Regulation of Murine Oocyte Meiosis: Evidence for a Gonadotropin-induced, cAMP-dependent Reduction in a Maturation Inhibitor

ROLF R. FRETER and RICHARD M. SCHULTZ
Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT We have developed an assay that can detect relative changes in the amount of a non-cAMP inhibitor of maturation present in cumulus cells (Eppig et al., 1983, *Dev. Biol.*, 100:39–49). Using this assay in which accelerated maturation of a group of treated cumulus cell-oocyte complexes relative to untreated complexes indicates a decrease in the amount of inhibitor, results of the experiments described here suggest a possible relationship between elevation of cAMP levels and subsequent decreased amounts of a non-cAMP inhibitor. Mouse oocytes obtained from cumulus cell-oocyte complexes treated with luteinizing hormone (LH) resumed meiosis prior to oocytes obtained from untreated complexes; the degree of acceleration of maturation was dependent on LH concentration. A similar result was obtained with follicle-stimulating hormone (FSH). Correlated with LH- or FSH-acceleration of maturation was an LH- or FSH-induced elevation of cumulus cell cAMP levels. Inhibiting LH-induced elevation of cumulus cell cAMP levels inhibited LH-induced acceleration of maturation. An initial incubation of complexes in medium containing dibutyryl cAMP (dbcAMP) also promoted acceleration of maturation. In contrast, maturation of denuded oocytes was not altered by treatment with either LH, FSH, or dbcAMP. Complexes initially incubated in dbcAMP-containing medium still demonstrated acceleration of maturation after a subsequent 2 h incubation in dbcAMP-free medium. Relative to untreated complexes, none of these treatments disrupted intercellular communication between cumulus cells and the oocyte. Elevating follicle cAMP levels with cholera toxin induced maturation of follicle-enclosed oocytes when cumulus cell-oocyte coupling was still fully maintained. These results are interpreted to indicate that gonadotropin-mediated acceleration of maturation is via a cAMP-dependent reduction in the level of a maturation inhibitor present in granulosa/cumulus cells.

Mammalian oocytes in nonatretic follicles are arrested in the first meiotic prophase; the preovulatory gonadotropin surge initiates resumption of meiosis of follicle-enclosed oocytes in vivo. Oocytes liberated from their follicles, however, spontaneously resume meiosis in vitro (14, 28). In contrast, cumulus cell-enclosed oocytes grafted to granulosa cells of experimentally opened follicles do not resume meiosis (33). If graft formation is prevented, however, resumption of meiosis occurs (33). Furthermore, cumulus cell-enclosed oocytes grafted to follicle wall hemisections resume meiosis in response to luteinizing hormone (LH)[1] (23). Results from the experiments described above suggest that (*a*) follicle cells produce an inhibitor of oocyte maturation, (*b*) a functional syncytium between granulosa cells, cumulus cells and the oocyte is required for maintenance of meiotic arrest, and (*c*) LH, acting on the granulosa/cumulus cells, promotes relief of meiotic inhibition. These conclusions are supported by other studies (9, 10).

Recently, an attractive hypothesis was formulated that envisioned that gap junction-mediated transmission of follicle cell cAMP to the oocyte inhibited maturation (9, 10). LH, which ultimately terminates cumulus cell-oocyte intercellular communication (16, 19) was proposed to initiate resumption of meiosis by terminating the flux of cAMP to the oocyte. LH-induced termination of communication, however, could not be the trigger for resumption of meiosis since intercellular communication between cumulus cells and the oocyte was maintained during a period of time in which oocytes underwent germinal vesicle breakdown (GVBD) (16, 26, 27);

