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Control Mechanisms of Ovulation by Pituitary Adenylate Cyclase-Activating Polypeptide

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Pituitary Adenylate Cyclase-Activating Polypeptide에 의한 배란 조절에 관한 연구

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배 경: Pituitary adenylate cyclase-activating polypeptide (PACAP)은 양의 시상하부에서 추출된 신경펩타이드 호르몬으로 난소에도 존재하여 배양된 과립막 세포에서 스테로이드합성과 cyclic AMP 형성을 촉진함이 보고되었다.

목 적: 흰쥐 난소를 실험 모델로 사용하여 배란시 황체화호르몬 (luteinizing hormone; LH)에 의해 유도된 PACAP과 PACAP 수용체의 유전자 발현양상과 신호 전달경로를 규명하고자 하였다.

재료 및 방법: 미성숙 흰쥐의 배란전 난포를 체외 배양하면서 LH로 처리하고 PACAP 및 PACAP수용체의 유전자 발현을 보기 위해서는 Northern blot 분석과 in situ hybridization (ISH)을, 그리고 단백질 수준의 PACAP 검색을 위해서는 enzyme linked immunosorbent assay (ELISA) 분석을 이용하였다.

결 과: LH 처리 후 Northern blot상의 PACAP 유전자 발현은 6~9시간에 일시적으로 최고치에 도달하였으며 ISH로 보아 과립막 세포에서 발현됨을 알 수 있었다. ELISA 분석 상 PACAP 단백질도 LH처리 후 6~12시간에 최고치를 나타내었으며, PACAP 수용체 mRNA 역시 3~9시간에 최고치로 과립막 세포에서 발현되었다. Adenylate cyclase (AC) 억제제인 MDL12330A 처리시 LH로 발현된 PACAP mRNA가 감소되며, AC의 활성제인 forskolin 처리에는 LH시와 유사한 PACAP mRNA의 발현양상을 나타내었다. 그러나 protein kinase C (PKC)의 억제제인 chelerythrine과 2-0-tetradecanolphorbol-13-acetate (TPA) 처리로는 PACAP의 유전자 발현에 영향을 주지 못하였다. 5-lipoxygenase의 억제제인 MK886이나 nordihydroguaiaretic acid (NDGA)로 처리한 결과 LH로 유도된 PACAP 유전자의 발현이 감소되었으나, cyclooxygenase의 억제제인 indomethacin은 별로 영향을 주지 못하였다. MEK와 p38의 억제제인 PD98059와 SB203580도 LH로 촉진된 PACAP의 유전자 발현을 농도 의존적으로 억제하였다.

결 론: 배란전 난포에서 PACAP과 PACAP 수용체의 유전자 발현은 모두 LH의 폭발적 분비에 의해 유도되어 일시적으로 과립막 세포에서 나타나 배란을 위한 국소적인 조절 작용을 할 것으로 추정되며, LH로 촉진된 PACAP 유전자 발현을 위한 신호전달은 cAMP-PKA, lipoxygenase 및 MAP kinase 경로를 통하는 것으로 사료된다.

Key Words: Pituitary adenylate cyclase-activating polypeptide (PACAP), 배란, 흰쥐

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Follicle of mammalian ovaries consists of an innermost oocyte, surrounding granulosa cells, and outer layers of theca cells. The maturation of ovarian follicles involves several stage-dependent events; initiation, growth, selection, ovulation, and luteinization. Basal concentrations of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), maintain growth up until the small antral stage. Small antral follicles are selected to continue growth by subtle increases in basal concentrations of gonadotropins, such as those that occur at menses in primates[1] or at the end of pregnancy in rodents.[2] Once selected, the growing dominant follicle acquires specific functional characteristics that permit it to differentiate to the preovulatory stage. Finally, the surge of LH triggers the preovulatory follicles to ovulate and luteinize. Follicles that are not selected or fail to ovulate become atretic.[3]

