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PACAP Induces Signaling and Stimulation of 5-Hydroxytryptamine Release and Growth in Neuroendocrine Tumor Cells

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Abstract

Neuroendocrine tumors, although rare, are currently diagnosed with increasing frequency, owing to improved imaging techniques and a greater clinical awareness of this condition. To date, BON is a very well established and characterized human pancreatic neuroendocrine tumor cell line used to study the signal transduction and genetic regulation of neuroendocrine tumors secretion and growth. The secretory activity of BON cells is known to release peptides, such as chromogranin A,

neurotensin, and biogenic amines, as 5-HT, permitting an assessment of their biological activity. The neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP), released from the enteric neurons in the gastrointestinal tract by binding to its high affinity receptor PAC1, has been previously shown to regulate the secretory activity and growth of the neuroendocrine-derived enterochromaffin-like cells in the stomach. This led us to speculate that PACAP might also play an important role in regulating the growth of human neuroendocrine tumors. Accordingly, in the current study, we have shown that BON cells express PAC1 receptors, which are rapidly internalized upon PACAP activation. Furthermore, PAC1 receptor activation, in BON cells, couple to intracellular Ca^{2+} as well as cAMP responses and induce the release of intracellular 5-HT, activate mitogen activated protein kinases, and stimulate cellular growth. These data indicate that PACAP functionally can stimulate 5-HT release and promote the growth of the BON neuroendocrine tumor cell line. Therefore, PACAP and its receptors regulate neuroendocrine tumor secretory activity and growth in vivo, and this knowledge will permit the development of novel diagnostic and therapeutic targets for managing neuroendocrine tumors in humans.

Keywords

BON cell; Carcinoid tumor; PACAP

Introduction

Neuroendocrine tumors are relatively rare neoplasms, mainly localized in the gastrointestinal tract (Creutzfeldt 1996; Halford and Waxman 1998; Langley 1994; Parekh et al. 1994; Pisegna and Sawicki 2001). Neuroendocrine tumors represent a heterogeneous group of tumors, characterized by combinations of argentaffin cells containing granules that stain positive for chromogranins, synaptophysins, or neurotensin (Lemmer et al. 2002; O'Connor and Deftos 1986).

Although rare, neuroendocrine tumors are being diagnosed with increasing frequency due to the improved imaging techniques and a greater awareness in the medical community (Hopfner et al. 2003; Langley 1994; Pisegna and Sawicki 2001). The incidence of neuroendocrine tumors is relatively low (1.5 per 100,000 population), although they comprise about 25% of all intestinal tumors (Halford and Waxman 1998). The exact cellular origin of the neuroendocrine tumors has not been clearly defined; however, it is presumed that they arise from the malignant transformation of enteroendocrine cells localized in the gastrointestinal tract and, thus, they would derive embryo-logically from the neuroectodermal cells of the neural crest (Kalcheim et al. 2002). The peculiar biological characteristic of neuroendocrine tumors is the hypersecretion of biogenic amines and peptides that leads to the development of a functional clinical syndrome characterized by flushing and diarrhea (Pisegna and Sawicki 2001). To date, the only established model for investigation of neuroendocrine tumors is the pancreatic endocrine tumor cell line, BON, obtained from C.M. Townsend Jr., (University of Texas, Galveston, TX, USA); these cells are derived from a lymph node metastasis of a pancreatic neuroendocrine tumor (Ahlund et al. 1989; Townsend et al. 1993; Tran et al. 2004). The understanding of the regulatory mechanisms underlying the synthesis, secretion, and growth of the BON cell line can

facilitate the study of neuroendocrine tumors. Microscopically, the BON cells show the presence of numerous dense core granules containing chromogranin A and C, neuron-specific enolase, synaptophysins, and bombesin (O'Connor and Deftos 1986; Carraway et al. 1994; Zhang et al. 1995). BON have also been shown to release growth factors, such as transforming growth factor, fibroblast growth factor, and epidermal growth factor receptor and peptides, such as pancreastatin, neurotensin, and the amine 5-HT (O'Connor and Deftos 1986; Kim et al. 2001a, b). Similarly, their immunoreactivity for peptide hormones, such as gastrin, vasoactive intestinal polypeptide (VIP), glucagon, and somatostatin has been already demonstrated (Lee et al. 1990; Ishihara et al. 1992; Lutz et al. 1993). Furthermore, the BON cells have been previously shown to express receptors for acetylcholine, 5-HT, and beta-adrenergic agonists like isoproterenol, somatostatin, and insulin-like growth factor-1 (Harmar et al. 1998; Lemmer et al. 2002); however, the expression of pituitary adenylate cyclase activating polypeptide (PACAP) has never been investigated (Miyata et al. 1990; Gottschall et al. 1993; Moody et al. 1993, 2002; Pisegna and Wank 1993, 1996; Journot et al. 1996; Pisegna et al. 1997; Le et al. 2002; Miampamba et al. 2002; Germano et al. 2001a, b, 2004). The release of secretory products, such as 5-HT, results in the symptoms of the carcinoid syndrome that is manifested by flushing and diarrhea (Ishizuka et al. 1992). Therefore, palliative treatment for these symptoms is aimed at reducing circulating levels of these biologically active agents.

