Published in final edited form as:

Ann N Y Acad Sci. 2000 ; 921: 233–241. doi:10.1111/j.1749-6632.2000.tb06971.x.

Role of PACAP₁ Receptor in Regulation of ECL Cells and Gastric Acid Secretion by Pituitary Adenylate Cyclase Activating Peptide

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Abstract

We previously reported that PAC1 is expressed on ECL cells resulting in stimulation of $[Ca^{2+}]_i$, histamine and acid secretion. The study reported here characterized the signaling by PAC₁ on ECL cells; determined the effects of PACAP on the gastric acid secretion *in vivo*, and determined the effects of chronic administration of PACAP-27 on ECL cell proliferation. PACAP-27 dose dependently stimulated ECL cell Ca^{2+} and AC with detectable stimulation at 1 nM and maximal stimulation at 100 nM (six-fold). In rats PACAP-27 administration (10 pmol/kg/h) increased the rate of gastric acid secretion when an antisomatostatin antibody was co-administered. Chronic administration of PACAP (10 pmol/h for seven days) via osmotic pump resulted in a more than twofold increase in BrdU incorporation into ECL cells. PAC-AP acting at the PAC1 results in dual signaling responses to both $[Ca^{2+}]_i$. AC in ECL cells stimulates gastric acid secretion via the actions of histamine acting at the parietal cell and in whole animals leads to proliferation of ECL cells when administered chronically.

INTRODUCTION

Pituitary adenylate cyclase activating polypeptides (PACAP-27 and PACAP-38), the most recently described members of the secretin-VIP-glucagon family of peptides, were isolated in 1989 from ovine hypothalamus. The PACAP-27 and PACAP-38 hormones have been demonstrated to be equipotent, both pharmacologically on cell systems and as biologically.¹ There are three types of receptors that have high affinity to the PACAP hormones and they are now pharmacologically classified as PAC₁ (Type I PACAP receptor), VPAC I (classical VIP receptor), and VPAC II for the helodermin-preferring receptor.² The PAC₁ has high affinity for only PACAP compounds, whereas, the VPAC I and VPAC II receptors have nearly equal affinity for both VIP and PACAP. The type I PACAP receptor (PAC₁) has been cloned, and four splice variants have been described coupled to both adenylate cyclase (cAMP) and phospholipase C with similar potencies (10–15). PAC₁ belongs to the Class II receptor family that includes VPAC I, VPAC II, secretin, and glucagon receptors based on their homologous amino acid structures. The nature of the PACAP receptors, their coupling

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mechanisms to activation of ECL cell histamine release, and their effects on parietal cell function have not been previously described.

PACAP hormone expression has been found in numerous tissues, such as the brain, gastrointestinal tract, adrenal gland, and testis, by the use of immunohistochemistry and radioimmunoassay.^{3–5} In the brain, PACAP has been shown to exhibit a growth-promoting effect. It also exerts protective effects against the neurotoxicity of the gp120 envelope protein of the human immunodeficiency virus (HIV).⁶

In the gastrointestinal tract, the effect of PACAP on physiology is less clearly defined. PACAP-staining nerve fibers have been identified in the esophagus, stomach, duodenum, liver, pancreas, and in the small and large intestines.^{7–10} It has also been demonstrated that PACAP is a potent nonadrenergic, noncholinergic inhibitory neurotransmitter that directly relaxes intestinal smooth muscle cells, an action that is believed to be mediated by apamin-sensitive potassium channels, suggesting the possible existence of a unique PACAP receptor subtype.¹¹ A reduction of PACAP-containing nerve fibers has also been shown in Hirschsprung's disease.¹²

Another important action of PACAP is its ability to stimulate cellular growth and differentiation. For example, in AR-42J cells where PACAP stimulates an increase in ornithine decarboxylase activity and cell proliferation.^{6,10} In the rat pheochromocytoma cell line, PC-12 PACAP also has been shown to stimulate neurite out-growth, and in the human neuroblastoma cell line, NB-OK1, PACAP stimulates growth.^{13,14} In other cell systems, such as the human non-small cell lung cancer cell line, NCI-H838, PACAP-27 (10 nM) stimulates an almost threefold increase, an effect that can be inhibited by the PACAP antagonist PACAP 6–38.¹⁵ Similarly, in nude mice, PACAP (6–38), given at a dose of 10 µg/day subcutaneously antagonized the effects of PACAP-27. Taken together, these results suggest that PACAP plays an important role in modulating cellular growth.¹⁶ In cultured ECL cells, PACAP has also been shown to stimulate the proliferation of ECL cells.

