Lack of effect of oocyectomy on expansion of the porcine cumulus

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Summary. Oocyte–cumulus cell complexes (OCC) and complexes with an attached piece of membrana granulosa (C + P), isolated from prepubertal or cyclic gilts stimulated with pregnant mares' serum gonadotrophin, were cultured in media supplemented with follicle-stimulating hormone (FSH; 0.01–1.0 µg/ml) or forskolin (50–100 µmol/l) for 24 and 32 h. FSH and forskolin each induced dose-dependent cumulus and membrana granulosa expansion. After 2 h of culture, FSH (0.1 µg/ml) or forskolin (100 µmol/l) increased the contents of intracellular adenosine 3',5'-phosphate (cAMP) in OCC from prepubertal gilts to almost 10 times that in unstimulated complexes. After 24 h of culture in media supplemented with FSH (0.1 µg/ml) or forskolin (100 µmol/l), the oocyectomized OCC and C + P showed similar expansion to that of the control groups. The intracellular cAMP contents in intact and oocyectomized OCCs were similar in all groups except those treated with FSH, in which the intact OCCs had significantly higher contents than their oocyectomized counterparts ($P < 0.01$). After hyaluronidase treatment, cumulus and membrana granulosa cells of intact and oocyectomized OCC and C + P were suspended, except for those of the innermost layers of the corona radiata. The results suggest that increases in cAMP contents and synthesis of an extracellular, hyaluronidase-sensitive mucus by pig OCC and C + P induced by FSH or forskolin are not dependent on the oocyte.

Keywords: FSH; cumulus expansion; hyaluronidase; oocyectomy; pig

Introduction

After the preovulatory surge of gonadotrophins, hyaluronic acid is produced and deposited extracellularly, which disperses the cumulus cells and embeds them in a mucous matrix (Eppig, 1979a). The time sequence of cumulus expansion is species specific: in mice (Eppig, 1980) and rats (Dekel *et al.*, 1979) it follows, but in pigs (Motlík *et al.*, 1986) it precedes, germinal vesicle breakdown. *In vitro*, cumulus expansion is induced by follicle-stimulating hormone (FSH; Thibault, 1972; Dekel & Kraicer, 1978). Mice cumuli expand exclusively under FSH stimulation (Eppig, 1979b), but, in pigs, cumulus expansion is induced with FSH or luteinizing hormone (LH) (Hillensjö & Channing, 1980). Gonadotrophins induce cumulus expansion via an increase in cAMP concentrations (Dekel & Kraicer, 1978; Eppig, 1979b, 1989).

It has been reported that expansion of mice cumuli induced by FSH and 8-bromo cyclic adenosine monophosphate (8-Br-cAMP) is dependent upon a specific factor(s) secreted by the oocyte (Buccione *et al.*, 1990; Salustri *et al.*, 1990). Thus, FSH and 8-Br-cAMP failed to stimulate

cumulus expansion after oocyectomy (Buccione *et al.*, 1990). Moreover, after mechanical dissociation of cumulus-oocyte complexes into cumulus cells and oocytes, neither FSH nor dibutyryl cAMP (dbcAMP) were able to stimulate the formation of mucus around isolated cumulus cells (Salustri *et al.*, 1990).

To test the hypothesis that a specific factor secreted by the oocyte is essential for cumulus expansion, intact and oocyectomized porcine oocyte-cumulus cell complexes (OCC) and complexes with an attached piece of membrana granulosa (C + P) were cultured in media supplemented with FSH or forskolin, a specific activator of adenylate cyclase (Seamon & Daly, 1981).

Materials and Methods

Source of oocytes and culture. Gilts from crosses between Minnesota and Göttingen strains of miniature pigs were stimulated with 500 iu pregnant mares' serum gonadotrophin (PMSG) (Antex, Leo, Copenhagen, Denmark) on Day 16 of the cycle; 72 h later, the gilts were slaughtered and ovaries were immediately excised and put in prewarmed phosphate-buffered saline (PBS, pH 7.4). The preovulatory follicles were opened and fragments of a membrana granulosa layer were released in PBS by scraping the inner surface of the follicular wall using a surgical blade. Under a dissecting microscope, the isolated oocyte-cumulus cell complexes and those with an attached piece of membrana granulosa (Fig. 1) were washed three times in culture medium. The same procedure was used for the isolation of OCC and C + P from follicles (5–7 mm in diameter) of slaughtered prepubertal gilts of the Large White breed.

