INHIBITORY ACTION OF PORCINE FOLLICULAR FLUID
UPON GRANULOSA CELL LUTEINIZATION IN VITRO:
ASSAY AND INFLUENCE OF FOLLICULAR MATURATION*

FLORENCE LEDWITZ-RIGBY,† B. W. RIGBY,† V. L. GAY,
MARGARET STETSON,‡ J. YOUNG,§
AND CORNELIA P. CHANNING

Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115,
Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh,
Pennsylvania 15261, and Department of Physiology, University of Maryland School of
Medicine, 660 West Redwood Street, Baltimore, Maryland 21201, U.S.A.

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SUMMARY

Culture medium 199 supplemented with follicular fluid from 1–2 mm antral porcine follicles inhibited spontaneous luteinization of granulosa cells from preovulatory porcine follicles in vitro. Three characteristics of luteinization were inhibited: morphological transformation, progesterone secretion, and accumulation of cyclic AMP in response to LH. The last was inhibited more effectively by culture media containing 50 % follicular fluid than by media containing 20 % follicular fluid. The inhibitory actions of the follicular fluid were not altered by charcoal or petroleum ether extraction. Follicular fluid from large follicles (6–12 mm) did not exhibit any of these inhibitory actions. These observations may indicate the presence of a luteinization inhibitor in the fluid of small follicles which (1) is lost by the time the follicle reaches the preovulatory stage, or (2) is overcome by a stimulatory agent which may accumulate as the follicle grows.

INTRODUCTION

Nalbandov and his colleagues have suggested that follicular fluid might either contain a luteinization inhibitor or lack a substance needed for luteinization (El-Fouly, Cook, Nekola & Nalbandov, 1970; Nekola & Nalbandov, 1971; El-Sheikh & Nalbandov, 1972). Granulosa cells harvested from preovulatory (large) monkey and porcine follicles luteinize spontaneously when cultured in vitro (Channing, 1970a, b). They accumulate more cyclic AMP in response to luteinizing hormone (LH) and secrete significantly larger quantities of progesterone during 18 h of culture than do granulosa cells harvested from smaller, less mature follicles (Channing, 1973; Channing & Ledwitz-Rigby, 1974). Granulosa cells from preovulatory follicles undergo morphological luteinization within 4–5 days of monolayer culture, but cells from medium-sized follicles will luteinize only if follicle-stimulating hormone (FSH) or LH is added to the culture medium; granulosa cells from small follicles will not luteinize in vitro even in the presence of FSH and/or LH (Channing, 1970a, b, c). It

* Presented in part at the 6th and 7th annual meetings of the Society for the Study of Reproduction held at Athens, Georgia (1973), and at Ottawa, Canada (1974) respectively. Address for reprints: F. Ledwitz-Rigby, Northern Illinois University, DeKalb, Illinois, 60115, U.S.A.

Present addresses: † Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115, U.S.A. ‡ University of Rochester School of Medicine, Rochester, NY 14627, U.S.A. § Saint Francis Hospital, Honolulu, Hawaii 96817, U.S.A.
is possible that there are luteinization inhibitors in follicular fluid which control this maturation of the granulosa cells within the follicle.

The present study therefore investigated the inhibitory effect of follicular fluid on luteinization in vitro. We examined the ability of follicular fluid collected from porcine follicles at various stages of maturity to inhibit spontaneous luteinization of porcine granulosa cells from preovulatory follicles in vitro. Three criteria of luteinization were examined: cyclic AMP accumulation, progesterone secretion, and cell morphology.

**MATERIALS AND METHODS**

**Hormones**

Ovine LH (NIH-LH-S17), ovine prolactin (NIH-P-S12) and rat LH (NIAMDD-RP-1) were obtained from the National Institute of Arthritis, Metabolic and Digestive Diseases, Bethesda, Maryland. The ovine LH had a potency of 1·01 NIH-LH-S1 units/mg and the rat LH a potency of 0·033 NIH-LH-S1 units/mg. The ovine prolactin had an activity of 35 i.u./mg. [3H]Progesterone (sp. act. 80–100 µCi/µg) was purchased from the New England Nuclear Corporation, Cambridge, Massachusetts and unlabelled progesterone from the Sigma Chemical Co.

