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Luteinizing Hormone-stimulated Pituitary Adenylate Cyclase-activating Polypeptide System and its Role in Progesterone Production in Human Luteinized Granulosa Cells

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Abstract. The present study examined the gonadotropin regulation of pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP type I receptor (PAC_1-R) expression, and its role in progesterone production in the human luteinized granulosa cells. The stimulation of both *PACAP* and *PAC_1-R* mRNA levels by LH was detected using a competitive reverse transcription-polymerase chain reaction (RT-PCR). *PACAP* transcript was stimulated by LH reaching maximum levels at 12 hours in a dose dependent manner. LH treatment also stimulated *PAC_1-R* mRNA levels within 24 hours. Addition of PACAP-38 (10^{-7} M) as well as LH significantly stimulated progesterone production during 48 hours culture. Furthermore, co-treatment with PACAP antagonist partially inhibited LH-stimulated progesterone production. Treatment with vasoactive intestinal peptide, however, did not affect progesterone production. Taken together, the present study demonstrates that LH causes a transient stimulation of PACAP and PAC_1-R expression and that PACAP stimulates progesterone production in the human luteinized granulosa cells, suggesting a possible role of PACAP as a local ovarian regulator in luteinization.

Key words: PACAP, PAC_1-R, LH/hCG, *In vitro* fertilization, Progesterone

PITUITARY adenylate cyclase-activating polypeptide (PACAP) is originally isolated from the ovine hypothalamus and considered a pleiotropic neuroendocrine polypeptide with a widespread distribution [1, 2]. PACAP exists in two biologically active amidated forms derived from a single precursor protein, PACAP-38 and a COOH-terminally truncated form PACAP-27 [1]. On the basis of sequence similarity, PACAP is the latest member of the secretin/glucagons/vasoactive intestinal peptide (VIP) family of peptides [3]. PACAP exerts its action by binding to three types of G-protein-coupled seven transmembrane PACAP receptors: PACAP type I receptor (PAC_1-R), which preferentially binds to PACAP and with lower affinity to VIP [4, 5], and two others ($VPAC_1$-R and $VPAC_2$-R), which bind to PACAP and VIP with equally high affinity [5, 6]. PAC_1-R is coupled to both the adenylate cyclase and phospholipase C pathways while $VPAC_1$-R and $VPAC_2$-R are coupled only to the adenylate cyclase pathway [6]. Studies of tissue distribution have shown that both PACAP and its receptors are present not only in the central nervous system but also in a variety of peripheral tissues such as lung, testis, adrenal, and ovary [7, 8], suggesting that PACAP may not play an exclusively neuroendocrine role. Indeed, PACAP and PAC_1-R are induced by LH/hCG in preovulatory follicles [9-12], and they play a role in immature follicle growth [13] and luteinization in rodents [14].

Several lines of evidence support for the role of PACAP in LH/hCG-induced acute periovulatory progesterone production and subsequent luteinization

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during the ovulatory process in rodents. The spatiotemporal expression of PACAP coincides with high expression of P450 side chain cleavage (P450scc) and the steroiogenic acute regulatory protein (StAR), enzymes from the periovulatory cascade involved in progesterone production [14-16]. Furthermore, PACAP is a potent stimulator of cyclic adenosine monophosphate (cAMP) and progesterone production in preovulatory follicles of rodent ovary [14, 17, 18], an action it shares with LH/hCG. Indeed, inclusion of neutralizing PACAP antibody to rat granulosa/lutein cells causes the impaired luteinization [14]. The lack of LH/HCG-stimulated PAC_1-R expression and PACAP-stimulated progesterone production has recently been reported in human luteinized granulosa cells [19]. The present study demonstrated the stimulation of both PACAP and PAC_1-R expression by LH/hCG using competitive RT-PCR method and of progesterone production by PACAP in human luteinized granulosa cells.

