Follicular fluid modulation of functional LH receptor induction in pig granulosa cells

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Summary. Follicular fluid was collected from small (1–2 mm), medium (3–5 mm) and large (6–12 mm) follicles of pigs, treated with charcoal to remove steroids, and tested for effects on the induction of functional LH/hCG receptors in cultures of granulosa cells from small antral pig follicles. Granulosa cells were cultured for 2, 4 or 6 days in Medium 199 + 10% pig serum. Granulosa cells cultured in the presence of purified human FSH (0.1 µg/ml, LER 8/117), insulin (1 mU/ml), cortisol (0.01 µg/ml) and thyroxine (10^{-7} M) accumulated a 4- to 8-fold increase in LH/hCG receptors compared to control cultures. The amounts of cyclic AMP and progesterone secreted after exposure to ovine LH (1 µg/ml: NIH-S19) were also increased 2–3-fold and 80–100-fold, respectively. Exposure to FSH alone resulted in lower amounts of LH/hCG receptors with a concomitant decrease in optimum LH responses.

Addition of 12.5–50% follicular fluid obtained from small (1–2 mm) follicles led to a dose-dependent inhibition of the FSH plus insulin, cortisol and thyroxine induction of LH/hCG receptors after 4 days of culture. Fluid from medium follicles showed reduced ability to inhibit LH/hCG receptor induction, and fluid from large follicles exerted only a slight inhibition or no inhibition of receptor induction. Fluid from medium-sized and large follicles exerted a progressive dose-dependent stimulation of progesterone secretion by the granulosa cell cultures. The inhibitory activity was precipitated primarily with 70% ethanol and to a lesser degree by 36 and 90% ethanol. These studies demonstrate that induction of functional LH/hCG receptors in cultures of pig granulosa cells from immature follicles is enhanced by including insulin, cortisol and thyroxine, in addition to FSH, in the culture medium, and that follicular fluid modulates both receptor induction and progesterone secretion as a function of follicular maturation.

Introduction

The mechanisms whereby certain follicles are chosen to mature, ovulate and luteinize are poorly understood. Granulosa cells from large, preovulatory pig follicles have 10–50-fold more luteinizing hormone/human chorionic gonadotrophin (LH/hCG) receptors than do cells from small follicles (Channing & Kammerman, 1973; Nakano, Akahori, Katayama & Tojo, 1977). Concomitant with an increase in receptors for LH, granulosa cells gradually develop the ability to secrete large amounts of progesterone and accumulate cyclic AMP in response to LH as follicular maturation progresses (Channing, 1970; Lindsey & Channing, 1979; May & Schomberg, 1984). Pig granulosa

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cells only express their full potential to secrete progesterone *in vitro* if they are exposed to the preovulatory LH surge *in vivo* (Channing, Brinkley & Young, 1980). Monkey granulosa cells will also secrete increasing amounts of progesterone *in vitro* as the follicles mature and therefore the primate follicle must also respond to the preovulatory LH/FSH surge *in vivo* to attain full capacity to secrete progesterone in culture (Channing, 1980).

Channing, Tsai & Sachs (1976) observed that granulosa cells obtained from small pig follicles, when exposed *in vitro* to LH, follicle-stimulating hormone (FSH), insulin (I), cortisol (F) and thyroxine (T), could maximally secrete progesterone compared to cells incubated in LH + FSH alone. May & Schomberg (1981, 1984) observed that addition of FSH plus insulin to pig granulosa cells in culture could stimulate LH/hCG receptor induction and progesterone secretion. However, components contained within pig follicular fluid also seem to influence granulosa cell maturation. Ledwitz-Rigby & Rigby (1979) observed that follicular fluid obtained from small follicles inhibited basal and LH-stimulated progesterone secretion, whereas follicular fluid from large follicles enhanced granulosa cell progesterone secretion. Ledwitz-Rigby, Rigby, Long, Stewart & McLean (1981) also reported that follicular fluid obtained from large follicles enhanced granulosa cell conversion of androgen to oestrogen. The present studies were designed to examine whether non-steroidal intrafollicular components modulate the induction of functional LH/hCG receptors and progesterone secretion in cultures of granulosa cells from immature pig follicles. Additionally, the induction of functional LH/hCG receptors with FSH, insulin, cortisol and thyroxine was validated as a bioassay for factors affecting granulosa cell maturation *in vitro*. The effects of Amicon PM-10 membrane filtration and ethanol precipitation upon these activities of pig follicular fluid were also examined to develop a standard preparation for analysis of further purification procedures.