**Chemicals**

[3H]Cyclic adenosine monophosphate (cyclic AMP) (sp. act. 27 Ci/mmol) was obtained from Schwarz/Mann, Orangeburg, New York, and unlabelled cyclic AMP from the Sigma Chemical Co. Other chemicals and solvents were of the highest purity commercially available.

**Ovaries**

Ovaries were obtained at a local slaughterhouse from non-pregnant pigs, 4 months old or older, within 20 min of death and were immediately placed on ice in 0·9% NaCl solution containing 100 i.u. penicillin/ml, 100 µg streptomycin/ml, and 25 µg amphotericin (Fungizone)/ml. Granulosa cells were harvested only from large (6–12 mm diameter) follicles and follicular fluid was collected from 1–12 mm follicles 1–4 h after death as described in detail by Channing & Kammerman (1973) and Channing & Ledwitz-Rigby (1975).

**Culture media**

Fluid samples from small (1–2 mm diameter), medium (3–5 mm) and large (6–12 mm) follicles are abbreviated as SFF1, MFF1, and LFF1 respectively. Follicular fluid and sera were diluted in culture medium 199 (v/v) before culture. A culture medium devoid of biological fluid (199D) was prepared by adding 0·2% lactalbumin hydrolysate (Grand Island Biological Co.), 0·4% bovine serum albumin (BSA) (GIBCO) and 1 µm insulin/ml (Squibb) to medium 199 (Channing & Ledwitz-Rigby, 1975). All media contained 50 µg gentamicin/ml and 2·5 µg Fungizone/ml.

The LH content of the fluid samples added to culture media was determined by radioimmunoassay. Only sera samples containing less than 1 ng LH (LER-788-4)/ml were employed. The concentration of LH in a pool of fluid from large follicles was 0·96 ng/ml; LH concentrations were undetectable (less than 0·3 ng/ml) in fluid from small follicles and in culture medium 199D.

**Culture procedures**

Granulosa cell morphology was studied in monolayer cultures grown in Leighton tubes with an initial inoculum of 1 x 10⁶–5 x 10⁶ cells. The culture medium was changed every 2 days for up to 6 days. At the end of the culture period, the cultures were fixed in Bouin’s solution and stained with haematoxylin and eosin (H and E) as described previously (Channing, 1970a, b).

For studies in which cyclic AMP accumulation was examined, granulosa cells were
incubated as short-term ‘suspension’ cultures in 5% CO₂ in air at 37 °C (Channing & Ledwitz-Rigby, 1975). Cell attachment did not occur. There were no significant alterations in cell number in suspension cultures during the 48 h incubation period ($P > 0.1$). Progesterone secretion was determined in both monolayer and suspension cultures.

**Cyclic AMP studies**

To examine the effects of follicular fluids on cyclic AMP accumulation, granulosa cells were first incubated in medium 199 supplemented with follicular fluid or serum. Following this pre-incubation the cells were centrifuged at 600g for 5 min, transferred to 1 ml of 1% BSA in Eagle’s medium, and incubated with shaking for 20 min at 37 °C as described by Channing & Ledwitz-Rigby (1975). For each treatment group, two control cultures were incubated without LH and two or three experimental cultures were incubated with 1 µg ovine LH/ml. At the end of the incubation the cultures were placed on ice, followed by separation of culture medium and cells by centrifugation for 10 min at 1000g. The cyclic AMP contents of both the cells and medium were then determined by competitive protein binding assay (Gilman, 1970) as modified by Kolena & Channing (1972) and by Mashiter, Mashiter, Hauger & Field (1973). Data are presented as total cyclic AMP in the cells and in the culture medium. Forty-eight hour pre-incubations were routinely used since preliminary experiments with pre-incubations of 20 min, 1, 6, 12, 18, 24, 36, and 48 h indicated that 48 h pre-incubations yielded the most consistent results (Channing, Thanki, Lindsey & Ledwitz-Rigby, 1977).