The preovulatory surge of LH induces the functional changes that occur in multiple ovarian cell types within the follicles characterized by four independent events; oocyte maturation, cumulus expansion, follicle rupture, and corpus luteum formation. Recent studies have demonstrated that the LH surge induces the specific genes that are obligatory for cumulus expansion to occur. These include (a) COX-2, the rate-limiting enzyme in the synthesis of prostaglandins, such as prostaglandin E_2 (PGE_2),[4] (b) HA synthase-2 (HAS-2), which catalyzes the production of HA[5], and (c) TSG-6, which is an HA binding protein.[6,7] Likewise, several studies have shown that LH surge induces the rapid, but transient, expression of specific genes associated with follicle rupture in preovulatory follicles. These ovulation-specific genes induced by the LH surge include PG endoperoxide synthase, P450scc and 17α-hydroxylase and transcription factor such as the early growth regulatory factor-1 (Egr-1), progesterone receptor (PR), CAAT enhancer binding protein β (C/EBPβ) and receptor-interacting protein 140.[8,9] Other transcription factors, such as the activator protein-1 family members (e.g, c-Fos, c-Jun, Fra2, and JunD), are induced rapidly and remain elevated during the postovulatory luteal phase.[10] Each of these mediators appear to be involved in the functional activity of granulosa cells of ovulating follicles. The net result of these studies, combined with information from recent knockout mouse models (and especially knockout models for progesterone receptor)[11,12] has brought about a dramatic change in concept of the ovulatory process. Ovulation is now considered to be the consequence of a highly complex assortment of gene expressions.[13,14]

Pituitary adenylate cyclase-activating polypeptide (PACAP), the latest member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family of peptides,[15,16] has the potential of being a local regulator of ovarian physiology[17~21] and periovulatory progesterone production, in particular.[22] PACAP was originally isolated from hypothalamus and exists in two biologically active forms, PACAP 27 and PACAP 38, of which PACAP 38 is the dominating form in tissue.[15,16,23] PACAP is also expressed in nonneuronal cells, as PACAP immunoreactivity (PACAP-IR) and PACAP mRNA have been shown in spermatogenic cells from the rat testis[24~26] and in steroidogenic cells from the rat ovary.[22] PACAP expression in ovarian steroidogenic cells is transient and confined to the periovulatory period.[22] The spatiotemporal expression of the peptide coincides with high expression of elements from the periovulatory cascade involved in progesterone production. Furthermore, PACAP is a potent stimulator of cAMP formation and is known to stimulate cAMP and steroidogenesis in ovarian cells.[7,20,21,27]

Several lines of evidence showing an ability of PACAP to stimulate cAMP formation and steroidogenesis suggest that PACAP could be an auto- or paracrine regulator of gonadotropin actions

during ovulation. To test this hypothesis, the present study examined: 1) whether LH surge induces PACAP gene expression and stimulates immunoreactive PACAP production and, 2) signal pathways for LH action on PACAP expression during the ovulatory process.

MATERIALS AND METHODS

1. Hormones and reagents

Ovine LH (LH-S-26; 2300 IU/mg) was obtained from the National Hormone and Pituitary Distribution Program, NIDDK, NIH (Baltimore, MD). Pregnant mares' serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), forskolin, and 2-0-tetradecanol phorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). PACAP 38 was obtained from Bachem (Torrance, CA). MDL-12330A and Chelerythrine (CE) Chloride were purchased from Calbiochem (San-Diego, CA). Indomethacin, nordihydroguaiaretic acid (NDGA) and MK-886 were purchased from BIOMOL Research Laboratories (Berlin, Germany).

2. Animals

Immature female rats of the Sprague Dawley strain were purchased from Daehan Laboratories (Chungpuk, Korea). They were housed in groups in a room with controlled temperature and photoperiod (10-h dark, 14-h light, with lights on from 0600~2000 h). The animals had *ad libitum* access to food and water. Ovaries were collection from 26-day-old immature rats at various times after treatment with 10 IU PMSG to induce multiple follicle growth. Some rats received a single ip (intraperitoneal) injection of 10 IU hCG to induce ovulation, and ovaries were obtained at different time intervals.

3. *In vitro* culture of preovulatory follicle

Preovulatony follicles (>800 μm in diameter) were dissected by fine forceps from ovaries collected at 48~52 h after PMSG injection for follicle culture. Fifteen to twenty follicles were cultured in glass vials containing 800 μl MEM (Life Technologies, Inc, Grand Island, NY) supplemented with penicillin, streptomycin, L-glutamine, and 0.1% BSA (wt/vol, Fraction V, Sigma) in the absence or presence of LH. Cultures were maintained for up to 24 h at 37℃ under 5% CO_2~95% O_2. Following incubation, follicles were snap-frozen for RNA isolation, or were fixed for *in situ* hybridization analysis.