Materials and Methods

Patients and culture of human luteinized granulosa cells

The use of human luteinized granulosa cells *in vitro* was approved by the Institutional Review Board of the Chonnam National University Hospital. Human luteinized granulosa cells were obtained from patients (25-42 year-old) undergoing an *in vitro* fertilization program. These women underwent a standard ovarian stimulation regimen involving pretreatment with GnRH agonist using a long or short protocol in combination with recombinant FSH and HMG followed by the administration with 10,000 IU hCG 34-36 hours before follicle retrieval. The number of retrieved oocytes was varied from 7 to 30.

Luteinized granulosa cells were obtained from follicular aspirates during oocyte retrieval and rinsed three times in prewarmed Ham's F-10 medium (GibcoBRL, Grand Island, NY). Cells were layered onto 0.5 mL Ficoll plaque (Amersham, Arlington Heights, IL) and centrifuged at 15,800 × g for 15 min to remove red blood cells. The granulosa cell layer was washed twice with 5-10 mL fresh DMEM/F-12 (GibcoBRL) containing 10% fetal bovine serum (FBS) (GibcoBRL) and centrifuged for 1 min at 15,800 × g. The granulosa cell pellet was dispersed by gentle pipetting in 1 mL culture medium containing 0.1% collagenase (Sigma Chemical Co., St. Loius, MO). The dispersed cells were centrifuged at 15,800 × g for 1 min, and cell pellet was rinsed twice in DMEM/F-12 medium. The viable cells were counted, pooled from 2-3 patients for each experiment and seeded at a density of 10^6 cells in 35 mm plastic culture dish (Falcon, Lincoln Park, NJ), or 2×10^5 cells in 24-well culture dish (Corning, Cambridge, MA) in DMEM/F-12 containing 10% FBS. Cells were allowed to adhere for 24 hours at 37°C in a humidified atmosphere of 5% CO_2-95% air. Cells were then rinsed twice to discard degenerated cells and remaining red blood cells, and cultured in serum-free DMEM/F-12 supplemented with 0.1% bovine serum albumin (wt/vol, Fraction V, Sigma) for up to 48 hours in the presence of ovine LH (NIDDK, NIH, Baltimore, MD), or PACAP-38, PACAP(6-38) and VIP (Bachem, Torrance, CA).

Construction of the native (target) and mutant (competitive) cDNA for hPACAP and hPAC$_1$-R

Using an internal primer pair based on the human *PACAP* cDNA sequence [20], a 226-bp fragment of native human *PACAP* was obtained by PCR amplification from human testis RNA (Fig. 1A). The PCR product was cloned into pGMTeasy vector (Promega Corp., Madison, WI) and confirmed by sequencing. Because there were no proper sites for restriction enzymes to generate mutant cDNA within a 226-bp fragment of native *PACAP* cDNA, cDNA of the neuroblastoma cell line SY-5Y was used to generate mutant cDNA using the identical primers. Following the first and second PCR reactions, fragments ranging 450-550 bps were subcloned into pGMTeasy vector and a 539-bp fragment was used for mutant human *PACAP* cDNA.

Using an internal primer pair based on the human *PAC$_1$-R* cDNA sequence [21], a 443-bp fragment of native human *PAC$_1$-R* was obtained by PCR amplification from human testis RNA (Fig. 1B). The mutant competitor cDNA for human *PAC$_1$-R* was generated by digesting the subcloned fragment of a 443-bp native human *PAC$_1$-R* cDNA with Hinc II and Ban II, resulting in the 223-bp mutant cDNA fragment, a 210-bp deleted cDNA of native clone. As a result, the mutant competitor retained the identical primer binding sites as the native human *PAC$_1$-R* cDNA.

Because identical primer sets were used both native and mutant human *PACAP* and *PAC$_1$-R* cDNA, mutant cDNA is suitable for an internal control for the competition.

