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Expression and Regulation of Pituitary Adenylate Cyclase-Activating Polypeptide in Rat Placental Cells

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first identified as a hypophysiotropic factor that regulates pituitary cell functions and has been subsequently shown to be widely distributed and have multiple functions. The PACAP is known to be expressed in placental tissues and is suggested to have a critical role in physiological function of the placenta. In addition to PACAP, the hypothalamic peptides kisspeptin and gonadotropin-releasing hormone (GnRH) are also expressed in placental cells. In this study, we used primary cultures of placental tissues from rats of 16 to 18 days gestation and examined the regulation and function of PACAP. The PACAP messenger RNA (mRNA) expression and PACAP-immunoreactive cells were detected in primary cultures of rat placental cells. The PACAP mRNA expression in placental cells was upregulated in the presence of the sex steroids estradiol and progesterone; however, their combined treatment failed to enhance their individual effects. When the cells were stimulated with kisspeptin, PACAP mRNA expression was increased. Similarly, GnRH had a stimulatory effect on PACAP expression. Conversely, kisspeptin expression in placental cells was increased by PACAP stimulation, whereas PACAP failed to stimulate GnRH mRNA expression in these cells. Finally, we found that PACAP had a stimulatory effect on human chorionic gonadotropin expression in placental cells. Our current observations suggest that the hypothalamic peptides PACAP, kisspeptin, and GnRH are interrelated and maintain placental functions.

Keywords

PACAP, GnRH, kisspeptin

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated as a novel hypothalamic neuropeptide based on its ability to stimulate cyclic adenosine monophosphate (cAMP) accumulation in anterior pituitary cells.[1] The PACAP is a highly conserved member of the vasoactive intestinal peptide/secretin/glucagon superfamily and is present in 2 bioactive forms, PACAP27 and PACAP38.[1] The PACAP binds to 3 types of receptors, PAC1, VPAC1, and VPAC2.[2] Among these receptors, PAC1 receptor (PAC1R) was identified as the predominant receptor for PACAP and couples mainly with Gs protein to induce rapid cAMP production.[2,3] The PACAP is expressed not only in the central nervous system but also in a variety of peripheral tissues including pituitary, lung, gut, nerve, gonad, and so on, and it exerts several functions on the nervous, immune, cardiac, gastrointestinal, respiratory, and endocrine system.[4] We have previously reported that PACAP exerts its action on gonadotropin-releasing hormone (GnRH)-producing neurons[5] as well as pituitary gonadotrophs.[6] The presence of PACAP and its action in the hypothalamus, pituitary, and gonads suggests its role in the reproductive system. In addition, it has been reported that PACAP or PAC1R knockout female mice have decreased fertility,[7,8] which is in part due to impaired implantation.[9]

The PACAP and its receptor PAC1R are also expressed in human and rat placentas.[10] The PACAP and PAC1R messenger RNAs (mRNAs) are expressed in decidual cells, chorionic vessels, and stromal cells of chorionic villi in rats[11] and stromal cells of stem villi and terminal villi in humans.[12] The physiological roles of PACAP expressed in the placenta are still largely unknown, but a recent study suggests that PACAP has

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roles in a variety of processes such as cell proliferation and angiogenesis in placental trophoblast cells.[13] In human placenta, PACAP expression has been shown to increase as gestation progresses.[12] In rat placenta, PACAP expression in decidual cells disappeared as gestation progressed, but its high expression became evident in chorionic vessels and stromal cells of chorionic villi.[11] These observations suggest that PACAP expression is regulated within placental tissues.

The placenta is an essential organ for fetal growth and the maintenance of pregnancy. Consistent with this notion, the placenta synthesizes growth hormone[14] and several growth factors such as insulin-like growth factor, fibroblast growth factor, and vascular endothelial growth factor.[15,16] In addition, several reports have demonstrated that the hypothalamic neuropeptides corticotropin-releasing hormone,[17] dynorphin A,[18] neurokinin B,[19] GnRH,[20] and kisspeptin[21] play roles in maintaining placental functions.

In this study, we focused on PACAP in placental cells and examined its regulation. Using primary cultures of rat placental tissues obtained from rats at 16 to 18 days of gestation, we examined the relationship between PACAP, GnRH, and kisspeptin within the placental cells.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: fetal bovine serum (FBS; Life Technologies, Carlsbad, California); Dulbecco modified Eagle medium (DMEM), water-soluble β-estradiol, progesterone, GnRH, and penicillin–streptomycin (Sigma-Aldrich Co, St Louis, Missouri); kisspeptin (Kp-10; ANA SPEC, Fremont, California); PACAP38 (Peptide Institute, Osaka, Japan); and DNase I (Promega Co, Madison, Wisconsin).