We have previously shown that the neuropeptide, PACAP, released from enteric neurons, regulates the secretion and growth of the neuroendocrine-derived enterochromaffin-like (ECL) cells of the gastric mucosa, by acting at its high affinity receptor, PAC1 (Zeng et al. 1991; Zanner et al. 2002). Thus, we have hypothesized that PACAP may exert a similar biological activity on the secretory activity and growth of neuroendocrine tumors. Accordingly, in the current study, we have characterized the expression and biological activity of PAC1 receptors on the BON neuroendocrine tumor cell line. PAC1 receptor is coupled to both G α s and G α q, leading to the activation of dual signaling pathways with stimulation of cAMP and Ca²⁺ (Spengler et al. 1993; Schomerus et al. 1994; Lyu et al. 2000; Germano et al. 2001a, b, 2004; Mergler 2003; Payet et al. 2003). The role of PACAP and PAC1 receptor in the regulation of human neuroendocrine tumors has never been previously studied. Understanding the signaling pathways triggered by PACAP binding to PAC1, in the BON cells, may provide new insights into the biology of neuroendocrine tumors and into the development of new therapeutic strategies for tumors not responding to conventional treatments. Thus, the overall aim of this study is to investigate the role of PACAP in regulating neuroendocrine tumors secretion and growth.

Materials and Methods

Cell Culture

BON carcinoid tumor cells were cultured in Dulbecco's modified eagle medium (DMEM) F: 12 (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), and the antibiotics kanamycin (Gibco, Carlsbad, CA, USA) and gentamicin (Sigma, St. Louis, MO, USA). Adherent confluent cells were washed with phosphate buffer solution (PBS),

detached with trypsin/ethylenediaminetetraacetic acid, then pelleted, resuspended in fresh medium, and incubated in a humidified atmosphere of 5% CO₂ at 37°C.

RT-PCR

Total RNA isolation from BON cells was performed using BD Biosciences' Nucleospin RNA II Kit (Palo Alto, CA, USA) according to the manufacturer's instructions. RNA (200–600 ng) was primed with oligo (dT) and reverse transcribed, and the resulting cDNA was amplified by polymerase chain reaction (PCR) in the presence of 1 µg of sense and antisense primers (Invitrogen). Reverse transcription (RT)-PCR was performed using HuPACAP TGA-AS (5'-GGAGGAGGGAGACAGGCCTCA-3')+HuPACAPATG-S (5'-TAGTGGCTGGGAAGCACCATG-3'), HuVIP1-R-S (5'-GAT GAG CAG CAG ACC ATG TTC TAC TAC CTG TCA GTG AAG GCC CTC-3')+HuVIP1-AS (5'-CTA CTC GTC GTC TGG TAC AAG ATG ATG GAC AGT CAC TTG CGG GAG-3'), and HuVIP2-R-S (5'-GAG GAT GAG AGC AAG ATC ACG 3')+HuVIP2-AS (5'-CTG TGG TCG TTT GTA TCC CAG C-3') primers, specific to PAC1, VPAC1, and VPAC2 receptors, respectively, and acidic ribosomal phosphoprotein (5'-GTTGAACATCTCCCCCTTCTC-3') + (5'-ATGTCCT CATCGGATTCCTCC-3') primers as control (Fig. 1). Each sample was initially heated to 94°C for 3 min. After 32 cycles of amplification, the samples underwent one cycle of incubation of 94°C for 45 s, 60°C for 20 s, and 72°C for 5 min. Lastly, the amplified bands were separated on 70% agarose gel, and photography was performed using Gel Expert Software and a Nucleotech digital imaging system.

Immunostaining

Confocal microscopy was used to characterize the expression of PAC1 receptors and intracellular PACAP using rabbit polyclonal anti-PAC1 receptors antibodies (CURE Antibodies Core), or anti-PACAP antibodies (Peninsula), respectively. BON cells, plated on polylysine-treated cover slips overnight, were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton-X for the anti-PACAP staining, then incubated 30 min at room temperature with goat serum, and later at 4°C with polyclonal rabbit anti-PAC1 (1:1,000) or anti-PACAP (1:1,000) antibodies overnight. As a negative control, some cells were incubated with rabbit IgG (1 µg/mL), overnight at 4°C. On the next day, cells were washed twice with PBS and then incubated with Alexa-488 conjugated goat anti-rabbit IgG antibodies (Molecular Probes) in PBS/1% bovine serum albumin (BSA)/goat serum. Samples were observed using a Zeiss laser scanning microscopy (LSM) 510 laser scanning microscope (Carl Zeiss, Thornwood, NY, USA). The cells were excited with an Ar-Kr laser at 488 nm. Emissions were captured by a photomultiplier tube through an LP 590 filter and saved as an LSM format images. The thickness of each optical slice was 0.5–1.5 µm. Images represent averages of four scans.

