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Neurotensin Regulates Growth of Human Pancreatic Cancer

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Objective

The effect of neurotensin (NT) on *in vitro* growth of human pancreatic cancer cells (MIA PaCa-2) was examined. Furthermore, the intracellular signal-transduction pathways by which neurotensin regulates growth of MIA PaCa-2 cells were determined.

Summary Background Data

NT is trophic for normal rat pancreas, but the effect of NT on growth of human pancreatic cancer is not known.

Methods

Effects of NT (10^{-12} to 10^{-6} mol/l) on growth of MIA PaCa-2 cells were determined by both count of cell numbers and 3H-thymidine incorporation. Action of NT on phosphatidylinositol (PI) hydrolysis, cyclic AMP production, and intracellular calcium level were determined by conventional methods. The effects of 8-bromo-cyclic AMP and prostaglandin E^2 on cell growth were determined.

Results

Low concentrations of NT (10^{-12} to 10^{-9} mol/l) stimulated growth in a dose-dependent manner, but higher concentrations of NT (10^{-8} to 10^{-6} mol/l) did not stimulate growth of MIA PaCa-2 cells. NT (10^{-12} to 10^{-6} mol/l) stimulated PI hydrolysis and increased intracellular calcium levels in a dose-dependent manner. High concentrations of NT (10^{-8} to 10^{-6} mol/l) stimulated production of cyclic AMP in a dose-dependent manner. 8-bromo-cyclic AMP inhibited growth of MIA PaCa-2 cells; prostaglandin E_2 did not affect growth of MIA PaCa-2 cells.

Conclusions

NT stimulates growth of MIA PaCa-2 cells through stimulation of PI hydrolysis and mobilization of calcium. Stimulation of the cyclic AMP pathway by high concentrations of NT abolishes the growth-stimulatory effect of NT that is mediated through PI hydrolysis or calcium mobilization.

Pancreatic cancer is the fourth leading cause of cancer death.[1] More than 25,000 new cases of pancreatic cancer are expected in the United States in the next year; most patients die within 6 months after diagnosis. Surgical resection is rarely useful; the average 5-year survival rate is less than 5%.[2] Systemically active therapy is required to improve survival of patients with pancreatic cancer. Understanding of the biology of the disease is essential to develop new strategies for treatment.

Neurotensin (NT), a tridecapeptide originally isolated from bovine hypothalami,[3] is found mainly in N-cells dispersed throughout the mucosa of the distal gut.[4,5] The

functions of NT include stimulation of pancreatic and biliary secretions,[6] stimulation of colonic motility[7,8] and inhibition of small bowel and gastric motility.[9] NT stimulates growth of numerous gastrointestinal tissues including pancreas,[10,11] gastric antrum,[10] small bowel,[12–14] and colon[15] as well as human small lung cell carcinoma.[16,17] However, the question of whether NT is trophic for human pancreatic cancer is still unanswered.

Binding of NT to its specific membrane receptor stimulates phosphatidylinositol (PI) hydrolysis in neuroblastoma cell line (N1E115)[18] and human colon cancer cell line (HT-29).[19–21] NT also increases intracellular calcium ($[Ca^{++}]_i$) in HT 29 cells[20,21] and some small cell lung cancer cell lines.[22,23] However, the signal-transduction mechanism by which NT regulates cell growth is not known. Therefore, in this study, we examined the effect of NT on *in vitro* growth of human pancreatic cancer cells (MIA PaCa-2) and further examined the intracellular signal-transduction mechanisms through which NT regulates growth of MIA PaCa-2 cells.

MATERIALS AND METHODS

The tissue culture cell line employed in this study was the MIA PaCa-2 human pancreatic cancer cell line that we obtained from American Type Culture Collection (Rockville, MD). To avoid changes of cell characteristics produced by prolonged culture, we used only cells from passages 108–112. MIA PaCa-2 cells had been maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Laboratories, Grand island, NY) containing 5% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan, UT) in a humidified atmosphere of 95% air and 5% CO_2 at 37 C. In stock cultures, the medium was changed every 3 days.

All experiments were performed using six dishes or wells (one coverslip for the calcium study) in each experiment and each study was repeated on at least three separate occasions. Representative data from each experiment are shown in this article.