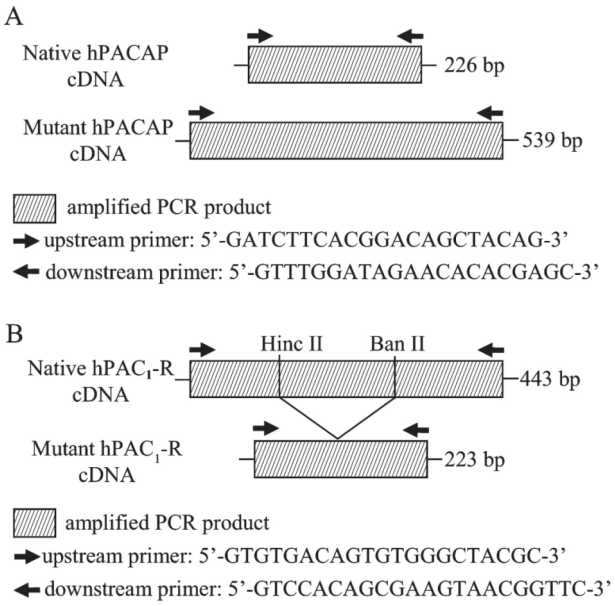

Fig. 1. Schematic diagram showing the cloning of native and mutant hPACAP (A) and $hPAC_1$-R cDNA (B). Mutant hPACAP cDNA was obtained by amplifying the neuroblastoma cell line SY-5Y cDNA using the same primers for the amplification of native hPACAP cDNA. Mutant $hPAC_1$-R cDNA was obtained by cutting native $hPAC_1$-R cDNA with Hinc II and Ban II, and by ligating with T4 ligase. Because both native and mutant cDNAs use identical primer sets, mutant cDNAs are suitable for an internal control.

Construction of standard curve for competitive RT-PCR

A constant amount of competitor mutant cRNA was added into a reaction mixture containing 3 µg random hexamer, 20 units RNase inhibitor, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 5 mM dNTP, and 200 U MML-V reverse transcriptase. This reaction mixture was then dispensed into 0.2 mL thin wall PCR tubes and known amounts of native RNA in 1 µL of RNase-free water were added individually to each tube. Twenty microliter of RT mixture was then overlaid by 50 µL light mineral oil (Sigma Chemical Co., St. Loius, MO) and RT reaction was carried out at room temperature for 10 min and at 42°C for 60 min followed by heating to 75°C for 15 min and final cooling to 4°C. Subsequently, RT products (1 µL) were added to 49 µL of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 15 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U Taq polymerase and 10 pmol of primer. To prevent hybrid formation of native and mutant *PAC_1-R* cRNA, PCR amplification was carried out with a two-step procedure. The first step was performed with denaturation at 94°C for 40 sec, primer annealing at 62°C and 64°C for 40 sec and 1 min, and primer extension at 72°C for 40 sec. The second step was performed with denaturation of only hybrid at 86°C for 1 min, primer annealing at 62°C and 64°C for 1 min, and primer extension at 72°C for 1 min.

Ten microliter aliquots of amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining under UV light. Quantitation was performed using a visual light densitometer (model 620, Bio-Rad Laboratories, Inc). A ratio was calculated for the intensity of native *versus* mutant bands on each lane of the gels. The logarithmic ratio of native to mutant was plotted against the logarithmic initial amounts of native to produce the standard curve.

Quantification of hPACAP and $hPAC_1$-R mRNA levels in human luteinized granulosa cells

Total RNA was extracted from cultured human luteinized granulosa cells using Tri Reagent Solution (Molecular Research Center Inc., Cincinnati, OH). Two micrograms of total RNA were subjected to RT. One microliter of RT was added to 49 µL of PCR mixture containing a constant amount of mutant cRNA. PCR amplification was carried out in 50 µL reaction containing 2.5 U of Taq polymerase (Takara Co. Ltd., Shiga, Japan), 0.2 mM dNTP, and 10 pmol of sense and antisense primer. Ten microliter aliquots of amplified PCR products were separated by gel electrophoresis, visualized under UV light and quantitated using a densitometer. A ratio was calculated for the intensity of native *versus* mutant bands on each lane of the gels and concentrations were obtained by comparison to the standard curve.

