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Neurotensin Stimulates Inositol Phospholipid Metabolism and Calcium Mobilization in Murine Neuroblastoma Clone N1E-115

R. M. Snider, *C. Forray, *M. Pfenning, and *E. Richelson

*Neuroscience Laboratory/Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan; and *Departments of Psychiatry and Pharmacology, Mayo Clinic and Foundation, Rochester, Minnesota, U.S.A.*

Abstract: Murine neuroblastoma cells (clone N1E-115) possess neurotensin receptors that mediate cyclic GMP synthesis. Because of the hypothesized relationship between phospholipid metabolism, intracellular Ca^{2+}, and cyclic GMP synthesis, we determined with these cells the effects of neurotensin on ^{32}P labeling of phospholipids, release of inositol phosphates, and intracellular Ca^{2+} (as determined with the use of Quin-2, a fluorescent probe sensitive to free Ca^{2+} levels). Neurotensin stimulated incorporation of ^{32}P into phospholipids, especially phosphatidylinositol and phosphatidate. Neurotensin also stimulated the release of [^{3}H]-inositol phosphates with an EC_{50} of about 1 n*M*. Mean basal Ca^{2+} concentration in these cells was 134 n*M* and this level was increased in a rapid and dose-dependent manner by neurotensin, with an EC_{50} of 4 n*M*. Since the EC_{50} for neurotensin in stimulating cyclic GMP synthesis is 1.5 n*M* and the K_D for binding of [^{3}H]neurotensin at 0°C is 11 n*M*, all these different effects appear to be shared proximal consequences of neurotensin receptor activation. **Key Words:** Neurotensin—Inositol phosphate—Cyclic GMP—N1E-115. **Snider R. M. et al.** Neurotensin stimulates inositol phospholipid metabolism and calcium mobilization in murine neuroblastoma clone N1E-115. *J. Neurochem.* **47,** 1214–1218 (1986).

Neurotensin is a basic tridecapeptide (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) that is localized in a wide range of CNS and peripheral tissues and has been shown to be implicated in a variety of physiological processes (Nemeroff and Prange, 1982; Goedert, 1984). Neurotensin-like immunoreactivity has been demonstrated in nerve terminals (see Uhl, 1982; Kahn et al., 1982) and neurotensin receptors have been localized in regions of the CNS (Uhl, 1982; Kanba et al., 1986). Neurotensin was recently shown to stimulate cyclic GMP formation (Gilbert et al., 1984) and inositol phospholipid hydrolysis (Goedert et al., 1984) in rat brain, confirming the existence of functional receptors for this peptide in the mammalian CNS.

Neuroblastoma clone N1E-115 is a widely studied neuronal clone that possesses a neurotensin receptor that mediates the stimulation of intracellular cyclic GMP synthesis (Gilbert and Richelson, 1984). Data from binding studies with radiolabeled neurotensin correlate well with measurements of cyclic GMP response for neurotensin as well as for several structural analogs of this peptide (Gilbert et al., 1986). Since it has been reported that many of the receptors that stimulate cyclic GMP formation also are effective in the activation of inositol phospholipid turnover (Berridge, 1981), we investigated the effect of neurotensin on inositol phospholipid metabolism and on Ca^{2+} mobilization (as measured with Quin-2) in N1E-115 cells.

MATERIALS AND METHODS

Cell culture

Murine neuroblastoma cells (clone N1E-115) were cultured in medium I consisting of Dulbecco's modified Eagle's

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Address correspondence and reprint requests to Dr. E. Richelson at Mayo Clinic/Foundation, Guggenheim 7, Rochester, MN 55905, U.S.A.
The present address of Dr. C. Forray is Instituto Mexicano de Psiquiatria, Mexico City, Mexico.

Abbreviations used: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PA, phosphatidate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

medium (GIBCO, Grand Island, NY, U.S.A.) without antibiotics and supplemented with 10% (vol/vol) fetal bovine serum (GIBCO). Cells (subculture numbers 9–16) were grown for 10–21 days in 20 ml of medium I in 75-cm^2/250-ml tissue culture flasks (Corning Glass Works, Corning, NY, U.S.A.) in an atmosphere of 10% CO_2/90% humidified air at 37°C. Subculture was achieved by aspirating medium I, and detaching the cells by incubation in modified Puck's D_1 solution followed by centrifugation of the cell suspension for 2 min at 300 *g*. The supernatant was removed and the cells resuspended in 10 ml of medium I which was then inoculated into flasks to give 4–8 × 10^5 cells/flask on day 0. The culture medium was changed on days 4 and 6 and daily thereafter by addition of 10 ml of fresh medium I and removal of 10 ml of medium I.