Materials and Methods

Granulosa cell preparation and culture procedures. Ovaries were collected from sows at a local slaughterhouse in 0.9% (w/v) NaCl plus 100 i.u. penicillin/ml + 100 µg streptomycin/ml in groups of 200–1000 and transported to the laboratory at room temperature. Within 3–5 h of ovary collection, granulosa cells were aspirated under aseptic conditions using a 20-gauge needle and 3-ml syringe from small (1–2 mm) follicles and separated from follicular fluid by centrifugation at 500 *g* (Channing & Ledwitz-Rigby, 1975). The cells were washed three times in TC medium 199 + 5% pig serum (Gibco, Grand Island, NY). Cells were separated from wash medium by an additional centrifugation at 500 *g*. The final washed granulosa cells were counted in a haemocytometer containing 0.06% trypan blue. Live cells (which excluded the dye) were then adjusted to a final concentration of 300 000 cells/culture for 2- and 4-day incubations or 150 000 cells/culture for 6-day incubations in Medium 199 containing 10% pig serum, 1 mM-glutamine (Gibco) and 50 µg gentamicin sulphate/ml (Schering Corp., Kenilworth, NJ) added immediately before use. The cells were cultured for 2, 4 or 6 days in 0.5 ml of the above culture medium containing no additions, purified human FSH (LER 8/117; 0.1 µg/ml), FSH with IFT (1 mU porcine insulin/ml: Lilly, Indianapolis, IN; 0.01 µg cortisol/ml; 10⁻⁷ M-thyroxine, Sigma, St Louis, MO), or IFT alone. The purified human FSH (895 U/mg) had been treated with α-chymotrypsin to inactivate the LH contaminant (Reichert, 1967) which was 5.7 i.u./mg. After 2 and 4 days of incubation in Falcon Multiwell plates (No. 3008; Oxnarel, CA) at 37°C with 95% air and 5% CO₂, some cells were removed from culture for subsequent analysis of binding of ¹²⁵I-labelled hCG. Other cells were additionally exposed to ovine LH (NIH-S19, 1 µg/ml) for 1 h before removal from culture and measurement of intracellular cyclic AMP accumulation. Some cultures were continued for 2 additional days in the presence of LH alone (same concentration as above) to determine the responsiveness of the granulosa cells in culture to LH in terms of progesterone secretion. Cultures containing follicular fluid (12.5%, 25% or 50%) were cultured for 4 days in the presence of FSH + IFT. Cells were assayed for DNA content using the method of Brunk, Jones & James (1979).

Progesterone. Progesterone concentrations in the media were determined by radioimmunoassay using an antiserum generated in sheep against an 11-hydroxyprogesterone-bovine serum albumin conjugate supplied by Dr Gordon Niswender. Culture medium was appropriately diluted and assayed without prior extraction with petroleum ether as described by Channing *et al.* (1976). The cross-reaction to 20 α -dihydroprogesterone was <5% compared to progesterone (Channing & Coudert, 1976).

LH/hCG binding. LH receptors on granulosa cells were measured utilizing ^{125}I -labelled hCG since LH and hCG are thought to share the same binding sites on ovarian cells. Addition of unlabelled LH and hCG can inhibit binding of ^{125}I -labelled hCG to granulosa cells (Sakai, Channing & Engel, 1977). Highly purified hCG obtained from Dr Robert Canfield (CR 117; 14 000 i.u./mg) was iodinated with 1 mCi ^{125}I (New England Nuclear, Boston, MA) in aliquants of 10 μg , utilizing the chloramine-T technique originally described by Greenwood, Hunter and Glover (1963) and modified by Sakai *et al.* (1977). The hormone (10 μg) plus ^{125}I was mixed with an equal amount of chloramine-T and then iodinated at 4°C for 30 sec. The hCG was iodinated to a specific activity of 25–50 $\mu\text{Ci}/\mu\text{g}$. Counting of ^{125}I was 67% efficient. Binding studies were carried out in siliconized 12 $\times$ 75 mm glass test tubes according to the methodology described by Sakai *et al.* (1977). Briefly, about 200 000 granulosa cells were incubated in the presence of 5 ng ^{125}I -labelled hCG with or without an additional 10 μg ovine LH (NIH-S19) for determination of non-specific binding. All incubations were carried out for 1 h at 37°C.

cAMP accumulation. The accumulation of intracellular cAMP was measured utilizing a radioimmunoassay kit for cAMP determination purchased from New England Nuclear, Boston, MA. The sensitivity of the assay was 0.1 pmol cAMP per tube.