4. Northern blot analysis

Total RNA from ovaries or cultured follicles was isolated using Tri-Reagent solution (Sigma). Twenty micrograms of total RNA were fractionated by electrophoresis on a 1% agarose gel containing formaldehyde and transferred to nylon membranes by capillary blotting with 10 × sodium citrate-sodium chloride (SSC). After an UV cross-linking and prehybridization, membranes were hybridized overnight at 42℃ in a solution containing 50% formamide, 5 × SSC, 1 mM EDTA, 250 μg/ml denatured salmon sperm DNA, 500 μg/ml yeast transfer RNA, and a total of 1×10^6 cpm of a ^{32}P-labeled rat PACAP and PACAP receptor (PACAPR) complementary DNA (cDNA) probe.[16] After hybridization, membranes were washed twice for 5 min at room temperature in 2 × SSC and 0.1% SDS, followed by 1 h at 65℃ in 0.5 × SSC and 0.1% SDS. Membranes were then exposed using Kodak RX films (Eastman Kodak Co., Rochester, NY) for 1 days at -80℃. The band intensities were subsequently measured using a phosphorimager (Bio-Rad Laboratories, Inc. Hercules, CA), and the signals were normailized to the rat glyceraldehyde-3-phosphate-dehydrogenase

(GAPDH) as an internal control.

5. Enzyme-linked immunosorbent assay

Ninety-six-well polyvinyl microtiter plates were coated with mouse monoclonal antibody anti-PACAP at a concentration of 250 ng/ml in carbonate-coating buffer. After adding 100 μl of the monoclonal antibody to each well, plates were sealed with parafilm and incubated overnight at 37℃. Plates were rinsed 3 times with PBS-Tween. Two hundred fifty microliter of PBS-BSA were added to each well and then plates were incubated overnight at 4℃ and either used immediately or stored for no more than 2 weeks with PBS-BSA containing 0.02% sodium azide at 4℃ until used. The coating of mouse monoclonal antibody anti-PACAP at the solid phase was monitored with anti-mouse IgG linked to peroxidase in wells taken at random.

Coated plates were washed twice with PBS-Tween and 100 μl of sample or standard PACAP diluted in PBS assay were added to each well. Plates were covered, incubated 16~24 h at 4℃ with gentle agitation and then washed 3 times with PBS-Tween. One hundred microliter of rat PACAP antibody was added to each well at a final dilution of 1:10,000 in PBS assay and the plates were rinsed 4 times with PBS-Tween. One hundred microliter of anti-rabbit IgG peroxidase conjugate diluted in PBS assay were added to each well. Plates were incubated 4 h at room temperature and washed 4 times with PBS-Tween. Substrates were then added to each well. After 30 min of incubation in the dark at room temperature, the reaction was stopped with 50 μl of stop solution. Optical density was then measured at 495 nm in a Boot-Celltech EL 310 microplate autoreader.

6. *In situ* hybridization analysis

Cryostat sections (14-μm thick) were mounted on poly-L-lysine (Sigma) coated microscope slides, fixed in 4% paraformaldehyde in PBS, and stroed at -80℃ until analyzed. Sections were pretreated serially with 0.2 M HCl, 2 × SSC, pronase (0.125 mg/ml), 4% paraformaldehyde, and acetic anhydride in triethanolamine. Hybridization was carried out at 52~55℃ overnight in the mixture containing ^{35}S-labeled rat PACAP and PACAPR complementary RNA (cRNA) probe (2×10^7 cpm/ml), 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1 × denhardt's solution, 10% dextran sulfate, 1 μg/ml carrier transfer RNA, and 10 mM dithiothreitol. Posthybridization washing was performed under stringent conditions that included ribonuclease A (25 μg/ml) treatment at 37℃ for 30 min and a final stringency of 0.1 × SSC. Slides were dipped into NTB-2 emulsion (Eastman Kodak Co.) and exposed at 4℃ and developed after 3~4 weeks. The slides were stained with hematoxylin and eosin and examined under the light microscope with bright- and dark-field illumination.