Placental Cell Culture

Placental tissue was processed to obtain trophoblast primary cultures as described previously.[22,23] Briefly, placental tissue obtained from rats at 16 to 18 days of gestation was cut into small pieces and digested for 30 minutes with 0.25% trypsin and DNase I (300 U/mL). Digested tissue was spun in a Percoll gradient, and the middle layer containing trophoblast cells was cultured in high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin–streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The isolated trophoblast cells were cultured in high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin–streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air.

Immunocytochemistry

The cells were fixed on coverslips by a 10-minute methanol treatment at −20°C. After 10 minutes of dehydration at 25°C, the cells were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. Nonspecific antibody binding was blocked by preincubation with 1% albumin, followed by incubation overnight at 4°C with an anti-PACAP antibody (1:50 dilution; Santa Cruz Biotechnology, Inc, Dallas, Texas). For preabsorption control, PACAP antigen peptide was added together with anti-PACAP antibody. To visualize PACAP, the cells were stained with secondary antibodies using a Histofine SAB-PO (MULTI) Kit (Nichirei Bioscience, Inc, Tokyo, Japan) according to the manufacturer's instructions. Briefly, the biotinylated secondary antibodies were coupled to streptavidin-biotinylated horseradish peroxidase, and PACAP-expressing cells were visualized using diaminobenzidine as the chromogenic substrate. Cell images were obtained with an Olympus BX41 microscope (Tokyo, Japan).

RNA Preparation, Reverse Transcription, Reverse Transcription-Polymerase Chain Reaction, and Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA from placental cells was extracted using TRIzol-S (Life Technologies) according to the manufacturer's instructions. To obtain complementary DNA (cDNA), 1.0 μg total RNA was reverse transcribed using an oligo-dT primer (Promega) and prepared using a First-Strand cDNA Synthesis Kit (Life Technologies). The preparation was supplemented with 10 mmol/L dithiothreitol, 1 mmol/L of each deoxynucleotide, and 200 U RNase inhibitor/human placenta ribonuclease inhibitor (Code No. 2310; Takara Bio, Tokyo, Japan) in a final volume of 10 μL. The reaction was incubated at 37°C for 60 minutes. The reaction mixtures were diluted 20-fold and then amplified by polymerase chain reaction (PCR) using PACAP-specific primers (forward: 5′-GATGTCGCCCACGAAATCCT-3′ and reverse: 5′-GTATGCTATTCGGCGTCCTT-3′). The PCR amplification was performed using the Program Temperature Control System PC-701 (ASTEC, Fukuoka, Japan). Cycles for each amplification consisted of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 40 seconds. The PCR products were resolved by electrophoresis in a 1.0% agarose gel and stained with ethidium bromide. Quantification of PACAP, GnRH, kisspeptin, and human chorionic gonadotropin (hCG) mRNA was obtained through quantitative real-time PCR using an ABI Prism 7000 (Applied Biosystems, Foster City, California) following the manufacturer's protocol (User Bulletin No. 2) and utilizing Universal ProbeLibrary Probes and Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for PACAP (forward: 5′-GATGTCGCCCACGAAATCCT-3′ and reverse: 5′-GTATGCTATTCGGCGTCCTT-3′), kisspeptin (forward: 5′-ATGATCTCGCTGGCTTCTTGG-3′ and reverse: 5′-GGTTCACCACAGGTGCCATTTT-3′), GnRH (forward: 5′-ACTGTGTGTTTGGAAGGCTGC-3′ and reverse: 5′-TTCCAGAGCTCCTCGCAGATC-3′), and hCG (forward: 5′-ACATGGGCATCCAAGGAGCCGCTT-3′ and reverse: 5′-CGCACATCGCGGTAGTTGCACA-3′), the simultaneous measurement of mRNA and glyceraldehyde-3-phosphate dehydrogenase permitted normalization of the amount of cDNA

