Both Ca\textsuperscript{2+}-Protein Kinase C Pathway and cAMP-Protein Kinase A Pathway are Involved in Progesterone Production in FSH- and LH-stimulated Cumulus Cells during In Vitro Maturation of Porcine Oocytes

Masayuki Shimada\textsuperscript{1,2*}, Naoko Samizo\textsuperscript{2}, Yasuhisa Yamashita\textsuperscript{1}, Kaori Matsuo\textsuperscript{1} and Takato Terada\textsuperscript{1,2}

\textsuperscript{1}Laboratory of Animal Reproduction, Graduate School of Biosphere Sciences,\textsuperscript{2}Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima, Hiroshima, 739-8528, Japan

Abstract: We investigated the role of cAMP-Protein kinase A pathway and Ca\textsuperscript{2+}-Protein kinase C pathway which were activated by FSH and/or LH in the progesterone production by cumulus cells during in vitro maturation of porcine oocytes. The level of progesterone in the medium in which COCs had been cultured for 24 hr without FSH was 7.8 ± 1.5 ng/ml. The addition of FSH significantly increased the progesterone level in a dose-dependent fashion during 24-hr cultivation of COCs; a plateau was detected in 0.02 µg/ml, but no significant increase in the level of progesterone was observed in the medium in which COCs had been cultured for 24 hr with 1 or 10 µg/ml LH. When COCs were cultured with both 0.02 µg/ml FSH and 1.0 µg/ml LH for 24 hr, the maximal level of progesterone was detected. The effects of the addition of LH to FSH-supplemented medium on the response of cumulus cells were affected by the suppression of the Ca\textsuperscript{2+}-Protein kinase C pathway. Forskolin-induced progesterone production was not affected by suppression of the Ca\textsuperscript{2+}-Protein kinase C pathway but was reduced by Protein kinase A inhibitor. These results showed that Ca\textsuperscript{2+}-Protein kinase C pathway in cumulus cells stimulated by FSH, enhanced LH-induced progesterone production via a cAMP-Protein kinase A dependent pathway.

Key words: LH, PKA, PKC, Progesterone, Cumulus cells

Progesterone is produced by cumulus cells during in vitro cultivation of COCs in the presence of LH and/or FSH in human [1], rat [2], cattle [3] and pig [4–6]. When porcine COCs were cultured with LH, FSH, and progesterone synthesis inhibitor, almost complete inhibition of both progesterone production and GVBD was detected, and this inhibitory effect on GVBD was overcome by additional progesterone [6]. Therefore, progesterone secreted by cumulus cells is associated with meiotic maturation of porcine oocytes. Moreover, we also showed that the binding of progesterone to its receptor induced the disappearance of connexin-43 in cumulus cells, resulting in closing gap junctional communication in outer layers of cumulus cells [6, 7]. In pigs, a significant positive correlation was seen between the proportion of GVBD oocytes and that of COCs exhibiting a loss of the gap junctional communication between the cumulus cells in outer layers [8, 9]. Thus, the binding of progesterone to its receptor in cumulus cells exhibits GVBD in porcine oocytes, through closing gap junctional communication in outer layers of cumulus cells.

The addition of adenylcyclase activator, forskolin, to the maturation medium has been reported to stimulate progesterone production in cumulus cells concomitantly with an increase in the cAMP level in the cells [10].
Progesterone production in FSH-stimulated bovine granulosa cells was suppressed by Protein kinase A (PKA) inhibitor, H89 [11], suggesting that a cAMP-PKA pathway was involved in the progesterone production.

The signal transduction of FSH has been considered to be mediated by the cAMP-PKA pathway, but the results of several studies showed that Protein kinase C (PKC) may also play an important role in the signal transduction of FSH [12, 13]. At least 6 members of PKC family were detected in cumulus cells and the addition of PKC activator to the hypoxanthine containing medium stimulated GVBD [14]. Recently, we showed that PKC inhibitor, Calphostin C suppressed the reduction in gap junctional protein connexin-43 in cumulus cells during meiotic resumption of porcine oocytes [7] and either forskolin, or PKC activator, 12-O-tetradecanoylphorbol-13-acetate, induced the reduction in connexin-43 in rat follicular cells [15]. These findings suggest that the PKC pathway in cumulus cells is one of the regulators of gap junctional communication during meiotic resumption of porcine oocytes, as well as the cAMP-PKA pathway through the function of progesterone, but the relationship between the cAMP-PKA pathway induced progesterone production and the PKC pathway in FSH-stimulated cumulus cells remains unclear.