Cell labeling with ^{32}P and lipid extraction

Cells were isolated from tissue culture flasks in modified Puck's D_1 solution as described above. The cells were pelleted by centrifugation (300 *g*, 2 min), the supernatant aspirated, and the cell pellet washed twice in phosphate-free physiological saline solution (solution I) of the following composition: 142 m*M* NaCl, 5.6 m*M* KCl, 1.0 m*M* $MgCl_2$, 2.2 m*M* $CaCl_2$, 3.6 m*M* $NaHCO_3$, 30 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 11.2 m*M* glucose. Solution I was adjusted before use to pH 7.4 and 340 mOsmol. On resuspension in solution I, viable cells were enumerated by trypan blue dye exclusion. The cells were then diluted in solution I to about 800,000 cells/ml and distributed in 350-μl portions into 10 × 75 mm plastic test tubes containing [^{32}P]orthophosphate (carrier-free, Amersham, Arlington Heights, IL, U.S.A.) and neurotensin (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.) in a final reaction volume of 500 μl at 37°C. The reactions were terminated after an incubation of 30 min with agonist by transferring the contents of each tube to a 16 × 125 mm glass test tube containing 1.5 ml of chloroform/methanol (1:2, vol/vol). The tubes were stored on ice for 30–60 min, after which time 0.5 ml of 2.4 *M* HCl and 1.0 ml of chloroform were added. The tubes were vigorously vortex-mixed for 20–30 s and the phases separated by centrifugation. The chloroform layer was removed with a Pasteur pipet, and the aqueous layer and cell debris were extracted with an additional 1.0 ml of chloroform. After centrifugation, the chloroform layers were combined and washed with 2.25 ml of water/methanol/2.4 *M* HCl (1:1:0.25). The tubes were vortex-mixed and centrifuged, and the aqueous (upper) layer was aspirated and discarded. The volume of the chloroform layer was measured and a portion (50–200 μl) was counted to estimate recovery following TLC. The remaining chloroform layer was evaporated under a stream of nitrogen and mild heating. The residue was redissolved in 80 μl of chloroform/methanol (2:1) and spotted onto TLC plates (Merck, Darmstadt; silica gel 60, 20 × 20 × 0.025 cm) that had previously been run in a solution containing 1.2 g potassium oxalate in 40 ml of methanol and 60 ml of water, then activated immediately before use by heating for 15 min at 110°C. Separation of the phospholipids was achieved by developing the plates in one dimension for 1–1.5 h in chloroform/acetone/methanol/glacial acetic acid/water (40:15:13:12:7). After TLC, the labeled phospholipids were localized by autoradiography with the use of Kodak XRP-5 film (exposure time approximately 16 h). For quantitation, the areas on the TLC plates corresponding to lipids of interest were scraped and counted for radioactivity. The data for phosphatidate (PA), phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2) were corrected for recovery of total lipids before analysis.

Assay for the release of [^3H]inositol phosphates

Confluent cells in one flask (250-ml/75-cm^2) were collected as described above, washed, and suspended in 2 ml of solution II, consisting of 110 m*M* NaCl, 5.3 m*M* KCl, 1.8 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 2.0 m*M* Na_2HPO_4, 25 m*M* glucose, and 70 m*M* sucrose (pH 7.35 and 340 mOsmol) to which was added 50 μCi *myo*-[^3H]inositol (15 Ci/mmol; American Radiolabelled Chemicals, St. Louis, MO, U.S.A.). This suspension was then incubated for 60 min at 37°C in a shaking water bath. At the end of this incubation period, cells were centrifuged and the supernatant was aspirated to remove excess radiolabel. The cells were then suspended in solution II to give about 6 × 10^5 cells/ml. Aliquots of 210 μl of this suspension were added to glass tubes (13 × 100 mm) containing 30 μl of 100 m*M* LiCl and incubated at 37°C in a shaking water bath. After a 30-min equilibration period, neurotensin in 60 μl of solution II was added and after 10 min, the reaction was stopped with the addition of 750 μl of chloroform/methanol (1:2). Each tube was then vortex-mixed and placed on ice. The phases were formed by the addition of 250 μl of chloroform and 250 μl of an aqueous solution containing L-*myo*-[^{14}C]inositol 1-phosphate (about 0.7 nCi, 55 mCi/mmol; Amersham) as an internal standard. After further vortex-mixing, the tubes were centrifuged at about 400 *g* for 5 min. A portion (600 μl) of the upper phase was transferred to a polystyrene (12 × 75 mm) tube that contained 2 ml of water. This solution was then applied to an AG 1-X8, 100–200 mesh, column (0.6 × 1.0 cm; converted to the formate form; Bio-Rad Laboratories, Richmond, CA, U.S.A.) and the inositol phosphates were eluted as described by Berridge et al. (1983). Thus, to this column was added 20 ml of 60 m*M* ammonium formate/5 m*M* sodium tetraborate, and the eluate was discarded. Total [^3H]inositol phosphates were then eluted with 4 ml of 1 *M* ammonium formate/100 m*M* formic acid. The eluate was combined with 16 ml of Safety Solve (RPI, Mount Prospect, IL, U.S.A.) and the radioactivity was determined by liquid scintillation spectrometry. Data were analyzed by computer and corrected for recovery of [^{14}C]inositol 1-phosphate which was about 50%.