Effect of NT on ^{3}H-Thymidine Incorporation

MIA PaCa-2 cells (1.5×10^4) grown in 24-well tissue culture dishes for 2 days with DMEM containing 5% FCS had medium replaced by the same culture medium containing 0.1% FCS. After 2 days, the medium was removed and fresh medium containing NT (10^{-12} to 10^{-6} mol/l in distilled water) (Peninsula Laboratories, Inc., Belmont, CA)) was added. Control cells received only distilled water. On the subsequent 2nd, 4th, 6th, and 8th days, cells were pulsed for 3 hours with ^{3}H-thymidine (1 μCi/ml) (Amersham Corporation, Arlington Heights, IL), then fixed with 5% trichloroacetic acid (TCA) and washed twice. Acid-insoluble material was collected with 0.1N NaOH and counted by liquid scintillation counter.

Effect of NT on Growth of MIA PaCa-2 Cells

MIA PaCa-2 cells (1.5×10^4) grown in 24-well tissue culture dishes for 2 days with DMEM containing 5% FCS had medium replaced by the same culture medium containing 0.5% FCS. After 2 days, medium was removed and fresh medium containing NT (10^{-12} to 10^{-6} mol/l) was added. Control cells received only distilled water. On the subsequent 2nd, 4th, 6th, and 8th days, total cell counts were performed by a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) which was electrically set to count cells under 100 μm size. Cell numbers determined by a Coulter counter were similar (less than 5% difference) to viable cell numbers determined by a dye (trypan blue) exclusion method using a hemocytometer; the coefficients of variance of low and high counts determined by this electric counter were less than 2%.

Effect of NT on PI Hydrolysis

PI hydrolysis in MIA PaCa-2 cells was measured as reported previously.[24] In brief, cells (3×10^5) were harvested by trypsin and cultured in 24-well tissue culture dishes with DMEM containing 0.5% FCS for 2 days. Then, myo-^{3}H-inositol (5 μCi/ml) (Amersham) was added for an additional 18 hours. Cells were incubated at 25 C for 10 minutes with oxygenated Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.4) containing 10 mmol/l Hepes, 0.1% bovine serum albumin (BSA), 2.5 mmol/l glucose, and 10 mmol/l LiCl. After 1 minute of incubation in the presence of NT (10^{-12} to 10^{-6} mol/l), cells were extracted with 10% perchloric acid and then immediately neutralized with 6N KOH. Fractions of inositol phosphates were extracted by anion exchange chromatography (Dowex AG-1 × 8; formate form; 200–400 mesh) (Bio-Rad Laboratories, Richmond, CA) using a mixture of formic acid and ammonium formate as eluents. After a fraction of inositol was eluted by distilled water, inositol monophosphate, inositol bisphosphate, inositol trisphosphate (IP_3), and inositol tetrakisphosphate were eluted by 0.1 mol/l formic acid with 0.2, 0.4, 1.0, and 1.6 mol/l of ammonium formate, respectively,

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in a stepwise manner. Radioactivity of each fraction was measured by liquid scintillation counter.

Effect of NT on Mobilization of $[Ca^{++}]_i$

Real-time recording of $[Ca^{++}]_i$ was performed in single cells as we reported previously.[25] In brief, MIA PaCa-2 cells were grown for 2 days on 25 mm diameter glass coverslips coated with 50% Matrigel (Collaborative Research, Inc., Bedford, MA) solution with DMEM containing 5% FCS in a humidified atmosphere of 95% air and 5% CO_2 at 37 C. Cells were washed with Krebs-Ringer-Henseleit (KRH) buffer (pH 7.4) containing 125 mmol/l NaCl, 5 mmol/l Kcl, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 2 mmol/l $CaCl_2$, 6 mmol/l glucose, and 25 mmol/l Hepes, then incubated for 60 minutes at 25 C with 2 ml of KRH buffer containing 10 μmol/l fura-2/acetoxyl-methyl ester (Molecular Probes, Eugene, OR) to minimize compartmentalization of fura-2. Loaded cells were washed twice with fresh KRH and reincubated in KRH with 0.1% BSA at 25 C for 60 minutes in the dark before fluorescence microscopy. Loaded cells attached to coverslips were placed in an open perfusion Micro-Incubator (PDMI-2) (Medical System Corp., Greenvale, NY) covered with 3 ml KRH containing 0.1% BSA. NT (concentrated 100 times, 30 μl) was added to obtain the desired concentration (10^{-12} to 10^{-7} mol/l). These experiments were done at 37 C. To examine the effect of NT on mobilization of calcium from intracellular stores, a calcium-free KRH solution with 1 mmol/l ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (Sigma) was employed after calcium-containing KRH was washed out. Fura-2 fluorescence intensity was measured every second using a Nikon Diaphot inverted microscope (Garden City, NY) coupled to a dual-wavelength excitation spectrofluorometer (CM111I, Spex Industries, Inc., Edison, NJ). The excitation light was obtained from xenon high-pressure lamp at wavelengths of 340 nm and 380 nm (band width: 10 nm). $[Ca^{++}]_i$ was calibrated by lysing cells with 0.33% Triton X-100 and by using 2.5 mmol/l EGTA. Intracellular concentration of calcium was calculated by the method of Grynkiewicz and colleagues[26] using a dissociation constant (Kd) for fura-2 and calcium of 224 nmol/l.