In this study we investigated the role of the cAMP-PKA pathway and Ca\(^{2+}\)-PKC pathway which were activated by FSH and/or LH in the progesterone production by cumulus cells during in vitro maturation of porcine oocytes. After COCs were cultured with either PKA inhibitor or PKC inhibitor in the presence of FSH, LH or forskolin, the cAMP level in cumulus cells of COCs and the progesterone level in the cultured medium were analyzed. The results showed that the Ca\(^{2+}\)-PKC pathway activated by FSH enhanced LH-induced progesterone production via a cAMP-PKA-dependent pathway.

**Materials and Methods**

**Isolation and culture of porcine cumulus oocyte complexes (COCs)**

Isolation of porcine COCs was described previously [7]. Briefly, porcine ovaries were collected from 5- to 7-month-old prepubertal gilts at a local slaughterhouse. Oocytes were collected with a surgical blade from the surfaces of intact healthy antral follicles measuring from 3 to 5 mm in diameter. Oocytes having evenly granulated cytoplasm with at least 4 layers of unexpanded cumulus oophorus cells were selected and were washed 3 times with maturation medium. The maturation medium was modified NCSU37 [16] supplemented with 10% (v/v) FCS (Gibco BRL, Grand Island, NY, USA) and 7 mM Taurine (Sigma, St. Louis, MO).

Twenty COCs were cultured for 24 hr with 0, 0.002, 0.02 or 0.2 \(\mu g/ml\) purified porcine FSH (NHPP, Torrance, CA, USA), 0.1, 1, 10 \(\mu g/ml\) purified porcine LH (NHPP), 0.02 \(\mu g/ml\) FSH + 1.0 \(\mu g/ml\) LH, or 0, 0.5, 1.0, 5.0, 10.0 or 30.0 \(\mu M\) forskolin (Sigma) in each well of a Nunc 4-well multidish (Nunc, Roskilde, Denmark) containing 500 \(\mu l\) of culture medium at 39°C in a humidified atmosphere of 5% CO\(_2\) in air. Control experiments demonstrated that the osmolarity of cultures maintained under these conditions varied by < 1% after 44 hr. Forskolin was dissolved in DMSO at 50 mM and stored at –20°C. The final concentration described above was obtained by dilution with the basic medium. This concentration of methanol or DMSO does not affect porcine oocyte maturation [17].

**Quantification of progesterone in medium by HPLC-UV analysis**

Quantification of progesterone by HPLC-UV was based on the procedures reported in our previous study [6]. Briefly, the medium in which COCs had been cultured for 24 hr was collected into plastic tubes and centrifuged at 10,000 \(\times g\) for 20 min. Progesterone was extracted from the medium by 5-min mixing with 10 ml dichloromethane (Nakalai, Kyoto, Japan). After centrifugation, 10 ml of the dichloromethane fraction was collected into a disposal tube and the solvent from this fraction was removed by vacuum extraction for 120 min at 5°C. Samples were reconstituted in 100 \(\mu l\) of 50% (v/v) methanol solution.

The samples were separated on a reverse-phase CAPCELL PAK column (2.0 \(\times\) 100 mm) (Shiseido, Tokyo, Japan). The solvent delivery system (DP 8020, TOSOH, Tokyo Japan) contained 50% (v/v) methanol solution. The detections of progesterone were performed at 240 nm with a UV detector (UV 8020, TOSOH) and peak heights were measured with a computer integrator (Sic chromatocorder 11,TOSOH). The standard curve of progesterone was linear, from zero to 800 ng/ml.

**Quantification of cAMP by HPLC-UV analysis**

Quantification of cAMP by HPLC-UV was based on the procedures reported in our previous study [18]. After COCs were cultured for 24 hr, cumulus cells were separated from COCs. The cumulus cells were
extracted in 100 µl assay buffer of 97.2% (v/v) 0.01M ammonium acetate (Nakalai) and 2.8% (v/v) acetonitrile (Nakalai), pH 6.7 containing 50 µM IBMX (Sigma). The cumulus cell extracts were separated on a reverse-phase Eicompak CA-5DS column (2.1 × 150 mm) (Eicom, Kyoto, Japan). The solvent delivery system contained 97.2% (v/v) 0.01M ammonium acetate and 2.8% (v/v) acetonitrile, pH 6.7. The detection was performed at 254 nm with a UV detector; peak heights were measured with a computer integrator.