RESULTS

1. Expression and regulation of PACAP and its receptor

To study the hormonal regulation of PACAP mRNA expression, preovulatory follicles obtained from ovaries of rats primed for 2 days with PMSG were incubated in serum-free condition. Northern blot analysis revealed that gonadotropin treatment resulted in a transient induction of PACAP mRNA (Figure 1A). Three different sizes of PACAP transcripts, 3.0, 2.4 and 1.2 kb, were detected in preovulatory follicles. PACAP expression was detected at 3 h and reached a peak at 6 h. The effect of LH on PACAP transcripts remained elevated at 12 h after LH treatment. PACAP transcripts were not detectable in follicles before culture and 24 h after gonadotropin addition. Cell types expressing PACAP mRNA were also determined by *in situ*

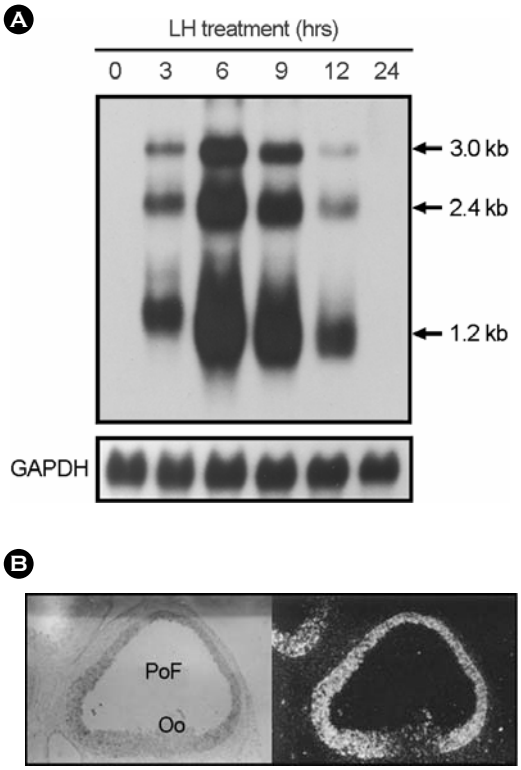

Figure 1. Stimulation of PACAP expression by gonadotropins in preovulatory follicles cultured *in vitro*. Preovultory follicles, obtained from ovaries of PMSG-primed immature rats, were cultured in serum-free conditions under 5% CO_2~95% O_2 at 37℃, in the presence of LH (200 ng/ml). **A,** Northern analysis showing the stimulation of PACAP expression by LH. **B,** In situ localization of PACAP mRNA in LH-treated cultured preovulatory follicles. PoF, Preovulatory follicles; Oo, oocyte.

hybridization. In follicle sections obtained from preovulatory follicles cultured in serum-free medium in the presence of LH for 6 h, high levels of PACAP mRNA were detected in mural granulosa cells (Figure 1B).

To determine PACAP immunoactivity in cultured preovulatory follicle, ELISA was performed. Consistent with results of Northern analysis, high levels of PACAP production were seen between 6 and 12 h after LH treatment in follicles (Table 1). However, PACAP production in medium was not highly stimulated.

Similar to the PACAP expression, Northern blot

Table 1. Measurement of PACAP immureactive production in cultured preovulatory follicles by enzyme-linked immunosorbent assay (ELISA)

LH treatment (hrs)	PACAP immunoreactive production (pg/follicle)	
	Follicle extract	Media
0	0.74±0.05	ND
3	2.50±0.08	0.15±0.02
6	21.59±5.95[a]	0.60±0.12[b]
12	20.74±6.40[a]	0.74±0.15[b]
18	9.46±2.32[a]	0.26±0.05[b]
24	5.36±0.86[a]	0.15±0.01

Preovulatory follicles, obtained from ovaries of PMSG-primed immature rats, were cultured in serum-free conditions under 5% CO_2~95% O_2 at 37℃, in the presence of LH (200 ng/ml). Enzyme-linked immunosorbent assay (ELISA) was performed to detect PACAP immunoactive products in culture media or follicles. Date points represent the mean ± SEM of triplicate cultures. ND, Not determined.
[a] $p<0.05$, vs. 0 h.p, [b] $p<0.05$, vs. 3 h.

