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A gap-junction-mediated signal, rather than an external paracrine factor, predominates during meiotic induction in isolated mouse oocytes

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Summary

This study was carried out to compare the possible role of a secreted paracrine factor versus that of a gap-junction-transmitted signal in mediating meiotic induction in isolated mouse oocytes from PMSG-primed, immature mice. In the first set of experiments, oocyte–cumulus cell complexes (OCC) were pretreated for 3 h with 2 mM dbcAMP or FSH, washed, and the oocytes then cultured for 17–18 h in 40 µl drops containing either 300 µM dbcAMP or 4 mM hypoxanthine (HX). Each set of pretreated oocytes was cultured under three different conditions: (1) intact cumulus-cell-enclosed oocytes (CEO); (2) denuded oocytes (DO), cultured alone after removal of cumulus cells; and (3) co-cultured cumulus cells and oocytes (CC/DO), where the cumulus cells were removed in the same drop with a mouth-operated pipette and cultured alongside the oocytes. When pretreated with high dbcAMP or FSH, maturation was stimulated in CEO when cultured in either inhibitor (by 41.4–53.7%). Pretreatment failed to affect the maturation rate in DO. DO maturation was not altered appreciably by co-cultured cumulus cells when arrest was maintained with dbcAMP. However, an increase in maturation of 21–23% was observed in CC/DO in the HX-containing cultures that was not dependent on prior treatment with a meiosis-inducing stimulus. When DO were co-cultured with intact, FSH-treated OCC, there was no evidence of a positive factor secreted by the stimulated complexes, despite the fact that oocytes within the OCC were induced to resume maturation. In a second series of experiments the gap junction inhibitor, 18α-glycyrrhetinic acid (GA), was utilised. An initial experiment determined that GA dose-dependently blocked OCC metabolic coupling (0.2% coupling at 10 µM compared with 13.6% in controls). When HX-arrested CEO and DO were cultured for 17–18 h in medium containing increasing concentrations of GA, meiotic maturation was induced in CEO but not DO, suggesting that the cumulus cells provided a positive stimulus in the absence of functional gap junctional communication. No effect of GA was seen in dbcAMP-arrested oocytes. A kinetics experiment showed that when CEO were cultured in dbcAMP ± FSH, meiotic induction was initiated after 3 h and germinal vesicle breakdown reached 60% by 6 h. When GA was added to the cultures at different times after the initiation of culture (0, 2, 3, 4 and 5 h), meiotic induction was immediately blocked. In addition, measurement of OCC coupling revealed that no reduction in coupling occurred during this induction period in the absence of GA. It is concluded that cumulus cells can secrete a positive factor, but that this is normally overridden by inhibitory influences transmitted through the gap junction pathway in intact complexes. Furthermore, upon exposure of complexes to a meiosis-inducing stimulus, a positive gap-junction-mediated signal now predominates to trigger germinal vesicle breakdown, and this signal is utilised throughout the induction period.

Key words: Cell–cell coupling, Co-culture, Meiotic maturation, Positive stimulus

Introduction

Mammalian oocytes remain arrested in prophase I of meiosis during their entire growth phase while achieving the competence to resume and complete meiotic maturation. During this time, oocytes communicate

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with the follicle through gap junctions that metabolically couple germ cells to all other granulosa cells and facilitate the rapid transfer of ions or small molecules between the two cell types. In healthy, developing follicles, oocytes remain in meiotic arrest by means of inhibitory factors provided by the follicle and do not resume nuclear maturation until stimulated by the preovulatory gonadotropin surge. The mechanism whereby meiotic resumption is brought about by this pulse of hormone is not known but continues to be an active area of research. Two basic models have gained prominence to explain this phenomenon: (1) a decrease or interruption in the flow of meiotic inhibitor to the oocyte or (2) a positive signal that overrides the action of a follicular inhibitor.

It has been hypothesised that gap junctions mediate the transfer of inhibitory molecules such as cyclic adenosine monophosphate (cAMP) to the oocyte and thereby facilitate meiotic arrest; in the first model, termination of cell–cell coupling after the hormone surge precedes, and is the causative force leading to, meiotic resumption (Dekel & Beers, 1978). This mechanism has gained support by the demonstration that elevation of cAMP in the cumulus cell compartment by agents such as FSH, forskolin or cholera toxin can result in a corresponding rise within the oocyte (Racowsky, 1984, 1985*a*, *b*; Bornslaeger & Schultz, 1985; Salustri *et al*., 1985; Sherizly *et al*., 1988). In addition, a loss in oocyte–cumulus cell coupling (Racowsky, 1984; Racowsky & Satterlie, 1985; Dekel *et al*., 1988) or decrease in gap junctions between cumulus granulosa and membrana granulosa cells (Larsen *et al*., 1987; Racowsky *et al*., 1989) coincident with germinal vesicle breakdown (GVB) has been reported. Thus, physical separation of the oocyte from the somatic compartment could eliminate inhibitory input and bring about GVB.

The second model involves the production of a positive factor within the granulosa cells that triggers GVB despite the continued presence of inhibitory follicular influences. This positive induction model is supported by several lines of evidence. First, stimulation of meiotically arrested cumulus-cell-enclosed oocytes (CEO) with FSH or EGF stimulates a significantly higher percentage of GVB than divesting the oocyte of its cumulus cells (Eppig & Downs, 1987; Downs *et al*., 1988). Thus, meiotic resumption must be induced by a positive cumulus cell signal rather than by simple removal of inhibitory input. Second, oocyte–cumulus cell coupling is not necessarily decreased prior to GVB (Eppig, 1982; Eppig & Downs, 1988), which is consistent with the need for gap junctional communication in the induction process. However, it could be argued that the loss of cumulus–cumulus or cumulus–membrana granulosa gap junctions is more important for meiotic induction or that the transfer of inhibitor could be affected differently from the radiolabelled markers used to measure coupling. Third, inhibitors of cell–cell coupling such as octanol or 18α-glycyrrhetinic acid block FSH-induced maturation in dbcAMP-arrested CEO (Fagbohun & Downs, 1991; Downs, 1995). This implicates the direct transfer of a meiosis-inducing substance to the oocyte through the gap junctional coupling pathway.

Yet evidence also suggests that a similar type of activity can be released into the medium by co-cultured cumulus granulosa cells. We showed that dissociated cumulus cells, but not complexes, could stimulate the maturation of co-cultured DO maintained in meiotic arrest with hypoxanthine (Downs and Mastropolo, 1994). More extensive co-culture experiments were carried out by Byskov and colleagues (Guoliang *et al*., 1994; Byskov *et al*., 1997), who reported the release of positive paracrine factors by isolated cumulus cells in response to stimulation by forskolin or FSH. This effect is apparently specific for cumulus granulosa cells, as membrana granulosa cells are a relatively poor source for the meiosis-inducing factor (Eppig & Downs, 1987; Byskov *et al*., 1997).