Follicular fluid collection and treatment. Follicular fluid was collected from small (1–2 mm), medium sized (3–5 mm) and large (6–12 mm) follicles by aspiration at 4°C. An attempt was made to avoid aspiration of grossly atretic, cloudy, follicles. All fluids were centrifuged for 20 min at 1000 *g* to remove cell debris and frozen at –35°C until use. Four pools of whole follicular fluid were made consisting of 5–10 days of collections. Immediately before freezing, the follicular fluid was treated with 2 mM-phenylmethylsulphonylfluoride (PMSF) for 30 min to inactivate available serine proteases. The PMSF was made as a 100-fold solution in absolute ethanol and added dropwise to the follicular fluid so that one part of the PMSF solution was added to 100 parts of follicular fluid.

Oocytes recovered from these pools were examined under the binocular microscope and evaluated for gross signs of atresia: <20–30% of the oocytes were denuded of granulosa cells (evidence of atresia). The other oocytes had healthy looking cumuli which were intact (in most instances) or partly removed. Therefore, <30% of the follicles that yielded these oocytes and fluid were grossly atretic. Oocytes recovered from follicles that had intact cumuli were cultured for 42–44 h in groups of 40–50 and had a 50–70% nuclear maturation rate (see Stone, Pomerantz, Kripner & Channing, 1978). Atresia was similar to that reported by Ryan (1981) and far less in some instances. Follicular fluid in the non-sterile state was only allowed to stand for 2–3 h at 4°C or room temperature since Sluss & Reichert (1983) have observed that raw non-sterile porcine follicular fluid allowed to stand at 4°C for 1–2 days develops bacterial growth which can generate an FSH-binding inhibitor. Our collection procedure, which involved placing the ovaries in sterile 0.9% NaCl plus penicillin and streptomycin, served to wash away bacteria which might be associated with the ovaries collected at the slaughterhouse. The follicular fluid was sterilized by filtration (0.22 μm ; Millipore-Millex HA, Bedford, MA) and the progesterone concentration of each pool was measured as detailed by Channing & Coudert (1976). Before the final 0.22 μm filtration the fluid was prefiltered through filters of 1.2, 0.8 and 0.45 μm . In all procedures the follicular fluid was kept under reasonably aseptic conditions and not allowed to stand. When untreated follicular fluid was added to granulosa cell cultures the progesterone content of the added follicular fluid was subtracted from subsequent progesterone secretion data. In additional experiments follicular fluid recovered from small (1–3 mm), medium-sized (3–5 mm) and large (6–12 mm) pig follicles were

treated with charcoal to remove steroids before addition to granulosa cell cultures. The follicular fluid pool (200 ml) was mixed with 10% (v/v) activated charcoal (Norite A, Fisher Pittsburgh, PA) and the mixture stirred overnight at 4°C. The following day, the mixture was centrifuged at 3000 *g* for 30 min, followed by centrifugation at 100 000 *g* for 60 min. The fluid was then serially filtered through a prefilter, and filters of 1.2, 0.8, 0.45 and 0.22 µm to remove charcoal and to render the fluid sterile. Charcoal treatment in this manner reduced the oestrogen and progesterone content of the fluid to non-detectable levels as determined by radioimmunoassay (<0.1 ng/ml for both). After charcoal treatment, the follicular fluid was extracted with diethyl ether or petroleum ether for oestrogen and progesterone determinations, respectively.

The charcoal-treated follicular fluid was further separated into the high and low molecular weight fractions by filtration on an Amicon PM-10 membrane. Each charcoal-treated follicular fluid pool was added in a 100 ml sample to a PM-10 membrane and the Amicon cell stirred overnight at 4°C under 0.7–1.4 kg N₂/cm². The filtrate was recovered and added to culture without further treatment after sterile filtration through a 0.22 µm filter. The retentate was recovered from the cell and reconstituted to half the original volume of the starting fluid with distilled water. Subsequently it was reconstituted to the original volume of follicular fluid with culture medium and filtered through a 0.22 µm filter preceded by filtration through filters of 1.2, 0.8 and 0.45 µm. Filtrate, retentate and follicular fluid were added at a dose of 50% the final culture volume.

Follicular fluid recovered from small follicles was also treated with various concentrations of ethanol to determine which concentration precipitated the bulk of luteinization inhibitory activity. Samples (50 ml) of fluid recovered from 1–2 mm follicles were mixed with cold absolute ethanol such that the final concentration of ethanol was 20, 30, 50, 70 or 90%: 100 ml fluid were brought to 5, 10 and 15%. The mixture was allowed to stand overnight at 4°C followed by centrifugation and freeze-drying of the precipitate to dryness. This precipitate was added to granulosa cell cultures at 1 or 10 mg/ml.