A standard solution of 100 µM cAMP (Sigma) was prepared in the assay buffer and kept frozen at –80°C. Samples were diluted in the assay buffer prior to analysis. The standard curve of cAMP for determination of the concentration was linear from zero to below 2 pmol.

Treatment of COCs with PKA inhibitor, H89
COCs were cultured with 0, 10, 20 or 50 µM H 89 (Sigma) in the presence of 0.02 µg/ml FSH, 1.0 µg/ml LH, 0.02 µg/ml FSH + 1.0 µg/ml LH, or 5 µM forskolin. H 89 was dissolved in methanol at 25 mM and stored at –80°C. Each final concentration mentioned above was obtained by dilution with each maturation medium. As a control, inhibitor-free medium was prepared by adding 0.1% (v/v) methanol to the basic maturation medium.

Treatment of COCs with PKC inhibitor, Calphostin C
COCs were cultured with 0.5 µM Calphostin C for 24 hr in the presence of 0.02 µg/ml FSH, 1.0 µg/ml LH, 0.02 µg/ml FSH + 1.0 µg/ml LH, or 5 µM forskolin. Calphostin C was dissolved in dimethyl sulfoxide (DMSO, Sigma) at 1 mM and stored at –20°C. The final concentration mentioned above was obtained by dilution with each maturation medium. As a control, inhibitor-free medium was prepared by adding 0.1% (v/v) DMSO to the basic maturation medium.

Treatment of COC with Ca²⁺ chelator BAPTA-AM
BAPTA/AM treatment was based on the procedures reported in our previous study [19]. COCs were loaded with 50 µM BAPTA-AM for 1 hr at 39°C. To enhance loading, the medium was further supplemented with 1 µl/ml of 25% (w/v, final concentration: 0.025%) dispersing agent Pluronic F-127 (Sigma) in DMSO. After the treatment, the COCs were cultured for 24 hr in the presence of 0.02 µg/ml FSH, 1.0 µg/ml LH, 0.02 µg/ml FSH + 1.0 µg/ml LH or 5 µM forskolin.

Treatment of COCs with PKC activator
COCs were cultured with PKC activator, 0, 0.5, or 1.0 µM phorbol 12-myristate 13-acetate (PMA, Sigma) for 24 hr. PMA was dissolved in DMSO at 1mM and stored at –20°C. The final concentration described above was obtained by dilution with the basic medium. 0.1% (v/v) DMSO was added to all of the treatment groups.

Statistical analysis
Statistical analyses of all data, including three or four replicates for comparison, were carried out by one-way ANOVA followed by a least significant difference test with STATVIEW (Abacus Concepts, Inc, Berkeley, CA, USA).

Results

Experiment 1: Effect of H89, Calphostin C or BAPTA-AM on the production of progesterone by FSH and/or LH-stimulated cumulus cells
The level of progesterone in the medium in which COCs had been cultured for 24 hr without FSH was 7.8 ± 1.5 ng/ml. The addition of FSH significantly increased the progesterone level in a dose-dependent fashion during 24-hr cultivation of COCs; a plateau of progesterone production (51.5 ng/ml) was detected in the 0.02 µg/ml FSH group (Fig. 1), but no significant increase in the level of progesterone was observed in the medium in which COCs had been cultured for 24 hr with 1 or 10 µg/ml LH (Fig. 1). When COCs were...
cultured with both 0.02 \( \mu g/ml \) FSH and 1.0 \( \mu g/ml \) LH for 24 hr, the maximal level of progesterone (86.5 \( \pm \) 4.3 ng/ml) was detected. The concentration was significantly higher than that in the medium in which COCs had been cultured with any concentration of either FSH or LH.

