Characterization of high affinity neurotensin receptor NTR1 in HL-60 cells and its down regulation during granulocytic differentiation

¹Se-Young Choi, ¹Hee-Don Chae, ¹Tae-Ju Park, ²Hyunjung Ha & ^{*}¹Kyong-Tai Kim

¹Department of Life Science, Pohang University of Science and Technology, Pohang, 790-784, Republic of Korea; ²Division of Life Sciences, Chungbuk National University, Cheongju 361-763, Republic of Korea

- 1 We investigated responses to neurotensin in human promyelocytic leukaemia HL-60 cells.
- 2 Neurotensin increased the cytosolic calcium concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner and also produced inositol 1,4,5-trisphosphate ($InsP_3$).
- 3 Among the tested neurotensin analogues, neurotensin 8-13, neuromedin-N, and xenopsin also increased $[Ca^{2+}]_i$, whereas neurotensin 1–11 and neurotensin 1–8 did not elicit detectable responses.
- 4 SR48692, an antagonist of NTR1 neurotensin receptors, blocked the neurotensin-induced $[Ca^{2+}]_i$ increase, whereas levocabastine, which is known as an NTR2 neurotensin receptor antagonist, did not attenuate the neurotensin-evoked effect.
- 5 The expression of NTR1 neurotensin receptors was confirmed by Northern blot analysis and reverse transcriptase-polymerase chain reaction (RT–PCR).
- 6 During 1.25% dimethylsulfoxide (DMSO)-triggered granulocytic differentiation of HL-60 cells, the neurotensin-induced $[Ca^{2+}]_i$ rise became gradually smaller and completely disappeared 4 days after treatment with DMSO. The mRNA level for neurotensin receptors was also decreased after differentiation.
- 7 The results show that HL-60 cells express NTR1 neurotensin receptors and suggest that granulocytic differentiation involves transcriptional regulation of the receptors resulting in down-regulation of the neurotensin-induced signalling.

Keywords: Neurotensin; cytosolic Ca^{2+} ; phospholipase C; human hemopoietic cells; differentiation

Introduction

Neurotensin, the tridecapeptide ELYENKPRRPYIL, has been studied as a modulator in neuronal and non-neuronal systems (Kitabgi *et al.*, 1985; Vincent, 1995). Neurotensin reveals its modulatory effect in almost every region of the nervous system including the cerebral cortex, terminals of striatal nucleus, hypothalamus, midbrain, cerebellar purkinje cells, retina, and spinal cord (Shi & Bunney, 1992a). In the central nervous system neurotensin is known to modulate the signals of dopaminergic neurons by regulating D_2 receptors. Therefore, neurotensin displays activities similar to antipsychotics in clinical trials involving schizophrenia, Parkinson's disease, and Alzheimer's disease (Nemeroff *et al.*, 1992). It has been also reported that neurotensin potentiates the barbiturate- and ethanol-induced sedation and that it induces antinociception, catalepsy, and hypothermia.

For non-neuronal systems, major roles of neurotensin have been demonstrated in the gastrointestinal and cardiovascular system. Neurotensin is localized at the intestinal mucosa (endocrine N-cells) where it is thought to be involved in intestinal contraction (Buhner & Ehrlein, 1989). Intravenous injection of neurotensin changes the concentration of insulin, glucagon, and pituitary hormones including thyroid releasing hormone (Kitabgi *et al.*, 1985; Schimpff *et al.*, 1995). It also induces vasodilatation and affects vascular permeability by way of vascular, portal vein, and smooth muscle contraction (Bachelard *et al.*, 1992). The

neurotensin effect on the cardiovascular system somewhat depends on the species, because neurotensin causes hypotension in dogs but hypertension in guinea pigs. It has been reported that endothelial cells of the umbilical vein also express the neurotensin receptor which is involved in the increase of intracellular Ca^{2+} (Schaeffer *et al.*, 1995) and the production of prostacyclin (Schaeffer *et al.*, 1997). However, it is not yet known what role neurotensin plays in hemopoietic or immune cells.

Neurotensin evokes two major actions at the cellular level. It causes inositol 1,4,5-trisphosphate ($InsP_3$) production and increase in the cytosolic calcium concentration ($[Ca^{2+}]_i$) through the activation of phospholipase C (PLC) (Goedert *et al.*, 1984; Bozou *et al.*, 1989; Hermans *et al.*, 1992). In another signalling pathway, neurotensin modulates the intracellular cyclic nucleotide level including cyclic GMP (Gilbert & Richelson, 1984; Amar *et al.*, 1985) and cyclic AMP (Bozou *et al.*, 1986; Yamada *et al.*, 1994).