The present study was carried out to examine the possible role of secreted paracrine factors in FSH- and dbcAMP-induced GVB in isolated mouse oocytes co-cultured with cumulus cells or oocyte–cumulus cell complexes. These experiments focus on (1) the need for hormonal stimulation in the release of such factors, (2) the generality of this paracrine effect using two different meiotic inhibitors, (3) the comparative kinetics of FSH-induced versus paracrine factor-induced maturation and (4) a further examination of the role of the coupling pathway in meiotic induction using the gap junction inhibitor, 18α-glycyrrhetinic acid. Although the release of a positive paracrine factor is indicated by the experiments performed herein, evidence fails to support this as a primary mechanism for hormone-induced maturation in isolated mouse oocytes.

Materials and methods

Oocyte isolation and culture conditions

Immature, female (C57BL/6J × SJL/J) F_1 mice, 20–23 days old, were used in all experiments. Two days after priming with 5 IU pregnant mare serum gonadotropin, mice were killed, and the ovaries were removed and placed in culture medium. The antral follicles were pierced with sterile needles and the oocyte–cumulus cell complexes released into the medium were collected, washed through several fresh changes of medium, and allocated to the appropriate test condition.

The medium used in this study was Eagle's mini-

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mum essential medium supplemented with 0.23 mM pyruvate, penicillin, streptomycin sulphate and 3 mg/ml crystallised lyophilised bovine serum albumin. In experiments involving glycyrrhetinic acid, the bovine serum albumin was replaced with 3 mg/ml polyvinylpyrrolidone to facilitate the action of this agent (cf. Downs, 1995). In most experiments, oocytes were cultured in 1 ml medium in capped plastic culture tubes, whereas in the co-culture experiments oocytes were cultured in 40 µl microdrops under oil in plastic Petri dishes. For the microdrop experiments which involved denuded oocyte culture, the pyruvate concentration was increased to 0.46 mM to help promote oocyte viability. All cultures were gassed with a humidified mixture of 5% O_2, 5% CO_2 and 90% N_2.

Co-culture experiments

Cultures were carried out in Petri dishes in 40 µl of medium overlaid with paraffin oil. In all treatment groups, the manipulations were done so that the final volume of the microdrop was held constant at 40 µl. Cumulus cell/oocyte co-cultures were prepared by denuding cumulus-cell-enclosed oocytes within the microdrop with a mouth-operated pipette. The level of the medium in the pipette tip was carefully monitored before and after denuding to ensure that no change in volume occurred in the microdrop. Cultures were carried out at 37 °C in modular incubators after gassing with the 5/5/90 gas mixture. In cultures containing both complexes and denuded oocytes, the two types of oocytes were carefully separated at the end of culture before assessment for germinal vesicle breakdown (GVB).

Oocyte–cumulus cell coupling

Oocyte–cumulus cell complexes were cultured for 1 h in medium containing 10 µCi [^{3}H]hypoxanthine and the coupling percentage was determined by methods previously described (Fagbohun & Downs, 1991).

Statistical analysis

Each oocyte maturation experiment was conducted at least three times with at least 30 oocytes per group per experiment. Data are reported as the mean percentage of GVB ± SEM. Maturation frequencies were subjected to arcsin transformation and analysed statistically by ANOVA followed by Duncan's multiple range test. Non-transformed coupling data were analysed by the same test and, where paired comparisons were made, by Student's *t*-test. A p value < 0.05 was considered significant.

Results

Effects of cumulus cell and OCC co-culture on meiotic arrest in denuded oocytes

Cumulus cell co-culture

Initial experiments were carried out to determine the effects of co-culture of denuded oocytes with dissociated cumulus cells on the meiotic arrest maintained by either dbcAMP or hypoxanthine. Fig. 1 shows the protocol schematic for these experiments. In the first set of experiments, cumulus-cell-enclosed oocytes (CEO) were pretreated for 3 h in 1 ml medium in culture tubes

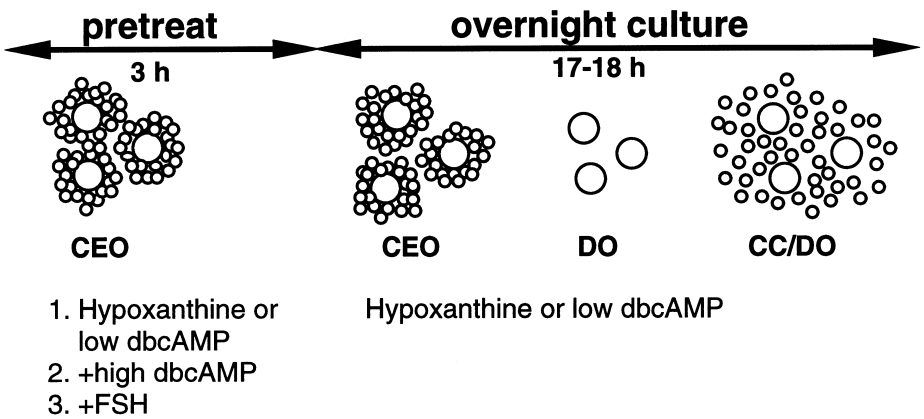

Figure 1 Protocol schematic for co-culture experiments shown in Fig. 2. These experiments were carried out in medium containing either 4 m M hypoxanthine or 300 µM dbcAMP (control medium). Oocyte–cumulus cell complexes were pretreated for 3 h in tubes containing one of three treatments: control medium or control medium supplemented with 2 mM dbcAMP or FSH. At the end of this period, 40 oocytes were then washed free of the high dbcAMP or FSH and transferred to 40–µl drops of the corresponding dbcAMP or hypoxanthine control medium under oil and culture continued for 17–18 h. Three different culture conditions were investigated: (1) intact cumulus cell-enclosed oocytes (CEO), (2) denuded oocytes (DO), or (3) cumulus cell-denuded oocyte co-cultures (CC/DO).