Quin-2 loading and measurement of $[Ca^{2+}]_i$

Cells were isolated as described above, resuspended in solution I (1 × 10^6 cells/ml; total volume 7–8 ml), and incubated with Quin-2 acetoxymethyl ester (Calbiochem-Behring; 50 μ*M*) for 30 min at 37°C in a shaking water bath. The cells were then diluted threefold and allowed to incubate 15–30 min longer at 37°C. After gentle centrifugation (300 *g*, 2 min), the supernatant was removed, and the cells were resuspended in fresh solution I (1 × 10^6 cells/ml) and kept at room temperature. Immediately before use, a 1-ml portion of the cellular suspension was centrifuged, the supernatant was removed, and the pellet was resuspended in fresh solution I to remove any extracellular Quin-2. Fluorescence measurements were made at 37°C with constant stirring in an Aminco-Bowman Spectrophotofluorometer (American Instruments, Silver Springs, MD, U.S.A.); excitation 339, emission 490 nm. Quantitation of calcium concentrations in intact cells was carried out as described by Tsien et al. (1982).

RESULTS

Stimulation of [^{32}P]inositol lipid labeling by neurotensin

When neuroblastoma cells were incubated under conditions in which neurotensin was added at the beginning of the incubation period together with ^{32}P (carrier-free), there was a substantial increase in the amount of ^{32}P localized in PI and PA (Table 1). Relatively minor changes were observed in PIP and PIP_2 (Table 1) whereas no alterations in the labeling of the quantitatively major phospholipids (phosphatidylethanolamine, -choline, and -serine) were found (data not shown).

Stimulation of the release of [^{3}H]inositol phosphates by neurotensin

The above results indirectly indicated that neurotensin stimulated the cleavage of inositol phosphates from phospholipids of N1E-115 cells. To obtain direct evidence for this mechanism, we tested the capacity of neurotensin to stimulate the release of [^{3}H]inositol phosphates from cells that had been prelabeled with *myo*-[^{3}H]inositol. Neurotensin rapidly stimulated this release, with peak responses occurring by about 2 min (Fig. 1). Dose–response curves for neurotensin and release of [^{3}H]inositol phosphates (Fig. 2) yielded an $EC_{50} \pm$ SEM = 0.9 ± 0.6 n*M* (n = 4) for this peptide. Neurotensin at a maximal concentration (10^{-7} *M*) increased release of [^{3}H]inositol phosphates over basal in the range of 50% at an incubation time with agonist of 10 min.

Effects of neurotensin on [Ca^{2+}]$_i$

Receptors that stimulate the turnover of inositol phospholipids also increase the intracellular concentration of Ca^{2+}. With the use of Quin-2, a calcium-sensitive fluorescent probe (Tsien et al., 1982), we found that neurotensin caused an increase in intracellular Ca^{2+} (Figs. 3 and 4). Mean basal [Ca^{2+}]$_i$ ± SEM was 134 ± 7 n*M* (n = 17) and the EC_{50} for neurotensin determined from a dose–response experiment (Fig. 4) was about 4 n*M*. Repeated addition of 10 n*M* or lower concentration elicited a reproducible elevation in [Ca^{2+}]$_i$ (Fig. 3). A 10-fold higher concentration of the peptide caused a rapid desensitization of the neurotensin receptor.

TABLE 1. *Neurotensin-stimulated changes in ^{32}P radioactivity in phospholipids*

	^{32}P Radioactivity[a]	
	Control	Neurotensin (10^{-7} *M*)
PIP_2	22,000 ± 700	26,000 ± 3,000
PIP	6,600 ± 200	8,000 ± 800
PI	6,000 ± 600	14,500 ± 1,000[b]
PA	6,800 ± 300	13,700 ± 600[b]

[a] De novo phospholipid labeling was carried out for 30 min as described in Materials and Methods. The radioactivity in the lipids shown accounts for 80–85% of the total radioactivity in the lipid extract. Values given are the $\bar{x}$ ± SEM of four separate experiments.
[b] $p < 0.01$ compared to control.