HL-60 cells have served as a good model in which to study signal transduction of various pharmacological receptors before and after differentiation of the hemopoietic cells (Klinker *et al.*, 1996). HL-60 cells can be forced to terminally differentiate into neutrophil-like cells by dibutyl cyclic AMP, DMSO, or retinoic acid treatment and into adherent macrophage-like cells by 1, 25-dihydroxy vitamin D_3 and phorbol esters (Klinker *et al.*, 1996; Collins *et al.*, 1978).

Here we report the expression of PLC-coupled neurotensin receptors in HL-60 human promyelocytic leukaemia cells and the down-regulation of neurotensin-induced signalling during granulocytic differentiation into neutrophil-like cells.

*Author for correspondence.

Methods

Cell culture

HL-60, U937, K562, Jurkat T, H9, and U266 cells were maintained at 37°C in RPMI 1640 supplemented with 10% (v/v) heat-inactivated bovine calf serum and 1% (v/v) penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was changed daily. We induced differentiation of HL-60 cells by incubating them in 1.25% DMSO for 6 days. We observed the morphology of the cells and monitored fMLP-induced [Ca²⁺]_i increase, which was only detectable in differentiated HL-60, as indication of differentiation into neutrophil-like cells. We also counted viable cells by the Trypan-blue exclusion method.

Isolation of human neutrophils

Human neutrophils were isolated from the peripheral blood of healthy donors as previously described (Pember *et al.*, 1983). Isolated cells were suspended in phosphate buffered saline/glucose (in mM: KCl 2.6, KH₂PO₄ 1.5, MgCl₂ 0.5, NaCl 136, Na₂PO₄ 8 and glucose 5.5, pH 7.4) and used immediately.

Measurement of [Ca²⁺]_i

The level of intracellular Ca²⁺ was determined using fura-2 tetraacetoxymethyl ester (fura-2/AM) as we have previously described (Choi & Kim, 1997). Briefly, the cell suspension was incubated in fresh serum-free RPMI 1640 medium with 3 µM fura-2/AM at 37°C for 60 min with continuous stirring. Sulphinpyrazone (250 µM) was added to prevent dye leakage. Changes in the fluorescence ratio were measured at dual excitation wavelengths of 340 and 380 nm and emission wavelength of 500 nm. The calibration of the fluorescence signal in terms of [Ca²⁺]_i was performed according to Grynkiewicz *et al.* (1985).

Measurement of InsP₃ production

InsP₃ production was determined by competition assay with [³H]-InsP₃ as described previously (Lee *et al.*, 1997). To determine InsP₃ production, 1 × 10⁶ cells were stimulated with drugs. The reaction was terminated by addition of ice-cold 5% trichloroacetic acid containing 10 mM EGTA. The supernatant of the cell lysate was saved and the trichloroacetic acid was removed by extraction with diethyl ether. The aqueous fraction was neutralized with 200 mM Trizma base and adjusted to pH 7.4. 20 µl of extract was added to 20 µl of assay buffer (100 µM Tris buffer containing 4 mM EDTA) and 20 µl [³H]-InsP₃ (0.1 µCi ml⁻¹). The mixture was incubated for 15 min on ice and then centrifuged at 2,000 × *g* for 10 min. One hundred µl water and 1 ml liquid scintillation cocktail were added to the pellet to measure the radioactivity. The InsP₃ concentration in the sample was determined by comparison to a standard curve and expressed as pmol mg⁻¹ of protein. The total cellular protein concentration was measured by the Bradford method (Bradford, 1976) after sonication of 1 × 10⁶ cells.