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under one of three conditions: (1) 300 µM (low) dbcAMP; (2) 2 mM (high) dbcAMP; or (3) 300 µM dbcAMP + FSH. Groups 2 and 3 were washed in 300 µM dbcAMP to remove FSH or the high concentration of dbcAMP. Each set of pretreated oocytes was subsequently cultured for 17–18 h in 40 µl drops of medium containing 300 µM dbcAMP covered with paraffin oil under three different conditions: intact CEO; denuded oocytes (DO), cultured alone after removal of cumulus cells; and co-cultured oocytes (CC/DO), where the cumulus cells were removed in the same drop with a mouth-operated pipette and cultured alongside the oocyte. Forty oocytes were included in each group. A second set of experiments was carried out in identical fashion to the above experiments, but 4 mM hypoxanthine was substituted for 300 µM dbcAMP. For these experiments, the second group was pretreated for 3 h in medium containing hypoxanthine plus 2 mM dbcAMP, washed and then returned to medium containing hypoxanthine alone.

dbcAMP-treated. When pretreated with high dbcAMP or FSH, maturation was stimulated in CEO maintained in meiotic arrest by dbcAMP (by 55.9–64.1%; Fig. 2, top panel). Pretreatment failed to affect the maturation rate in DO. DO maturation was also not appreciably altered by co-cultured cumulus cells when arrest was maintained with dbcAMP: an increase of 7–11% was observed that was not significant.

Hypoxanthine-treated. In hypoxanthine-arrested oocytes, maturation was stimulated in CEO by prior exposure to high dbcAMP or FSH (by 41.4–53.7%; Fig. 2, bottom panel). Although maturation in DO was not affected, significantly higher frequencies of maturation were observed in CC/DO compared with DO (increases of 21–23%), but this effect was not dependent upon prior stimulation with FSH or high dbcAMP.

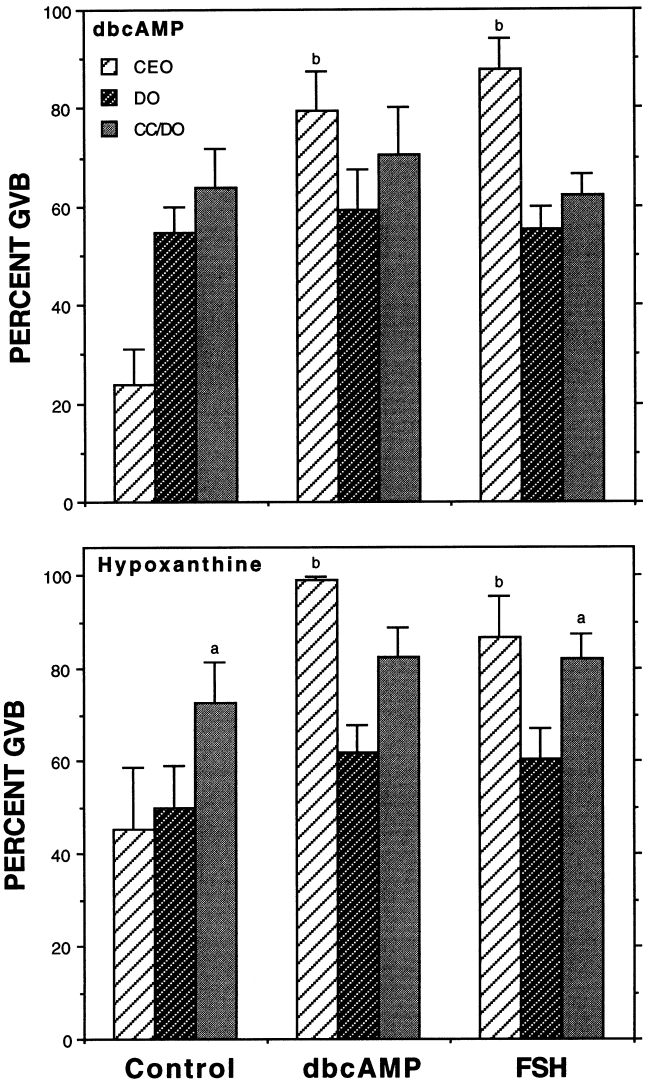

Figure 2 Effects of cumulus cell co-culture on the meiotic maturation of denuded oocytes. Experiments using 300 µM dbcAMP as the control medium are shown in the top panel, while those utilising 4 mM hypoxanthine are in the bottom panel. A letter at the top of a CEO bar denotes a significant difference from the control CEO group. A letter at the top of a CC/DO bar denotes a significant difference from the corresponding DO group for that particular treatment.

OCC co-culture

The above results suggested that dissociated cumulus cells can release a factor that acts in a positive capacity on DO to stimulate meiotic resumption. We next carried out an experiment to ascertain whether intact oocyte–cumulus cell complexes (OCC) had a similar effect when co-cultured with DO. Forty DO were cultured for 17–18 h in 40 µl drops of medium under oil either in the presence or absence of 40 CEO. Medium was supplemented with dbcAMP or hypoxanthine, and cultures were carried out in the presence or absence of FSH.

dbcAMP-treated. As shown in Fig. 3 (top panel), dbcAMP maintained about 60% of the DO in meiotic arrest. Interestingly, FSH tended to inhibit maturation when added to cultures of DO, but this effect was not significant. In the absence of FSH, co-culture with OCC reduced the maturation percentage in DO from 41% to 15%. The addition of FSH to the co-culture system negated this inhibitory effect of the OCC but did not stimulate maturation beyond the percentage observed in the –OCC/–FSH group. That complexes were properly stimulated by FSH was evident by an FSH-stimulated increase in GVB from 26% to 95% within CEO.

Hypoxanthine-treated. An identical pattern was observed in hypoxanthine-treated oocytes, although no differences in maturation percentages between any of the four groups was statistically significant (Fig. 3, bottom panel). It is important to note that while cumu-

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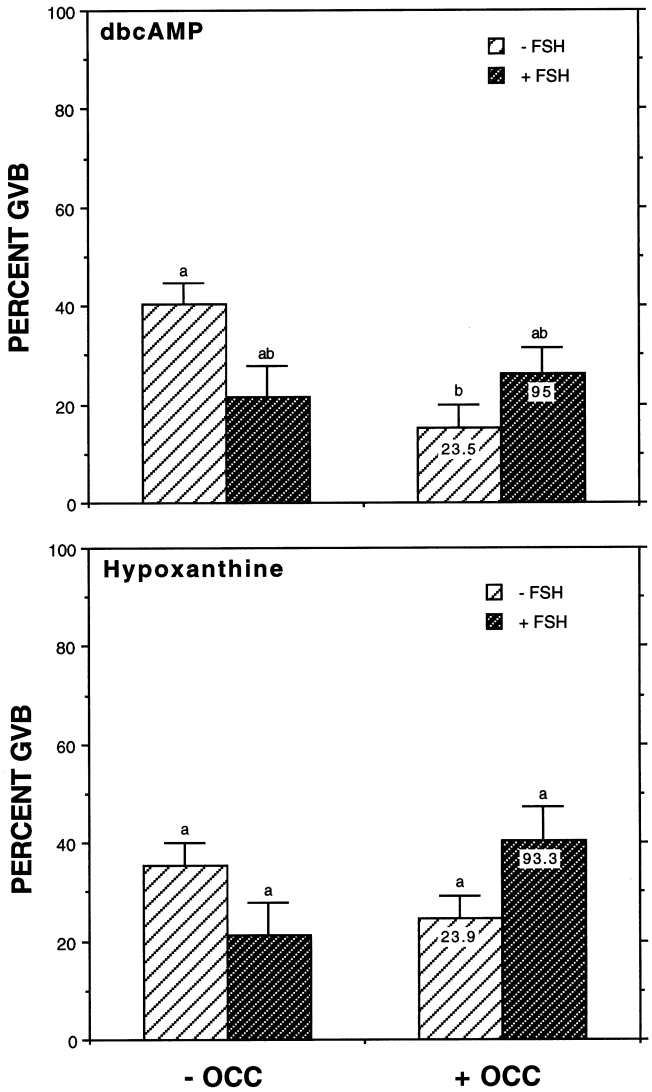