PAC1 Receptor Internalization

Alexa-488 conjugated PACAP-38 (Fluor-PACAP) was used to study PAC1 receptor internalization. Fluor-PACAP was previously shown to have a pharmacological and biological activity identical to the native peptide (Germano et al. 2001a). BON cells were cultured on cover slips, as described previously (Germano et al. 2001a), then BON were incubated for 1 h with 100 nM of Fluor-PACAP on ice in the dark in buffer A, containing

140 mM, 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM HEPES, 11.10 mM d-glucose, and 1% BSA. After, cells were washed with PBS, placed in buffer A, warmed up to 37°C in a thermostatically controlled chamber, and observed in time series images by confocal microscopy.

cAMP Assay

To demonstrate that PAC1 receptors expressed on the BON cells are functionally coupled to adenylyl cyclase, BON cells were stimulated with PACAP-38 in a dose-dependent manner and the cAMP levels were measured, as previously described (Solomon et al. 1974; Lyu et al. 2000). Briefly, the cells were seeded on 24-wells culture dishes overnight (approximately 200,000 cells/well) with DMEM F:12 containing 10% FBS, kanamycin, and gentamicin, in the presence of [^3H]-adenine (Amersham, Arlington Heights, IL, USA) at a concentration of 2 mCi/ml. The cells were then washed with DMEM F:12, added with 1% BSA, incubated with or without the indicated concentration of PACAP-38 in DMEM F:12 with 1% BSA and 2.5 mM 3-isobutyl,1- β -methyl-xanthine (Sigma) for 30 min, and then aspirated. The cells were lysed by a 100- μl 2% sodium dodecyl sulfate (SDS) and 1 mM cAMP solution. Total [^3H]-cAMP was assayed by consecutive Dowex AG-50W-X4 resin (Bio-Rad, Richmond, CA, USA) and aluminum oxide (Sigma) column chromatography according to a modification of the procedure described by Solomon et al. (1974). Eluates were collected in vials and counted using a Beckman liquid scintillation counter (Beckman, Fullerton, CA, USA).

Ca^{2+} Imaging

To further investigate whether PAC1 receptors, expressed by BON cells, could mediate an increase in cytosolic [Ca^{2+}]_i, calcium imaging was performed. Cells were seeded on glass cover slips overnight at a density of 50–100 thousand cells/ml, then washed twice with PBS and incubated at 37°C for 30 min in buffer A, containing 140 mM, 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM HEPES, 11.10 mM d-glucose, and 1% BSA, and 2 μM fluo-4 acetoxymethyl ester (fluo-4/AM; Molecular Probes, Eugene, OR, USA), then washed and imaged in a chamber thermostatically controlled at 37°C within 1 h from the loading. PACAP-38 (100 nM) was directly added into the incubation chamber after 850 s. Ionomycin (10 μM) was used as a positive control and administered in the same manner after 1,650 s. Imaging was performed using a Zeiss LSM 510 laser scanning microscope at $\times 20$ magnification. Ca^{2+} response was expressed as an average of cell fluorescence intensity change relative to the maximal fluorescence intensity increase induced by ionomycin.

MTT Proliferation Assay

To investigate the effect of PACAP on BON growth, approximately 20,000 BON cells were plated per well in 96-well plates overnight at 37°C. The following day, the cells were activated with PACAP-38 in a logarithmic dose-response manner for approximately 20 h. Ten microliters of 12 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) was added to each well and incubated for 4 h at 37°C. Following this incubation, 100 μl of 0.01 M HCl/10% SDS solution was added to lyse the cells in each well and incubated overnight at 37°C. The colorimetric reaction was read the day after at 570 nm in an MRX Revelation enzyme-linked immunosorbent serologic assay (ELISA) Reader

(Dyner Technologies, Vista, CA, USA). The colorimetric reaction was expressed in optical density as a change in the percentage of color's intensity of the unstimulated cells. MTT incorporation is proportional to the number of viable cells in each well.

5-HT Release Assay

In 12-well/plate, 10^6 cells/well were seeded, changed medium. Next day, cells were washed twice with 0.5 ml OptiMEM, incubated in OptiMEM for 30 min at 37°C and activated with increasing logarithmic concentration of PACAP, as indicated, and incubated for 20 min. Supernatants were collected, and 5-HT release was measured by an enzyme immunoassay using an ELISA kit (Coulter Corp., Miami, FL, USA) according to the manufacturer's instructions. The absorbance was measured at 405 nm, and the 5-HT concentration was determined from a standard curve, as described previously (Kim et al. 2001a). The sensitivity of the assay is approximately 0.5 nM (1.8 pg/well).