Measurement of cyclic AMP

Intracellular cyclic AMP was determined by measuring the formation of [³H]-cyclic AMP from [³H]-adenine nucleotide pools as previously described (Choi & Kim, 1997). Briefly, cells were loaded with [³H]-adenine (1 µCi ml⁻¹) in complete

medium for 24 h. After the loading, the cells were washed two times with Locke's solution (in mM: NaCl 154, KCl 5.6, MgCl₂ 1.2, CaCl₂ 2.2, HEPES 5, and glucose 10, pH 7.3). 1 × 10⁶ cells were aliquoted into Eppendorf tubes for stimulation. The stimulation reaction was stopped by addition of 1 ml ice-cold 5% (v/v) trichloroacetic acid containing 1 µM unlabelled cyclic AMP. [³H]-cyclic AMP and [³H]-ATP were separated using sequential chromatography on Dowex AG50W-X4 (200–400 mesh) cation exchanger and a neutral alumina column. The increase in intracellular cyclic AMP concentration was presented as [³H]-cyclic AMP/([³H]-ATP + [³H]-cyclic AMP) × 10³.

RNA isolation and RT-PCR

Total cellular RNA was prepared from undifferentiated and DMSO-induced differentiated HL-60 cells by the method of Chomczynski & Sacchi (1987). Poly(A)⁺-RNA was isolated from total RNA by oligo(dT)-cellulose column chromatography (Boehringer Mannheim). One µg of poly(A)⁺ mRNA was used as template for the first-strand cDNA synthesis using SUPERScript II reverse transcriptase (GIBCO-BRL) in a final volume of 40 µl supplemented with 50 ng of random hexamer and 500 ng of oligo(dT)12-18. Three µl of the reverse transcriptase reaction mixture was amplified with 20 pmol of subtype specific oligonucleotide primers (human NTR1: 1090-1732, 25mer; 5-aaCACCTTCATGTCCTTCATATTCC; 3-gcggatccTCTGCCTGACCCCCATC; Vita *et al.*, 1993) using Pfu polymerase. The cycle profile was as follows: 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 35 cycles, and finally, a 10-min extension at 72°C. The PCR products and 1 Kb ladder (GIBCO-BRL) were electrophoresed in a 1.5% agarose gel and visualized using ethidium bromide. The DNA fragments were inserted into the *Hinc*II site of the pBluescript II SK(+) vector and sequenced to confirm the authenticity of the neurotensin receptor gene.

Northern blot analysis

Poly (A)⁺-RNA (10 µg per lane) was resolved by electrophoresis through 1% agarose gel containing 660 mM formaldehyde and transferred to a nylon membrane (ICN, East Hills, NY). The blot was hybridized with each human neurotensin receptor cDNA probe (listed above) labelled by random primer extension with [α -³²P]dCTP and Klenow enzyme. The hybridization was performed at 65°C in a solution containing 10% polyethylene glycol, 7% SDS, EDTA 10 mM, NaCl 250 mM, Na₂HPO₄ 85 mM (pH 7.2), denatured salmon sperm DNA (100 µg ml⁻¹) and the radiolabelled probes (5 × 10⁵ c.p.m. ml⁻¹). After hybridization, the blot was washed twice in 1 × saline-sodium citrate (SSC; 300 mM NaCl and 30 mM sodium citrate) solution containing 0.1% SDS at room temperature, three times in 0.2 × SSC with 0.1% SDS at 65°C, twice in 0.1 × SSC at room temperature. The membrane was later reprobbed with the cDNA of the human ribosomal large subunit protein 32 (a gift from Dr H.S. Shin) as a control.

Materials

RPMI 1640 and penicillin-streptomycin were purchased from GIBCO (Grand Island, NY, U.S.A.). Bovine calf serum was obtained from HyClone Laboratories (Logan, UT, U.S.A.). Neurotensin, neurotensin fragments 1–8, 1–11 and 8–13, neuromedin N, xenopsin, cyclic AMP, EGTA, trichloroacetic acid, isobutyl methyl xanthine (IBMX), fMLP, DMSO, and sulphinpyrazone were purchased from Sigma Chemical Co.

(St. Louis, MO, U.S.A.). Ionomycin and PMA were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). [^3H]-adenine and [^3H]-InsP₃ were purchased from NEN (Boston, MA, U.S.A.). Fura-2/AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). Levocabastine (Schotte *et al.*, 1986) was a gift from Dr Marcel Janssen (Janssen Research Foundation) and SR48692 (Gully *et al.*, 1993) was a gift from Dr Danielle Gully (Sanofi Recherche).

Analysis of data

All quantitative data are expressed as the means $\pm$ s.e.mean. The results were analysed for differences using unpaired Student's *t*-test. We calculated half maximal effective concentration (EC₅₀) and half maximal inhibitory concentration (IC₅₀) with the AllFit program (De Lean *et al.*, 1978).