Figure 3 The effect of FSH and co-culture of oocyte–cumulus cell complexes and denuded oocytes on the maturation of denuded oocytes. Forty freshly isolated denuded oocytes were cultured for 17–18 h in 40 µl medium drops under oil in the presence or absence of FSH. In some cultures an equal number of oocyte–cumulus cell complexes (OCC) was included. The culture medium contained either 300 µM dbcAMP (top panel) or 4 mM hypoxanthine (bottom panel). A different letter at the top of the bar denotes a significant difference. The numbers at the top of the bars represent the mean maturation percentage exhibited by the co-cultured CEO.

lus cells induced meiotic resumption in co-cultured DO, intact complexes had no such effect.

Effects of glycyrrhetinic acid on meiotically arrested oocytes

A series of experiments with the uncoupling agent 18α-glycyrrhetinic acid (GA) was carried out to test the idea that blocking cumulus cell–oocyte coupling would prevent the transport of inhibitory factors through the coupling pathway but allow secreted paracrine factors of cumulus cell origin to act on the oocyte in a meiosis-inducing capacity. A dose response experiment was first conducted to determine the concentration of GA that effectively blocked coupling. In the absence of this agent an average coupling percentage of 13.6% was obtained. Coupling was reduced to 5.8% at 1 µM, and coupling was nearly eliminated at 5 µM (0.3% coupling; Fig. 4). Upon determining the range of effective doses that block coupling, GA dose response experiments were subsequently performed on both hypoxanthine- and dbcAMP-arrested CEO and DO cultured for 17–18 h in plastic tubes.

dbcAMP-treated. When oocytes were maintained in meiotic arrest with dbcAMP, GA had no effect on either CEO or DO in a range of doses that eliminated coupling (Fig. 5, top panel).

Hypoxanthine-treated. In hypoxanthine-arrested DO, GA also had no effect on meiotic resumption. However, this agent dose-dependently induced maturation in CEO. In the absence of GA, 43.5% of the CEO underwent GVB, but this frequency was increased to 67.8% and 77.6% GVB, respectively, at 5 and 10 µM GA (Fig. 5, bottom panel). Interestingly, 2 µM GA had no effect on hypoxanthine-arrested CEO, despite the fact that it reduced the coupling percentage from 13.8% to only

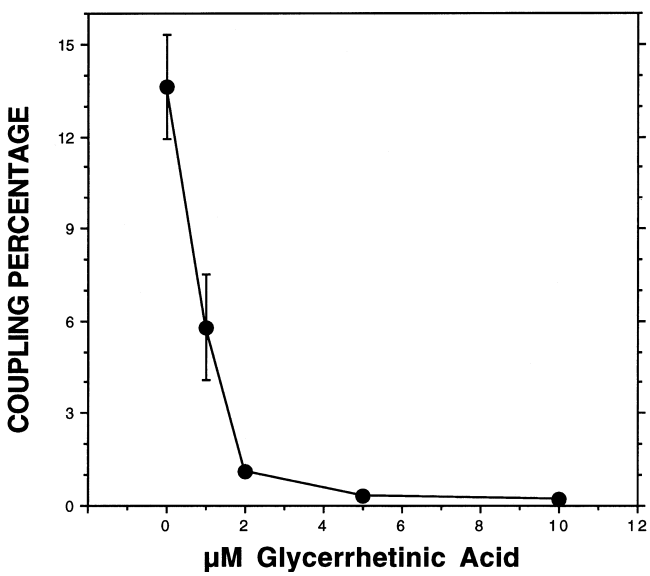

Figure 4 Effect of glycyrrhetinic acid (GA) on metabolic coupling. OCC were cultured for 15 min in medium containing 0, 1, 2, 5 or 10 µM GA. Radiolabelled hypoxanthine was then added to each tube and cultures were continued for 1 h, after which the coupling percentage was determined. Data are presented as the mean percentage coupling ± SEM of three determinations.

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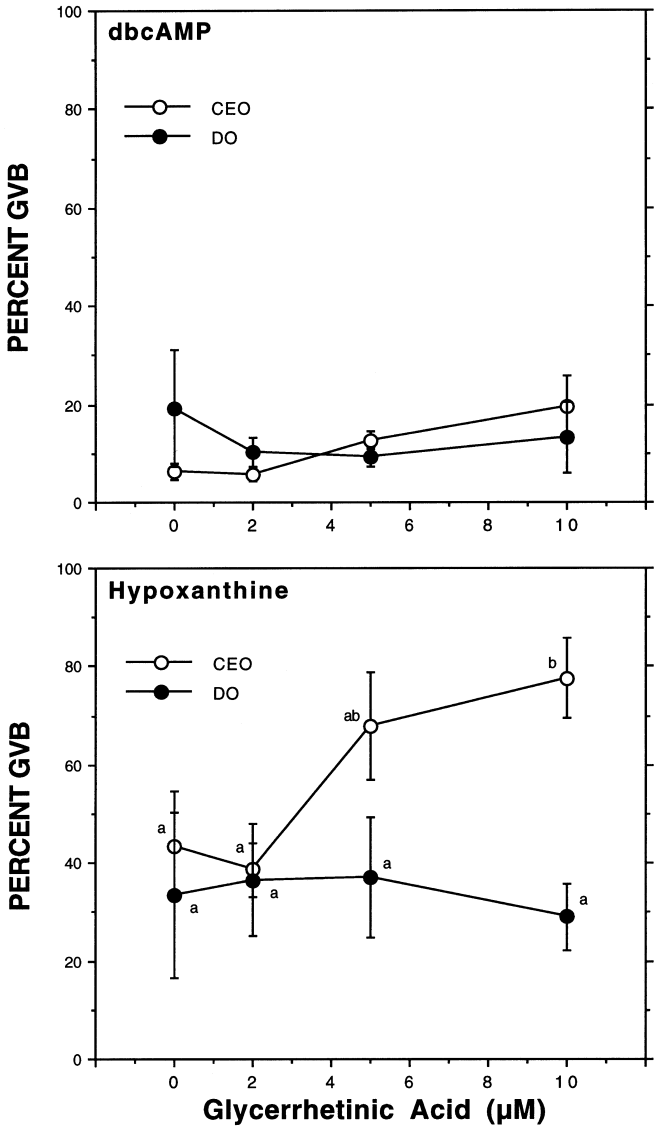