Statistical analysis of data. Data were subjected to Analysis of Variance and Duncan's New multiple range test. Cochran's test for heterogeneity was applied and if the data were heterogeneous the data were transformed to the logarithm before analysis. A *P* value of <0.05 was considered significant. Each experiment was repeated on 2–6 separate collections of granulosa cells to confirm reproducibility.

Results

Binding of hCG

As shown in Text-fig. 1 the amount of ¹²⁵I-labelled hCG specifically bound to granulosa cells from small follicles increased 4–5-fold above Day 0 values by Day 2 when the cells were cultured in the presence of FSH or FSH + IFT (*P* < 0.01). Addition of IFT alone did not bring about a significant increase in hCG binding. After 4 days of culture, FSH + IFT brought about an 8-fold increase in hCG binding (*P* < 0.05) compared with control value.

Preliminary observations demonstrated that addition of 0.1 or 1.0 µg FSH both resulted in similar increases in hCG binding (M. Loeken & C. P. Channing, unpublished data). In most experiments the specific binding was 80–90% of total binding.

Cyclic AMP accumulation

As shown in Text-fig. 2, after 2 days of culture with FSH or FSH + IFT, granulosa cells accumulated significantly more intracellular cyclic AMP than did Day 0 cells when exposed to LH for 1 h (*P* < 0.05). The LH response of cells incubated in control or IFT media for 2 days did not significantly change from Day 0 values. The amount of cyclic AMP accumulated in response to LH did not differ between FSH and FSH + IFT at Day 2, but was highest in FSH + IFT containing cultures on Day 4 (*P* < 0.05).

Text-fig. 1. Effect of insulin, cortisol and thyroxine (IFT) and FSH + IFT upon induction of ability of pig granulosa cells to bind iodinated hCG. Granulosa cells from small antral follicles were cultured for 0, 2 or 4 days in Medium 199 and 10% pig serum containing no exogenous hormones (control), hFSH (LER 8/117; 0.1 µg/ml), hFSH with IFT (1 mU insulin/ml, 0.01 µg cortisol/ml, 10⁻⁷ M-thyroxine) or IFT alone. At the end of each incubation the cells were removed from the wells by scraping with a rubber policeman into saline (9 g NaCl/l). Aliquants of the cells were taken for determination of ¹²⁵I-labelled hCG binding and for DNA content. The values represent the mean ± s.e.m. (*n* = 8) of 4 separate experiments. Day 0 values are those of ¹²⁵I-labelled hCG bound to the cells on the day of collection.

Text-fig. 2. Effect of FSH, insulin, cortisol and thyroxine (IFT) and FSH + IFT upon the ability of pig granulosa cells to respond to LH by accumulation of cyclic AMP. Granulosa cells from small antral follicles were cultured for 0, 2 or 4 days in Medium 199 and 10% pig serum containing no exogenous hormones (control), hFSH (LER 8/117; 0.1 µg/ml), hFSH with IFT (1 mU insulin/ml, 0.01 µg cortisol/ml, 10⁻⁷ M-thyroxine) or IFT alone. At the end of each incubation the cells were removed from the wells by scraping with a rubber policeman into saline (9 g NaCl/l). Aliquants of the cells were taken and incubated for an additional 1 h in the absence or presence of ovine LH (NIH-S19, 1 µg/ml) for determination of intracellular cyclic AMP. Separate aliquants were taken for DNA determination. The values represent the mean ± s.e.m. (*n* = 4).

Progesterone secretion

As shown in Text-fig. 3, granulosa cells from small follicles secreted significantly more progesterone than control cultures when cultured in FSH or FSH + IFT (*P* < 0.01). FSH + IFT-containing cultures secreted greater than 100-fold more progesterone than the control cells. Cultures grown for 4 days exhibited similar increases in progesterone secretion over control levels when grown with both FSH and FSH + IFT. Progesterone secretion was significantly greater in cultures containing FSH + IFT than in cultures containing FSH alone at both culture periods