The production of progesterone by COCs cultured with FSH or FSH+LH was significantly reduced by the additional of H89 with increasing concentrations of the drug (Fig. 2). Treatment with BAPTA-AM or addition of Calphostin C to the medium significantly decreased the level of progesterone when COCs were cultured with FSH or FSH+LH (Fig. 3), but there is no significant difference in the progesterone concentration among the treatment groups of COCs which were cultured in the presence of LH (Figs. 2, 3).

**Experiment 2: Effect of H89, Calphostin C or BAPTA-AM on the production of progesterone by forskolin-stimulated cumulus cells**

In order to find the effective concentration of forskolin that stimulates progesterone production, COCs were cultured for 24 hr with 0, 0.5, 1.0, 5.0, 10.0 or 30.0 \( \mu M \) forskolin. The level of progesterone in the cultured medium was increased by the addition of forskolin in a dose-dependent manner (Fig. 4A). The stimulatory effects reached their maximum at 5.0 \( \mu M \), and the level of progesterone in the medium where COCs had been cultured with 30 \( \mu M \) forskolin was significantly lower than the maximum level observed at 5.0 \( \mu M \) (Fig. 4A). Accordingly, the concentration of forskolin added to the maturation medium in other experiments was 5.0 \( \mu M \).

The high concentration of progesterone was significantly decreased by supplementation with H89, but no significant difference was observed when COCs were cultured with various concentrations of Calphostin C or treated with BAPTA-AM (Fig. 4B, C).

To examine whether PKC can directly stimulate progesterone production in cumulus cells, COCs were cultured with 0.5 or 1.0 \( \mu M \) PMA for 24 hr. The results showed that the addition of 0.5 or 1.0 \( \mu M \) PMA to the basic maturation medium cannot significantly affect the production of progesterone (Fig. 5).

**Experiment 3: Effect of Calphostin C or BAPTA-AM on the level of cAMP in cumulus cells**

The level of cAMP in cumulus cells just after collection from the follicles was very low, whereas a significant increase in cAMP in cumulus cells cultured for 24 hr with FSH was noted (Fig. 6). Additional LH in the FSH-containing medium significantly increased the level of cAMP in cumulus cells as compared with that of COCs cultured with FSH alone (Fig. 6). There is no significant difference in the level of cAMP in cumulus cells of COCs between those cultured with FSH+LH and forskolin (Fig. 6). In contrast, a significantly lower level of cAMP was observed in COCs cultured with LH alone, as compared with that of COCs cultured with FSH, FSH+LH or forskolin (Fig. 6). The low level in cumulus...
cells of COCs was comparable to that of COCs just after collection from the follicles.

The increase in the cAMP level in cumulus cells of COCs stimulating by FSH or FSH+LH, was significantly suppressed by either the addition of Calphostin C or treatment with BAPTA-AM (Fig. 6). But the forskolin-induced increase in the cAMP level in cumulus cells was not affected by either Calphostin C or BAPTA-AM (Fig. 6).

**Discussion**

In granulosa cells, either LH or FSH promotes a cAMP increase and increases progesterone production in a PKA-dependent manner [20, 21]. In this study, the addition of FSH to the maturation medium produced a significant increase in the level of cAMP and progesterone production in COCs. An increase in progesterone production was also observed in the cultured medium where COCs had been cultured with forskolin. The progesterone production in FSH- or forskolin-stimulated COCs is reduced by H 89. Therefore, FSH stimulates progesterone production in cumulus cells via a cAMP-PKA dependent pathway, as well as in granulosa cells.

When COCs were cultured with both 0.02 µg/ml FSH and 1.0 µg/ml LH for 24 hr, the maximal level of progesterone was detected. The concentration was significantly higher than that in the medium where COCs had been cultured with any concentration of either FSH or LH. The effects of the addition of LH to the FSH-supplemented medium on the response of cumulus cells were affected by the suppression of the Ca²⁺-PKC pathway. The signal transduction of FSH was considered to be mediated by cAMP-PKA, but the results of several studies showed that protein kinase C (PKC) may also play an important role in the signal
transduction of FSH [12, 13]. FSH receptor is coupled to the adenylcyclase or phospholipase C signal pathway, inducing the production of cAMP or both inositol phosphates (IP₃) and diacylglycerol as intracellular signal molecules [22]. Judging from these reports and our present results, it is estimated that FSH stimulates both the cAMP-PKA pathway and the Ca²⁺-PKC pathway in cumulus cells, and the Ca²⁺-PKC pathway enhances the LH-induced increase in the cAMP level and progesterone production in cumulus cells (Fig. 7). Our idea is supported by the studies reported by Budnik et al. [23] and Rajkumar et al. [24], indicating that pre-treatment of the cells with PKC activator produces a great amount of cAMP in LH-stimulated bovine and porcine luteal cells.