**Progesterone secretion studies**

Granulosa cells were incubated for 48 h in medium 199 supplemented with follicular fluid or serum. The progesterone secreted into the culture medium during the entire 48-h incubation was determined as described below.

In the first experiments, the progesterin content of the incubation medium and follicular fluid was measured using the competitive protein-binding assay developed by Johansson, Neill & Knobil (1968) and modified for culture medium by Channing (1970a). In later experiments, the progesterone contents of the culture media, serum, and follicular fluid were determined by radioimmunoassay after extraction with petroleum ether (Thorneycroft & Stone, 1972) using an antiserum made against 6-OH progesterone linked to BSA (generously donated by Dr Gordon Niswender). The progesterone radioimmunoassay was validated for culture medium by Channing, Tsai & Sachs (1976).

**Oestrogen radioimmunoassay**

The oestrogen content of follicular fluid was determined by radioimmunoassay after diethyl ether extraction according to the method originally described by Hotchkiss, Atkinson & Knobil (1971) and later validated by Channing & Coudert (1976).

**LH radioimmunoassay**

The LH concentrations of follicular fluid, serum and culture medium were measured by radioimmunoassay (Niswender, Reichert & Zimmerman, 1970) using NIH-LH-RP-1 for the standard curve. These values were converted to and are expressed in terms of porcine LH (LER-778-4) which has a potency of 0.5 NIH-LH-S1 units/mg (Niswender et al. 1970). Both LH preparations gave a parallel dose–response curve in the radioimmunoassay.

**Charcoal and petroleum ether extraction of follicular fluid and serum**

Follicular fluid and sera added to culture media employed in progesterone secretion studies were pretreated with either charcoal or 10 volumes petroleum ether to remove steroids.
Follicular fluid (5 ml) was added to a 12 ml graduated centrifuge tube containing 0.5 ml charcoal (Norit A). The fluid and charcoal were mixed vigorously with a Vortex mixer and separated by centrifugation for 10 min at 2000 g. The remaining charcoal was removed from the fluid by filtration through 0.45 and 0.22 micron Millipore filters. The extraction was necessary because 100–700 ng progestins/ml were present in SFF1 (n = 4) and 300–1400 ng progestins/ml in LFF1 (n = 4). The extraction removed 99% of the progestins. Oestrogen content of the SFF1 was also reduced from 2–5 ng/ml to 0.010–0.016 ng/ml. In LFF1 it was reduced from 8–108 ng/ml to 0.032–0.200 ng/ml (n = 3).

Statistics
Data have been analysed for differences between means with paired and unpaired Student’s t-tests. For the paired t-tests, different treatment groups within the same experiment were compared. Analysis of variance was employed when comparisons of multiple treatments were made.

RESULTS
Morphology of cultures grown in Leighton tubes
Morphological luteinization of cells from preovulatory follicles was seen after culture for 5 days in 20% serum or 20% LFF1 (Fig. 1c, d). Cells grown in 20% MFF1 luteinized partially but were slightly abnormal in appearance (Fig. 1e), whereas cells cultured in 20% SFF1 failed to luteinize and became necrotic after 5 days (Fig. 1f). Granulosa cells cultured in 50% SFF1 also failed to luteinize. They attached to the cover-slip, grew well and appeared healthy for 2 days (Fig. 1a), but after 4–6 days exhibited pronounced membrane blebbing and separated from the cover-slip. Granulosa cells grown in 50% LFF1 began to luteinize by day 2 of culture, appearing more epithelioid and containing more lipid droplets than cells cultured in 50% SFF1 (Fig. 1b).

Morphology of cells incubated as short-term cultures
Granulosa cells appeared healthy under ×450 magnification after 48 h in short-term suspension cultures in 50% serum, SFF1, MFF1, LFF1 and 199d and demonstrated viability by excluding lissamine green and trypan blue. Cells incubated in 50% SFF1 had slightly fewer lipid droplets than cells incubated in 50% LFF1.