The culture medium was TCM 199 buffered with 25 mmol Hepes/l (Gibco, Grand Island, NY, USA) supplemented with fetal calf serum (10%, v/v) (Filtron, Australia), sodium pyruvate (0.2 mg/μl) (Wako Pure Chemical Ind. Ltd, Osaka, Japan) and antibiotics with or without 0.01–1.0 μg FSH/ml or 50–100 μmol forskolin/l (Serva, Heidelberg, Germany). The OCC and C + P from PMSG-stimulated and prepubertal gilts were cultured in 1.0 ml and 0.5 ml medium, respectively, under paraffin oil at 38.5°C in air containing 5% CO₂ for 24 and 32 h.

At the end of the culture period, OCC and C + P were assessed for expansion according to a subjective scoring system in which 0 indicates no detectable response and +1, 3, 5 and +2, 4, 6 indicate degrees of OCC and C + P expansion, respectively (see Fig. 1 and Table 1). To assess cumulus expansion precisely, OCC and C + P were scored for expansion by microscopical examination and then treated for 10 min with hyaluronidase (247 U/ml).

Oocyectomy. This was accomplished by the use of two Leitz micromanipulators according to Buccione *et al.* (1990). Each oocyte-cumulus complex was attached to a holding pipette. A glass needle was then introduced through the cumulus cells and the oocyte into the holding pipette. After withdrawal of the needle, the ooplasm, but not the zona pellucida, was aspirated into the holding pipette by a burst of negative pressure (Fig. 2e). Evacuated zonae usually collapsed after this procedure, but within a few minutes they returned to a normal spherical shape. Ooplasm was removed from OCC and C + P by this technique. A set of ~15 oocyectomized complexes was prepared within 20 min and immediately placed into culture. The medium used for micromanipulation was that already described.

Determination of cAMP by radioimmunoassay. The intact or oocyectomized OCCs isolated from prepubertal gilts were cultured for 2 h in the medium supplemented with 3-isobutyl-1-methylxanthine (IBMX) (1 mmol/l), FSH (0.1 μg/ml) or forskolin (100 μmol/l). At the end of culture, OCC were washed in the IBMX medium to minimize cAMP hydrolysis within the cells and transferred immediately to 20 μl 0.5% sodium dodecyl sulphate (SDS, w/v; Racowsky, 1984). When lysis was completed, the samples were capped and stored at –80°C. Before extraction with 100 μl 6% trichloroacetic acid (TCA) at 4°C, 10 μl bovine serum albumin (10 μg/ml) was added to each microcap to act as carrier protein. The extracted samples were centrifuged at 2000 g for 5 min and supernatants were removed and lyophilized. TCA extracts were diluted and assayed after acetylation. Cyclic AMP was measured by radioimmunoassay using kits (UVVVR, Prague, Czechoslovakia).

Statistical analysis. The effects of IBMX, FSH, forskolin and oocyectomy upon cAMP content in OCC were assessed by analysis of variance (ANOVA). Significant differences were evaluated by Student's *t* test.

Results

Effects of FSH and forskolin on expansion of OCC and C + P

After 24 h in culture, 1.0 μg FSH/ml induced full expansion in OCC and C + P isolated from prepubertal gilts (Fig. 1). After hyaluronidase treatment, all granulosa cells were dispersed. After 24 h in culture, 0.1 μg FSH/ml induced expansion of membrana granulosa and cumulus granulosa cells but not of the corona radiata (Fig. 1). After 32 h in culture, full expansion was achieved;

Fig. 1. Effect of FSH and forskolin on the expansion of porcine (a) oocyte-cumulus cell complexes and (b) complexes with an attached piece of membrana granulosa after 24 and 32 h of culture: (□) expansion limited to the peripheral layers, (▨) cumulus expansion of all except the corona radiata cells, (■) total expansion including corona radiata.

Table 1. Effect of follicle-stimulating hormone (FSH) and forskolin on the expansion of intact and oocyctomized complexes of porcine oocyte-cumulus cells after 24 h of culture

Culture medium	No. of complexes	Type of complex*	Degree of expansion†
Modified TCM 199 (control)	40	OCC	0
	42	OCC after Ect	0
	40	C + P	0
	45	C + P after Ect	0
TCM 199 + FSH (0.1 µg/ml)	48	OCC	+3
	60	OCC after Ect	+3
	46	C + P	+4
	58	C + P after Ect	+4
TCM 199 + Forskolin (100 µmol/l)	45	OCC	+1
	56	OCC after Ect	+1
	45	C + P	+2
	60	C + P after Ect	+2

*OCC, oocyte-cumulus cell complex; Ect, oocyctomy; C + P, oocyte-cumulus cell complex with an attached piece of membrana granulosa.

†0, no detectable response; 1, expansion limited to the peripheral layers of the cumulus mass; 2, expansion limited to the peripheral layers of cumulus and membrana granulosa cells; 3, cumulus expansion, except the corona radiata cells; 4, expansion of cumulus and membrana granulosa cells, except the corona radiata.