It was found that when porcine cumulus oocyte complexes (COCs) from the antral follicles were cultured with LH, the level of cAMP in the cumulus cells was not increased [25]. This shows that LH receptors may be expressed at a very low level in cumulus cells which are commonly used for in vitro maturation of porcine oocytes. Chen et al. [26] investigated the quantification of LH receptor in mouse COCs by the radioligand receptor analysis method, to demonstrate the role of LH in meiotic maturation of mouse oocytes. They provided biochemical evidence that the weak expression of LH receptors was seen in cumulus cells immediately recovered from their follicles, the formation of the receptors was increased by FSH, and LH produced the great stimulation of cumulus expansion upon binding to these acutely formed receptors. In our previous study (unpublished data), FSH addition to the maturation medium induced a significant increase in the level of LH receptors in porcine COCs. These findings imply that a Ca²⁺-PKC dependent pathway in FSH-stimulated cumulus cells induced the formation of LH receptors, and the activation of the LH receptors raises the level of cAMP, which induces a further increase in the progesterone production.

In our previous study [27], we showed that progesterone secreted by COCs was absorbed by mineral oil which covered the medium, and both meiotic resumption and activation of p34cdc2 kinase in oocytes cultured with the oil covering were delayed as compared with those of oocytes cultured without mineral oil. Furthermore, the absence of mineral oil which covers the maturation medium improves the rate of early embryonic development to the blastocyst stage after IVF, concomitantly with an increase in the level of progesterone [27]. These results suggested that progesterone which is secreted by cumulus cells plays an important role in nuclear maturation and developmental competence of in vitro matured porcine oocytes. Therefore, the activation of both the cAMP-PKA pathway and the Ca²⁺-PKC pathway in cumulus cells is essential for in vitro maturation of porcine oocytes.

---

**Fig. 6.** Effects of PKC inhibitor, Calphostin C or intercellular Ca²⁺ chelator, BAPTA-AM on the cAMP level in porcine COCs. a–c: Columns with no common superscript are significantly different within the control group (p<0.05). †, ‡; The level of cAMP in COCs which were cultured with Calphostin C or treated with BAPTA-AM were significantly lower than that of COCs cultured without any drug (control) (p<0.05). (Free): COCs were cultured for 24 hr, (control) COCs were cultured with 20 ng/ml FSH, 1.0 µg/ml LH, 20 ng/ml FSH and 1.0 µg/ml LH, or 5.0 µM forskolin. (Calphostin C): COCs were cultured with 0.5 mM Calphostin C for 24 hr. (BAPTA-AM): After COCs were treated with 50 µM BAPTA-AM, the COCs were cultured for 24 hr.

**Fig. 7.** Schematic diagram of mechanisms involved in progesterone production in porcine cumulus cells.
In conclusion, FSH stimulates progesterone production in COCs via a cAMP-PKA dependent pathway. The additional LH in the FSH containing medium produces a significant increase in the cAMP level and progesterone production in COCs. The significant increases were suppressed by the cultivation of COCs with PKC inhibitor or treatment with Ca^{2+}-chelator, but the progesterone production in forskolin-stimulated COCs was not affected by suppression of the Ca^{2+}-PKC pathway but reduced by PKA inhibitor. These results suggested that the Ca^{2+}-PKC pathway in cumulus cells surrounding oocytes stimulated by FSH enhanced LH-induced progesterone production via a cAMP-PKA dependent pathway.

**Acknowledgement**

Porcine FSH and LH, and hCG were kindly provided by Dr. A.F. Parlow of the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Disease. The authors are grateful to Dr. M. Fujita of Laboratory of Animal Management, Hiroshima University, for technical advice on the use of HPLC-UV analysis. They thank the staff of the Meat Inspection Office in Hiroshima City for supplying the porcine ovaries.

This work was partly supported by Grant-in-Aid for Scientific Research M.S. No.14760179 from the Japan Society for the Promotion of Science.

**References**