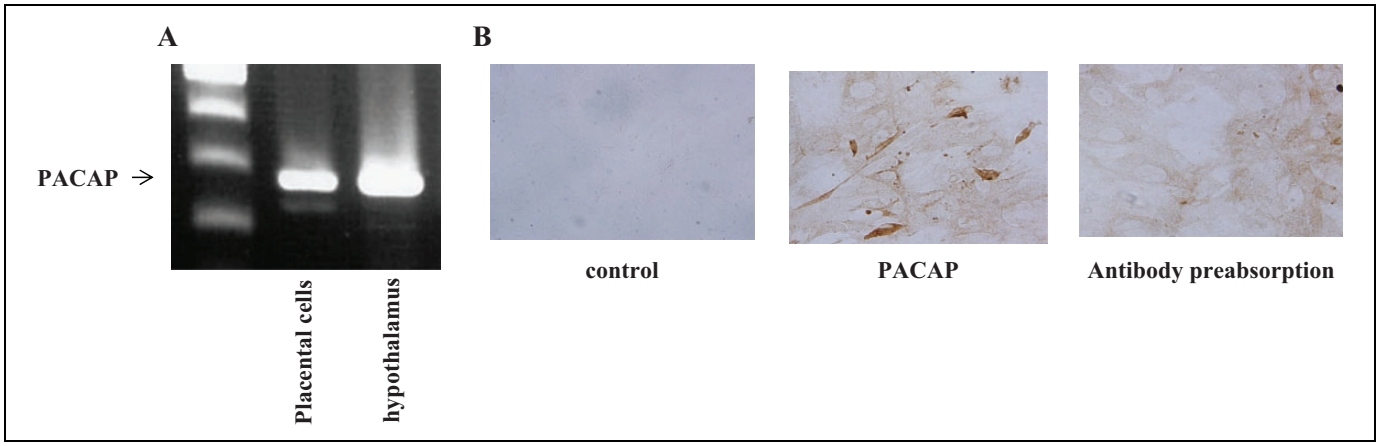

Figure 1. Pituitary adenylate cyclase-activating polypeptide (PACAP) expression in placental cells. A, Total RNA prepared from placental cells from rats at 18 weeks of gestation were reverse-transcribed and reverse transcription-polymerase chain reaction (RT-PCR) was performed using PACAP-specific primers. Hypothalamic extracts were used as a positive control. B, Staining of rat placental cells for PACAP. Cultures of placental cells were immunostained using anti-PACAP antibodies. The negative control (the primary antibody was omitted) is indicated as "control." In "Antibody preabsorption," PACAP antibody was incubated in the presence of PACAP antigen peptide. The color was developed by streptavidin-biotinylated horseradish peroxidase bound to biotinylated primary antibody. Magnification: ×20.

added per sample. For each set of primers, a no-template control was included. The specificity of each assay was confirmed by dissociation curve analysis and agarose gel electrophoresis of the PCR product. Assay performance was validated by assessing amplification efficiencies by means of calibration curves and ensuring that the plot of log input amount versus δCq had a slope with an absolute value of less than 0.1. Thermal cycling conditions were as follows: 10 minutes denaturation at 95°C, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The crossing threshold was determined using PRISM 7000 software, and postamplification data were analyzed using the ΔΔCT method in Microsoft Excel.

Statistical Analysis

All experiments were repeated independently at least 3 times, and each experiment was performed in duplicate for each experimental group. Data are expressed as means ± standard error of the mean. Statistical analysis was performed using 1-way analysis of variance, followed by Newman-Keuls multiple comparison test.

Results

Expression of PACAP in Rat Placental Cells

Primary cultures were produced from rat placental cells collected from rats at 16 to 18 days of gestation. The majority of cells were considered to be trophoblasts according to the protocol used for placental cell collection. The PCR analysis demonstrated that PACAP mRNA was present in primary cultures of rat pituitary cells (Figure 1A). Immunostaining analysis using a specific antibody for PACAP also showed that PACAP-expressing cells were present among these cells (Figure 1B).

Effect of Sex Steroids on PACAP mRNA Expression in Placental Cells

We examined how the expression of PACAP in placental cells is influenced by the sex steroids estradiol and progesterone. After treatment with 10 μmol/L estradiol for 24 hours, PACAP mRNA expression was significantly increased by 2.51 ± 1.39-fold compared to nonstimulated cells. Progesterone also significantly stimulated PACAP mRNA expression. Although a combined treatment with estradiol and progesterone significantly increased PACAP mRNA expression, they failed to potentiate their individual effects (Figure 2).

Expression of PACAP mRNA is Stimulated by Kisspeptin and GnRH

In addition to PACAP expression, the hypothalamic neuropeptides kisspeptin[24] and GnRH[20] are also expressed in placental cells. To examine how these neuropeptides affect placental PACAP expression, placental cells were stimulated with kisspeptin and GnRH. Kisspeptin and GnRH slightly but significantly increased PACAP mRNA expression in placental cells by 1.51 ± 0.20-fold and 1.65 ± 0.05-fold, respectively (Figure 3).