Results

We examined the neurotensin-induced responses in HL-60 cells. Neurotensin (300 nM) triggered the [Ca^{2+}]_i rise in the presence or absence of extracellular Ca^{2+} in fura-2-loaded undifferentiated cells (Figure 1A). Neurotensin elevated the

Figure 1 Neurotensin-induced increase in cytosolic Ca^{2+} in undifferentiated HL-60 cells. (A) Fura-2/AM-loaded HL-60 cells were challenged with 300 nM neurotensin with (left) or without (right) 2.2 mM extracellular Ca^{2+} . The level of cytosolic Ca^{2+} was measured as described in Methods. A typical trace from more than 20 separate experiments is shown. (B) Fura-2/AM-loaded HL-60 cells were treated with the indicated concentrations of neurotensin. The peak height of each stimulation was monitored. Each result is the means $\pm$ s.e.mean from triplicate assays. The result is representative of five separate experiments. The results were reproducible.

[Ca^{2+}]_i in a concentration-dependent manner at maximal and half-maximal effective concentrations (EC₅₀) of 300 and 38.5 ± 3.0 nM, respectively (Figure 1B). We tested the production of InsP₃ upon neurotensin treatment, because neurotensin elicited [Ca^{2+}]_i rise in the absence of extracellular Ca^{2+} . Figure 2 shows that treatment with 300 nM neurotensin evoked production of InsP₃ with the maximum effect seen 15 s after treatment.

To compare the potency of neurotensin analogues in elevating [Ca^{2+}]_i, we used several neurotensin peptide fragments and related peptide hormones. As shown in Figure 3, neurotensin fragment 8-13, neuromedin N, and xenopsin increased the [Ca^{2+}]_i with an EC₅₀ of 9.9 ± 3.2 nM, 40.2 ± 8.3 nM and 37.5 ± 4.8 nM, respectively. However, neurotensin fragments 1-8 and 1-11 did not elicit an increase in [Ca^{2+}]_i at concentrations up to 10 μM . We further tested whether the neurotensin response overlapped the responses elicited by other peptide hormones. Among the tested peptide hormones bradykinin, substance P, angiotensin II, somatostatin, galanin, vasoactive intestinal peptide (VIP), cholecystokinin, and endothelin did not change the [Ca^{2+}]_i, but thrombin did increase the [Ca^{2+}]_i in HL-60 cells (Table 1). However, thrombin pretreatment did not block a subsequent response to neurotensin. In addition, 300 μM extracellular ATP, 300 μM histamine, 3 μM platelet-activating factor, and 100 nM small peptide WKYMVm (Seo *et al.*, 1997), which are all known to elicit [Ca^{2+}]_i rise in HL-60 cells, also did not overlap with neurotensin in its [Ca^{2+}]_i elevating effect (data not shown). The results suggest that neurotensin has its own receptor on HL-60 cells and elevates [Ca^{2+}]_i through activation of PLC.

To identify the neurotensin receptor subtype expressed on HL-60 cells, subtype-specific antagonists were tested. It is generally accepted that SR48692 (Gully *et al.*, 1993) and levocabastine (Schotte *et al.*, 1986) block the neurotensin receptors NTR1 and NTR2, respectively. SR48692 inhibited the neurotensin-induced [Ca^{2+}]_i increase in a concentration-

Figure 2 Neurotensin-induced InsP₃ generation in undifferentiated HL-60 cells. Cells were stimulated with 300 nM neurotensin for the indicated times. The levels of InsP₃ produced were measured as described in Methods. Each point is the means $\pm$ s.e.mean of triplicate measurements. The result is representative of six separate experiments and were reproducible.

dependent manner with an IC_{50} of 14.0 ± 0.2 nM (Figure 4). However, levocabastine did not inhibit the neurotensin-induced effect even at millimolar concentrations. The results suggest that HL-60 cells express NTR1 neurotensin receptors.

It has been reported that neurotensin receptors are also coupled to adenylyl cyclase (Yamada *et al.*, 1994). So we tested whether neurotensin regulates cyclic AMP signalling in HL-60 cells. 300 nM neurotensin did not have any effect on cyclic AMP production, whereas prostaglandin E_2 did increase the cyclic AMP level (Figure 5A). In addition, simultaneous treatment or pretreatment with neurotensin did not affect the prostaglandin E_2 -induced cyclic AMP formation (Figure 5B).