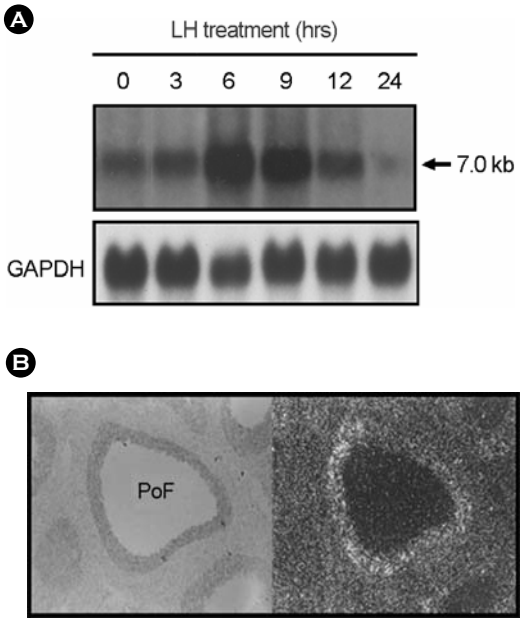

Figure 2. Northern blot analysis **(A)** and *in situ* localization **(B)** showing the stimulation of PACAPR expression by LH in preovulatory follicles cultured *in vitro*. Preovultory follicles, obtained from ovaries of PMSG-primed immature rats, were cultured in serum-free conditions under 5% CO_2~95% O_2 at 37℃, in the presence of LH (200 ng/ml). PoF, Preovulatory follicle.

analysis revealed that LH treatment resulted in the stimulation of PACAPR mRNA within 6 h which persisted at 9 h after the treatment and declined at 12 h in cultured preovulatory follicles (Figure 2A). Cell types expressing PACAPR mRNA were also determined by *in situ* hybridization. In ovarian sections obtained from 2 days after PMSG treatment injected at 26 days of age, high levels of PACAPR mRNA were mainly detected in large preovulatory follicles, but not in small growing follicles (Figure 2B).

2. Signaling pathways for LH-stimulated PACAP expression

In addition to the stimulation of cAMP-PKA pathway, the actions of LH are additionally medicated by other intracellular messengers, such as those derived from the phospholipase C pathway. To determine the signaling pathway for PACAP mRNA induction by LH, preovulatory follicles were incubated for 6 h in the presence of 10 μM MDL, an inhibitor of adenylate cyclase, or 10 μM

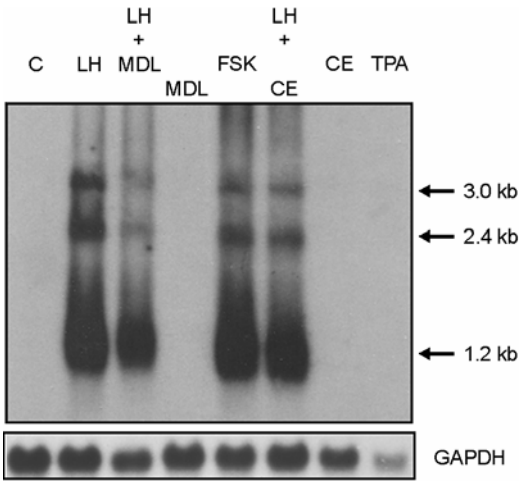

Figure 3. Effects of adenylate cyclase activation on LH-stimulated PACAP mRNA expression. Preovulatory follicles were incubated, under serum-free conditions, in the absence (control; C) or presence of MDL (10 μM), forskolin (FSK; 10 μM), CE (10 μM) and TPA (200 μM), with or without LH (200 ng/ml) for 6 h. Twenty micrograms of follicular total RNA were analyzed by Northern blotting using a cDNA probe for rat PACAP. The estimated sizes of PACAP transcripts are indicated to the right. The expression of GAPDH was used as an internal standard. Data are representative of two separate experiments.