Figure 5 Effect of glycyrrhetinic acid (GA) on meiotic arrest maintained by hypoxanthine and dbcAMP. CEO were cultured for 17–18 h in medium containing 300 μM dbcAMP (top panel) or 4 mM hypoxanthine (bottom panel) plus increasing concentrations of GA. Data are presented as the mean percentage GVB ± SEM of at least three determinations. DO and CEO were analysed separately, and groups without an identical letter are significantly different. No differences existed between any of the dbcAMP-treated groups.

1.1%. This suggests that virtually complete uncoupling must be achieved to bring about a reversal of meiotic arrest.

Cell–cell coupling and meiotic induction

Because GA had no effect on dbcAMP-arrested CEO, dbcAMP could be used to test the importance of cell–cell coupling in FSH-induced meiotic maturation. Thus, CEO were cultured for 6 h in medium containing 75 μM dbcAMP, and meiotic maturation was stimulated by the addition of FSH. GA was added to FSH-treated groups at increasing concentrations from 2 μM to 10 μM. FSH stimulated an increase in GVB from 26.4% to 72.1%, and GA dose-dependently prevented this response (Fig. 6). Partial inhibition was achieved at 2 μM GA, and essentially complete suppression of meiotic induction was realised at 5 and 10 μM.

Suppression of FSH-induced maturation with GA supports the idea that gap junctions mediate a positive stimulus that is transferred from the cumulus cells to the oocyte. Since this would require patent coupling between the two cell types, we next compared the coupling kinetics in complexes that were treated with 75 μM dbcAMP ± FSH. Both coupling and oocyte maturation were assayed at 2 h intervals up to 6 h of culture. When CEO were examined after 2 h of culture, FSH suppressed GVB, but thereafter meiotic induction was observed, with 25% and 46% more maturing oocytes in FSH-treated than in untreated OCC at 4 and 6 h, respectively (Fig. 7*A*). Control maturation levels rose only slightly during this period, from 37% to 51%.

The coupling percentage in freshly isolated complexes was 13.2%, and this remained stable throughout the 6 h of culture in dbcAMP, with a small, but insignificant, decrease (to 10%) at 6 h (Fig. 7*B*). Treatment with FSH increased the coupling percentage at 2 and 4 h (15.6 and 16.5%, respectively), but this value dropped

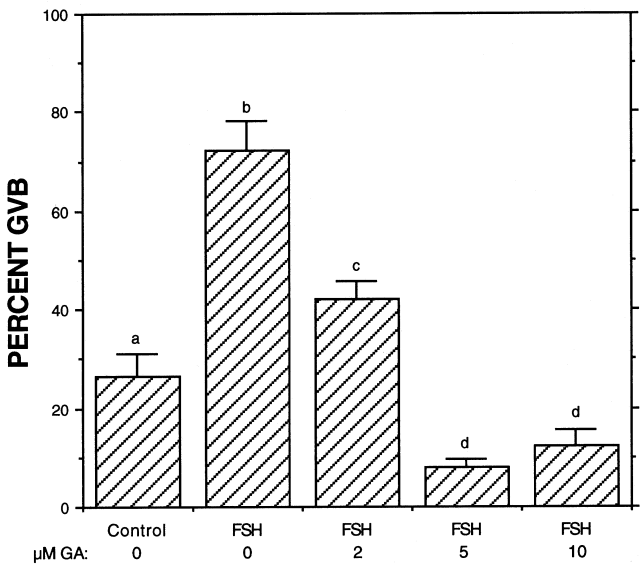

Figure 6 Effect of glycyrrhetinic acid (GA) on FSH-induced GVB in dbcAMP-arrested CEO. CEO were cultured for 6 h in 75 μM dbcAMP plus or minus FSH. FSH-containing cultures were exposed to 0, 2, 5 or 10 μM GA. Data are presented as the mean percentage GVB ± SEM of three determinations. A different letter at the top of a bar denotes a significant difference.

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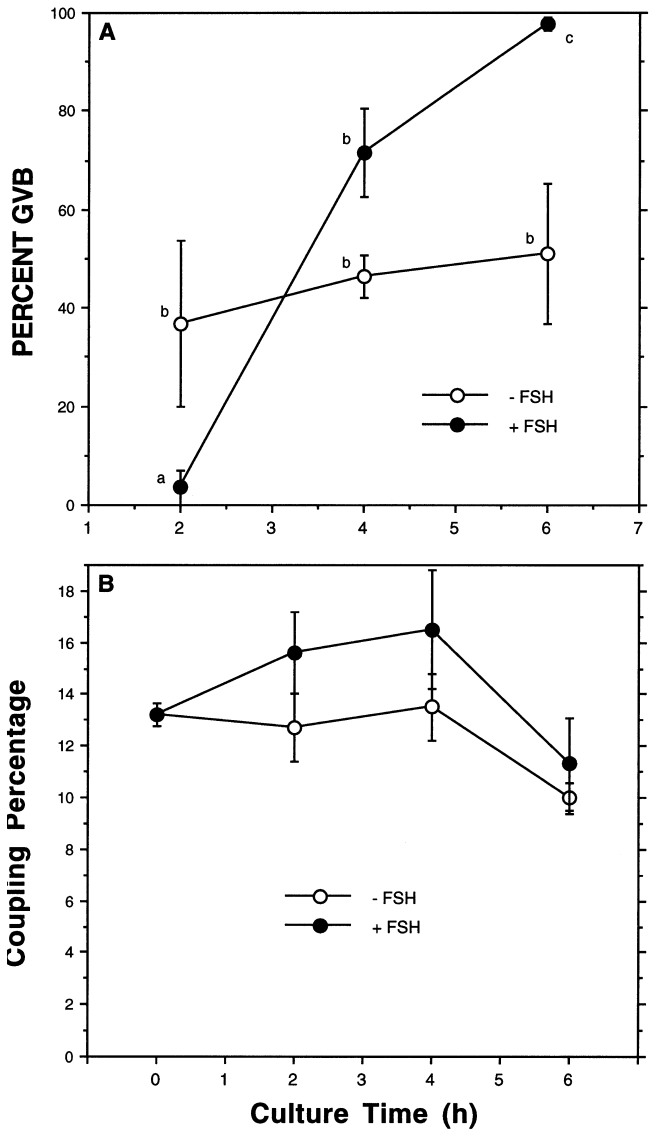

Figure 7 Oocyte–cumulus cell coupling during meiotic induction *in vitro*. The kinetics of FSH-induced maturation were examined in (*A*). CEO were cultured for 6 h in 75 μM dbcAMP plus or minus FSH and oocytes were assessed for GVB at 2, 4 and 6 h. A different letter denotes a significant difference. In (*B*), the oocyte–cumulus cell coupling percentage was determined for these groups and compared with an additional freshly isolated group. No significant differences were observed between any of the groups by ANOVA, but the 4 h FSH group was different from both the 0 h control and 6 h FSH groups by Student's *t*-test.

to 11.3% by 6 h. Hence, the coupling percentage remained high during meiotic induction but began to decrease after meiotic resumption.