Cyclic AMP accumulation
Granulosa cells pre-incubated for 48 h in 50% LFF1, 50% serum or medium 199d accumulated similar amounts of cyclic AMP during 20 min incubations with LH (P > 0.4) (Table 1). Pre-incubation in 50% SFF1 decreased the ability of granulosa cells to accumulate cyclic AMP in response to LH by 71% (P < 0.001). Fifty per cent MFF1 decreased LH-induced cyclic AMP accumulation by only 25% (P < 0.02). Pre-incubation in 50% SFF1 was more effective in inhibiting LH-induced cyclic AMP accumulation than pre-incubation in 20% SFF1 (P < 0.02). In the case of LFF1, the opposite effect was observed. Cells pre-incubated in 50% LFF1 accumulated more cyclic AMP in response to LH than did cells pre-incubated in 20% LFF1 (P < 0.01). Although the accumulation of cyclic AMP in response to LH appeared greater following incubation with 50% MFF1 than 20% MFF1, the great variability observed for MFF1 rendered the difference non-significant (P > 0.1). None of the basal cyclic AMP levels were significantly different from each other (P > 0.05 for 20% MFF1 v. 50% MFF1 and 20% LFF1 v. 50% LFF1 and P > 0.2 for all other comparisons using paired t-tests). The apparent increase in basal cyclic AMP for cells incubated in 20% MFF1 and in 20% SFF1 and LFF1 were due to a few experiments in which basal levels were raised in all treatment groups. Luteinizing hormone stimulation of cyclic AMP accumulation
Fig. 1. (a, b) Influence of 50\% follicular fluid upon the morphology of porcine granulosa cell monolayer cultures. Porcine granulosa cells obtained from large follicles were cultured for 2 days in 50\% porcine follicular fluid from large (a) and small (b) follicles. (c–f) Influence of 20\% follicular fluid and serum upon the morphology of porcine granulosa cell monolayer cultures. Porcine granulosa cells were cultured in Leighton tubes for 5 days in 20\% porcine serum (c) or 20\% follicular fluid obtained from large (6–12 mm) (d), medium (3–5 mm) (e) or small (1–2 mm) (f) follicles in a balance of medium 199. The cells were obtained from large (6–12 mm) follicles. (Haematoxylin and eosin staining: \times 450 magnification.)
in these experiments was not different from those of the experiments with low basal cyclic AMP levels ($P > 0.2$).

The inhibitory actions of SFFl depended more on the size of the follicles from which the fluid was collected than on the stage of the oestrous cycle. SFFl was collected and pooled from (1) ovaries containing no follicles larger than 5 mm, with or without corpora lutea or (2) ovaries containing follicles larger than 6 mm. The ovaries with no follicles greater than 5 mm were assumed to be from pigs between days 2 and 15 of the oestrous cycle (day 1 = day of ovulation) while ovaries containing follicles greater than 6 mm were assumed to be from pigs in the late follicular phase (days 16–21) (Robertson, 1969). Granulosa cells pre-incubated for 48 h in 50 % SFFl from the early part of the oestrous cycle accumulated 30.0 ± 2.6 (s.e.m.) % ($n = 7$) of control cyclic AMP levels in response to LH as compared with 35.0 ± 4.0 % ($n = 7$) accumulated by cells pre-incubated in 50 % late follicular phase SFFl ($P > 0.8$). Granulosa cells pre-incubated in 50 % LFFl collected from the same ovaries served as controls. Basal cyclic AMP levels were not significantly different from each other ($P > 0.2$) and ranged from 7.0 to 10.8 % of LH-stimulated control levels.

Charcoal or petroleum ether extraction of the follicular fluids did not alter the inhibitory influence of follicular fluids on granulosa cell accumulation of cyclic AMP. Cells pre-incubated in 50 % extracted SFFl accumulated 21.2 ± 2.8 % of control cyclic AMP ($n = 6$) during the 20-min incubation with LH as compared with 29.8 ± 7.5 % ($n = 5$) accumulated by cells pre-incubated in 50 % unextracted SFFl ($P > 0.2$). Cells pre-incubated in 50 % extracted LFFl accumulated 92.3 ± 20.8 % of control cyclic AMP ($n = 6$) in response to LH as compared with 100 ± 11.9 % ($n = 6$) accumulated by the control cells pre-incubated in 50 % unextracted LFFl ($P > 0.4$). None of the basal cyclic AMP levels was significantly different from each other ($P > 0.3$).
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Influence upon progesterone secretion

