in a stably transfected cell line, also had the ability to stimulate PI hydrolysis and cAMP formation.[1] These findings strongly support the hypothesis that an amino acid or group of amino acids in this conserved domain (amino acids 270–282) play an important role in coupling of the receptor to the G protein that induces PI hydrolysis.

[1] Yamada, M., Mi. Yamada, K. Grosham, and E. Richelson, unpublished observations.

Using many types of brain preparations, researchers reported that neurotensin receptors do not mediate cAMP synthesis (33–37). Only indirect evidence suggests that the neurotensin receptor stimulates the formation of cAMP in brain (5). More recently, it was shown for MIA PaCa-2 human pancreatic cancer cells that neurotensin (10 nM to 1 μM) stimulates production of cAMP in a dose-dependent manner (4). In our previous study, we proposed that cAMP formation mediated by the rat neurotensin receptor in CHO-K1 cells occurs by coupling to a G protein (e.g., G_s) and not secondary to an increase in the intracellular Ca^{2+} concentration resulting from PI hydrolysis (18). These conflicting results may be due to differences in signal transduction systems present within the cells expressing neurotensin receptors.

On the other hand, in the present study receptor-mediated cAMP formation was observed for all of our mutants using membrane preparations. These results suggest that we have identified a domain essential for coupling to the G protein that induces PI hydrolysis; however, it is likely that there are different domains that are essential for G proteins that induce cAMP formation. These two different signal transduction systems may be induced by different G proteins. Another coupling site could be in the remaining domain of the putative i-3 loop or a domain of another intracellular loop. A point mutation in the putative i-3 loop of the rat thyrotropin receptor also results in a loss of ability to induce PI hydrolysis but not cAMP formation (38). These observations strongly support the hypothesis that different types of G proteins couple to the same receptor in different ways.

Additionally, CHO-K1 cells expressing wild-type and mutant receptors (except mutant I) showed a positive correlation between the level of cAMP formation and the number of binding sites. To compare the pharmacological properties of mutant receptors, it is ideal to use cells expressing comparable levels of each receptor. However, our findings may suggest that mutant I and II receptors were not impaired in their ability to stimulate cAMP formation. On the other hand, despite its high B_{max} value (Table 3), the magnitude of the cAMP response mediated by mutant I, with the largest deletion (31 amino acids), in membrane preparations was small, compared with that of the wild-type receptor. This suggests that the relationship between receptor expression and coupling to cAMP is not always linear. However, our results may suggest that the putative i-3 loop of the rat neurotensin receptor also, in part, plays a role in the mechanism of cAMP formation. An alternative interpretation is that the large i-3 loop deletion affected the function of other intracellular domains of this receptor protein. However, this receptor was not likely present on the surface membrane of the cell, making it more difficult for it to associate with G proteins at that site. For the rat tachykinin (neurokinin

TABLE 5

Deduced amino acid sequences of the putative i-3 loop from molecularly cloned rat and human neurotensin receptors, in the region of amino acids 270–282 and the surrounding domain

The putative i-3 loop of rat wild-type and mutant neurotensin receptors is contained between predicted transmembrane regions 5 and 6. In the human neurotensin receptor, four amino acids (amino acids 283–286, **HNGL**) found in the rat receptor are missing. The bold letters represent amino acids differing between rat and human receptors.

	Deduced amino acid sequence
Rat receptor[a]	5th←/ANKLTVMVH/Q^{270}AAEQGRVCTVGT^{282}/HNGLEHSTFNMTIE^{296}/PGRV/→6th
Human receptor[b]	5th←/ANKLTVMVR/Q^{270}AAEQGQVCTVGG^{282}/----EHSTFSMAIE^{296}/PGRV/→6th

[a] Sequence from Ref. 15.
[b] Sequence from Ref. 32.

type 2) receptor, researchers have proposed that the carboxyl tail is important for coupling to G proteins mediating cAMP formation (29). A similar function has been proposed for the carboxyl-tail domain in other G protein-coupled receptor systems (16). We are planning to investigate the role of the carboxyl-tail domain in neurotensin receptor-mediated cAMP formation.

Additionally, cAMP formation was not detected in the CHO-K1 cell line expressing mutant I receptor, using intact cells. It is still not clear why cAMP formation is detectable using membrane preparations but is not detectable using intact cells. One may speculate that some mutant I receptors are expressed on the cell surface membrane at low levels. However, combined with the data obtained from PI hydrolysis and binding studies, our results support the hypothesis that mutant I receptors are not expressed on the cell surface (or are expressed at undetectable levels). How and where this mutant receptor is expressed are still largely unknown.

The internalization of neostriatal neurotensin receptors, followed by retrograde axonal transport to the substantia nigra pars compacta, occurs in rat brain (13, 14). The mechanisms involved in neurotensin receptor internalization *in vivo* may be similar to those involved in receptor sequestration *in vitro*. Previously we showed that the rapid sequestration of neurotensin receptors occurred after exposure to 100 nM neurotensin at 37° in N1E-115 neuroblastoma cells (6, 8, 9). Both the sequestration and the recovery of neurotensin receptors were temperature dependent (8). Those data suggested that a certain degree of phospholipid fluidity or activity of some enzymes (e.g., G proteins, protein kinases, or phosphatases) is required for these processes to occur.

The aminosteroid U-73122 [1-[6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-1*H*-pyrrole-2,5-diane] inhibits agonist-induced sequestration of the neurotensin receptor in N1E-115 cells (6, 9). This compound also inhibits PI hydrolysis elicited by neurotensin or sodium fluoride, which stimulates G proteins. PI hydrolysis elicited by a calcium ionophore was not affected by U-73122. These data suggested that G proteins play a role in the mechanism of agonist-induced sequestration of this receptor in N1E-115 cells.

We found that U-73122 inhibited the sequestration of rat wild-type neurotensin receptors expressed in CHO-K1 cells (data not shown). To study further the relationship between agonist-induced sequestration and G proteins, we studied the sequestration of neurotensin receptors using i-3 loop deletion mutants of this receptor. For many receptors that couple to G proteins, protein phosphorylation may be involved in receptor regulatory processes. Isoforms of PKC induced after PI hydrolysis, cAMP-dependent protein kinase, β-adrenergic receptor kinases (e.g., β-adrenergic receptor kinase I and β-adrenergic receptor kinase II), or other Ca^{2+}-dependent protein kinases may play a role in the mechanism of neurotensin receptor sequestration in transfected CHO-K1 cells.

The putative i-3 loop is important for sequestration of the Hm1 muscarinic receptor (39, 40). However, we found that deletion of part of the putative i-3 loop of the neurotensin receptor had no effect on the ability of neurotensin to cause sequestration of these receptors (Table 3). Our data suggest that sequestration of the rat neurotensin receptor does not require the activation of a G protein mediating PI hydrolysis and subsequent activation of PKC. These results support the findings of others showing that agonist-stimulated internalization of neurotensin receptors is not mediated by PKC activation in human colonic adenocarcinoma HT-29 cells (12).

In conclusion, we suggest that the putative i-3 loop and surrounding structural regions of the rat neurotensin receptor play an important role in coupling to certain G proteins mediating PI hydrolysis but not in coupling to G proteins mediating cAMP formation or ligand binding. We also suggest that neurotensin receptor sequestration is independent of agonist-induced PI hydrolysis. Not only the primary structure of the receptor protein but also its distinct secondary and tertiary structures are important for receptor functions. Further mutational analysis is being conducted to determine specific amino acids that may comprise the actual residues that are important for neurotensin-mediated signal transduction systems and receptor sequestration.

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