The last experiment was carried out to test how long after the onset of culture GA could still block meiotic induction. CEO were cultured in 150 μM dbcAMP + FSH, and in one set of cultures GA was added 0, 2, 3, 4 or 5 h after the onset of culture. The oocytes in all these groups were examined after 6 h of culture for GVB. The other set of cultures did not receive GA but were examined for GVB at the same time GA was administered to the groups in the other set. A higher dbcAMP concentration was used to achieve a greater range of stimulation in response to FSH. As shown in Fig. 8 (open circles), meiotic induction was initiated after 3 h and GVB reached 55.4% by 6 h. Maturation of control oocytes not treated with FSH was 8.2% at 6 h. When GA was added to the cultures at different times after the initiation of culture, meiotic induction was immediately blocked regardless of the time of GA administration, i.e. the percentage GVB observed at 6 h was identical to the maturation percentage that had been reached in FSH-treated CEO at the time of GA administration (filled circles). These results demonstrate that meiotic induction in isolated CEO is a protracted process that requires gap junctional patency up until the time of GVB.

Discussion

Evidence has been presented that cumulus cells release factors into the culture medium that influence the

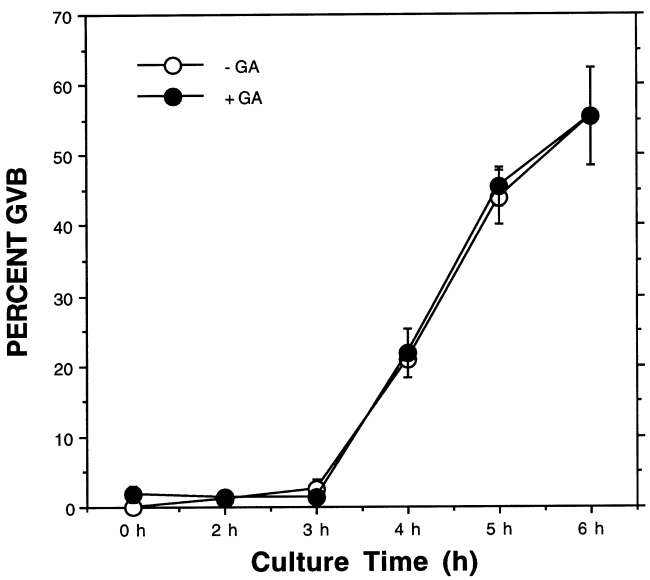

Figure 8 Effect of adding glycyrrhetinic acid (GA) after the onset of culture on FSH-induced maturation. CEO were cultured for 6 h in medium containing 150 μM dbcAMP plus FSH, and the percentage GVB was assessed at different intervals up to 6 h (open circles). GA was added to the cultures at 0, 2, 3, 4 or 5 h after the onset of culture and these groups were assessed for GVB after 6 h (filled circles). The figure shows that GA could block further meiotic maturation at each time point it was administered. CEO not exposed to FSH exhibited 8% GVB after 6 h.

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nuclear maturation of DO in either a positive or negative capacity. Dissociated cumulus cells trigger GVB in hypoxanthine- but not dbcAMP-arrested DO, and this action does not require hormonal stimulation. Yet unstimulated, intact oocyte–cumulus cell complexes are inhibitory to maturation when co-cultured with DO, but not in the presence of FSH. These results suggest that communication between the oocyte and surrounding follicle cells is important in determining what factors the latter cells release into the culture medium. Gap junctions are also vital for the meiosis-inducing influence of FSH-treated cumulus cells in isolated OCC and are apparently required for the duration of the induction period instead of mediating a transitory stimulatory signal. It is concluded that, at least under the conditions employed in this study, meiotic resumption in response to gonadotropin stimulation does not occur principally by the release of diffusible, cumulus-cell-derived inducing agents but, rather, by the production of stimulatory factors that reach the oocyte through the cell–cell coupling pathway.

Evidence for paracrine factors of cumulus cell origin

Co-culture experiments were carried out in 40 µl volumes to help concentrate putative paracrine factor(s) that might be released into the medium. Under these conditions, a consistent increase was observed in the maturation of hypoxanthine-arrested DO when co-cultured with dissociated cumulus cells, confirming the results of several previous studies (Downs & Mastropolo, 1994; Guoliang *et al*., 1994; Byskov *et al*., 1997). Of particular significance was the finding that this positive effect was not dependent upon stimulation of the complex with FSH or high levels of dbcAMP prior to denudation and co-culture. Further evidence of this phenomenon was provided by the experiment in which GA dose-dependently induced maturation in hypoxanthine-arrested CEO. The lower frequency of maturation in DO compared with CEO indicated the response to GA was not simply due to loss of a cumulus cell inhibitory factor transferred through gap junctions or to a direct action of GA on the oocyte.

It is proposed that blocking the gap junctional transfer of inhibitory components such as ATP (cf. Downs, 1995) or cAMP from the cumulus compartment to the oocyte permits the positive action of cumulus cell paracrine factor(s) to be manifested in the intact complex. In keeping with this idea, the same results were seen in the co-culture experiments upon physical separation of the cumulus cells by denudation. Thus, the ability of cumulus cells to induce GVB by extracellular factors appears to be an innate quality of these cells but is overridden by inhibitory signals transmitted by gap junctions (Fig. 9*A*). It is also important to note that the most effective meiotic induction by FSH or dbcAMP occurred in the intact complex. Although reducing the volume of the culture environment may increase the efficacy of the cumulus cell co-culture effect, optimal meiotic induction still required an intact cell–cell coupling pathway.