Effect of PACAP on Kisspeptin and GnRH Expression

Conversely, to examine how PACAP affects kisspeptin and GnRH expression, placental cells were stimulated with PACAP. After stimulation of the placental cells with PACAP for 24 hours, kisspeptin mRNA expression was significantly increased by 1.78 ± 0.02-fold (Figure 4A). On the other hand, GnRH mRNA expression was unchanged by PACAP stimulation (Figure 4B).

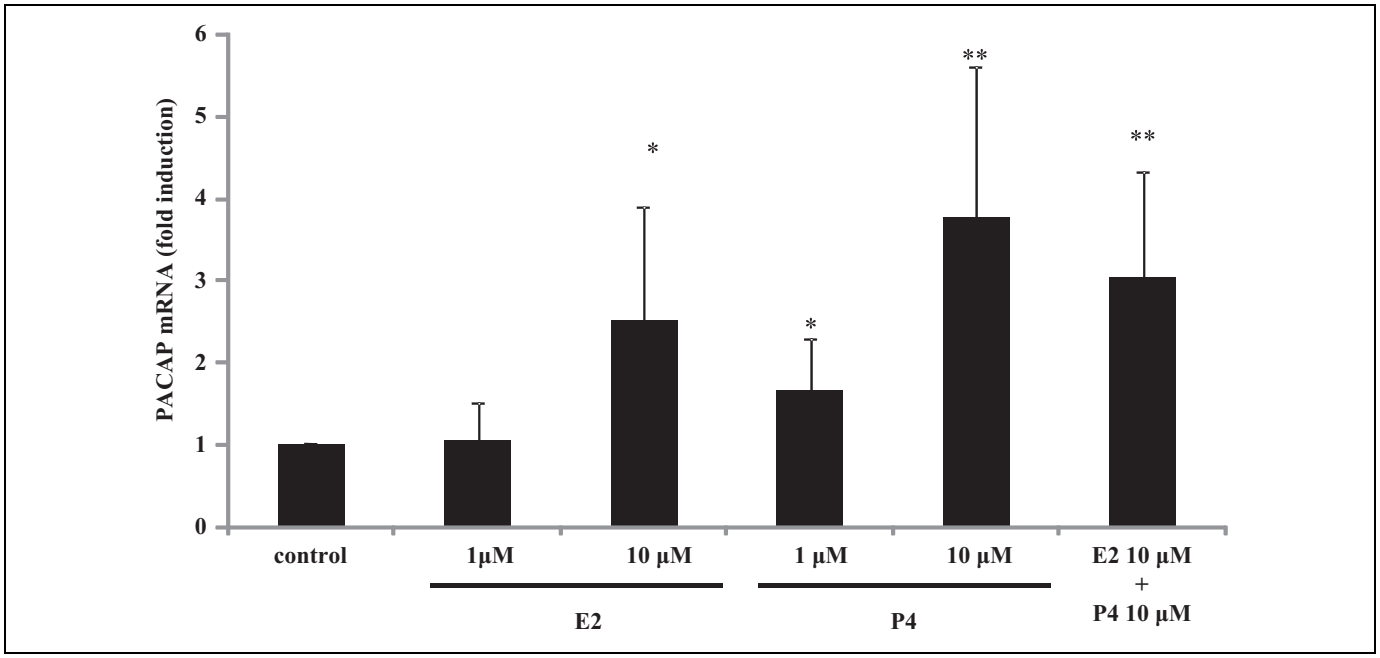

Figure 2. Effect of sex steroids on pituitary adenylate cyclase-activating polypeptide (PACAP) expression in placental cells. Primary cultures of rat placental cells were cultured in the presence or absence (control) of 1 µmol/L or 10 µmol/L estradiol (E2) and progesterone (P4) for 24 hours. Then, PACAP messenger RNA (mRNA) levels were measured by quantitative real-time polymerase chain reaction (PCR) after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated group/control. Values are the means ± standard error of the mean (SEM) of fold stimulation from 3 independent experiments. $^{**}P < .01$, $^{*}P < .05$ versus control.