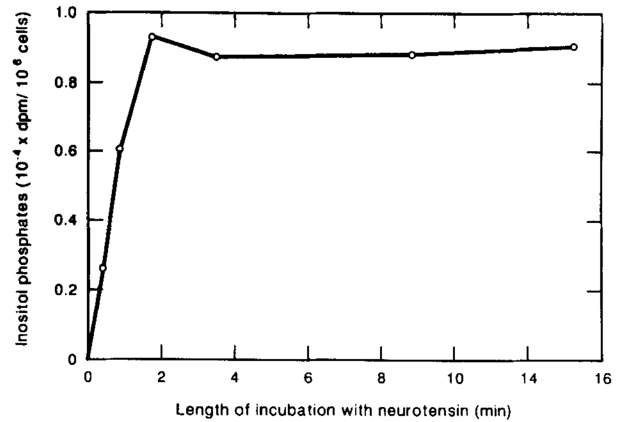

FIG. 1. Time course for neurotensin-stimulated release of [^{3}H]-inositol phosphates. Neurotensin (10 n*M*) was incubated with cells (about 100,000/tube) prelabeled with *myo*-[^{3}H]inositol as described in Materials and Methods for the indicated times and release of [^{3}H]inositol phosphates was measured. Unstimulated levels of release of [^{3}H]inositol phosphates at each time point (around 15,000 dpm/10^6 cells) have been subtracted. Data are from one of two independent time-course experiments, each point of which was determined in triplicate.

DISCUSSION

These data show that neurotensin, presumably by activating its receptors on clone N1E-115, stimulated inositol phospholipid metabolism and increased [Ca^{2+}]$_i$ with similar dose–response profiles. Pre-

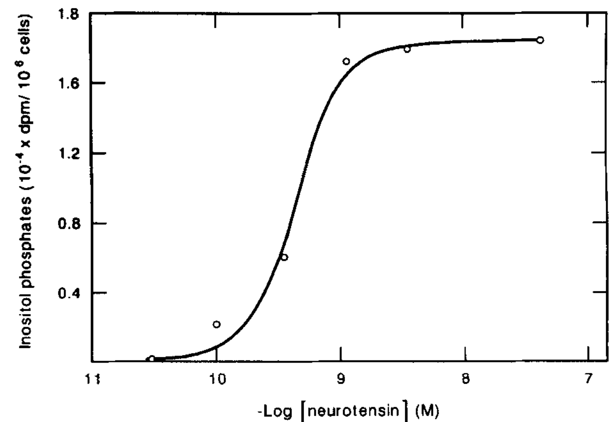

FIG. 2. Dose–response for neurotensin-stimulated release of [^{3}H]inositol phosphates from clone N1E-115 cells. Neurotensin at the indicated concentrations was incubated for 10 min with cells prelabeled with *myo*-[^{3}H]inositol as described in Materials and Methods and release of [^{3}H]inositol phosphates was measured. Release in the absence of neurotensin (40,000 dpm/10^6 cells) has been subtracted from each point. Data are representative results from one of four independent dose-response experiments, each experimental point determined in triplicate.

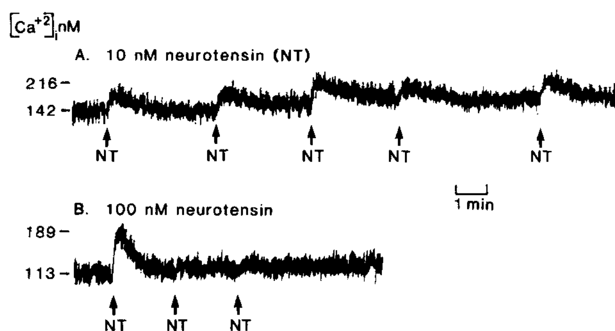

FIG. 3. Neurotensin-mediated increases in intracellular Ca^{2+} of N1E-115 cells loaded with Quin-2. Changes in the fluorescence intensity of Quin-2 were taken as a measure of the concentration of intracellular Ca^{2+} as described by Tsien et al. (1982).

viously, we showed that neurotensin acting on N1E-115 cells and on rat brain slices also mediates cyclic GMP synthesis (Gilbert and Richelson, 1984; Gilbert et al., 1984, 1986), the first known biochemical event associated with neurotensin receptors. In addition, Goedert et al. (1984) showed that neurotensin stimulates the release of inositol phosphate in rat brain slices. All these data support the hypothesis that this tridecapeptide serves as a neurotransmitter or neuromodulator in the nervous system.