We previously reported that the PAC I is expressed on gastric ECL cells by immunocytochemistry and RT-PCR.¹⁷ In this study, the expression of PAC I was shown by Western Blot analysis, as well as by RT-PCR using specific primers for the PAC I on ECL cells isolated from the rat stomach. In the pure (95%) preparation, identification of PAC I was also demonstrated using a polyclonal antibody directed against the C-terminus of the receptor. Primers for VPAC₁ and VPAC₂ were used and no amplification of product was detected by agarose gel electrophoresis. These results suggested that PAC I expressed on the gastric ECL cells may play an important role in regulating the physiologic functions in the stomach, such as gastric acid secretion and proliferation of the endocrine cell components of the stomach. The identification of PACAP immunoreactivity on gastric enteric neurons and the expression of receptor on specific cellular components make it intriguing to speculate that the neuropeptide PACAP may play an important role in the regulation of gastric function.¹⁸ There has been conflicting data concerning the effects PACAP administration in the regulation of gastric acid secretion. In one study, the central injection of PACAP stimulates acid secretion.¹⁹ Similarly, it has been shown that PACAP stimulates histamine release from ECL cells.^{20–23} Furthermore, these preliminary studies have shown that, not

only is PACAP a potent and effective stimulant of histamine release from ECL cells,^{20,21} but also that it is coupled to the stimulation of intracellular calcium release.²¹ Accordingly, our current study was undertaken to explore the role of PAC I as a molecule that stimulates the ECL cell to signal and stimulate gastric acid secretion and to undergo proliferation in response to exogenously administered PACAP.

MATERIALS AND METHODS

Immunohistochemistry

Paraffin-embedded tissue sections from rat stomach were treated with Triton X-100 to enhance antibody penetration, and incubated with rabbit-derived antibody for 24–48 hours. The antibody peroxidase–antiperoxidase method or the avidin–biotinylated horseradish peroxidase methods were used for immunohistochemistry. Controls consisted of using antibody dilutions that result in loss of immunostaining and nonimmune rabbit antibodies. The immunohistochemical studies were performed in the Histology Core Facility of the Morphology CORE at CURE: VA/UCLA DDRC.

Isolation and Purification of ECL Cells

Rat gastric ECL cells were isolated by a combination of elutriation and density gradient centrifugation as described elsewhere, using a combination of elutriation and density gradient centrifugation obtained from approximately five rat stomach.^{24,25} Cellular purity was determined by using an anti-HDC antibody and immunocytochemistry. Approximately 65% of cells in the isolated cell population were identified as ECL cells. These isolated ECL cells were rinsed by gentle centrifugation in growth medium containing DME/F12 supplemented with 2 mg/ml BSA, 2.5% fetal calf serum (FCS), 100 uM hydrocortisone, 1% penicillin, streptomycin, 5 mg/ml insulin, 5 mg/ml transferrin, and 5 ug/l sodium selenite. Enrichment of the primary culture was performed by plating the cell suspension onto glass coverslips coated with Cell-Tak and incubating the cells at 37°C for 45 min. The ECL cell population was enriched to more than 85% by using these techniques.

Measurement of Intracellular Adenylyl Cyclase

Isolated ECL cells were plated on 24-well culture dishes (Cell-Tak®) with DMEM/10% calf serum in the presence of [³H]-adenine (Amersham, Arlington Heights, IL) at a concentration of 2 mCi/ml. The cells were washed with DMEM, incubated with or without the indicated concentrations of PACAP-38 and PACAP-27 in DMEM containing BSA 1 mg/ml and 2.5 mM 3-isobutyl, 1–1-methyl-xanthine (IBMX) for 30 minutes. and then aspirated. One hundred µl of 2% SDS/1 mM cAMP solution was used to lyse the cells. Adenylyl cyclase was assayed by consecutive Dowex AG-50W-X4 resin (BioRad, Richmond, CA) and aluminum oxide (Sigma, St. Louis, MO) column chromatography using a modification of the procedure described by Salomon. [³H]-cAMP was measured using a liquid scintillation counter (Packard, Downers Grove, IL). Preliminary studies indicate that maximal stimulation occurs following incubation with ligand for 30–60 minutes.

In vivo Measurements of Gastric Acid Secretion

Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), approximately 300 grams in weight, were anesthetized with Inactin®, placed on a heated table to maintain rectal temperature at 37°C and tracheotomized (PE-240) according to established Animal Use Protocols (VA Greater Los Angeles Health Care System, West Los Angeles). Catheters were placed into the femoral veins and laparotomy was performed in order to place a gastric cannula. Following the establishment of stable basal gastric acid secretion, rats received either an infusion of antisomatostatin antibody or sham treatment and the indicated secretagogues (10 nmol/kg/h) using a peristaltic pump (speed, 0.8 ml/h) for two hours from the start of the experiment. Gastric acid output was measured using the titration method with 10 mM NaOH as the titrant.