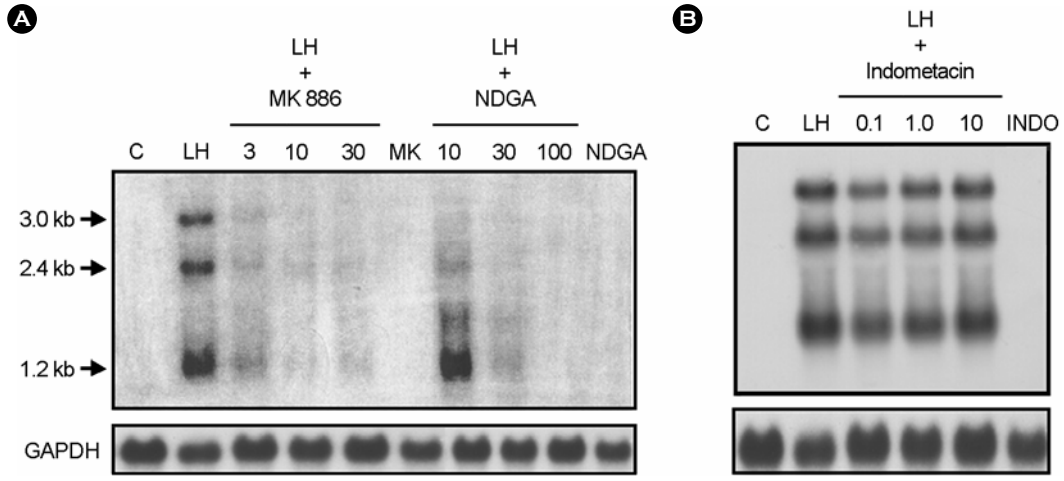

Figure 4. Involvement of lipoxygenase pathway in LH-induced PACAP gene expression in cultured preovulatory follicles. Preovulatory follicles were cultured in the absence (control; C) or presence of LH with or without lipoxygenase inhibitor, MK886 or NDGA, or cyclooxygenase inhibitor, indomethacin. Total RNA (20 μg/lane) was analyzed for PACAP mRNA levels by Northern blotting using a rat PACAP cDNA probe. The estimated sizes of PACAP transcripts are indicated. The expression of GAPDH was used as an internal standard. Data are representative of two separate experiments.

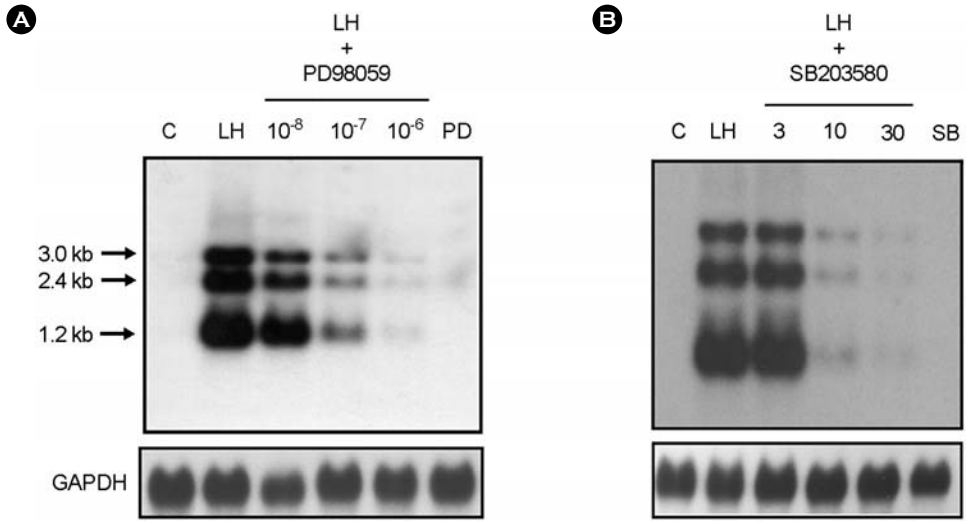

Figure 5. Inhibition of LH-stimulated PACAP expression by ERK **(A)** or p38 kinase inhibitor **(B)**. Preovulatory follicles, obtained from ovaries of PMSG-primed immature rats, were preincubated in serum-free conditions under 5% CO_2~95% O_2 at 37℃ with an increasing dose of PD98059 or SB203580 for 30 min. Follicles were then cultured in the absence (Control; C) or presence of LH (200 ng/ml) for 6 h. Twenty micrograms of follicular total RNA were analyzed for PACAP mRNA levels by Northern blotting using a rat PACAP cDNA probe. The estimated sizes of PACAP transcripts are indicated. The expression of GAPDH was used as an internal standard. Data are representative of three separate experiments.

CE, an inhibitor of protein kinase C, with or without LH (200 ng/ml). Some follicles were treated with 10 μM forskolin or 200 μM TPA alone for 6 h. The addition of CE did not affect the LH action on stimulation of PACAP mRNA. Moreover, forskolin treatment stimulated the induction of PACAP transcripts, whereas treatment with the phorbol eater TPA had no effect. Inclusion of MDL markedly decreased LH-induced PACAP transcrips (Figure 3), indicating a role of cAMP-PKA pathway in LH-stimulated PACAP expression.