Text-fig. 3. Effect of FSH, insulin, cortisol and thyroxine (IFT) and FSH + IFT upon the ability of cultured pig granulosa cells to (a) secrete progesterone and (b) respond to LH with increased progesterone secretion. Granulosa cells from small antral follicles were cultured for 2 or 4 days in Medium 199 and 10% pig serum containing no exogenous hormones (control), hFSH (LER 8/117; 0.1 μ g/ml), hFSH + IFT (1 mU insulin/ml, 0.01 μ g cortisol/ml, 10^{-7} M-thyroxine), IFT alone or LH (NIH-S19, 1 μ g/ml). In (b) ovine LH (NIH-S19, 1 μ g/ml) was added to the cultures for an additional 2 days. The spent medium was then removed and the content of progesterone determined by specific radioimmunoassay. The values represent the mean $\pm$ s.e.m. for 4 experiments (a) or 2 experiments (b).

examined ($P < 0.05$). Additionally, cultures containing LH secreted significantly more progesterone than did control cultures ($P < 0.05$) while cultures containing IFT alone did not secrete more progesterone than control cultures. However LH alone was much less effective in inducing progesterone secretion than was FSH alone or FSH + IFT.

Additional granulosa cell cultures were preincubated for 2 or 4 days with the various FSH and IFT combinations before being incubated for 2 more days in the presence of LH alone (Text-fig. 3). Cells preincubated with FSH alone or FSH + IFT secreted significantly more progesterone during 2 days exposure to LH than did control cultures or cultures containing only IFT at both culture periods examined ($P < 0.05$). Progesterone secretion in cultures containing FSH + IFT was greater than in cultures containing FSH alone. Cultures treated with IFT alone (no FSH) did not respond to LH with progesterone secretion above control values at either time period examined.

Follicular fluid effects

Neat follicular fluid. The effects of neat follicular fluid from small (1–2 mm) and large (6–12 mm) follicles on FSH + IFT-induced LH/hCG receptor generation and stimulation of progesterone

secretion are shown in Table 1. Follicular fluid from small follicles significantly inhibited progesterone secretion in granulosa cell cultures below control values even at the most dilute (12.5%) concentration ($P < 0.01$). However, a dose-dependent effect was indicated since at 12.5% the inhibition of progesterone secretion was significantly less than at 50% follicular fluid ($P < 0.05$). Follicular fluid obtained from large follicles significantly stimulated (50% fluid) progesterone secretion ($P < 0.05$) or had no effect compared to control cultures (25% and 12.5% fluid). Cultures containing 50% fluid from large follicles also secreted significantly more progesterone ($P < 0.05$) than did cultures containing 12.5% fluid.

Table 1. The effect of neat follicular fluid from small or large pig follicles on progesterone secretion and FSH-induced acquisition of LH/hCG receptors by pig granulosa cells *in vitro*†

Follicle size	Fluid conc.	Specific binding of ¹²⁵ I-labelled hCG (% of control)‡	Progesterone secretion§ (ng/culture/day)
Control		100	206.9 ± 15.3
Small	50%	22.6 ± 2.6**	68.4 ± 6.8*
	25%	30.1 ± 2.9**	80.1 ± 7.7*
	12.5%	36.2 ± 2.7**	116.6 ± 8.6*
Large	50%	61.5 ± 5.2*	285.1 ± 26.9*
	25%	67.2 ± 5.9*	226.5 ± 23.9
	12.5%	75.2 ± 12.7	176.7 ± 24.4

† Granulosa cells from small antral follicles were cultured for 2 or 4 days in Medium 199 and 10% pig serum containing FSH (LER 8/117; 0.1 µg/ml), insulin (1 mU/ml), cortisol (0.01 µg/ml) and thyroxine (10^{-7} M).

‡ After 4 days of culture. Values represent the mean ± s.e.m. for 3 determinations from 3 separate culture experiments.

§ From Days 0 to 2 of culture. Values represent the mean ± s.e.m. for 4 determinations from 3 separate experiments.

Compared with control value: * $P < 0.05$, ** $P < 0.01$.

Fluid from small or large follicles inhibited the induction of LH/hCG receptors by FSH + IFT below the 95% confidence limits of cultures containing FSH + IFT alone. However, fluid from small follicles inhibited receptor induction significantly below the inhibition observed with the same dose of fluid from large follicles ($P < 0.01$). The most dilute dose of fluid from small follicles was significantly less effective in inhibiting LH/hCG receptor induction when compared to the 50% dose ($P < 0.05$).