Incubation of granulosa cells in 50% SFF1 for 2 days in either suspension or monolayer cultures depressed the secretion of progesterone into the culture medium by 50 to 95% as compared with cells cultured in 50% LFF1, serum or medium 199d (Table 2). To determine whether our observations of SFF1 inhibition of progesterone secretion might be due to the action of prolactin, porcine granulosa cells from large preovulatory follicles were cultured in monolayer in 10% pig serum in medium 199 with 0, 5, 10, 25, 50 or 100 ng ovine prolactin/ml (NIH-P-S12). The addition of 5 to 50 ng prolactin/ml significantly enhanced progesterone secretion over a 2-day incubation period ($P < 0.05$). Only the highest concentration of prolactin (100 ng) produced even a small decrease (69% of control; $P < 0.05$) in progesterone secretion.

Table 2. Influence of follicular fluid upon progesterin secretion during 48-h incubations (means ± S.E.M.)

<table>
<thead>
<tr>
<th>Expt</th>
<th>Type of culture</th>
<th>Medium:</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50% SFF1</td>
<td>50% LFF1</td>
</tr>
<tr>
<td>1*</td>
<td>Monolayer</td>
<td>4700±400 (3)</td>
<td>120000 (3)</td>
</tr>
<tr>
<td>2*</td>
<td>Monolayer</td>
<td>1100±43 (3)</td>
<td>2334±90 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(50% serum)</td>
</tr>
<tr>
<td>3‡</td>
<td>Suspension</td>
<td>36±6 (5)</td>
<td>74±8 (4)</td>
</tr>
<tr>
<td>4‡</td>
<td>Suspension</td>
<td>81±5 (4)</td>
<td>202±39 (3)</td>
</tr>
<tr>
<td>5*</td>
<td>Suspension</td>
<td>5656±548 (4)</td>
<td>40121±2167 (4)</td>
</tr>
<tr>
<td>6*</td>
<td>Monolayer</td>
<td>423±7 (3)</td>
<td>10595±429 (3)</td>
</tr>
</tbody>
</table>

Granulosa cells obtained from large (6–12 mm) porcine follicles were incubated for 48 h as monolayers or suspensions. Follicular fluids and sera were pretreated to remove endogenous progesterone. The number of cultures used to obtain the mean is indicated in parentheses. See text p. 176 for descriptions of culture media.

* Progesterin secretion measured by radioimmunoassay.
† Progesterin secretion measured by competitive protein-binding assay.

DISCUSSION

Follicular fluid from small porcine follicles inhibited the spontaneous luteinization in vitro of granulosa cells obtained from large follicles: failure of morphological luteinization, diminished accumulation of cyclic AMP in response to LH and decreased secretion of progesterin were observed. Follicular fluid from medium-sized follicles was less inhibitory than fluid from small follicles; 50% LFF1 was as permissive of luteinization as 50% serum or culture medium 199d. These last two media have been previously shown to support morphological luteinization and progesterone secretion by monkey and porcine granulosa cells (Channing et al. 1977; F. Ledwitz-Rigby & C. P. Channing, unpublished observations) while medium 199 plus 50% pig serum or 50% LFF1 or medium 199d supported similar accumulation of cyclic AMP in response to LH. These findings suggest that there is a luteinization "inhibitor" present in fluid from small follicles which is gradually lost as the follicle matures, but this does not rule out the possibility that fluid from large follicles contains a stimulatory substance which overcomes the action of an inhibitor or, alternatively, acts alone.

The observations that granulosa cells luteinize in defined medium (199d) and in media containing serum could be interpreted as supporting the existence of an inhibitory substance(s) in SFF1. It is possible, however, that these two culture media contain a molecule permissive
of luteinization, similar to one which might exist in LFFI. The greater inhibitory influence of 50 % SFFI as compared with 20 % SFFI suggests that inhibition is dose dependent. However, granulosa cells accumulated more cyclic AMP in response to LH when pre-incubated in 50 % LFFI than in 20 % LFFI. This may be due to a non-specific effect of increased protein concentration in the culture media or to a dose-dependent action of a specific permissive molecule. The ability of 50 % serum to enhance granulosa cell responsiveness to LH as compared with 20 % serum, supports the former hypothesis (F. Ledwitz-Rigby & C. P. Channing, unpublished observations).