Radioimmunoassay

Progesterone concentrations in media were measured by radioimmunoassay without further extraction using [1,2,6,7-^{3}H] progesterone (99 Ci/mmol, Amersham, Buckinghamshire, England) and antibody against progesterone (provided by Dr. H.B. Kwon, Chonnam National University, Kwangju, Korea) as previously described [22]. Between and within assay coefficients of variation were 9.4% and 9.2%, respectively.

Data analysis

The amount of human *PACAP* and *PAC_1-R* transcript was calculated based on the ratio of the target

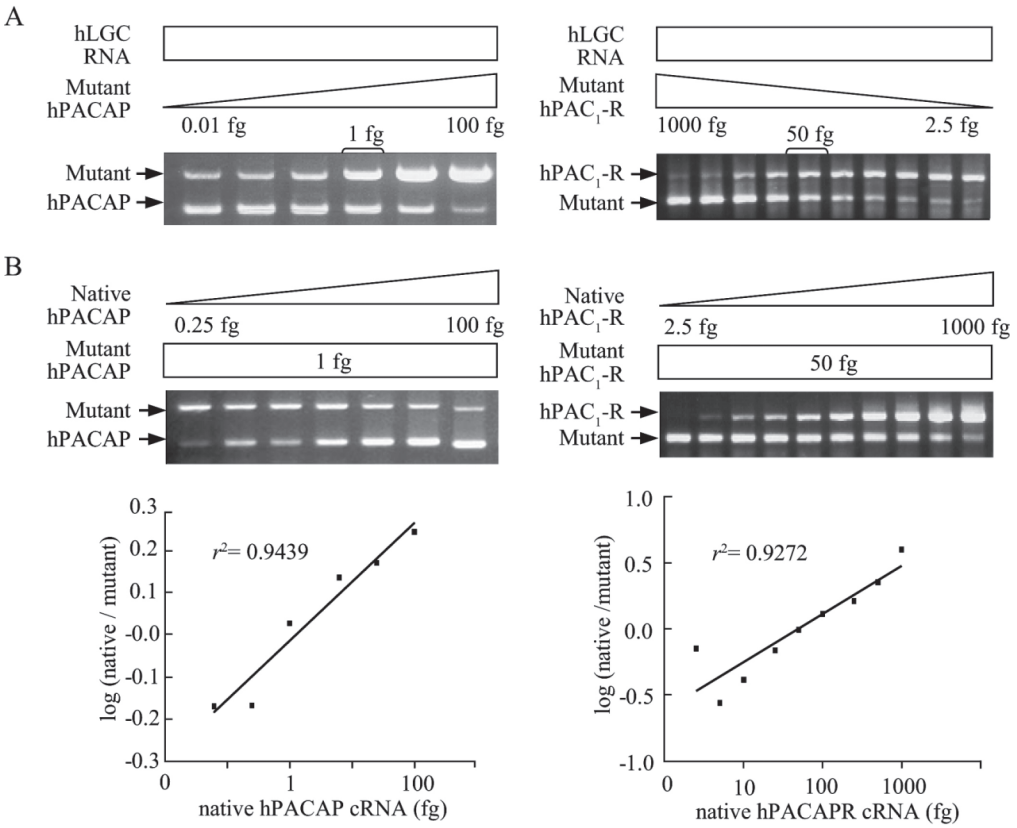

Fig. 2. Titration (A) and standard curve (B) of competitive RT-PCR for hPACAP and $hPAC_1$-R transcript in human luteinized granulosa cells. (A) A constant amount of mRNA from human luteinized granulosa cells was coamplified with a serial dilution of mutant cRNA of hPACAP and $hPAC_1$-R (0.01-100 fg and 2.5-1000 fg respectively). Thirty five cycles of PCR are used for amplification of RNA from human luteinized granulosa cells. (B) A constant amount of mutant hPACAP (1 fg) or $hPAC_1$-R cRNA (50 fg) was coamplified with increasing concentrations of native hPACAP or $hPAC_1$-R cRNA. PCR product was separated on 1.5% agarose gel. A linear relationship was observed when the ratio of native to mutant signals was plotted against different amounts of respective native cRNAs.

to competitive cRNA. Data depicted as the mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (PRISM GraphPad version 2). A value of $P < 0.05$ was considered statistically significant.