Charcoal-treated Amicon-filtered follicular fluid. Addition of charcoal-treated follicular fluid (50%) recovered from follicles of all sizes and its high and low molecular weight fractions led to a significant depression ($P < 0.05$) in ¹²⁵I-labelled hCG binding to the cells as compared to control cultures incubated with FSH + IFT alone (Table 2). However, both whole follicular fluid and the PM-10 retentate ($M_r > 10\,000$) from small follicles inhibited LH/hCG receptor induction significantly ($P < 0.05$) compared to the level observed with whole fluid or retentate from large follicles. The PM-10 filtrates ($M_r < 10\,000$) of follicular fluid from small, medium or large follicles were similar in their effect on induction of LH/hCG receptors. Nevertheless, the PM-10 filtrate could contain some toxic low molecular weight material and further studies are being undertaken. Whole follicular fluid from medium-sized follicles was not different from whole fluid from large follicles with regard to the induction of LH/hCG receptors; however, the PM-10 retentate of fluid from large follicles permitted more receptor induction than did the PM-10 retentate from medium-sized follicles ($P < 0.05$).

Table 2. Effect of 50% charcoal-treated follicular fluid fractions from small, medium or large pig follicles on progesterone secretion and FSH-induced acquisition of LH/hCG receptors by pig granulosa cells over 4 days *in vitro* (see text)

	Specific binding of ¹²⁵ I-labelled hCG (d.p.m./μg DNA)†	Progesterone secretion (ng/day/μg DNA)‡
Control (no hormones added)	300 ± 25 ^g	127 ± 15 ^f
FSH + IFT alone	1620 ± 57 ^a	365 ± 37 ^c
Fluid from small follicles		
Whole	312 ± 20 ^g	277 ± 35 ^d
Filtrate	962 ± 71 ^d	192 ± 37 ^e
Retentate	487 ± 28 ^f	299 ± 39 ^d
Fluid from medium follicles		
Whole	725 ± 29 ^e	536 ± 40 ^{a,b}
Filtrate	1050 ± 72 ^c	176 ± 47 ^{e,f}
Retentate	869 ± 29 ^d	525 ± 36 ^{a,b}
Fluid from large follicles		
Whole	847 ± 39 ^d	478 ± 50 ^{b,c}
Filtrate	1018 ± 57 ^c	172 ± 32 ^{e,f}
Retentate	1138 ± 50 ^b	692 ± 79 ^a

^{a-g} $P < 0.05$ compared with other values. Values having different letters differ ($P < 0.05$).

† On day of collection (Day 0) specific binding was 445 ± 48 d.p.m./μg DNA. Values are mean ± s.e.m. for 3 determinations from 3 separate cultures.

‡ Values, from Days 2 to 4 of culture, are mean ± s.e.m. for 6 determinations from 3 separate cultures.

Quantitatively, the PM-10 retentate had more inhibitory activity compared to filtrate and the inhibitory activity of the retentate changed with the size of follicles used as a source of fluid, i.e. there was more inhibitory activity in fluid recovered from small than from large follicles.

As was also shown in Text-fig. 1, addition of FSH + IFT alone significantly increased basal progesterone secretion in cultures of granulosa cells from the small follicles compared to control cultures between 2 and 4 days of culture (Table 2). Whole follicular fluid from small follicles significantly inhibited progesterone secretion in these cultures while the high molecular weight

Table 3. Ethanol precipitation of pig follicular fluid

Volume of fluid applied (ml)	% of ethanol achieved	Dry wt (mg) precipitate	Supernatant	Dry wt precipitate as % or total dry wt in follicular fluid
100	5	290	7520	3.6
100	10	400	6750	5.0
100	15	410	6450	5.1
50	20	980	3400	24.5
50	36	2000	1780	50.0
50	50	3010	418	75.2
50	70	2720	402	68.0
50	90	3380	359	84.5

Dry weight of 50 ml starting follicular fluid from small (1–2 mm) follicles was 4000 mg. Aliquants of each precipitate were assayed in 2 bioassays, the data of which are shown in Text-fig. 4.

Text-fig. 4. Effect of ethanol precipitation of follicular fluid from small pig follicles upon inhibitory activity upon FSH induction of ^{125}I -labelled hCG binding. Conditions of the experiment are described in the text: 1/10 is equivalent to 1 mg/ml and 1/1 is equivalent to 10 mg/ml ethanol precipitate. All cultures contained FSH + IFT except the control (C). The zero hour binding was 268 d.p.m./ μg cell DNA. S represents starting follicular fluid. F $\oplus$ refers to a control culture with FSH + IFT. Data are from 2 experiments and represent the mean $\pm$ s.e.m. of 4 individual values.

fraction (PM-10 filtrate) from all follicular fluids tested significantly inhibited progesterone secretion below the level observed with FSH + IFT alone. However, both whole fluid and PM-10 retentate from medium or large follicles stimulated progesterone secretion significantly above the level of cultures containing FSH + IFT alone. The highest level of progesterone secretion was observed in cultures containing PM-10 retentate from large follicles.