MAPKs Activation

BON cells were plated in 6-well tissue culture plates. Cells were activated in wells with 100 nM PACAP-38 in a time-response manner for 9, 27, or 60 min. Then, the supernatants were suctioned; the wells were washed with 3 mL of cold PBS, added with a mixture of proteases inhibitors (Sigma) and 1 μ M NaVO₄. One hundred twenty microliters of boiling SDS sample buffer containing 2% β -mercaptoethanol was pipetted into each well to lyse the cells. Samples were then transferred into Eppendorf tubes and boiled for 10 min. Solubilized proteins were separated by electrophoresis onto NuPAGE™ 10% Bis/Tris gels (Novex-Invitrogen) and then transferred onto nitrocellulose membranes. Phosphorylated extracellular-signal-regulated kinase (ERK) 1/2 were detected by immunoblotting with anti-phospho ERK 1/2 antibodies (Calbiochem). Nitrocellulose membranes were blocked with Tris-Tween buffered saline (TTBS) added with 4% BSA for 30 min at room temperature, and probed with rabbit anti-phospho ERK1/2 antibodies (1:1,000) overnight at 4°C. Membranes were rinsed with TTBS buffer added with 4% BSA. The chemiluminescent reaction was revealed by an ECL kit on Hyperfilm ECL (Amersham Pharmacia Biotech). Equivalent loading and transferring of the samples in all the lanes was ensured by Coomassie blue staining (Novex, Invitrogen kit) of the gels and Ponceau S red (ICN Biomedicals) staining of the membranes.

Results

RT-PCR

From BON cells grown to confluence, RNA was extracted to determine the relative levels of expression of PACAP receptors (Fig. 1). Specific primer pairs to detect expression of PAC1 and VPAC receptors were chosen for these studies, as indicated in Fig. 1. Transcripts for PAC1 were identified in BON (lane 3) as well as in NIH3T3 cells, transfected with the HOP variant of PAC1 receptor (lane 2). VPAC1 receptor transcripts were also identified in BON (lane 5) as well as in VPAC1-transfected NIH3T3 cells (lane 4), whereas, VPAC2 transcripts were found only in VPAC2-transfected NIH3T3 (lane 6) but not in BON cells (lane 5). NIH-3T3 cells stably transfected with PAC1, VPAC1, or VPAC2 were used as positive controls for these studies to corroborate both the expression and size of the expected PCR

products. The experiment shown in Fig. 1 is one representative of five experiments showing the same results.

Immunocytochemistry

To further confirm that the BON cells express PAC1 and PACAP receptors, immunofluorescent staining, revealed by confocal laser imaging microscopy, was used to visualize surface expression of PAC1 receptors and intracytoplasmic expression of PACAP (Fig. 2). The cells were incubated either with polyclonal rabbit anti-PAC1 (CURE), or rabbit anti-PACAP antibodies (Peninsula), or rabbit IgG, followed by goat anti-rabbit fluorescein isothiocyanate-conjugated antibodies. Immunoreactivity for PACAP was shown to be present in the cytoplasm of BON cells permeabilized with 1% Triton (panel B). Surface expression of PAC1 receptors was detected in BON cells, not previously permeabilized (Panel C). As a negative control, we utilized rabbit IgG, followed by the secondary antibodies, as shown in Panel A. This data confirms cell surface expression of PAC1 receptors, as well as the presence of immunoreactive PACAP in the cytoplasm of BON cells.

Internalization of PAC1 Receptors in BON Cells

We next studied the localization of PACAP receptors in BON cells, following ligand binding, to determine the time course for receptors internalization (Fig. 3). To perform these studies, we utilized confocal imaging series techniques, using Fluor-PACAP, as previously described in other cell systems (Germano et al. 2001a). BON cells were initially incubated at 37°C with red LysoTrack that is incorporated in the lysosomes and then with Fluor-PACAP on ice for 1 h. We observed Fluor-PACAP internalization starting after 30 s upon warming the cells at 37°C. The internalization was complete after 3–6 min; the green fluorescence of Fluor-PACAP co-localized with the red LysoTrack into the lysosomes, suggesting lysosomal sequestration of the ligand-receptor complex.

Adenylyl Cyclase Assay

To investigate whether PAC1 receptors are functionally coupled to the stimulation of adenylyl cyclase, intracellular cAMP levels were measured (Fig. 4). PACAP-38 elevated cAMP levels in a dose-dependent fashion, with detectable stimulation with a dose of 0.1 nM of PACAP-38, following a 30-min incubation. The data shown represent the mean of three experiments performed in triplicate. In previous time-course experiments (data not shown), a 30-min stimulation time was selected for providing the maximal stimulation of adenylyl cyclase. Half maximal concentrations, expressed as EC₅₀, for PACAP-38, were detected at approximately 1 nM, and maximal stimulation was noted at 100 nM (Fig. 3). In other experiments, the potency for activation of adenylyl cyclase was consistent with the stimulation observed in NIH-3T3 cells stably transfected with PAC1 receptor (Germano et al. 2001a, b) in the general shape of the activation curve. However, the half maximal (EC₅₀) of PAC1-transfected NIH3T3 cells were at 3 nM. These results were consistent with the previously described pharmacology of the PAC1 receptors and indicated that these receptors exhibit signal transduction coupled to adenylyl cyclase (Pisegna and Wank 1996).