Results

Validation of competitive RT-PCR for hPACAP and $hPAC_1$-R transcript

Human *PACAP* and *PAC_1-R* mRNA levels were analyzed using competitive RT-PCR. To determine the competition range between biological RNA samples and mutant cRNA, RNA extracted from human luteinized granulosa cells was reverse transcribed and subsequently coamplified with serial dilutions of mutant cRNA (Fig. 2A). This titration experiment indicated that 1 fg of mutant *hPACAP* cRNA and 50 fg of mutant *$hPAC_1$-R* cRNA were optimal to compete with the respective native cRNA for the measurement of mRNA levels in human luteinized granulosa cells. Based on titration result, a standard curve of RT-PCR for *hPACAP* and *$hPAC_1$-R* was constructed by coamplification of a constant amount of mutant cRNA with serial dilutions of the native cRNA (Fig. 2B). When the different amounts of native cRNA were plotted as a function of the ratio of native to mutant signals, a linear relationship were observed with a dilution range of 0.25-100 fg for *PACAP* and 2.5-1000 fg for *PAC_1-R*.

Stimulation of hPACAP and $hPAC_1$-R mRNA levels by LH in human luteinized granulosa cells

Using the competitive RT-PCR analysis, the effect of LH on mRNA levels of human *PACAP* and

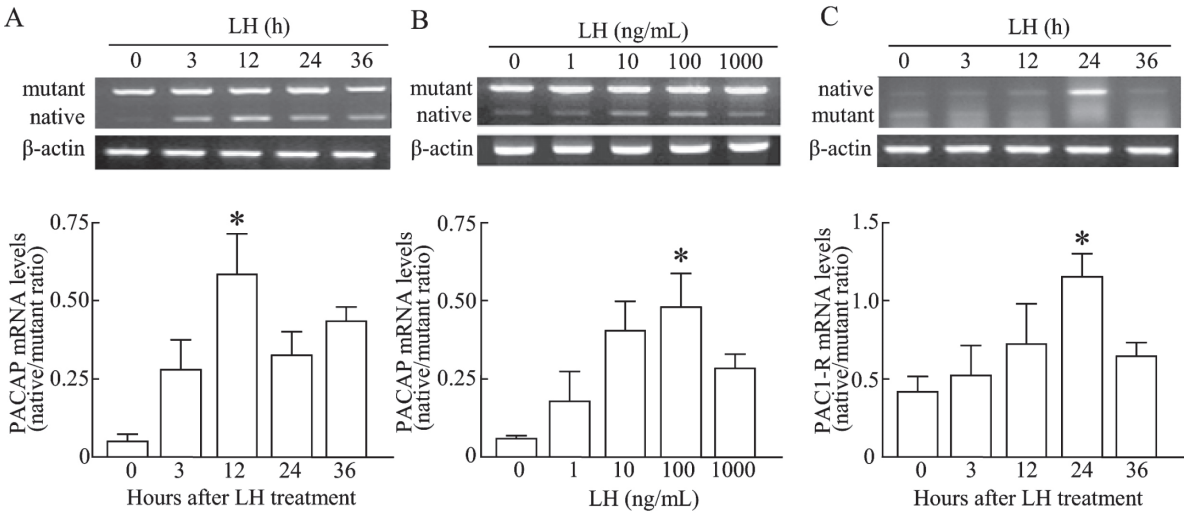

Fig. 3. Time and dose-dependant stimulation of hPACAP (A and B) and hPAC$_1$-R mRNA levels (C). Human luteinized granulosa cells were pooled from 2-3 patients for each experiment and precultured for 1 day in 10% fetal bovine serum. Cells were then treated in serum-free media with 100 ng LH for up to 36 h, or treated with different doses of LH. Two micrograms of total RNA from each cultured cells were assigned for the determination of mRNA levels by competitive RT-PCR. Transcript levels were normalized against β-actin mRNA levels. Each data point represents the mean ± SEM from three (for A and B) or four (for C) independent cultures. *, $P < 0.05$, compared with the value obtained from cells cultured without LH (0 hour or 0 ng/mL).