ECL Proliferation Studies

BrdU incorporation in ECL cells was assessed in Sprague-Dawley rats that received chronic administration of PACAP-38 via osmotic pump for a period of seven days (10 pmol/h). ECL cells were resuspended in growth media in wells containing Cell-Tak and cultured for 24 hours at 37°C. The medium was then aspirated and replaced by growth medium containing BrdU (1: 200 dilution) and PACAP-27 or PACAP-38 in either the presence or absence of PACAP 6–38 for 24 hours. PACAP 6–38 was added at least 10 minutes prior to the mitogens, PACAP-27 or PACAP-38. Gastrin 17–1 (10 nM) was used as a positive control in the assay. Following incubation, the cells were fixed in ethanol and incubated with an anti-BrdU antibody for 60 min at room temperature. After washing, a rabbit antimouse secondary antibody conjugated to peroxidase was applied to the cells. Binding of the secondary antibody was assessed using *o*-phenylenediamine(OPD) dihydrochloride as a peroxidase substrate. Spectrophotometric analysis of the end products (405 nm) was accomplished by means of a 96-well microplate reader.

RESULTS

Immunohistochemistry

Immunohistochemistry was performed by using a polyclonal antibody directed against the C-terminus of the rat PAC I as described elsewhere.¹⁸ In preliminary studies, we showed that the antibody was specific only for the PAC I, but not the VPAC I or VPAC II. Immunostaining of gastric mucosal sections demonstrates specific staining of enterochromaffin-like (ECL) cells and not parietal cells. No neuronal staining was identified. These results confirm previous studies indicating that PAC I can be detected on ECL cells of the stomach.

Adenylyl Cyclase Stimulation

To determine whether PACAP administration of ECL cells results in intracellular signaling, adenylyl cyclase measurements in response to PACAP administration were performed. Isolated ECL cells were exposed to increasing concentrations of PACAP-27. PACAP dose dependently increased the stimulation of cAMP in isolated ECL cells with observable stimulation at 1 nM and a half maximal stimulation at 10 nM. No stimulation was detected

after incubation with VIP at 100 nM. At 100 nM, PACAP-27 resulted in nearly a six-fold stimulation of cAMP. These results indicate that PACAP, but not VIP, is able to stimulate adenylyl cyclase in isolated ECL cells.

Gastric Acid Stimulation by PACAP in Rats

To determine whether PACAP-27 is able to stimulate gastric acid secretion, PACAP was administered to anesthetized rats and gastric output was measured via a gastric cannula. Because, in previous studies, intravenous PACAP had variable effects on gastric acid secretion, a neutralizing antibody to somatostatin was used in the experimental protocol. Following an equilibration period, either an antisomatostatin antibody or vehicle was administered as indicated by the arrow. No significant change in the amount of acid output was observed in either group of rats. Following the administration of PACAP-27, a significant increase in gastric acid output was observed in the group of rats pretreated with the antisomatostatin antibody when compared to the control group. There was an approximately threefold increase in the level of gastric acid secretion in rats receiving both the antisomatostatin antibody and PACAP-27.

Stimulation of ECL Cell Proliferation by PACAP

To determine whether chronic, exogenous administration of PACAP leads to proliferative changes in the ECL cell population, an experimental design was employed in which PACAP-27 was administered to rats *in vivo* for of seven days (10 pmol/h). BRDU incorporation was assessed in rats treated with PACAP compared to sham treated rats. PACAP-27 increased the level of BRDU incorporation in ECL cells that was approximately twofold greater than that in sham control rats. These results indicate that proliferation of ECL cells in rats occur following chronic or acute administration of PACAP-27, as shown by others.³¹

DISCUSSION

Pharmacological and cloning studies have revealed that there are three receptors with high affinity to PACAP-27 and PACAP-38: PAC I, VPAC I and VPAC II. The regulation of gastric acid secretion is complex and involves an interplay between neuronal and paracrine factors. The development of isolation techniques to study the cellular components of this process has permitted a more detailed knowledge to be developed about the processes that regulate gastric acid secretion and cellular proliferation.