Ca²⁺ Response to PACAP-38

Since it has been established in stably transfected cell systems that PAC1 receptor is capable of activating a dual signal transduction pathway, leading to the activation of intracellular Ca²⁺ release, we next examined whether PAC1 receptors, expressed by BON cells, were capable of a similar coupling to a dual signaling pathway (Germano et al. 2004). To determine this, we studied PACAP-induced Ca²⁺ response. Fluo-4 loaded BON cells were activated with 100 nM PACAP-38 (Fig. 4). Following PACAP-38 addition, the average of [Ca²⁺]_i response in activated cells, expressed as increase of fluorescent intensity, reached a peak after 120 s of stimulation and returned to baseline level after 280 s. As shown in Fig. 5, PACAP-38, added to the cells, induced an increase of fluorescent intensity that reached a peak at 980 s and returned to a more elevated baseline level at 1,000 s. The response of the BON cells to ionomycin, added at the end of the experiment, allowed us to calculate the maximal release of calcium from the cells, which was used to express the PACAP-induced Ca²⁺ release as a percentage of the maximum release induced by ionomycin.

Cells were activated at 860 s with PACAP-38, which elicited a lower, earlier, peak response after 20 s, and a higher, later, peak response after 120 s. The intensity of the peaks of activation fell to a baseline that was more elevated than the initial. The addition of ionomycin at 1,600 s revealed the maximal calcium response capability of BON cells that was considered as 100% of the response.

5-HT Release Assay Results

To determine whether PACAP activation in BON cells results in a secretive biological response, we measured the release of 5-HT (Fig. 6). In BON cells, PACAP at increasing logarithmic concentrations, dose-dependently stimulated serotonin release (Fig. 6). The observed EC₅₀ was approximately 100 nM, and the maximal activation occurs at 1,000 nM. The sensitivity of the assay is approximately 0.5 nM (1.8 pg/well). With a sample range between 4.5 and 5.5 nM, the inter-assay standard deviation and coefficient of variation were 0.4 nm and 8.9%, respectively.

BON Cell Proliferation Results

The effect of PACAP-38 on the regulation of growth in BON cells was investigated using the MTT assay. Following approximately 20 h of PACAP-38 activation in a dose-response manner, cellular proliferation was assessed using the well-established MTT assay (Fig. 7). An increase of the percentage of viable cells, upon 4 h of MTT incorporation, was detected already at 1 nM PACAP-38, but a maximal percentage increase of 30% of growth was obtained stimulating the cells with 100 nM PACAP-38 (EC₅₀=10 nM). These results were expressed as a percentage of change compared to the baseline values, and they were confirmed by measuring cell counts, using a cell-counting chamber, under an optic microscope (data not shown).

PACAP Phosphorylation of ERKs in BON Cells

To ascertain whether the effects of PACAP stimulation on BON growth were mediated by a PACAP-induced signaling cascade leading to the phosphorylation of mitogen activated protein kinases (MAPKs), we performed Western blotting analysis of the expression of

phosphorylated ERK 1 and 2 in BON cells activated by 100 nM PACAP-38 (Fig. 8). Activation of MAPKs was identified as early as 9 min, and it appeared not only to persist but to be more intense at 27 min for the 42 kDa and at 1 h for the 44 kDa band, as shown also in Fig. 8, with the relative densitometric analysis in Table 1.

Discussion

PACAP is a member of the VIP family of peptides (Harmar et al. 1998). Specific binding sites for PACAP have been identified in the human hypothalamus, brain stem, cerebellum, and cortex (Gottschall et al. 1993). The cloning of the high affinity receptor for PACAP (PAC1), which is specific for PACAP-27 and PACAP-38, has identified this as a member of the seven transmembrane G protein-coupled receptors. PAC1 are highly homologous to the receptors for VIP and secretin (Pisegna and Wank 1993). The pharmacological characterization of receptors within this super-family has demonstrated that PACAP hormone binds with a high affinity to PAC1 and with a lower affinity to the VPAC1 and VPAC2 receptors (Pisegna and Wank 1996).

Since the discovery of PACAP, several studies have identified specific PACAP receptors in normal gastric and colonic mucosa and tumoral tissues (Gottschall et al. 1993; Miampamba et al. 2002). The cloning of the rat and human PACAP receptor, PAC1, has lead to the development of molecular and antibody probes to specifically localize these receptors (Miampamba et al. 2002). PAC1 receptors have been detected on several cancer cell lines, derived from breast, gastric, pancreatic, lung tumors, and ECL cells, in which they appear to regulate cellular proliferation (Lee et al. 1990; Moody et al. 1993; Zia et al. 1995; Journot et al. 1996; Leyton et al. 1999; Germano et al. 2001b, 2004; Le et al. 2002). The finding of PACAP receptors expression in BON cells suggests that PACAP may regulate the growth of human carcinoid tumors.