The present study was also designed to test the role of signal pathway of PLA_2 activation in LH-induced PACAP gene expression in cultured preovulatory follicles. Treatment with MK886, a selective inhibitor of 5-lipoxygenase, suppressed LH-induced PACAP gene expression whereas indomethacin, an inhibitor of cyclooxygenase, did not have an effect (Figure 4). Another general lipoxygenase inhibitor, NDGA, also suppressed PACAP expression. This result suggests that 5-lipoxygenase, but not cyclooxygenase, play a crucial role in mediating LH-induced PACAP expression.

To study the role of MAP kinase in gonadotropin-induced PACAP gene expression, total RNA extracted from preovulatory follicle was analyzed by Northern blotting. Pretreatment of cultured preovulatory follicles with PD98059, an inhibitor of MEK, decreased the LH-induced PACAP gene expression in a dose-dependent manner (Figure 5A). Similarly, treatment with SB203580, an inhibitor of p38 kinase, also caused the inhibition of the LH-stimulated PACAP gene expression (Figure 5B).

DISCUSSION

In the present study, an *in vitro* follicle culture model was established to study the hormonal regulation of PACAP gene expression in the rat ovary. The present study demonstrates that gonadotropins cause the transient induction of PACAP mRNA in

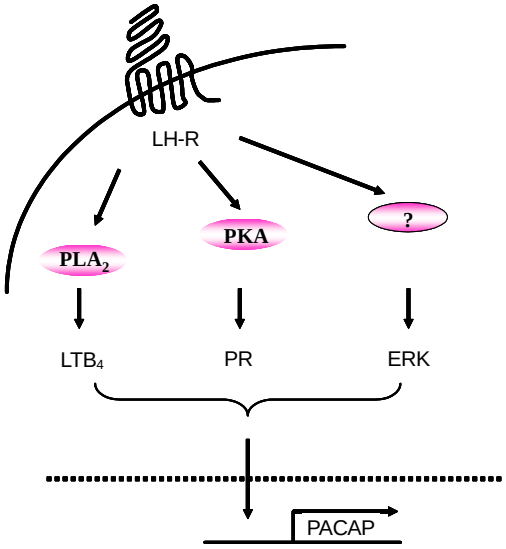

Figure 6. Signaling pathways for LH-stimulated PACAP expression in granulosa cells of preovulatory follicles. Activation of cAMP-PKA pathway by LH is known to activate progesterone receptor and in turn it promotes PACAP gene expression. Other signal pathways including PLA2-LTB4 and ERK activation also play a role in LH-stimulated PACAP expression.

granulosa cells of preovulatory follicles through multiple signal pathways, including progesterone receptor via cAMP-PKA pathway, PLA_2-LTB_4 activation, and ERK activation (Figure 6).

Treatment with exogenous LH or FSH in cultured follicles could induce a transient expression of PACAP mRNA in a dose-dependent manner, mimicking the expression pattern of PACAP mRNA after hCG injection *in vivo*. Northern blot analysis revealed three different PACAP transcripts. One transcript, 1.2 kb in size, has been reported to be expressed in the rat testis.[25] The other two transcripts (2.4 and 3.0 kb in size) were similar to those observed in nervous tissue.[28] The major cell type expressing PACAP mRNA in cultured preovulatory follicles was granulosa cells. However, unlike *in vivo* hCG-treated ovaries, theca cells were devoid of PACAP-specific signal in response to LH. Furthermore, PACAP mRNA remained elevated for up to 12 h in preovulatory follicles cultured *in vitro* with LH, compared with ovaries treated with hCG *in vivo*, indicating that the mechanism mediating the rapid decline in PACAP mRNA after hCG treatment *in vivo* is reduced *in vitro* after LH treatment. A similar observation has been reported in the induction of PG endoperoxide synthase.[29] Accordingly, one possible explanation for the difference is that cAMP production seems to be sustained longer *in vitro* after LH treatment.[30]

PACAP can interact with specific receptors. The type I receptor binds preferentially to PACAP, and the type II binds PACAP and VIP with similar affinity.[23] Of further interest are the present findings demonstrating the stimulation of PACAPR mRNA in granulosa cells of preovulatory follicles by LH/hCG. Thus, the present findings suggest that the direct PACAPR-mediated actions of PACAP on ovarian function may be restricted to specific developmental windows. Transient appearance of PACAP may therefore play a role as a novel intracellular factors and the regulatory processes during the periovulatory period. It has been demonstrated that PACAPR gene is expressed in the rat ovary in a stage- and cell-specific manner during follicle development. The increased expression of PACAPR is restricted to granulosa cells of preovulatory follicles after gonadotropin stimulation. Further studies are needed to determine whether PACAP is involved in the regulation of ovulation.