Figure 3 Concentration dependent increases in cytosolic Ca^{2+} by neurotensin and its analogues. Undifferentiated HL-60 cells were treated with various concentrations of neurotensin, neurotensin 8–13 fragments, 1–11 fragments, 1–8 fragments, neuromedin N, and xenopsin. The peak height of each stimulation was monitored. Each result is the means $\pm$ s.e. mean from triplicate assays. The result is representative of four separate experiments. The results were reproducible.

Table 1 Cytosolic Ca^{2+} -mobilizing activity of major peptide agonists in HL-60 cells

Peptide (concentration)	Changes in $[Ca^{2+}]_i$	Cross-reactivity with neurotensin (300 nM)
Bradykinin (10 μ M)	ND	ND
Substance P (10 μ M)	ND	ND
fMLP (1 μ M)	ND*	ND
Thrombin (50 U ml $^{-1}$)	Increase	ND
Angiotensin II (10 μ M)	ND	ND
Somatostatin (1 μ M)	ND	ND
Galanin (10 nM)	ND	ND
VIP (100 nM)	ND	ND
Cholecystokinin (300 nM)	ND	ND
Endothelin (20 μ M)	ND	ND

Fura-2/AM-loaded undifferentiated HL-60 cells were challenged with the indicated concentrations of peptide agonist. The experiments were performed more than four times independently and the results were reproducible. VIP, vasointestinal peptide; ND, not detected. *Increase in $[Ca^{2+}]_i$ was detected in the DMSO-triggered differentiated HL-60 cells.

We further analysed changes in the neurotensin-induced effect during DMSO-induced differentiation. HL-60 cells had morphologically and physiologically differentiated into neutrophil-like cells after incubation with 1.25% DMSO for 6 days (so called granulocytic differentiation; Collins *et al.*,

Figure 4 The effects of SR48692 and levocabastine on neurotensin-induced $[Ca^{2+}]_i$ rise in HL-60 cells. SR48692 or levocabastine were added to fura-2-loaded cells at the indicated concentrations. After 3 min the cells were challenged with 300 nM neurotensin. The peak heights of neurotensin-induced $[Ca^{2+}]_i$ rise were monitored. The experiments were performed three times independently, and the results were reproducible. Each result is the means $\pm$ s.e. mean from triplicate assays.

Figure 5 Lack of neurotensin-induced changes in cyclic AMP level in HL-60 cells. (A) $[^3H]$ -adenine-loaded cells were preincubated with 1 mM IBMX for 15 min, then stimulated for 15 min with 300 nM neurotensin (NT) or 10 μ M prostaglandin E_2 (PGE2) in the presence of 1 mM IBMX. (B) Cells were preincubated with or without 300 nM neurotensin, then challenged with 10 μ M prostaglandin E_2 in the presence of 1 mM IBMX. The levels of cyclic AMP produced were measured as described in Methods. We expressed the cyclic AMP level as cyclic AMP/(cyclic AMP + ATP) $\times$ 1000. Each point is the means $\pm$ s.e. mean of triplicate measurements. The result is representative of six separate experiments. The results were reproducible.

Figure 6 Changes in neurotensin-induced $[Ca^{2+}]_i$ increase in HL-60 cells during DMSO-induced differentiation. Undifferentiated HL-60 cells were cultured with 1.25% DMSO containing medium for the indicated times, then 300 nM neurotensin-induced $[Ca^{2+}]_i$ rise was examined. The peak height of the $[Ca^{2+}]_i$ increase was recorded. The experiments were performed three times independently, and the results were reproducible. Each result is the means $\pm$ s.e. mean of triplicate assays.

1978). In the neutrophil-like HL-60 cells a neurotensin-induced $[Ca^{2+}]_i$ increase was not detected. During the time course of the DMSO incubation the extent of the neurotensin-induced $[Ca^{2+}]_i$ increase became gradually smaller and disappeared completely after 4 days of incubation with DMSO (Figure 6).