Effect of NT on the Production of Cyclic AMP

MIA PaCa-2 cells (5×10^5), harvested by trypsin and cultured in 35-mm culture dishes with DMEM containing 0.5% FCS for 2 days, were incubated for 10 minutes at 25 C in oxygenated KRBB (pH 7.4) containing 10 mmol/l Hepes, 0.1% BSA, 2.5 mmol/l glucose, and 0.1 mmol/l isobutylmethylxanthine (IBMX). After another 30-minute incubation in the presence of NT (10^{-12} to 10^{-6} mol/l), cells were extracted with 10% TCA and neutralized with an excess of $CaCO_3$.[27] Samples were acetylated with acetic anhydride and triethylamine (1:2 vol/vol) and intracellular levels of cyclic AMP were measured by radioimmunoassay kit (Amersham).

Effect of 8-Bromo-Cyclic AMP

The effect of 8-bromo-cyclic AMP (0.1 and 0.5 mmol/l) (Sigma Chemical Co., St. Louis, MO), a synthetic, membrane-permeable cyclic AMP, on growth of MIA PaCa-2 cells was examined, as well as the effects of 0.1 mmol/l 8-bromo-cyclic AMP alone or combined with NT on $[Ca^{++}]_i$. Experiments were performed by methods described earlier.

Effect of Prostaglandin E_2 on Growth of MIA PaCa-2 Cells

Effect of prostaglandin E_2 (10^{-8} and 10^{-6} mol/l) (Sigma) on growth of MIA PaCa-2 cells was examined by methods described earlier.

Statistical Analysis

Results are expressed as the mean ± SEM and analyzed using Student's unpaired *t*-test and significance was assumed for p value less than 0.05.

RESULTS

Effect of NT on ^{3}H-Thymidine Incorporation and Cell Growth

NT regulated both ^{3}H-thymidine incorporation by and growth of MIA PaCa-2 cells in a dose-dependent fashion; that is, low concentrations of NT (10^{-12} to 10^{-8} mol/l) stimulated, but high concentrations (10^{-7} and 10^{-6} mol/l) of NT did not stimulate ^{3}H-thymidine incorporation (Fig. 1) and also low concentrations (10^{-12} to 10^{-9} mol/l) of NT stimulated, but high concentrations (10^{-8} to 10^{-6} mol/l) of NT did not stimulate cell growth (Fig. 2). The stimulatory effect of NT on both ^{3}H-thymidine incorporation and cell growth was not observed on the 2nd and 4th days, but became apparent on the 6th day after addition of NT and persisted through the 8th day of analysis.

Effect of NT on PI Hydrolysis

NT increased the intracellular level of IP_3, a byproduct of PI hydrolysis, in a dose-dependent manner (Fig. 3).