When dbcAMP was employed as inhibitor, no meiotic induction was observed in DO co-cultured with cumulus cells, despite the fact that FSH still effectively induced maturation in intact complexes. The reason for this is not known, but the fact that the presumptive paracrine effect was manifested in hypoxanthine-containing, but not dbcAMP-containing, cultures demonstrates that this is not a universal phenomenon. A similar discrepancy was seen in the ability of GA to promote GVB in hypoxanthine-arrested, but not dbcAMP-arrested, CEO and raises doubt as to the physiological importance of the paracrine effect.

It is interesting that a meiosis-inducing effect was observed with dissociated cumulus cells but not intact complexes. Rather, unstimulated OCC increased the meiotic arrest maintained in DO with either dbcAMP or hypoxanthine by 10–25%. Although this inhibition was reversed by FSH treatment, gonadotropin-stimulated OCC failed to induce GVB in co-cultured DO. This suggests that communication between the oocyte and cumulus cells, or maintaining the physical integrity of the cumulus oophorus, determines whether the action of co-cultured cumulus cells on DO is inhibitory or stimulatory. The lack of a stimulatory effect of complexes on DO might be due to the lack of priming of the oocytes with cumulus cells prior to denudation; Guoliang *et al*. (1994) reported greater induction of maturation by co-cultured cumulus cells in primed DO compared with unprimed DO. Yet this is doubtful, since the paracrine effect of cumulus cells observed herein did not require prior stimulation with FSH or dbcAMP.

Byskov *et al*. (1997) carried out an extensive series of co-culture experiments that examined the secretion of a positive meiosis-inducing factor by cumulus cells. Unlike our results, their data indicated that FSH or forskolin treatment stimulated the production of a positive paracrine factor by cumulus cells. However, their data also showed an apparent stimulation of GVB in DO by co-cultured cumulus cells from unstimulated complexes. Hence, in their system, hormone or forskolin stimulation was not required to obtain a positive effect and in this sense agrees with our results.

It should be noted that the meiosis-inducing effect of cumulus cell co-culture was modest, with never more than a 23% increase in meiotic resumption, and is less than that reported by Guoliang *et al*. (1994) and Byskov *et al*. (1997). This may be related to the different culture system utilised; our co-culture experiments were conducted in 40 µl drops of MEM under oil, while

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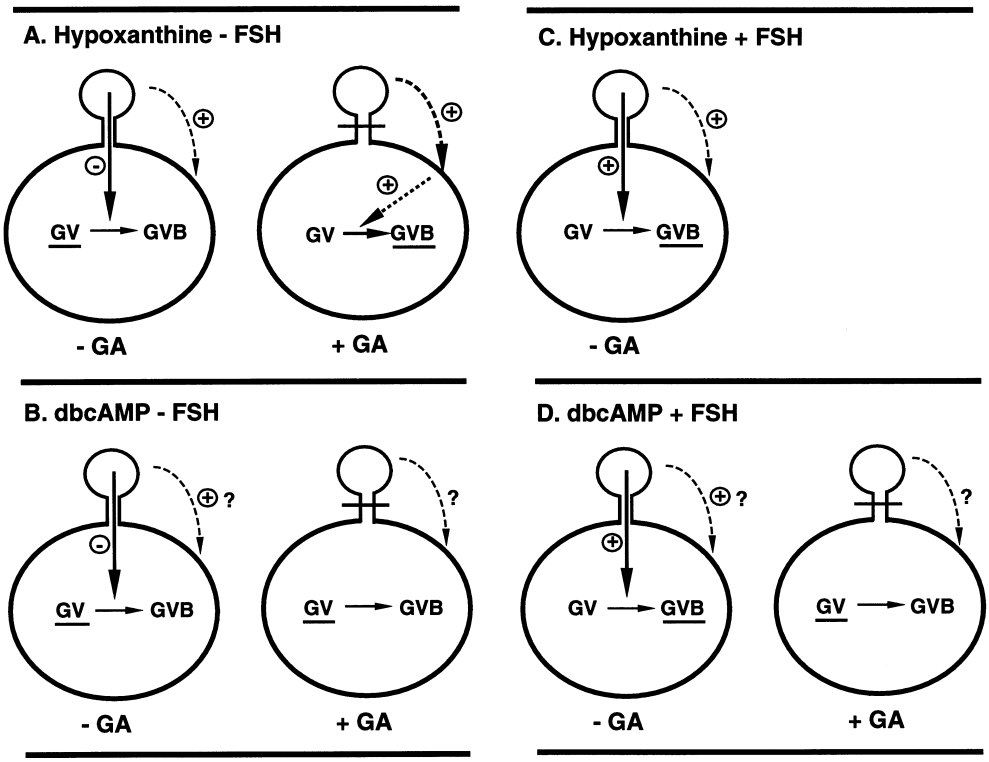

Figure 9 Proposed model for meiotic control. (*A*) When maintained in meiotic arrest with hypoxanthine, a cumulus cell inhibitory factor enters the oocyte through gap junctions and blocks meiotic resumption. A positive factor is also apparently secreted but is unable to override this inhibition. Blocking the coupling pathway with glycyrrhetinic acid (GA) prevents the transfer of inhibitor and the positive paracrine factor now predominates, resulting in meiotic induction. (*B*) This positive factor is either not produced in sufficient quantities or is ineffective in dbcAMP-treated CEO, since the germinal vesicle (GV) stage is maintained whether or not GA is present. (*C*) A paracrine factor may contribute to meiotic induction in FSH-treated, hypoxanthine-arrested CEO. GA would not be expected to block such induction, since it was stimulatory in the absence of FSH. (*D*) When dbcAMP-arrested CEO are treated with FSH, a positive stimulus is generated in the cumulus cells that is transferred to the oocyte via gap junctions to trigger germinal vesicle breakdown (GVB). The positive paracrine factor might contribute to meiotic induction but is insufficient alone to generate a meiotic response. Blocking the coupling pathway with GA prevents the influx of positive signal and thereby blocks FSH-induced maturation.

those from Byskov's laboratory were carried out in 0.4–0.5 ml MEMα or McCoy's 5A medium without an oil overlay (Guoliang *et al*., 1994; Byskov *et al*., 1997). Nevertheless, despite low percentages of meiotic induction in the co-cultured DO, under the same co-culture conditions meiotic resumption in hormone-treated CEO was stimulated to a much greater extent. These results are consistent with the idea that, although secreted paracrine factors can affect the meiotic status of the oocyte in a positive manner, they are not the principal means by which meiotic resumption is mediated in the intact complex.

What paracrine factors influence meiotic maturation?