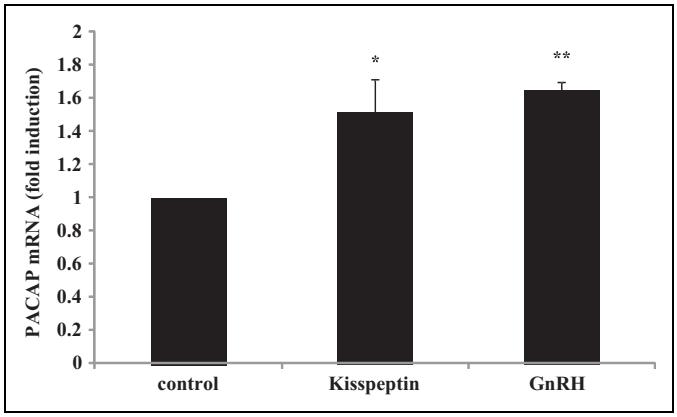

Figure 3. Effect of kisspeptin and gonadotropin-releasing hormone (GnRH) on pituitary adenylate cyclase-activating polypeptide (PACAP) messenger RNA (mRNA) expression in placental cells. Primary cultures of rat placental cells were treated with 100 nmol/L kisspeptin or gonadotropin-releasing hormone (GnRH) for 24 hours. Then, PACAP mRNA levels were measured by quantitative real-time polymerase chain reaction (PCR) after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated group/control. Values are the means ± standard error of the mean (SEM) of fold stimulation from 3 independent experiments. $^{**}P < .01$, $^{*}P < .05$ versus control.

The PACAP Stimulates hCG Expression in Placental Cells

The PACAP itself stimulated the expression of hCG mRNA in placental cells. The PACAP increased the level of hCG mRNA up to 1.29 ± 0.08-fold, which was statistically significant (Figure 5).

Discussion

Several reports have shown evidence for the expression of PACAP and its PAC1R in the placenta, but few studies have examined the role of PACAP in placental tissues. Because many cell types exist in the placenta, including trophoblasts, fibroblasts, muscle cells, vascular endothelial cells, neurons, and so on, several studies have used immortalized choriocarcinoma cells or invasive proliferative extravillous cytotrophoblast cell lines as models for placental cells. In this study, we used primary cultures of rat placental cells, which were obtained according to methods that lead to a relatively pure population of placental trophoblasts.[22,23] Using these placental cells, we planned to evaluate how PACAP is regulated and ascertain its function in the placenta.

After the identification of PACAP and its PAC1R in primary cultures of rat placental cells, we first examined the effects of sex steroids on PACACP expression in placental