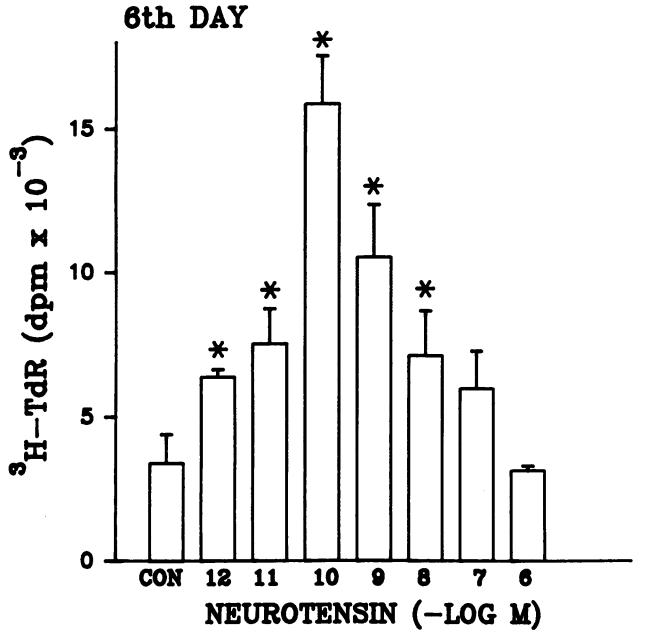

Figure 1. Effect of NT on ^{3}H-thymidine incorporation (^{3}H-TdR) into MIA PaCa-2 cells (*p < 0.05 vs. control).

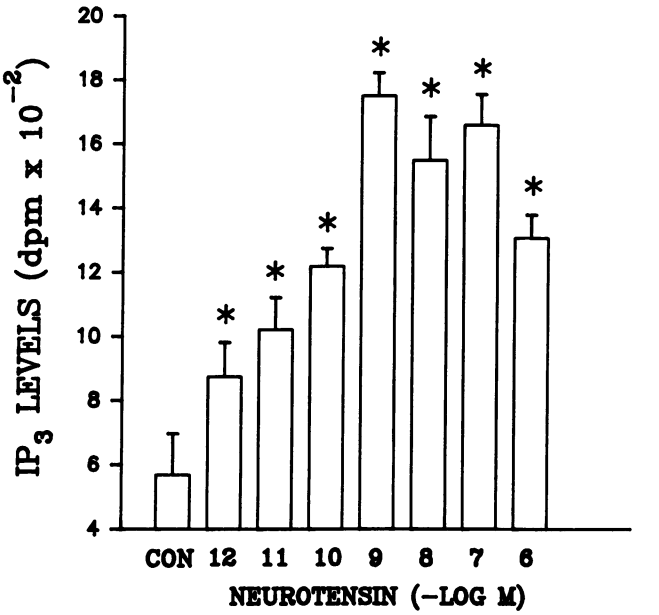

Figure 3. Effect of NT on PI hydrolysis (IP_3 accumulation) in MIA PaCa-2 cells (*p < 0.05 vs. control).

Effect of NT on Mobilization of $[Ca^{++}]_i$

NT increased intracellular levels of calcium. NT-stimulated mobilization of $[Ca^{++}]_i$ was composed of two phases: the first a transient peak and the second a maintained elevation over the basal level of $[Ca^{++}]_i$ (Fig. 4). The stimulatory effect of NT on mobilization of $[Ca^{++}]_i$ was dose-dependent (Fig. 5). In Ca^{++}-free medium, NT stimulated mobilization of $[Ca^{++}]_i$, but only the first peak of $[Ca^{++}]_i$ was observed without the maintained elevation of $[Ca^{++}]_i$ (Fig. 6). These results suggest that NT stimulates $[Ca^{++}]_i$ by both the release of Ca^{++} from intracellular stores (the transient peak of $[Ca^{++}]_i$) and Ca^{++}-influx from the extracellular space (the maintained elevation of $[Ca^{++}]_i$).

Effect of NT on the Production of Cyclic AMP

NT stimulated the production of intracellular cyclic AMP in a dose-dependent manner. In contrast to the effect on PI hydrolysis and increases in $[Ca^{++}]_i$ however, high concentrations (10^{-8} to 10^{-6} M) of NT stimulated, but low concentrations (10^{-12} to 10^{-9} M) of NT had no effect on cyclic AMP levels (Fig. 7).

Effect of 8-Bromo-Cyclic AMP

The cyclic AMP analog, 8-bromo-cyclic AMP, inhibited growth of MIA PaCa-2 cells in a dose-dependent

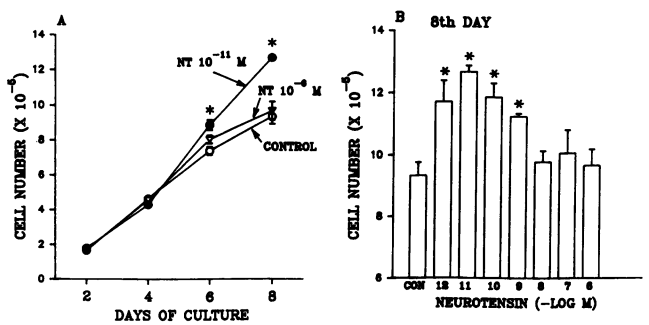

Figure 2. Effect of NT on growth of MIA PaCa-2 cells. A: Representative growth curve of MIA PaCa-2 cells and B: cell numbers on the 8th day after addition of NT. Error bars indicate standard error of the mean (*p < 0.05 vs. control).