To confirm the expression and down-regulation of the neurotensin receptor in differentiated cells, PCR with specific probes (Vita *et al.*, 1993) after a reverse transcriptase reaction with purified mRNA of HL-60 cells and Northern blot analysis were carried out. A specific DNA band was obtained, and the DNA fragment was confirmed by the digestion patterns obtained after reactions with *HinfI* (358 and 284 bp) and *XhoI* (467 and 175 bp) and by direct sequencing. The results clearly indicate that HL-60 cells express NTR1 neurotensin receptors (Figure 7A). The Northern blot analysis showed that the amount of neurotensin receptor mRNA in 6-day differentiated cells dramatically decreased in comparison to untreated control cells (Figure 7B). The result suggests that the signalling of neurotensin is down-regulated *via* the reduction of receptor expression during granulocytic differentiation.

Furthermore, we tested the neurotensin signalling in hemopoietic cell lineages of various types of human lymphocytes or cell lines. As shown in Table 2, we could detect a neurotensin-evoked $[Ca^{2+}]_i$ rise in human histiocytic leukaemia U937 cells which are closely related to HL-60 cells in their differentiation phylogeny. However, there was no detectable Ca^{2+} mobilization in isolated human neutrophils which are the final form of granulocytic differentiation. In addition, human myelogenous leukaemia K562 cells, human T cell leukaemia Jurkat T cells and H9 cells, and human B lymphoma U266 cells, which are all of a distinct differentiation lineage, did not exhibit response to neurotensin treatment.

Figure 7 RT-PCR and Northern blot analysis of the expression of neurotensin receptors in undifferentiated and DMSO-induced differentiated HL-60 cells. (A) RNA extracted from undifferentiated HL-60 cells were reverse-transcribed into cDNA. Then amplification was performed using NTR1 neurotensin receptor specific primers and Pfu polymerase as described in Methods. Lane 1, 1 kb ladder; lane 2, PCR product (642 bp); lane 3, PCR product digested with *HinfI* (358 and 284 bp); lane 4, PCR product digested with *XhoI* (467 and 175 bp). (B) Poly(A)⁺-RNA (10 μ g per lane) from control (lane C) and DMSO-induced differentiated HL-60 cells (lane D) was electrophoresed, transferred to a membrane, and hybridized with the human NTR1 neurotensin receptor cDNA (NTR1) for Northern blot analysis. The position of the 28S ribosomal RNA is indicated by an arrowhead. The human ribosomal large subunit protein 32 (RPL32) was used as a marker for RNA level.

Table 2 The neurotensin-triggered Ca^{2+} responses of cells in hemopoietic lineage

Cells in hemopoietic lineage	Changes in $[Ca^{2+}]_i$
HL-60 (human promyelocytic leukaemia cells)	Increase
U937 (human histiocytic leukaemia cells)	Increase
Human neutrophils	ND
K562 (human myelogenous leukaemia cells)	ND
Jurkat T (human T cell leukaemia cells)	ND
H9 (human T cell leukaemia cells)	ND
U266 (human B lymphoma cells)	ND

Fura-2/AM-loaded cells were challenged with 300 nM neurotensin, and changes in $[Ca^{2+}]_i$ were monitored. The experiments were performed more than three times independently and the results were reproducible. ND, not detected.

Discussion

Neurotensin is one of the important peptide hormones for both neuronal and non-neuronal systems that remain to be studied with regard to its mechanism of action. Here we demonstrate that human myeloid leukaemia cells, HL-60, express NTR1 neurotensin receptors. This is the first evidence that the NTR1 neurotensin receptor is expressed in hemopoietic cells.

At the present time, neurotensin receptors are believed to exist in three types: a high affinity brain-type neurotensin receptor (NTRH or NTR1), a low affinity brain-type receptor (NTRL or NTR2), and a low affinity mastocyte or macrophage neurotensin binding site. Since the NTR1 neurotensin receptor had been purified from rat brain (Mazella *et al.*, 1985), it was cloned from rat brain tissue (Tanaka *et al.*, 1990) and human colon adenocarcinoma HT-29 cells (Vita *et*

al., 1993). Mazella *et al.* (1988) purified two distinct neurotensin-binding sites from an adult mouse brain homogenate, each of which differed in binding affinity and sensitivity to levocabastine. Based on the above results, NTR2 was then cloned from mouse brain (Mazella *et al.*, 1996) and rat brain (Chalon *et al.*, 1996). In addition, a third type of receptor was also identified as a low affinity binding site on mastocytes/macrophages; not only neurotensin but also bradykinin or substance P could bind to this receptor (Lazarus *et al.*, 1977; Goldman & Bar-Shavit, 1983). It remains unclear whether this low affinity binding site's function is solely directed at neurotensin exactly because of its very low binding affinity for neurotensin and its cross-reactivity with bradykinin and substance P.