Research in folliculogenesis has shown that paracrine factors play an important role in the development of the oocyte. One of the best studied of these factors is KIT ligand (KL), a protein known to be synthesised and secreted by granulosa cells that promotes oocyte growth (Manova *et al*., 1993; Huang *et al*., 1993; Packer *et al*., 1994; Elvin *et al*., 1999) and is, in turn, under reciprocal control by oocyte secretions (Joyce *et al*., 1999). An additional function has been suggested for KL, that of meiotic regulation of rat oocytes, and the proposed action is to maintain the oocyte in meiotic arrest at prophase I (Ismail *et al*., 1996, 1997). Thus, KL could conceivably contribute to the inhibitory constraint on meiotic resumption imposed by the follicle during oogenesis. It remains to be determined whether the suppressive effect of OCC on oocyte maturation demonstrated in this study is due to production and secretion of inhibitory factors such as KL.

Attention has recently focused on intermediates in the sterol biosynthetic pathway, termed meiosis-activating sterols (MAS), that have been isolated from follicular fluid and stimulate meiotic resumption in

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hypoxanthine-arrested DO during 24 h cultures (Byskov *et al*., 1995). Further, this same group has demonstrated meiotic induction in mouse DO by co-cultured cumulus cells under a variety of conditions and has proposed that MAS released by the cumulus cells acts directly on the oocyte to trigger GVB (Guoliang *et al*., 1994; Byskov *et al*., 1997). Confirmation of this mechanism must await demonstration of MAS accumulation in the conditioned medium. We have found that although MAS effectively stimulates GVB in hypoxanthine-arrested DO, its physiological importance in meiotic regulation remains speculative, particularly since it is relatively ineffective in CEO and failed to trigger maturation in dbcAMP-arrested DO (Downs *et al*., 2001). Interestingly, the inability of MAS to stimulate dbcAMP-arrested oocytes parallels our results herein with GA and cumulus cell co-culture and indicates that the type of meiosis-arresting agent may affect the oocyte response to paracrine factors. It is therefore possible that MAS-like factors are released by dissociated cumulus cells.

There are other candidate molecules that may serve in a paracrine capacity to trigger GVB. As a result of glycolytic activity, cumulus cells produce significant amounts of pyruvate (Biggers *et al*., 1967; Donahue & Stern, 1968; Leese & Barton, 1985; Downs *et al*., 1996), an energy source previously shown to induce meiotic resumption in arrested mouse oocytes (Downs & Mastropolo, 1994). Like MAS, this substrate can induce maturation in DO (Downs & Mastropolo, 1994), but neither the generation of pyruvate in response to gonadotropin stimulation nor its oxidation appears to mediate meiotic resumption (Downs *et al*., 1996, 1997). Future work will determine whether cumulus cell synthesis and secretion of pyruvate could contribute to the co-culture effect on meiotic maturation.

The dimeric protein activin is a member of the transforming growth factor β superfamily that is secreted by granulosa cells (Bicsak *et al*., 1988; Turner *et al*., 1989; Sidis *et al*., 1998). This protein has been reported to augment the spontaneous maturation of human (Alak *et al*., 1998), bovine (Stock *et al*., 1997), rhesus monkey (Alak *et al*., 1996), and rat (Itoh *et al*., 1990; Sadatsuki *et al*., 1993) oocytes, but conflicting data also exist (O *et al*., 1989; Stock *et al*., 1997). The ability of activin to induce maturation in meiotically arrested oocytes *in vitro* has not been thoroughly investigated, but in one study it failed to reverse the meiotic arrest maintained in rat oocytes by dbcAMP (Itoh *et al*., 1990). Interestingly, Pang and Ge (1999) have presented evidence that activin mediates the stimulatory action of gonadotropin on zebrafish oocyte maturation. Such a role for activin in mammalian oocytes has yet to be addressed.

While small culture volumes were used in an effort to concentrate putative paracrine factors, it is important to caution that these culture conditions could also promote the loss of medium components that contribute to an inhibitory action on oocyte maturation. The metabolism or consumption of such components by co-cultured cumulus cells could deplete them below an inhibitory threshold, leading to meiotic resumption through a disinhibition mechanism, without production of a positive paracrine factor. At this time, the contribution of such a mechanism to the meiotic induction observed in co-culture conditions cannot be discounted.

Evidence that gap junctions mediate the meiosis-inducing action of FSH

Because GA did not reverse the meiotic arrest maintained by dbcAMP, this cAMP analogue was used to test the importance of gap junctions in meiotic induction. At 2 μM, GA significantly reduced meiotic resumption in response to FSH, and this response was completely eliminated at 5 μM GA or higher. These concentrations of GA were associated with a reduction in oocyte–cumulus cell coupling of over 90%, supporting the idea that gap junctional transfer of a stimulatory signal triggered GVB (Fig. 9*C*, *D*), in agreement with previous studies (Fagbohun & Downs, 1991; Downs, 1995; Coskun & Lin, 1994). It is interesting that while 2 μM GA effectively blocked meiotic induction by FSH in dbcAMP-arrested CEO, higher concentrations were required to stimulate maturation in hypoxanthine-arrested CEO not exposed to gonadotropin (Fig. 5, bottom panel). These results suggest that a more complete blockage of gap junctional patency is required to reduce or eliminate the effect of a cumulus cell inhibitory signal compared with that of a stimulatory signal. It is also important to note that the presumptive paracrine factor released by cumulus cells may not be the same signal as that transmitted through gap junctions.

Oocyte–cumulus cell coupling was maintained throughout the period of meiotic induction and did not decrease until after meiotic resumption had been initiated, in agreement with previous studies in the mouse (Eppig, 1982; Salustri & Siracusa, 1983; Eppig & Downs, 1988), sheep (Moor *et al*., 1981) and pig (Motlik *et al*., 1986). In addition, GA was able to block meiotic induction at any point prior to meiotic resumption. This latter result implies that meiotic induction in isolated complexes is not brought about by a brief positive signal but, rather, by the protracted delivery of a positive factor up until the time of GVB. Further support for this comes from the finding that a 3 h pretreatment of complexes with FSH followed by denudation did not significantly increase the level of GVB in dbcAMP- or hypoxanthine-arrested DO. Similar results were reported by Byskov *et al*. (1997). It is important to point

out that meiotic induction *in vivo* occurs with faster kinetics, which is probably due to the contribution of an inducing signal by both the membrana granulosa and cumulus granulosa cells.

In conclusion, the results of this study suggest a positive paracrine effect of co-cultured cumulus cells on meiotic maturation. However, it does not appear to be the primary mechanism mediating meiotic induction by FSH in isolated CEO, since hormonal stimulation was not required for the co-culture effect and much higher GVB percentages were observed in intact complexes. Rather, evidence supports the generation of a positive signal of cumulus cell origin that requires the coupling pathway to reach the oocyte. It will be important in future studies to identify these putative paracrine factors and further define the conditions under which they operate.

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