Fig. 2. (a), (b), (c), (d), (e) and (f).

expansion induced by 0.01 µg FSH/ml was limited to the outer layers of the cumulus and expansion of membrana granulosa cells was less evident.

After incubation in media supplemented with forskolin (50–100 µmol/l), the corona radiata remained unexpanded. Only cumulus and membrana granulosa cells expanded in a dose- and time-dependent manner (Fig. 1).

Effect of oocyectomy on expansion induced by FSH or forskolin

OCC and C + P isolated from prepubertal and PMSG-stimulated gilts were used for microsurgical removal of the oocyte. The expanded OCC and C + P from PMSG-stimulated gilts (Fig. 2) were larger than those from prepubertal gilts, but the type of expansion was similar. Therefore, data for OCC and C + P from both sources were grouped in Table 1. After 24 h of culture of the oocyectomized OCC and C + P in 0.1 µg FSH/ml, the cumulus cells, but not the corona radiata, expanded considerably as did the membrana granulosa cells of C + P (Fig. 2a). Also forskolin (50 µmol/l) apparently stimulated expansion of cumulus and membrana granulosa cells in the oocyectomized complexes (Fig. 2b). The extent of expansion was limited to the outer layers of the cumulus. Exposure of the oocyectomized OCC and C + P to hyaluronidase led to the complete dissociation of the membrana granulosa cells, the outer layers (forskolin group) or the whole cumulus (FSH group) except for a thin layer of corona radiata cells (Fig. 2f). The degree of expansion in the oocyectomized OCC and C + P did not differ from that in the control complexes.

To exclude the possibility that the rest of the oocyte cytoplasm, which was not removed by oocyectomy (Fig. 2f), could influence expansion, the membrana granulosa of C + P complexes was detached from the cumulus. In all cases (42), the piece of membrana granulosa expanded in media supplemented with FSH or forskolin. In contrast, pieces of membrana granulosa (48) isolated from other parts of the follicular wall did not expand.

Effect of oocyectomy on cAMP content in OCCs

After 2 h of incubation in control medium, there was 37.5 fmol cAMP/OCC (Fig. 3, Table 2). The medium supplemented with 1 mmol IBMX/l did not change the cAMP content. In contrast, 0.1 µg FSH/ml and 100 µmol forskolin/l increased the cAMP content to 453.3 and 352.9 fmol/OCC, respectively. Oocyectomy did not change the cAMP contents in OCC cultured in control media or those supplemented with IBMX or forskolin, but cAMP content in the oocyectomized OCC cultured in FSH-supplemented medium was significantly ($P < 0.01$) lower than in the other media.

Discussion

In vitro, the expansion of pig cumulus has been observed in media containing PMSG (Meinecke & Meinecke-Tillmann, 1979) or hCG (Minato & Toyoda, 1982). Highly purified FSH and ovine LH induced expansion of pig OCC isolated from medium-sized follicles (Hillensjö & Channing, 1980).

Fig. 2. Porcine oocyte–cumulus complex (OCC) with an attached piece of membrana granulosa (MG) (a) isolated from preovulatory follicle 72 h after stimulation with pregnant mares' serum gonadotrophin; $\times 150$; (b) cultured in medium supplemented with 0.1 µg follicle-stimulating hormone (FSH)/ml for 24 h; corona radiata cells remained unexpanded; $\times 50$; (c) cumulus (except corona radiata) and membrana granulosa cells expanded after 24 h of culture in 0.1 µg FSH/ml $\times 50$; (d) cultured in 50 µmol forskolin/l for 24 h; inner layer of cumulus and corona radiata remained unexpanded; $\times 100$; (e) ooplasm (†) is aspirated into the holding pipette; collapsed zona pellucida (▲); $\times 150$; (f) after hyaluronidase treatment of FSH-stimulated complexes, cumulus and membrana granulosa cells, except corona radiata, were dispersed; (†) the rest of the oocyte cytoplasm which was not removed by oocyectomy; $\times 200$.

Fig. 3. Effect of 1 mmol/l IBMX, 0.1 µg follicle-stimulating hormone/l (FSH) and 100 µmol forskolin/l on the content of adenosine 3',5'-phosphate (cAMP) of porcine oocyte-cumulus complexes. The intact (□) and oocyctectomized (▨) complexes were cultured for 2 h in the control supplemented medium. Bars represent mean $\pm$ s.e.m. from at least 3 experiments.