It has been indicated that low concentrations of LH preferentially activate adenylate cyclase, whereas higher concentrations of the hormone also increase intracellular calcium and activate protein kinase C (PKC).[31] The present findings, showing the inhibition of LH action on the induction of PACAP mRNA by adenylate cyclase inhibitor, but not by PKC inhibitor, provide evidence to hypothesize a primary role for cAMP in PACAP mRNA expression in preovulatory follicles. Indeed, it has been reported that the 5'-flanking region of the hPACAP gene contains sequence motif homolo-

gous to cAMP response element.[32]

The present study demonstrating that treatment of cultured preovulatory follicles with MEK inhibitor decreased the LH-induced PACAP gene expression suggests the possible role of ERK in LH-induced PACAP gene expression. Interestingly, the timing of ERK activation was different between cell types within preovulatory follicles. It has been reported that the activation of ERK occurs within 15 min after LH/hCG in cultured granulosa cells.[33] In oocyte-cumulus complexes, however, ERK is activated 2~4 h after gonadotropin treatment.[34] Therefore, it is likely that mural and cumulus granulosa cells might have a different machinery to activate ERK in response to ovulatory dose of LH. Lastly, the activated ERK by LH was responsible for PACAP expression. The involvment of p38 kinase in LH-induced PACAP gene expression supports the possible role of p38 kinase. The physiological function of these two kinases remains to be investigated.

The results of the present study indicates that the signal pathway activating phospholipase A_2-leukotriene (LT)B_4 also plays an important role in LH-induced PACAP gene expression in preovulatory follicles. The presence of five mammalian extracellular phospholipase A_2 (PLA_2) enzymes, cytosolic ($cPLA_2$; type IV), secretory ($sPLA_{2s}$; types IIA, V and IIC), and Ca^{2+}-independent PLA_2 ($iPLA_2$; type VI) regulate arachidonic acid release. Previous results demonstrate the correlation among PLA_2-I (type I) gene expression, estrus cycle, and progesterone secretion in rat ovaries and suggest a new function of PLA_2-I as an intragonadal regulatory factor for steroidogenesis. In cultured granulosa cells of immature follicles, the present data showing the inhibition of LH-induced PACAP gene expression by an inhibitor of phospholipase A_2 indicate an importance of PLA_2 pathway. Indeed, follicular $cPLA_2$ expression is regulated by gonadotropins and may involve a cell- and hormone-dependent mechanism.[35]

The product of arachidonic acid metabolism formed via the lipoxygenase pathway, as well as the cyclooxygenase pathway, have been shown to play a role in the regulation of reproductive function.[36] Numerous studies suggest a role for the lipoxygenase system in ovulation. Previous results demonstrate that the products of the lipoxygenase pathway, especially LTB_4, are important in the process of ovulation in cyclically ovulating species.[37] The intrabursal injection of specific inhibitors of lipoxygenase has been shown to block follicle rupture at ovulation in rat ovaries.[38] The lipoxygenase activity in rat preovulatory follicles has been shown to increase significantly within 6 h of hCG administration.[39] A number of inhibitors for lipoxygenase pathways of arachidonic acid have been reported.[40] Among them, MK886 has been shown to be the selective inhibitor of 5-lipoxygenase in human leukocytes and platelets.[40] In the present study, treatment with MK886, suppressed LH-induced PACAP gene expression.

In conclusion, LH/hCG induces a rapid and transient expression of both PACAP and PACAPR in granulosa cells of preovulatory follicles, implicating a role of PACAP system in the ovulatory process. The LH action on the stimulation of PACAP expression involves multiple signal pathways including cAMP-PKA, PLA_2-LTB_4 and MAP kinase activation.

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