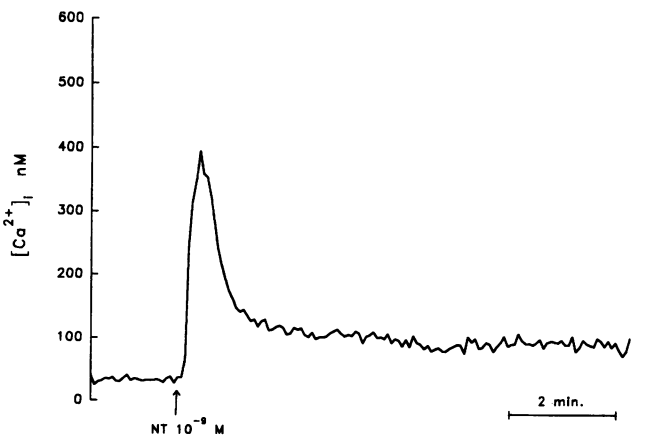

Figure 4. Representative mobilization of intracellular calcium in response to 10^{-9} mol/l NT in MIA PaCa-2 cells.

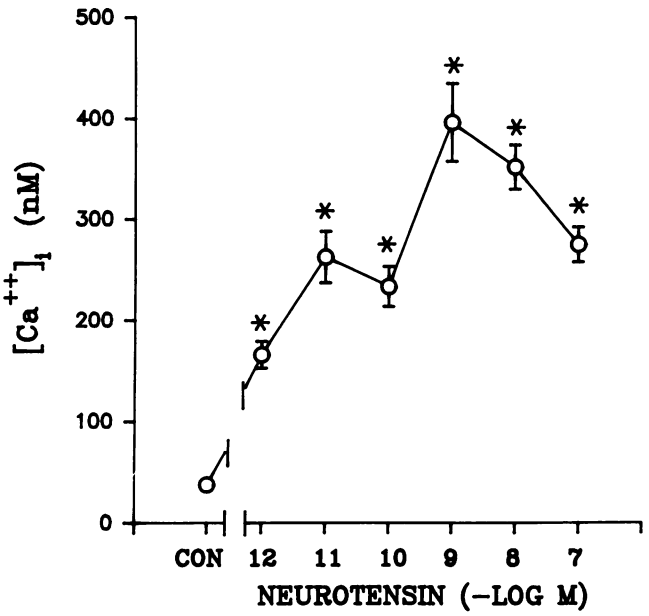

Figure 5. Dose-response curve of NT-induced mobilization of intracellular calcium in MIA PaCa-2 cells (*p < 0.05 vs. control).

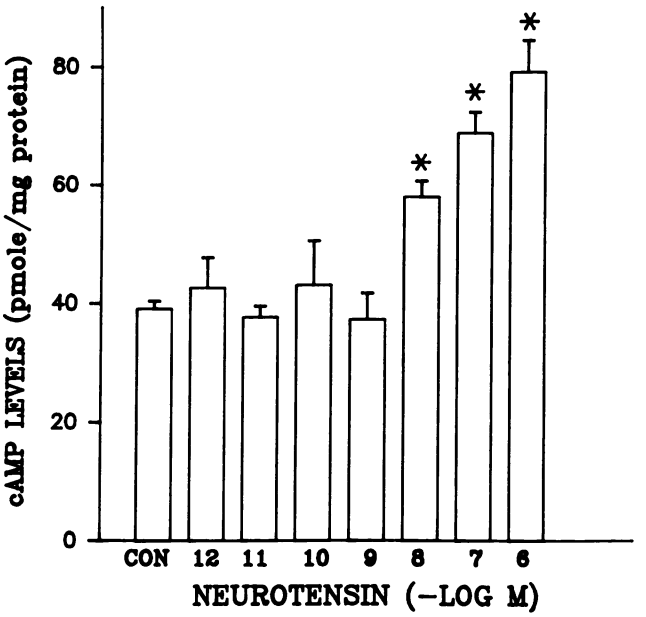

Figure 7. Effect of NT on the production of cyclic AMP (cAMP) in MIA PaCa-2 cells (*p < 0.05 vs. control).

manner. Persistent inhibitory effects became apparent on the 4th day after addition of 0.5 mmol/l 8-bromo-cyclic AMP and on the 6th day with both 0.1 mmol/l and 0.5 mmol/l 8-bromo-cyclic AMP (Fig. 8). 8-bromo-cyclic AMP did not affect $[Ca^{++}]_i$ in either basal or NT-stimulated conditions (data not shown).