Other authors (Hofslis et al. 2003) have previously reported the expression of PAC1, but not of VPACs receptors in BON cells. Here, we show that BON cells express not only PAC1 but also VPAC1, but not VPAC2 receptors. These data are supported by the VIP-induced cAMP response that we have observed in BON (unpublished data).

In our current study, we report the presence of PAC1 receptors and also of PACAP hormone in BON cells, a pancreatic neuroendocrine tumors cell line, and show PACAPs effects on signal transduction, secretion, and proliferation. The presence of both, PAC1 receptor and PACAP peptide, in BON cells, a neuroendocrine carcinoid tumor cell line, suggests a potential role for PAC1 and PACAP for an autocrine and/or paracrine regulation mechanism of gastric endocrine secretion. This possible autocrine regulatory role for PACAP could have clinical significance in regulating the growth or secretion of neuroendocrine tumors in humans.

We have identified, by RT-PCR and immunohistochemical staining, the expression of PAC1 receptors in BON cells. Immunohistochemical analyses using confocal laser scanning microscopy, performed in BON cells, using specific rabbit polyclonal anti-PAC1 and anti-PACAP antibodies, have provided evidence of PAC1 expression on the membrane and

PACAP expression within the cytoplasm. The simultaneous presence of both PACAP in the cytoplasm and its receptors on the membrane of BON cells suggests that PACAP may serve as an autocrine extracellular signaling molecule that affects growth as well as 5-HT release.

To determine whether activated PACAP receptors expressed on the surface of BON cells undergo cellular internalization following ligand exposure, we observed the time course of Fluor-PACAP internalization by imaging the cells. The pattern of internalization in BON resulted similar to the one previously demonstrated in PAC1 stably transfected NIH-3T3 cells, in which the receptors rapidly translocate to the poles of the cells, resulting in a nearly complete internalization within approximately 5 min (Germano et al. 2001a; Lyu et al. 2000; Germano et al. 2004). Similarly to our observations in colon cancer cells, PACAP receptors on BON cells appear to co-localize within lysosomes within 3–6 min (Germano et al. 2004). Characterizing the kinetics of PAC1 internalization in BON cells, using Fluor-PACAP and anti-PAC1 antibodies, elucidated the potential differences in internalization and/or desensitization of native PAC1 expressed on tumoral cells compared to transfected receptors, which has been shown previously (Germano et al. 2004).

To investigate whether PAC1 receptor is capable of activating two distinct G protein-signaling pathways, we examined both ligand-activated adenylyl cyclase and Ca^{2+} stimulation. PACAP-38 increased cAMP levels in a dose-dependent manner, with a half maximal stimulation (EC_{50}) of approximately 1 nM, which is similar to the potency of PACAP-38 stimulation of PAC1 in NIH 3T3 cells expressing either the rat or human PAC1 receptors (Pisegna and Wank 1996). As demonstrated previously, the potency of adenylyl cyclase activation is equivalent to the radioligand displacement shown in NIH-3T3 cells stably expressing PAC1 receptors, suggesting that there are spare receptors (Pisegna and Wank 1996). Activation of the phospholipase C pathway was examined by measuring PACAP-induced $[\text{Ca}^{2+}]_i$ release studies. We observed that the activation of $[\text{Ca}^{2+}]_i$ occurred approximately one log unit to the right compared to the stimulation of intracellular adenylyl cyclase; this is consistent with our previous observations in stably transfected NIH 3T3 cells (Germano et al. 2004). Hence, from these positive studies for Ca^{2+} and cAMP, the PAC1 receptors found on BON cells appear to be coupled to both G_α_s and G_α_q signaling pathways.

To examine whether PAC1 receptor signaling in BON cells is functionally coupled to secretive biological responses, we studied 5-HT release, which has already been shown to be a very sensitive index of BON cell secretory activity (Kim et al. 2001a). BON cells have been recognized to have mechano-sensitive properties that are coupled to the activation of G_α_q , phospholipase C, Ca^{2+} , and 5-HT release (Kim et al. 2001b). In the current study, the BON cells were not exposed to mechanical stimulation (Kim et al. 2001b); however, activation with PACAP specifically induced 5-HT release from BON cells in a concentration-dependent manner. PAC1 receptors expressed on BON cells are able to activate Ca^{2+} and, thus, the G_α_q pathway. Previous studies have determined that G_α_q is the predominant pathway for secretory activity in BON cells. For example, protein kinase C (PKC) isoforms such as α and δ play a role in the stimulated release of neurotensin in BON cells (Li et al. 2002, 2004), and similarly, protein kinase D (PKD) has been shown to mediate phorbol ester or bombesin-stimulated neurotensin secretion, which is regulated by

PKC- α , - δ , and the Rho/Rho kinase pathway (Li et al. 2004). Also, it should be noted that histamine secretion from ECL cells, which are the most similar to BON cells, is also regulated by G α_q pathway (Zeng et al. 1991; Zanner et al. 2002). Thus, these results support the reasonable conclusion that the predominant secretory mechanism by which PACAP affects 5-HT release in BON cells involves the G α_q pathway.