Ethanol precipitation of follicular fluid recovered from small follicles. As is evident from data presented in Text-fig. 4, inhibitory activity on FSH induction of hCG/LH binding sites precipitated primarily in 70% ethanol with activity precipitating to a lesser degree in 20, 36 and 90% ethanol. Much of the dry weight from follicular fluid was precipitated by 70% ethanol (Table 3).

Discussion

The observations reported in the present study demonstrate that the addition of either FSH alone or a mixture of FSH + IFT can induce functional LH/hCG binding sites on pig granulosa cells, *in vitro*. Functional LH/hCG binding sites were assessed by LH stimulation of intracellular cyclic AMP accumulation and progesterone secretion. The addition of FSH + IFT resulted in, not only a significant 8-fold increase in ¹²⁵I-labelled hCG binding (Text-fig. 1), but a concomitant 2–3-fold increase in the ability of LH to stimulate cyclic AMP accumulation (Text-fig. 2) and up to an 80–100-fold increase in LH stimulation of progesterone secretion (Text-fig. 3). The synergism of IFT on FSH induction of LH/hCG receptors was significant after 4 days of incubation. Preincubation of cells for 2 days with IFT maintained LH responsiveness in terms of cyclic AMP accumulation, but apparently this LH induced elevation in cyclic AMP was not transmitted to the steroidogenic response since there was no increase in progesterone secretion during a 2–4-day post-incubation with LH (Text-fig. 3). Both FSH and LH stimulated progesterone secretion during 0–2 and 2–4 days of culture, but the progesterone response was greatest if FSH was added (Text-fig. 3). The different responses to gonadotrophic stimulation may be due to the reported predominance of FSH receptors

compared to LH receptors in granulosa cells from small pig follicles (Nakanon *et al.*, 1977). To obtain greater responsiveness to LH in granulosa cells from small follicles, pretreatment with FSH is required to induce functional LH/hCG receptors (compare Text-figs 3a & 3b). We think the loss of LH/hCG receptors in control cultures, those without FSH + IFT, is probably due to a lack of hormones which are required to maintain existing levels of receptors as well as induce new ones. It is also possible that changes in surface area during culture, i.e. having half the cell exposed to a surface, could block surface receptors for hCG.

Previous studies using pig granulosa cells to investigate induction of LH/hCG receptors in monolayer culture with media containing 10% fetal calf serum revealed that insulin is required for the induction of functional LH/hCG receptors (in terms of hCG-stimulatable progesterone secretion) by FSH (May, McCarty, Reichert & Schomberg, 1980; May & Schomberg, 1981, 1984). In contrast, the present study demonstrates that FSH by itself can induce the appearance of new functional LH/hCG receptor in the absence of other exogenous hormones (IFT) after 2 and 4 days of incubation in media containing 10% porcine serum. However, in the presence of IFT, FSH induction of functional LH/hCG receptors was greatly enhanced. The difference between the present study and others employing the monolayer culture system may stem from dissimilarity in the type of serum used in the incubation media.