Effect of Prostaglandin E_2 on Growth of MIA PaCa-2 Cells

Both 10^{-8} and 10^{-6} mol/l of prostaglandin E_2 did not affect growth of MIA PaCa-2 cells (data not shown).

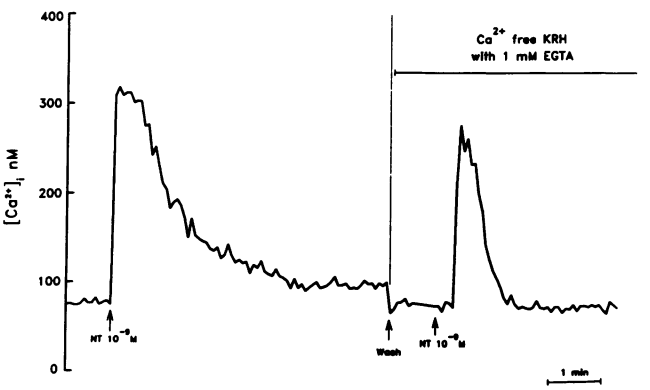

Figure 6. Mobilization of intracellular calcium in MIA PaCa-2 cells in response to NT in calcium (2 mmol/l)-containing KRH and in calcium-free KRH with 1 mmol/l EGTA.

DISCUSSION

There is no question that cell growth is regulated by various growth factors through their specific receptor-linked signal-transduction pathways.[28] A given peptide may act through a single receptor or multiple receptors. A peptide growth factor may act through different receptors coupled to different post-receptor signal-transduc-

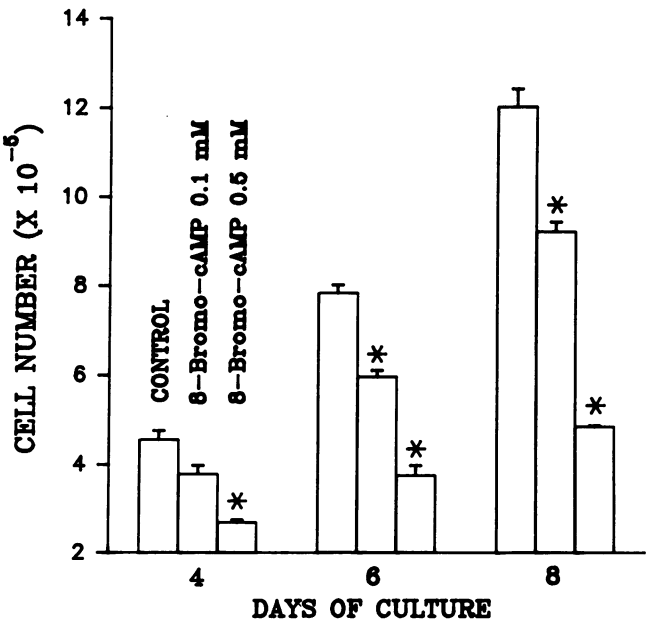

Figure 8. Effect of 8-bromo-cyclic AMP on growth of MIA PaCa-2 cells (*p < 0.05 vs. control).

tion pathways[24] or the same receptor for a given peptide growth factor may be coupled to different post-receptor signal-transduction pathways by crosstalk.[29] Therefore, detailed studies to examine the mechanism of mitogenic signalling at the receptor and post-receptor levels are required to understand which specific pathway is involved for the agent of interest. Elucidation of the mechanism of mitogenic signaling may result in development of novel therapy for patients with cancer.

NT stimulates growth of normal pancreas,[10,11] normal small intestinal mucosa,[12–14] colonic mucosa,[15] and clonal growth of some small cell lung cancer cells.[16,17] We have not found any previous determination of whether NT stimulates growth of human pancreatic cancer cells. Furthermore, the mechanism by which NT acts to regulate growth has been incompletely defined.