hPAC$_1$-R was examined in luteinized granulosa cells cultured in serum-free conditions. LH treatment increased human *PACAP* mRNA levels, reaching a maximum induction at 12 hours (Fig. 3A; 11.9-fold increase) ($P < 0.01$) and then declined during 24-36 hours culture. As shown in Fig. 3B, LH treatment for 12 hours stimulated *PACAP* mRNA levels in a dose-responsive manner. A 9.4-fold increase ($P < 0.05$) in *PACAP* mRNA levels was observed with a dose of 100 ng/mL of LH. Treatment with 1000 ng/mL LH, however, exerted a less stimulatory effect than that observed with 100 ng/mL of LH. Similarly, as observed in *PACAP* expressions, *PAC$_1$-R* mRNA levels increased with time in culture by 100 ng/mL of LH (Fig. 3C). A maximum stimulation (2.7-fold) was detected at 24 hours and then declined to control values by 36 hours after treatment.

Effect of PACAP on progesterone production

PACAP is known to stimulate steroid production in immature [23] and mature follicles in rodent ovary [9]. To test if PACAP-38 also stimulates steroid production in human, luteinized granulosa cells were cultured in serum-free conditions, and progesterone concentrations in media were measured. As shown in Fig. 4A, progesterone production was not increased by LH or PACAP-38 during the 24 hours culture of human luteinized granulosa cells. During the 48 hours culture, however, progesterone production was significantly stimulated by treatment with 10^{-7} M PACAP-38 ($P < 0.05$) as well as with LH. Co-treatment with LH (200 ng/mL) and PACAP (10^{-7} M) did not show the synergistic action. Addition of PACAP(6-38), a PAC$_1$-R antagonist, partially suppressed the stimulatory action of LH on progesterone production (Fig. 4B). PAC$_1$-R antagonist alone had no effect. In contrast to the stimulatory action of PACAP, treatment with VIP (10^{-7} M) had a marginal stimulatory effect on progesterone production. Co-treatment with LH and VIP did not have the synergistic effect.

Discussion

The present study demonstrates the transient stimulation of *PACAP* and *PAC$_1$-R* mRNA levels by LH/hCG and the increased production of progesterone by PACAP in human luteinized granulosa cells. The expression of both *PACAP* and *PAC$_1$-R* was up-regulated 12-24 hours post-LH treatment. Similarly, gonadotropin stimulation of *PACAP* and *VPAC$_{1/2}$-R* but not *PAC$_1$-R* expression has been reported in human luteinized granulosa cells [19]. In the rodent ovary, PACAP and its receptors are expressed in granulosa cells of preovulatory follicles in which it modulates