There are many differences in the composition of follicular fluid obtained at different stages of the reproductive cycle (Edwards, 1974). The concentrations of oestrogen, progesterin and LH increase with the increasing size of porcine follicles (Channing, 1973; Eiler & Nalbandov, 1973 and the present observations) whereas concentrations of Na+, K+ (Schuetz & Anisowicz, 1974; F. Ledwitz-Rigby & C. P. Channing, unpublished observations), total proteins (Shalgi, Kraicer, Rimon, Pinto & Soferman, 1973) and insulin (F. Ledwitz-Rigby & C. P. Channing, unpublished observations) do not change. The differences in oestrogen and progesterin concentrations between SFFI and LFFI do not appear to be critical for either SFFI inhibition or LFFI support of luteinization. Charcoal and petroleum ether extraction removed 99 % of the oestrogen and progesterin from the follicular fluid without affecting their influence on granulosa cell luteinization. These extraction procedures should also have removed the majority of unbound androgens from the fluid. Thus, a difference in androgen concentration in SFFI and LFFI should not be the cause of SFFI inhibitory actions.

The importance of the higher LH concentration in fluid from large follicles (0.96 ng/ml) as compared with undetectable levels in SFFI (less than 0.3 ng/ml) remains to be determined. It is significant, however, that medium 199D which is permissive of luteinization, also has undetectable levels of LH.

McNatty, Hunter, McNeilly & Sawers (1975) have found that prolactin concentrations decrease in human follicular fluid from the early to late follicular phase. There was no difference, however, between prolactin concentrations in fluid from different sized follicles. McNatty, Sawers & McNeilly (1974) reported that high concentrations of prolactin (25–100 ng/ml) depressed progesterone secretion by human granulosa cells in vitro. Interestingly, removal of prolactin from their control culture medium also depressed progesterone secretion. Our data on the effect of prolactin on porcine granulosa cells in vitro suggest that only concentrations of prolactin as high as 100 ng/ml inhibit progesterone secretion. Even this high level of prolactin was less inhibitory than 50 % SFFI. To our knowledge, no one has assayed prolactin in porcine follicular fluid. Unless porcine follicular fluid contains concentrations of prolactin at least three times greater than those reported in human follicular fluid (McNatty et al. 1975), our data would suggest that prolactin is not the inhibitory factor.

In preliminary experiments we have determined that dialysis of SFFI or heating it to 60 °C did not alter its ability to inhibit LH-induced cyclic AMP accumulation (F. Ledwitz-Rigby, V. L. Gay & C. P. Channing, unpublished observations). Bernard (1973) has reported that dialysis of porcine follicular fluid does not interfere with its inhibition of morphological luteinization by rat granulosa cells whereas heating to 80 °C does. These observations suggest that the luteinization inhibitor(s) is a relatively heat-stable molecule of greater molecular weight than 10000.

The physiological significance of inhibition of luteinization by SFFI but not by LFFI may be that SFFI interferes with the earliest onset of luteinization. Although luteinization is generally considered to be a postovulatory process, several stages of granulosa cell maturation can be defined. These steps include an increase in available binding sites for LH,
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increased ability of LH to stimulate cyclic AMP accumulation, increased ability to secrete progesterone when the cells are cultured in vitro, and a decrease in the hormonal requirements for luteinization in vitro (Channing & Tsafirri, 1977). It may be necessary for SFFL to change into LFFL with loss of the luteinization inhibitory substance(s) before these maturational processes can occur. Failure of a follicle to alter the composition of follicular fluid in the normal sequence might result in follicular atresia. The variability of the MFFI inhibitory action upon luteinization may be due to a mixed population of viable and atretic medium-sized follicles. Knowledge of the differences in follicular fluid composition which occur during follicular maturation may enhance our understanding of follicular atresia.

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