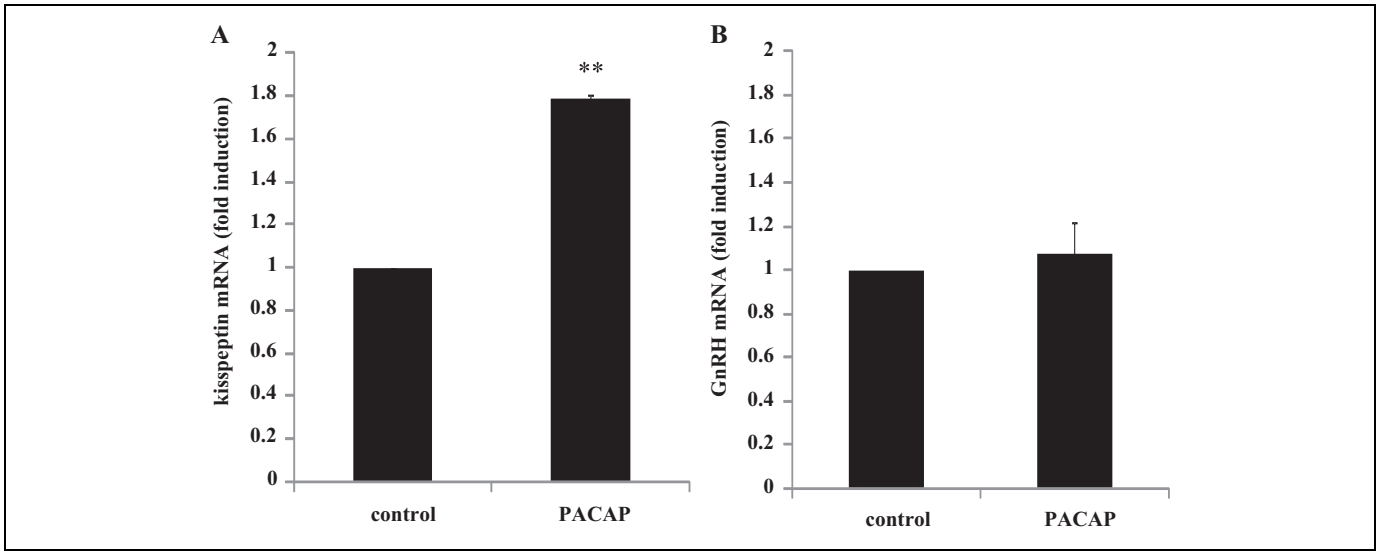

Figure 4. Effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on kisspeptin and gonadotropin-releasing hormone (GnRH) messenger RNA (mRNA) expression in placental cells. Primary cultures of rat placental cells were treated with PACAP (100 nmol/L) for 24 hours. Then, mRNA levels for kisspeptin (A) and gonadotropin-releasing hormone (GnRH; B) were measured by quantitative real-time polymerase chain reaction (PCR) after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Results are expressed as fold stimulation over the unstimulated group/control. Values are the means $\pm$ standard error of the mean (SEM) of fold stimulation from 3 independent experiments. $^{**}P < 0.01$ versus control.

cells. Both estradiol and progesterone increased PACAP mRNA expression in placental cells, and the stimulatory effect of progesterone was greater than that of estradiol (Figure 2). Previous studies have demonstrated that PACAP expression increases in the placenta during pregnancy.[11,25] Because serum levels of sex steroids increase during pregnancy, it is plausible that PACAP expression is increased as gestation progresses. Combined stimulation with estradiol and progesterone, however, failed to potentiate their individual effects. These observations suggest that these sex steroids individually maintain PACAP expression.

In this series of experiments, we examined the effect of kisspeptin and GnRH on PACAP expression. Kisspeptin, which was previously known as metastin, was first identified in the placenta as a product of the metastasis suppressor gene *Kiss-1*.[21] Subsequently, identification of the obligate role of kisspeptin in human puberty enhanced our understanding of the neuroendocrine regulation of reproduction.[26,27] Currently, it is generally agreed that kisspeptin expressed in the hypothalamus acts upstream of GnRH. In short, kisspeptin is a potent stimulator of the hypothalamic–pituitary–gonadal (HPG) axis that directly acts on GnRH neurons through the kisspeptin receptor. The GnRH is subsequently released into the portal circulation, which in turn stimulates the secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary.[28] Both LH and FSH coordinately act on the gonads to stimulate follicular growth, maturation of oocytes, and sex steroid synthesis. Kisspeptin and GnRH, both of which play pivotal roles in the HPG axis, also exist in the placenta. Kisspeptin has been reported to be expressed in the syncytiotrophoblast and is believed to negatively control trophoblast invasion of maternal sites.[29] It has been hypothesized that GnRH produced within the placenta might be involved in the autocrine/paracrine regulation of hCG synthesis.[30,31] In our experiments, both kisspeptin and GnRH stimulated the expression of PACAP in placental cells (Figure 3). These observations suggest that players in the HPG axis could maintain PACAP expression levels in the placenta. Interestingly, kisspeptin, but not GnRH expression, was increased by PACAP (Figure 4), indicating that kisspeptin and PACAP share an autocrine loop that maintains their individual expression levels in placental cells. In addition, the observation that PACAP increased hCG mRNA expression supports the hypothesis that both kisspeptin and GnRH indirectly maintain hCG expression by augmenting the expression of PACAP.

We have previously reported that kisspeptin and GnRH expressed in placental cells are upregulated by estradiol. In addition, we found that both kisspeptin and GnRH could increase placental hCG expression (manuscript in submission). Considering previous and current observations, it is plausible that the hypothalamic peptides PACAP, kisspeptin, and GnRH expressed in placental cells are under the regulation of estradiol and maintain hCG expression by mutual association in a paracrine manner.

It is obvious that kisspeptin and GnRH are gatekeepers of reproduction, in which kisspeptin neurons activate GnRH neurons in the hypothalamus.[32] In addition, mounting evidence shows the involvement of PACAP in the central control of reproductive function. Intravenous administration of PACAP inhibits gonadotropin release mediated through corticotropin-releasing