In this study we report, for the first time, that NT stimulates *in vitro* growth of MIA PaCa-2 human pancreatic cancer cells through receptor-linked specific intracellular signal-transduction systems. Our findings suggest that: 1) NT binds to at least two different types of NT receptors with high and low affinities, either of which is linked to a specific intracellular signal-transduction pathway; 2) binding of NT to receptors with high affinity stimulates PI hydrolysis and mobilization of $[Ca^{++}]_i$, stimulation of which may play a role in growth-stimulation of MIA PaCa-2 cells; 3) binding of NT to receptors with low affinity stimulates the production of cyclic AMP, stimulation of which may play a role in growth-inhibition of MIA PaCa-2 cells.

Two different types of NT receptors (with high and low affinities) have been identified in rat brain tissue[30] and in plasma membranes of rat fundic smooth muscle.[31] Our study shows that MIA PaCa-2 human pancreatic cancer cells have at least two functionally different types of NT receptors. We know of no example in which an agent acting through a single receptor activates multiple signal-transduction pathways, but as reported by Rozengurt,[32] the production of cyclic AMP might be due to the action of prostaglandins, derivatives of arachidonic acid (arachidonic acid is derived from diacylglycerol which is a byproduct of PI hydrolysis). Our findings do not support this possibility, since addition of exogenous prostaglandin did not affect growth of MIA PaCa-2 cells.

In contrast, the phenomenon of multiple receptors for a single agent linked to specific signal-transduction pathways is well known. We have reported that in human pancreatic carcinoid cells (BON), 5-hydroxytryptamine (5-HT) both stimulates PI hydrolysis through $5\text{-}HT_{1A}$ receptors and inhibits cyclic AMP production through $5\text{-}HT_{1C/2}$ receptors.[24] Our current findings are consistent with the presence of two types of NT receptors on MIA PaCa-2 cells; one type of NT receptor with high affinity (low concentrations of NT bind to this receptor) linked to PI hydrolysis and $[Ca^{++}]_i$ mobilization and another NT receptor with low affinity (high concentrations of NT bind to this receptor) linked to cyclic AMP. Another possibility is that two different types of NT receptors are present on different types of cells, because MIA PaCa-2 cell line is composed of a heterogenous population of cells; because growth is not stimulated by high concentrations of NT, this is unlikely.

PI hydrolysis and/or $[Ca^{++}]_i$ mobilization plays an important role in the growth of certain cells.[33–36] Both pathways usually work together to regulate growth, although growth of human stomach cancer (AGS) cells is stimulated through $[Ca^{++}]_i$ mobilization pathway, independent of PI hydrolysis pathway.[25]

Cyclic AMP plays an important role in the regulation of cellular growth.[24,37–41] However, a given level of cyclic AMP may not always produce the same effect on cell growth. Vasoactive intestinal polypeptide (VIP) inhibits growth of AGS cells through VIP-stimulated production of cyclic AMP[37] and 5-HT stimulates growth of BON cells through 5-HT-induced decrease of cyclic AMP production.[24] Furthermore, we have found that human gastrin-17 stimulates growth of two different types of human colon cancer (LoVo and COLO 320) cells through gastrin-stimulated production of cyclic AMP,[41] whereas human gastrin-17 inhibits growth of human colon cancer (HCT116) cells through gastrin-stimulated production of cyclic AMP (unpublished data). Experiments that examine the effects of addition of exogenous 8-bromo-cyclic AMP have proved that cyclic AMP pathway definitely plays a role in growth-regulation of cancer cells.[24,25,37,41]

Stimulation of growth of MIA PaCa-2 cells requires both PI hydrolysis and $[Ca^{++}]_i$ mobilization pathway; growth is not stimulated by increased levels of cyclic AMP. The absence of growth stimulation of MIA PaCa-2 cells by higher concentrations of NT can be explained by interaction (crosstalk) between these two pathways.

In summary, growth of human pancreatic cancer cells was stimulated by NT, through specific, functional NT receptors, linked to different intracellular signal-transduction pathways. The MIA PaCa-2 cell line appears to be an unique model to elucidate the role of intracellular signal-transduction pathways and to examine the crosstalk of signal-transduction pathways involved in the stimulation of cell proliferation. Full understanding of these mechanisms will be beneficial for development of novel therapy for patients with cancer.

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