Table 2. The effect of 3-isobutyl-1-methylxanthine (IBMX) and forskolin on cAMP content of the pig oocyte-cumulus complex

Culture conditions	Type of complex	
	Intact Mean $\pm$ s.e.m.	Oocyctectomized Mean $\pm$ s.e.m.
Control	37.5 $\pm$ 2.1	47.8 $\pm$ 7.2
IBMX	32.0 $\pm$ 3.7	38.5 $\pm$ 5.5
Follicle-stimulating hormone	453.3 $\pm$ 44.4	218.4 $\pm$ 12.4**
Forskolin	352.9 $\pm$ 55.0	362.3 $\pm$ 39.6

**Different from means for row ($P < 0.01$)

In the present experiments, purified porcine FSH at 1.0 µg/ml induced full expansion of OCCs and of an attached piece of membrana granulosa (C + P) after 24 h in culture. In media with a low dose of FSH (0.1 µg/ml), membrana granulosa cells and the whole cumulus, except the corona radiata layers, expanded after 24 h in culture; full expansion required 32 h of culture. The time sequence of cumulus expansion at this FSH concentration resembled that observed intrafollicularly after stimulation with human chorionic gonadotrophin (Motlík *et al.*, 1986), and, for that reason, 0.1 µg FSH/ml was used in further experiments.

In experiments by Racowsky (1985), the proportion of expanded (partially and fully) cumuli in pigs reached a maximum of ~90% when stimulated by 6.25 µmol forskolin/l; with higher concentrations of forskolin, cumulus expansion was not as great. Under our culture conditions, forskolin (50 and 100 µmol/l) induced the expansion of peripheral layers and the whole cumulus, except the corona radiata, after 24 and 32 h of culture, respectively. Forskolin stimulated a dose-dependent increase in the cAMP content of cumulus masses, with a maximum response to 100 µmol forskolin/l (Racowsky, 1985; present data). These results are consistent with previous studies indicating a central regulatory function of cAMP in cumulus expansion (see Salustri *et al.*, 1985).

Although 90% of mouse OCCs expanded in 1 µg o-FSH/ml, the oocyctectomized complexes failed to expand in response to FSH stimulation (Buccione *et al.*, 1990). Though the contents of cAMP in oocyctectomized complexes were the same as those in intact complexes, the oocyctectomized complexes had only a minimal activity of hyaluronic acid synthesis after FSH stimulation (Buccione *et*

al., 1990). The authors concluded that mouse oocytes exert an action on the cumulus cells that induces synthesis of hyaluronic acid and consequent cumulus expansion (Buccione *et al.*, 1990; Salustri *et al.*, 1990). In contrast, in the present study, pig oocyctomized complexes responded to highly purified porcine FSH and forskolin by increased cAMP production and cumulus expansion. We did not measure activity of hyaluronic acid synthesis, but the expanded pig complexes, either intact or oocyctomized, were sensitive to hyaluronidase treatment. These results imply that oocyctomy did not change the ability of pig cumulus granulosa cells to respond to FSH and forskolin by increased cAMP contents, hyaluronic acid synthesis and consequent cumulus expansion. Under in-vitro conditions, pig OCC expansion did not require a cumulus-expansion-enabling factor, which was postulated for the mouse model (Buccione *et al.*, 1990).

The formation of a follicular antrum, which is under the control of FSH and oestradiol, enables reorganization of the granulosa in cumulus granulosa cells and membrana granulosa cells adjacent to the basal lamina (see Gore-Langton & Daniel, 1990). The long-term culture of preantral follicles with FSH induced antrum formation in hamster follicles (Roy & Greenwald, 1989) and antrum-like reorganization of granulosa cells in rat follicles (Gore-Langton & Daniel, 1990). Mouse oocyte-granulosa cell complexes from preantral follicles cultured on a collagen membrane differentiated into cumulus cells and a stalk of granulosa cells adherent to the collagen membrane (Vanderhyden *et al.*, 1990). Only cumulus cells responded to FSH; the granulosa cells comprising the stalk did not expand.

In contrast, in pig preovulatory follicles after hCG stimulation, the intercellular deposit of a mucous matrix is first seen amongst the cells forming the connection of the cumulus oophorus with membrana granulosa (Fléchon *et al.*, 1986; Motlík *et al.*, 1986). At 24 and 32 h after hCG, the expanded cumulus is connected with the follicular wall by a firm, elastic stalk which makes contact with the parietal granulosa by a foot of $\sim 5 \text{ mm}^2$ in diameter. The granulosa cells in this area also expand (Motlík *et al.*, 1986). The experiments reported here demonstrate that, *in vitro*, FSH and forskolin induce expansion not only of cumulus but also membrana granulosa cells attached to OCCs. Neither oocyctomy nor mechanical separation of the OCC and the piece of membrana granulosa impaired the response of membrana granulosa cells to FSH or forskolin. However, pieces of membrana granulosa isolated from other parts of the follicular wall of rats (Yanagishita & Hascall, 1979), mice (Vanderhyden *et al.*, 1990) and pigs (this study) did not expand in response to FSH. Taken together, these results suggest that a limited area of the pig granulosa cells connected with OCC possesses cells with the characteristic of cumulus granulosa cells.

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