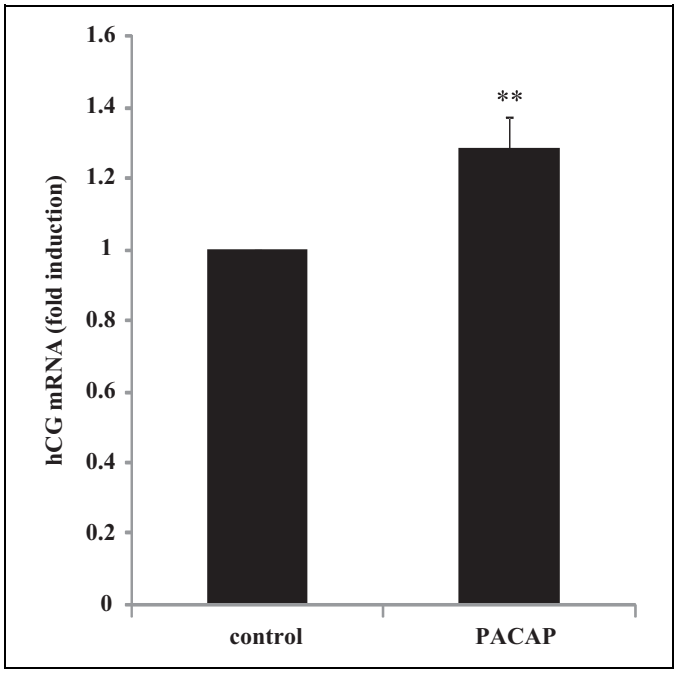

Figure 5. Human chorionic gonadotrophin (hCG) messenger RNA (mRNA) expression by pituitary adenylate cyclase-activating polypeptide (PACAP) stimulation. Primary cultures of rat placental cells were cultured in the presence or absence (control) of PACAP (100 nmol/L) for 24 hours. Then, hCG mRNA levels were measured by quantitative real-time polymerase chain reaction (PCR) after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Results are expressed as fold stimulation over the unstimulated group/control. Values are the means $\pm$ standard error of the mean (SEM) of fold stimulation from 3 independent experiments. $^{**}P < .01$ versus control.

factor and endogenous opioids. In addition, PACAP administration to neonatal female rats was reported to delay puberty.[33] Moreover, defects of PACAP or its receptor have been reported to impair reproductive function in female mice.[7,34]

In this study, we examined PACAP and its regulation in primary cultures of rat placental cells. The PACAP was found to be expressed in placental cells and upregulated by sex steroids. Pivotal players in the HPG axis, kisspeptin and GnRH, stimulated the expression of PACAP, which was found to stimulate expression of kisspeptin as well as hCG. Taken together, our results suggest that these neuropeptides work cooperatively within the placenta to maintain placental functions.

Declaration of Conflicting Interests

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References

1. Miyata A, Arimura A, Dahl RR, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun*. 1989; 164(1):567-574.
2. Harmar AJ, Fahrenkrug J, Gozes I, et al. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR review 1. *Br J Pharmacol*. 2012;166(1):4-17.
3. Spengler D, Waeber C, Pantaloni C, et al. Differential signal transduction by five splice variants of the PACAP receptor. *Nature*. 1993;365(6442):170-175.
4. Vaudry D, Falluel-Morel A, Bourgault S, et al. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev*. 2009;61(3):283-357.
5. Kanasaki H, Mijiddorj T, Sukhbaatar U, Oride A, Miyazaki K. Pituitary adenylate cyclase-activating polypeptide (PACAP) increases expression of the gonadotropin-releasing hormone (GnRH) receptor in GnRH-producing GT1-7 cells overexpressing PACAP type I receptor. *Gen Comp Endocrinol*. 2013;193:95-102.
6. Kanasaki H, Oride A, Kyo S. Role of pituitary adenylate cyclase-activating polypeptide in modulating hypothalamus-pituitary neuroendocrine functions in mouse cell models. *J Neuroendocrinol*. 2015;27(1):1-7.
7. Jamen F, Rodriguez-Henche N, Pralong F, et al. PAC1 null females display decreased fertility. *Ann N Y Acad Sci*. 2000; 921:400-404.
8. Reglodi D, Kiss P, Szabadfi K, et al. PACAP is an endogenous protective factor-insights from PACAP-deficient mice. *J Mol Neurosci*. 2012;48(3):482-492.
9. Isaac ER, Sherwood NM. Pituitary adenylate cyclase-activating polypeptide (PACAP) is important for embryo implantation in mice. *Mol Cell Endocrinol*. 2008;280(1-2):13-19.
10. Scaldaferri ML, Modesti A, Palumbo C, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP-receptor type 1 expression in rat and human placenta. *Endocrinology*. 2000;141(3):1158-1167.
11. Koh PO, Kwak SD, Kim HJ, et al. Expression patterns of pituitary adenylate cyclase activating polypeptide and its type I receptor mRNAs in the rat placenta. *Mol Reprod Dev*. 2003;64(1):27-31.
12. Koh PO, Won CK, Noh HS, Cho GJ, Choi WS. Expression of pituitary adenylate cyclase activating polypeptide and its type I receptor mRNAs in human placenta. *J Vet Sci*. 2005;6(1):1-5.
13. Horvath G, Reglodi D, Brubel R, et al. Investigation of the possible functions of PACAP in human trophoblast cells. *J Mol Neurosci*. 2014;54(3):320-330.
14. Ogilvie S, Buhi WC, Olson JA, Shiverick KT. Identification of a novel family of growth hormone-related proteins secreted by rat placenta. *Endocrinology*. 1990;126(6):3271-3273.
15. Correia-da-Silva G, Bell SC, Pringle JH, Teixeira N. Expression of mRNA encoding insulin-like growth factors I and II by uterine tissues and placenta during pregnancy in the rat. *Mol Reprod Dev*. 1999, 53(3):294-305.