We next explored the PACAP-induced effects on growth in BON cells. Our proliferation data show that PACAP-38 has the capability to induce a 25% increase in the number of viable cells, compared to unstimulated cells, after approximately 20 h of culture in media. These findings, in addition to the localization of endogenous intracytoplasmic PACAP in BON cells, suggest that PACAP may promote the growth and survival of human neuroendocrine tumors in vivo, possibly acting through an autocrine mechanism. Other authors (Hofsli et al. 2003) have described a twofold increase, in BON, of DNA synthesis with a dose of 0.1 nM PACAP, but lower with doses higher than 10 nM, by BrdU incorporation. In our experiments, the growth curve reaches a plateau at a 10-nM dose of PACAP. These differences could be perhaps attributed to the different methods used to study PACAP-induced effect on growth: BrdU incorporation used to measure DNA synthesis and MTT and cell counts to quantify the number of viable cells.

Our results are consistent with previous studies demonstrating an autocrine stimulatory pathway for PACAP in colonic tumoral cell lines (Le et al. 2002). Taken together, the activation of the PAC1 receptor triggers 5-HT release and proliferation within the cells. The increased extracellular levels of 5-HT may promote the malignant proliferatory response of the tumor (Ishizuka et al. 1992).

We next investigated whether PACAP stimulation in BON cells could induce phosphorylation of MAPKs. Phosphorylation of ERK 1 and 2 was observed in a time-dependent fashion, with a peak phosphorylation band at 27 min for ERK 1 and 1 h for ERK 2. However, it is still unclear whether the pathway mediating this effect in BON cells derives from G α_q or G α_s activation. For example, in the lung cancer cell systems, we have previously shown that the stimulating effects of PACAP on growth were dependent on G α_q activation (Moody et al. 2002). Yet, in other cell systems, such as the cerebellar granular cells, PACAP has the ability to induce phosphorylation of MAPK through a cAMP-dependent pathway (Journot et al. 1996). The presence of PAC1 receptors, together with the observed Ca²⁺ response, and the increase in the phosphorylation of MAPKs, serotonin release, and mitosis in BON cells suggest that PACAP receptors are expressed on the membrane and function inducing signal transduction, secretion, and growth. Consequently, membrane expressed PAC1 receptors may be a potential targets to regulate secretion and growth in neuroendocrine tumors. In a previous study, we hypothesized that PACAP released from colonic cancer cells through an autocrine mechanism could stimulate its specific receptors on the same cells and recruit phospholipase C, PKC, and MAPKs, causing cell proliferation (Germano et al. 2004). This hypothesis can be extended to neuroendocrine tumors. PAC1 receptors have been shown to regulate tyrosine phosphorylation of MAPK in a MAPKK-dependent manner that enhances the activation of immediate early genes, *c-fos*, *c-myc*, and *c-jun* and, at the same time, inhibits the apoptotic pathways mediated by caspases (Wallach et al. 1999; Moody et al. 2002; Sippel et al. 2003; von Wichert et al.

2005). Furthermore, the role of PKC has been explored previously in lung cancer cell lines, in which the inhibition of PKC results in a reduction of MAPK activity (Zia et al. 1995; Kuroda and Tanizawa 1999). These observations support our hypothesis that proliferation of BON cells is mediated through the PAC1 receptors and Gαq pathway. From these current and previous findings, we propose a potential role for PACAP as a stimulator of 5-HT secretion and proliferation of human neuroendocrine tumors. PACAP could function through an autocrine mechanism to activate PAC1 expressed on the surface of BON cells, transducing a signal through the Gαq pathway that would lead to serotonin secretion, cell growth, and survival.

In conclusion, the present study demonstrates a physiologic role for PAC1 receptor on BON cells, a model for the study of neuroendocrine tumors. BON cells express PAC1 receptors that are functionally coupled to dual signaling pathways leading to the release of 5-HT and cellular proliferation. These results suggest that in neuroendocrine tumors, PAC1 expression may play an important role in promoting the release of bioactive amines such as 5-HT and exerting a proliferative effect on BON cell growth. These data, therefore, may have important clinical consequences for enabling the localization of carcinoid tumors by providing novel therapeutic targets to regulate the release of 5-HT in patients with carcinoid syndrome.

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Figure 1.

mRNA expression of PAC1, VPAC1, and VPAC2 in neuroendocrine tumor cells. Total RNA was isolated from BON using a BD Biosciences Nucleospin RNA II kit. RNA was primed with oligo (dT) and reverse transcribed and amplified. The mRNA expression of PAC1 (lane 3), VPAC1 (lane 5), and VPAC2 (lane 7) was evaluated in BON cells, where PAC1 and VPAC1 transcripts were identified. Acidic ribosomal phosphoprotein PO housekeeping gene primers were used as control (lane 8); 1 Kb ladder (lane 1) was used to mark the size of the bands. NIH-3T3 cells stably transfected with PAC1 (lane 2), VPAC1 (lane 4), and VPAC2 (lane 6) were used as positive controls. The experiment in this figure is representative of a series of five experiments showing the same results

Figure 2.