Schaeffer *et al.* (1995) have suggested major characteristics for the NTR1 neurotensin receptors: (i) a more potent effect by neurotensin fragments 8–13 than by neurotensin, (ii) lack of inhibition by levocabastine, (iii) lack of cross-binding other peptide hormones such as bradykinin and substance P and (iv) mRNA detection with specific NTR1 neurotensin receptor probes. Our results meet the above criteria for the expression of NTR1 neurotensin receptors in HL-60 cells. Neurotensin produced InsP_3 and increased $[\text{Ca}^{2+}]_i$ at nanomolar concentrations in HL-60 cells (Figure 1). Neurotensin fragments 8–13 was more potent in elevating $[\text{Ca}^{2+}]_i$ than neurotensin (Figure 2). Levocabastine did not inhibit the neurotensin-evoked response, but NTR1 receptor antagonist SR48692 completely blocked it (Figure 3). The receptor did not cross-react with bradykinin and substance P (Table 1). Finally, the expression of the NTR1 receptor was detected by PCR and confirmed by sequencing (Figure 5).

Though the neurotensin receptor in HL-60 cells is clearly distinguishable from the mastocyte/macrophage neurotensin binding sites, it is of value to exclude the possibility that neurotensin may act on other receptors. HL-60 cells have several receptors which are coupled to Ca^{2+} mobilization such as receptors for extracellular ATP, histamine, platelet-activating factor, and WKYMVm. However, neurotensin independently elicited $[\text{Ca}^{2+}]_i$ rise when cells were sequentially treated with the above stimulants (data not shown).

It has been suggested that neurotensin performs its role not only by elevating $[\text{Ca}^{2+}]_i$ but also by regulating cyclic AMP levels. The neurotensin-induced increase in cyclic AMP and the subsequent activation of protein kinase A inhibit the dopaminergic signalling in the midbrain (Shi & Bunney, 1992b). In CHO-K1 cells expressing rat NTR1 neurotensin receptors, neurotensin increased the intracellular cyclic AMP level (Yamada *et al.*, 1994). In N1E115 cells, neurotensin

decreased the basal and the G-protein-mediated elevated cyclic AMP level (Bozou *et al.*, 1986). However, neurotensin-induced changes in the cyclic AMP level were not detected in HL-60 cells (Figure 4). The result suggests that neurotensin may use different signalling pathways in different cells and tissues.

In addition, HL-60 cells are of hemopoietic lineage and can be triggered to differentiate into neutrophils or macrophages. It has been reported that the neurotensin receptors were undetectable in undifferentiated primary cultured neurons but did increase during differentiation (Chabry *et al.*, 1990). Because the level of expression of the receptor is closely linked to the size of the physiological effect, it was valuable for us to test the expression of the neurotensin receptor in HL-60 cells during differentiation to neutrophils. We show here clearly that the signalling of neurotensin decreases during the DMSO-induced granulocytic differentiation. The down-regulation of the Ca^{2+} signalling correlated with a gradual decrease in the expression of neurotensin receptor mRNA. In contrast, the fMLP-induced $[\text{Ca}^{2+}]_i$ elevation increased during the differentiation process (data not shown). Whenever there is a bacterial infection, the fMLP receptor is activated evoking superoxide production in granulocytes and macrophages. It has been generally accepted that PLC-coupled receptors are involved in defence mechanisms of the immune system such as chemotaxis, phagocytosis, and secretion of cytokines. However, it is still unclear what the physiological function of the down-regulation of the neurotensin receptor during differentiation may be. Neurotensin presumably plays a critical role in the early stages of hemopoietic differentiation and loses its physiological effect after differentiation. Cytokines including IL-3, granulocyte/macrophage-CSF, and macrophage-CSF, and granulocyte-CSF, play important roles in the early stages of differentiation from bone marrow cells. It is generally assumed that Ca^{2+} signalling in bone marrow cells regulates the effect of the cytokines described above (Cook *et al.*, 1989). Moore *et al.* (1989) reported that neurotensin regulates the macrophage colony stimulating factor-induced myelopoiesis. Thus, it is possible to propose the interesting hypothesis that neurotensin modulates other differentiation signals such as are sent by cytokines or growth factors during differentiation.

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