As is the case for the effects of a diverse group of agents, the effects of neurotensin on its receptor appear to result in the breakdown of inositol lipids and mobilization of Ca^{2+} as early events (Berridge and Irvine, 1984). In N1E-115 cells neurotensin stimulated labeling of $^{32}P_i$ primarily into PA and PI (Table 1). These data suggest that, as in other tissues, breakdown of polyphosphoinositides precedes stimulated labeling of PI and PA, which labeling can be regarded as the restorative phase of a closed cycle that eventually resynthesizes PIP_2. Receptor-stimulated release of [^{3}H]inositol phosphates from prelabeled N1E-115 cells provided more direct evidence of the phosphodiesteratic cleavage of phosphoinositides.

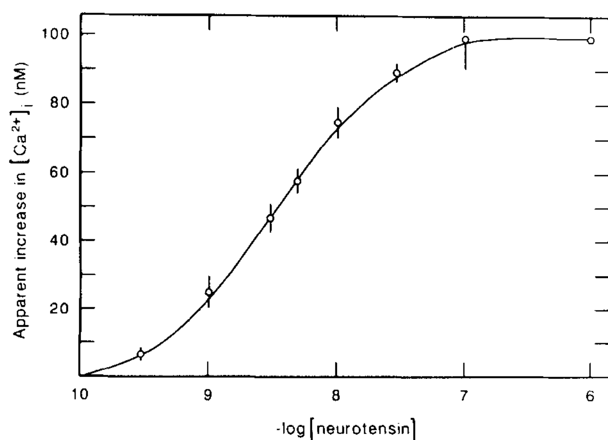

FIG. 4. Dose–response for neurotensin and the increase in intracellular Ca^{2+} of N1E-115 cells. Cells loaded with Quin-2 were incubated with the indicated concentrations of neurotensin and the increase in intracellular Ca^{2+} was calculated from the peak of the increases in fluorescence (Tsien et al., 1982). For each concentration, from three to six different groups of cells were tested. Means ± SEM are presented.

The dose–response relationships for neurotensin's stimulation of the release of [^{3}H]inositol phosphates and the increase in $[Ca^{2+}]_i$ were within the same range (Figs. 2 and 4). Since the K_D for [^{3}H]neurotensin and its receptors on these cells is 11 nM at 0°C (Gilbert et al., 1986) and the EC_{50} for stimulation of intracellular cyclic GMP formation is 1.5 nM, these events appear to be mediated by the same receptor site and may constitute early consequences of neurotensin receptor activation.

Of interest is the relationship between the release of inositol phosphates, the rise in intracellular Ca^{2+}, and the increased synthesis of cyclic GMP. It has long been hypothesized that increased intracellular Ca^{2+} stimulates guanylate cyclase, the enzyme that synthesizes cyclic GMP from GTP (Richelson and El-Fakahany, 1981). Some of the evidence in support of a direct role for Ca^{2+} in receptor-mediated cyclic GMP synthesis is that this response is absolutely dependent on the presence of extracellular Ca^{2+}, and guanylate cyclase in these cells is largely a soluble enzyme and is stimulated by this cation (Bartfai et al., 1978). In addition, our results showing that receptor-mediated inositol phosphate release in clone N1E-115 cells is also dependent on extracellular Ca^{2+} (Forray et al., manuscript in preparation) suggests a link between release of inositol phosphates, Ca^{2+} mobilization, and cyclic GMP synthesis.

However, previously we proposed (Snider et al., 1984) that neurotransmitters mediate cyclic GMP synthesis by stimulating the release of arachidonic acid from phospholipids (possibly by activating phospholipase A_2) followed by the metabolism of arachidonic acid by lipoxygenase(s) to a product that stimulates guanylate cyclase. Thus, for example, eicosatetraynoic acid (ETYA), a lipoxygenase inhibitor, blocks receptor-mediated cyclic GMP synthesis in clone N1E-115 cells (Snider et al., 1984), but has little effect on receptor-mediated release of inositol phosphates in these cells (McKinney and Richelson, 1986). Therefore, it is likely that release of inositol phosphates and the probable subsequent rise of $[Ca^{2+}]_i$ are not sufficient for receptor-mediated cyclic GMP synthesis.

In conclusion, our data show that the neurotensin-induced increases of [^{3}H]inositol phosphates, intracellular Ca^{2+}, and cyclic GMP all occurred within a similar time frame and with similar dose–response curves. However, we can draw no inference yet from these studies as to the sequential or causative relationships of these events.

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