Expression of PAC1 receptors and PACAP in carcinoid BON cells. Immunoreactivity in the BON cells was found predominantly localized in the cytoplasm for PACAP and on the cell membrane for PAC1 receptors. Confocal images show the expression of PAC1 receptor (c) on the impermeabilized cell surface and PACAP (b) in the cytoplasm of permeabilized BON cells. The cells were incubated first with anti-PACAP, or anti-PAC1 receptor, rabbit polyclonal antibodies, and then with Alexa-488-conjugated goat anti-rabbit IgG as secondary antibodies. **a** Cells were incubated with rabbit IgG, followed by Alexa-488-conjugated goat anti-rabbit IgG, as negative control

Figure 3.

Internalization of PAC1 receptor on BON cells. The internalization of PAC1 receptor was assessed by confocal microscopy series of images at a magnification of $\times 100$ using immersion oil. Cells were pre-loaded with red Lysotracker to visualize lysosomes. Confocal imaging, using PACAP conjugated with Alexa-488 (Fluor-PACAP), revealed PAC1 receptor internalization in BON cells. Fluor-PACAP internalization, at 37°C , started at 30 s and was completed within 6 min. The images series of the internalization process show the green fluorescence of Fluor-PACAP merging with the red Lysotracker, revealing the co-localization of the PACAP receptors with the lysosomes. This is one series of images that is representative of several experiments that have been performed

Figure 4.

Adenylyl cyclase response induced by PACAP-38. cAMP response was determined in BON cells as a function of PACAP-38 concentration by activating cells for 30 min at different logarithmic doses. A consecutive Dowex-Alumina column chromatography method was employed. Stimulation of cAMP occurred in a dose-dependent fashion with an EC₅₀ of approximately 1 nM and a maximal activation occurring at 100 nM, with a 1.3 maximum fold increase in cAMP response. The values represent the averages of six experiments performed in triplicate

Figure 5.

Ca^{2+} signaling response to PACAP stimulation. Laser scanning microscopy techniques were used to study the Ca^{2+} signaling response to PACAP stimulation in BON cells. Cells, upon activation with 100 nM PACAP-38 demonstrated an intracellular $[\text{Ca}^{2+}]_i$ elevation with an earlier peak of response at 880 s and a later peak at 980 s. The intensity of the peak fell to a new elevated baseline, and the addition of Ionomycin at 1,600 s, serving as positive control, revealed the maximal calcium response capability of BON cells. These data represent the mean reactive oxygen intermediates fluorescence intensity values of at least 80 activated cells selected from the field

Figure 6.

5-HT release assay results. The effects of PACAP on 5-HT release in BON cells were measured by a dose-dependent stimulation of BON cells in culture under static conditions, without mechanical stimulation. In BON cells, PACAP dose-dependently stimulated 5-HT release. Values represent the percentage of total 5-HT concentration in wells ($n=6$) over the control basal release with respect to the increasing concentrations of PACAP administered after 20 min

Figure 7.

Proliferation of BON cells upon activation with PACAP-38. Proliferation of BON cells was assessed by MTT assay. An approximately 25% average increase in MTT activity, over basal, was observed in BON cells activated with increasing logarithmic concentrations of PACAP for approximately 20 h. The Formazan forming reaction correlates with the number of viable cells. After approximately 20 h of PACAP-38 activation, in a dose-response manner, an increase of MTT incorporation was observed, with a maximum increase of cell concentration of approximately 30% over basal, occurring with 100 nM PACAP-38. *T* test was used to assess the significance of the results ($n=3$); the values occurring with 100 nM, $p=0.0454$, and 10 nM, $p=0.0366$ were found to be statistically significant. The results represent the averages of three experiments performed in triplicate

Figure 8.

PACAP activation of phosphorylated ERK1/2 in BON cells. BON cells were activated with 100 nM PACAP-38 for 9, 27, and 60 min. Cellular extracts were solubilized in 2× sodium dodecyl sulfate (SDS) sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane. Equivalent gel loading and membrane transferring of the samples was ensured by Coomassie blue gel (Novex, Invitrogen kit) and membrane Ponceau S red (ICN Biomedicals) staining. Membrane probing with polyclonal anti-phospho ERK1/2 antibodies indicated the presence of two specific bands of 42 and 44 kDa, phosphorylated upon PACAP activation, as shown also by the relative densitometric analysis in Table 1. This figure shows one representative blot of three replicated experiments

Table 1

Densitometry quantification

		Time (min)			
		0	9	27	60
Intensity	ERK1 p42	1,209	3,278	5,198	19,771
	ERK2 p44	4,917	8,394	10,948	3,186