Our previous demonstration that receptors for PACAP (PAC I) were expressed on the enterochromaffin-like cells of the stomach provided a foundation from which to further define the signaling of these receptors in isolated cell preparations. The ECL cell is an important regulator of gastric acid secretion, through the release of histamine in response to gastrin and other ligands.²⁶ This study demonstrated that PAC₁ receptors are expressed on gastric ECL cells using the recently developed antibody directed against the C-terminus of the rat PAC I. This antibody permitted the detection of only the expression of PAC I on ECL cells on rat gastric mucosal sections. No staining was present on parietal cells or on neuronal elements. No receptor expression was identified on the circular or longitudinal smooth

muscles of the stomach. These findings are consistent with studies previously performed by our group that demonstrate the expression of PAC I on ECL cells isolated from the fundic mucosa using the same antibody and immunocytochemical techniques. Similarly, we have also demonstrated the expression of PAC I on ECL cells using a fluorescent PACAP ligand. These results are also consistent with the identification of splice variants for the PACAP receptor (data not shown).

The role of PACAP in the physiologic regulation of the stomach has been unclear previously. The neuropeptide, PACAP, is present in the enteric nervous system of the stomach as well as on gastric nerve fibers, and this the possible colocalization of the hormone and receptor near the ECL cell provides an anatomic basis for the theory that PACAP is a central mediator of gastric acid secretion. The experimental data presented in this study not only demonstrates that PAC I is present on ECL cells of the stomach, but that signaling via phospholipase C as well as adenylyl cyclase, leads to activation and release of histamine to stimulate gastric acid secretion. PAC-AP activates adenylyl cyclase on isolated ECL cells in a dose dependant manner. The half maximal stimulation (EC50) is approximately 10 nM, which is similar to the half maximal stimulation observed for the activation of adenylyl cyclase in cells transfected with the rat Type I PACAP receptor.²⁷ These results are also similar to the stimulation that has been reported for the stimulation of intracellular Ca^{2+} on isolated ECL cells using the identical isolation techniques.²¹ Thus, PACAP, as has been demonstrated previously by Deutsch and Sun on PC-12 cells, is capable of activating a dual signaling cascade leading to the activation of both PLC and AC.¹³ The activation of both signaling cascades may have important implications for the ability of PACAP to stimulate secretion and cellular proliferation.

Intravenous administration of PACAP in whole animals has led to conflicting results with respect to gastric acid secretion. We presumed that somatostatin, released from gastric D cells, acts by inhibiting the stimulation of gastric acid secretion that can be accounted for by a dual effect of PACAP administration: stimulation of histamine release from ECL cells and stimulation of somatostatin release from D cells. The net result of this would be no overall change in the stimulated gastric acid secretion. Therefore, a somatostatin neutralizing antibody was used to inhibit the inhibitory effect of somatostatin in the net gastric acid secretion. In the current study, we demonstrated that administration of PACAP-27 resulted in nearly a threefold increase in gastric acid output. The observation that the antisomatostatin could unmask the PACAP stimulatory effect on gastric acid secretion supports the theory that PACAP activates somatostatin release from D cells. Although this study did not specifically address the role of PACAP as a central regulatory of gastric acid secretion, it does support the model that PACAP release in the CNS leads to regulation of gastric acid secretion. Similarly, other neural peptides, such as galanin, are present in the enteric nervous, system suggesting the possibility of interplay between PAC-AP and galanin in mediating the cephalic phase of acid secretion. Unlike PACAP, galanin inhibits ECL cell function, by reducing gastrin-induced calcium signaling and histamine release.^{20,28,29} Similar to our previous report, RT-PCR has been used to suggest that Gal₁ and Gal₂ receptors might be implicated in this ECL cell response.

That PACAP influences tumoral growth was shown previously in the small cell carcinoma cell line and in breast cancer cells.³⁰ These studies have indicated that PACAP stimulates the activation of c-Fos and c-Myc signaling cascades, presumably linked to the activation of PLC and protein kinase C. Recently, using BRDU-incorporation studies, *in vitro*, it was reported that PACAP is a trophic factor for ECL cells.³¹ The use of chronic PACAP administration as a model for studying the effects of PACAP on the proliferation of ECL cells has the advantage of being performed in the whole animal rather than *in vitro*. Thus, in the absence of gastrin stimulation, PACAP may play a significant role in mediating the proliferative response of ECL cells.

Based on these data from both *in vitro* and *in vivo* experiments, the neuropeptide, PACAP, is an important component gastric acid secretion and may be involved in regulation of the proliferative response of ECL cells. In conclusion these results show, for the first time, that PAC I receptors are expressed on tissue sections and are localized to ECL cells. The release of PACAP is coupled to increases in cAMP and to the stimulation of gastric acid output in the whole animal. Furthermore, the chronic administration of PACAP *in vivo* leads to nearly a twofold increase in the proliferation of ECL cells. These studies suggest a significant role for PACAP in regulating gastric physiological function.

ACKNOWLEDGMENTS

This work was supported by NIH DK 52274-01, AGA IRSA, and a VA Career Development Award (JRP), by US VA SMI funds, and by NIH Grants DK40615, 41301 and 17294 (GS).

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