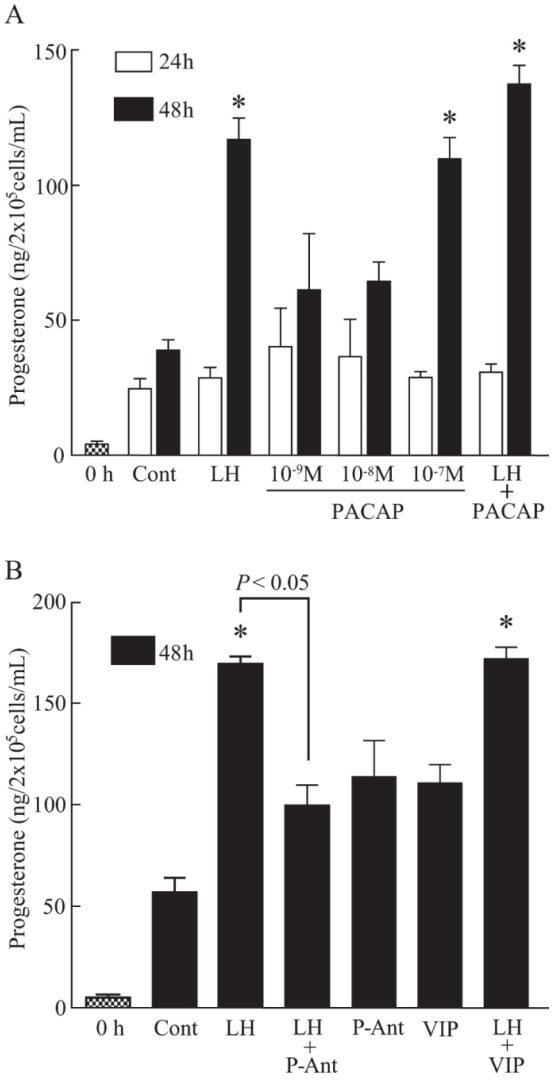

Fig. 4. Effect of PACAP-38 on progesterone production by cultured human luteinized granulosa cells. The 2×10^5 cells pooled from 2-3 patients were cultured for 24 hours or 48 hours, in the absence (Control; CONT) or presence of LH (200 ng/mL), and increasing doses of PACAP-38 (**A**), 10^{-6} M PAC_1-R antagonist PACAP(6-38) or 10^{-7} M VIP (**B**). Progesterone concentrations (mean ± SEM) in culture media were determined by RIA (n=3-6). *, $P < 0.05$ compared with the corresponding control value.

parameters for the ovulation process [9, 10, 12, 14, 18]. Thus it appears that PACAP system exists in both preovulatory and early luteal phase.

The stimulation of PACAP system and progesterone production by LH indicates that luteinized granulosa cells used in the present study can respond to the gonadotropins during the culture period. Indeed, an increase in the expression of LH-R with preculturing time in hormone-free medium is observed in human luteinized granulosa cells obtained from IVF programs [19]. These human luteinized granulosa cells produce steroid hormones and relaxin in response to gonadotropins *in vitro* in patterns similar to those observed during the luteal phase of a normal menstrual cycle [24], suggesting that these cellular system can serve as a useful model for studying the regulatory mechanism of luteinization *in vitro*. The present observation of PACAP action on the stimulation of progesterone production therefore suggests that PACAP system may be one of the regulatory factors for luteinization. Recent report demonstrating the suppression of apoptosis by PACAP in human luteinized granulosa cells [19] further supports this idea for PACAP system to maintain and/or to promote luteinization.

The induction of PACAP and PACAP/VIP receptors by LH/hCG in preovulatory follicles and its autocrine/paracrine role at the time of ovulation are supported by several findings in the rat [10, 14, 18] and mouse ovary [12, 17]. LH/hCG-dependent stimulation of PACAP and PAC_1-R in the present study suggests that these molecules may also play a role in human preovulatory follicles. Morelli *et al.* [19] indeed demonstrate a high expression of PAC_1-R in human cumulus cells and suggest a role of PACAP in the mediation of LH action in human preovulatory follicles during the ovulation process.

With regard to the expression of PACAP/VIP receptors, *PAC_1-R* expression was maximally stimulated at 24 hours following LH treatment. However, similar studies in human luteinized granulosa cells demonstrate that *$VPAC_{1/2}$-R* is predominantly present with LH/hCG-responsive manner whereas *PAC_1-R* is present with undetectable levels [19]. This difference in *PAC_1-R* expression between two groups would arise from the preculturing time used in each experimental protocol (24 h *vs.* 7 days) and/or from sensitivities of RNA detection techniques. In the present culture system with shorter preculturing time, it is possible that *PACAP* and *PAC_1-R* system may be functionally dominant in human luteinized granulosa cells based on the present observations showing the inhibition by PAC_1-R antagonist of LH action and the absence of VIP action on progesterone production. These cells may dominantly express VIP and $VPAC_{1/2}$-R with increasing preculturing time.

In summary, PACAP system including human PACAP and PAC_1-R exists in human luteinized granu-

losa cells with LH/hCG-responsive manner. PACAP stimulates progesterone production, suggesting that PACAP may act as a local regulator for luteinization in the human ovary. It would be of interest to investigate in the future the possibility for the use of PACAP as a biomarker for the patients suffering from luteal phase dysfunction.

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