16. Srivastava RK, Gu Y, Ayloo S, Zilberstein M, Gibori G. Developmental expression and regulation of basic fibroblast growth factor and vascular endothelial growth factor in rat decidua and in a decidual cell line. *J Mol Endocrinol*. 1998; 21(3):355-362.
17. Emanuel RL, Robinson BG, Seely EW, et al. Corticotrophin releasing hormone levels in human plasma and amniotic fluid during gestation. *Clin Endocrinol (Oxf)*. 1994;40(2):257-262.
18. Agbas A, Ahmed MS, Millington W, et al. Dynorphin A(1-8) in human placenta: amino acid sequence determined by tandem mass spectrometry. *Peptides*. 1995;16(4):623-627.
19. Torricelli M, Giovannelli A, Leucci E, et al. Placental neurokinin B mRNA expression increases at preterm labor. *Placenta*. 2007; 28(10):1020-1023.
20. Khodr GS, Siler-Khodr TM. Placental luteinizing hormone-releasing factor and its synthesis. *Science*. 1980;207(4428): 315-317.
21. Ohtaki T, Shintani Y, Honda S, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature*. 2001;411(6837):613-617.
22. Pennington KA, Schlitt JM, Schulz LC. Isolation of primary mouse trophoblast cells and trophoblast invasion assay. *J Vis Exp*. 2012;8(59):e3202.
23. Wu X, Jin LP, Yuan MM, Zhu Y, Wang MY, Li DJ. Human first-trimester trophoblast cells recruit CD56brightCD16- NK cells into decidua by way of expressing and secreting of CXCL12/ stromal cell-derived factor 1. *J Immunol*. 2005;175(1):61-68.
24. Hiden U, Bilban M, Knöfler M, Desoye G. Kisspeptins and the placenta: regulation of trophoblast invasion. *Rev Endocr Metab Disord*. 2007;8(1):31-39.
25. Brubel R, Boronkai A, Reglodi D. Changes in the expression of pituitary adenylate cyclase-activating polypeptide in the human placenta during pregnancy and its effects on the survival of JAR choriocarcinoma cells. *J Mol Neurosci*. 2010;42(3):450-458.
26. Seminara SB, Messager S, Chatzidaki EE, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med*. 2003;349(17):1614-1627.
27. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A*. 2003;100(19):10972-10976.
28. Skorupskaite K, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. *Hum Reprod Update*. 2014;20(4):485-500.
29. Bilban M, Ghaffari-Tabrizi N, Hintermann E, et al. Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. *J Cell Sci*. 2004; 117(Pt 8):1319-1328.
30. Currie WD, Steele GL, Yuen BH, Kordon C, Gautron JP, Leung PC. LHRH- and (hydroxyproline9) LHRH-stimulated hCG secretion from perifused first-trimester placental cells. *Recent Prog Horm Res*. 1993;48:505-509.
31. Siler-Khodr TM, Khodr GS, Valenzuela G, Rhode J. Gonadotropin-releasing hormone effects on placental hormones during gestation: II. Progesterone, estrone, estradiol and estriol. *Biol Reprod*. 1986;34(2):255-264.
32. Roa J, Castellano JM, Navarro VM, Handelsman DJ, Pinilla L, Tena-Sempere M. Kisspeptins and the control of gonadotropin secretion in male and female rodents. *Peptides*. 2009;30(1):57-66.
33. Koves K, Kántor O, Molnár J, et al. The role of PACAP in gonadotropic hormone secretion at hypothalamic and pituitary levels. *J Mol Neurosci*. 2003;20(2):141-152.
34. Shintani N, Mori W, Hashimoto H, et al. Defects in reproductive functions in PACAP-deficient female mice. *Regul Pept*. 2002; 109(1-3):45-48.