The concept that intragonadal factors contribute a significant role in reproductive processes is gaining acceptance and momentum. Follicular fluid from immature pig follicles has been shown to inhibit progesterone secretion in cultures of pig granulosa cells from large preovulatory follicles (Ledwitz-Rigby *et al.*, 1977, 1981; Ledwitz-Rigby & Rigby, 1979). "Luteinization inhibitors" have also been reported from bovine (Amsterdam, Riesel, Mintz, Shemesh & Salomon, 1979; Shemesh, 1979; Bernard, 1973, 1975) and human (Hillensjö *et al.*, 1978; Kraiem, Druker & Lunenfeld, 1978; Kumari, Vohra & Rayhavan, 1983) follicular fluid. Follicular fluid from large pig follicles has also been shown to stimulate progesterone secretion in cultures of granulosa cells from immature follicles (Ledwitz-Rigby & Rigby, 1979; Channing *et al.*, 1982). The relation between the inhibitory protein isolated by DiZerega, Goebelsmann & Nakamura (1982) from human follicular fluid and the luteinization inhibitor is not known since these authors used aromatase as an end point of inhibitory activity, and we used inhibition of progesterone secretion and FSH induction of LH/hCG receptor as end points of action. Nevertheless, the protein isolated is of interest since it has been partly purified by gel-exclusion HPLC and orange A gel-adsorption chromatography and has a molecular weight between 14 000 and 18 000. The present study confirms the follicular fluid effects on progesterone secretion by cultures of immature granulosa cells and extends the effects of follicular fluid on luteinization processes to include modulation of LH/hCG receptor induction *in vitro* by FSH + IFT. Addition of 50% follicular fluid obtained from large follicles enhanced progesterone secretion above that of cultures containing FSH + IFT alone. This stimulation of progesterone secretion *in vitro* is in sharp contrast to the inhibitory effect of fluid from small follicles observed with only 12.5% fluid added to the cultures. Follicular fluid also drastically altered the induction of LH/hCG receptors in cultured granulosa cells. Although fluid from large follicles inhibited the LH/hCG receptor induction of FSH + IFT, the inhibitory effect obtained with fluid from small follicles was significantly greater in all cultures at each concentration of fluid investigated. Follicular fluid exerted approximately the same effects upon FSH induction of hCG/LH binding sites and progesterone secretion whether or not it was charcoal treated, demonstrating that the effects of the fluid were not due solely to its steroid content or to other components removed by charcoal. These results indicate that the follicular fluid environment of maturing granulosa cells may be an important factor in the control of granulosa cell maturation and eventual luteinization. Whether or not the same factors within follicular fluid are responsible for both inhibition of progesterone secretion and inhibition of LH/hCG receptor induction is not known. Neither is it known whether the stimulatory activity of fluid from large follicles on progesterone secretion by cultures of immature granulosa cells reported here and by others (Ledwitz-Rigby & Rigby, 1979; Ledwitz-Rigby *et al.*, 1981) is involved in regulation of LH/hCG receptor induction by FSH. Since Ledwitz-Rigby & Rigby (1979) observed a more than additive

stimulatory effect of follicular fluid from large follicles upon LH and FSH-mediated granulosa secretion of progesterone, it is unlikely that the stimulator is merely FSH or LH in the fluid. The values of LH and FSH measured in pig follicular fluid by radioreceptor assay were too low to account for the present effects (Kolena & Channing, 1985).

The bulk of inhibitory activity upon FSH induction of hCG/LH binding sites exerted by fluid recovered from small and medium follicles appears to reside in the fraction of molecular weight decrease as the follicle develops from small to large. The relatively smaller amount of inhibitory activity upon FSH induction of hCG/LH binding sites present in the filtrate, which is fairly constant as a function of follicular maturation, could represent some other component of the fluid or a low molecular weight component which binds to a high molecular weight complex or be a breakdown product of the substance found in retentate. If this were the case, high molecular weight material could be retained in the follicle more readily to maintain an inhibitory action on the small follicle to prevent it from luteinizing. The FSH-binding inhibitor isolated from cow follicular fluid by Darga & Reichert (1978), Fletcher, Dias, Sanzo & Reichert (1982) and Sluss, Fletcher & Reichert (1983) and from human follicular fluid by Daume, Chari, Hopkinson & Sturm (1979) is of low molecular weight and probably not the same as the "luteinization inhibitor" detected in follicular fluid from small pig follicles. FSH binding inhibitor is a low molecular weight inhibitor (M_r 1000) of FSH binding to granulosa or testis cells (Sluss *et al.*, 1983). In the present studies we strived to avoid grossly atretic follicles, i.e. those visibly cloudy, and based on oocytes recovered from a pool of small follicles about 60–70% of the oocytes had compact cumuli of healthy appearance. The FSH binding inhibitor is present in higher concentration in atretic than in non-atretic follicles. In addition, fluid from atretic follicles of oestrous mares contains greater inhibitory activity on the secretion of progesterone than does fluid from non-atretic follicles (Channing, Batta, Condon, Ganjam & Kenney, 1981).

The inhibitory activity upon FSH induction of hCG/LH binding sites was precipitated primarily with 70% ethanol which served to increase the ability of the inhibitor to act compared to the ability of the raw follicular fluid used as starting material. This 70% ethanol precipitate would therefore serve as a useful starting material for purification of the putative luteinization inhibitor.

We thank Ms Pat Gagliano, Mr Timothy Conn and Mr Joaquin Vallejo for technical assistance, and Mrs Gail Matthews for typing; and Dr Gordon Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins, Colorado 80523, for a generous supply of progesterone antiserum; and Dr Robert Canfield, Department of Medicine, Columbia University, New York, NY 10032, for a supply of highly purified hCG. Supported in part by NIH grant HD08834 (C.P.C.) and NIH Training Grant HD00435, with additional support from USDA Cooperative Agreement CA-58-32440-158 (L.E.R.).

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Received 14 August 1984