[1] *Abbreviations used in this paper:* CHM, cyclohexamide; COCs, cumulus cell-oocyte complexes; CT, cholera toxin; DO, denuded oocyte; dbcAMP, dibutyryl AMP; FSH, follicle-stimulating hormone; GVBD, germinal vesicle breakdown; IBMX, 3-isobutyl-1-methyl xanthine; LH, luteinizing hormone; MEM/PVP, minimal essential medium with polyvinylpyrollidone; TLCK, $N\alpha$-p-tosyl-L-lysine chloromethyl ketone.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 MARCH 1984 1119–1128
© The Rockefeller University Press · 0021-9525/84/03/1119/10 $1.00

GVBD is the first easily observable manifestation of resumption of meiosis. A paradoxical situation thus arises; while cAMP inhibits spontaneous oocyte maturation in vitro (6, 31, 36), LH, which elevates follicle cAMP, initiates resumption of oocyte maturation in the follicle during a time when cumulus cell-oocyte intercellular communication is still present. A possible resolution of this paradox is that elevating cumulus/granulosa cell cAMP does not result in any detectable rise in oocyte cAMP; cumulus cell cAMP is apparently compartmentalized (30, 31). Rather, it is more likely that a change occurs in the nature and/or amount of a non-cAMP signal involved in regulation of resumption of meiosis and transmitted from follicle cells to the oocyte.

We have obtained results supporting the existence of a non-cAMP inhibitor of oocyte maturation which originates in follicle cells (17, 30). We proposed a model (17) that envisioned continuous production by the granulosa/cumulus cells of an inhibitor of oocyte maturation. The putative maturation inhibitor would enter the oocyte via the follicular network of gap junctions present between granulosa cells, cumulus cells, and the oocyte that renders the entire mass into a functional syncytium (1, 19).

In this communication, we present further evidence suggesting that elevation of cumulus cell cAMP promotes generation of a maturation inhibitor from an inactive precursor and that once generated, the maturation inhibitor is subsequently inactivated. Results from these experiments are incorporated into our previous model (17) and a model is presented for maintenance of meiotic arrest and LH-induced resumption of meiosis. As will be discussed, this model resolves the paradoxical situation in which detecting the maturation inhibitor requires elevating cAMP (17, 30); yet elevating cAMP can also induce resumption of meiosis by reducing the amount of maturation inhibitor.

MATERIALS AND METHODS

Collection and Culture of Oocytes and Follicles: Follicles, cumulus cell-oocyte complexes, and oocytes freed of their attached cumulus cells (denuded oocytes) were obtained from randomly bred, Swiss albino mice 24-d old (Swiss Webster; Ace Animal Farm, Boyertown, PA) that were primed 48 h previously with 5 IU pregnant mare's serum gonadotropin (Sigma Chemical Co., St. Louis, MO) as previously described (30). The culture medium used was Eagle's minimal essential medium (MEM; Gibco Laboratories, Grand Island, NY) containing Earle's salts, pyruvate (100 μg/ml), 10 mM HEPES, pH 7.2, polyvinylpyrollidone (3 mg/ml), and gentamicin (10 μg/ml) (MEM/PVP).

Cumulus cell-oocyte complexes (COCs) were collected in MEM/PVP containing 0.2 mM 3-isobutyl-1-methyl xanthine (IBMX). This concentration of IBMX reversibly inhibits initiation of maturation. COCs and denuded oocytes were incubated in MEM/PVP at 37°C in agarose-coated petri dishes (35 mm; Falcon Labware, Oxhard CA) in a humidified atmosphere of 5% CO_2 in air.

Preovulatory antral follicles with a minimum of associated connective tissue were incubated in stoppered 50-ml Erlenmeyer flasks containing 3 ml of MEM/PVP in which the bicarbonate was replaced by 25 mM HEPES, pH 7.2. Incubations were carried out at 37°C in a water bath with gentle agitation.

Measurement of Intercellular Communication Between Cumulus Cells and Oocytes: Intercellular communication between cumulus cells and oocytes was measured as previously described (30). The data are expressed as a coupling index, which is the percentage of radiolabeled uridine (and its metabolites) present in the oocyte relative to that in the intact COC. At least six samples of denuded oocytes (five oocytes/sample) and complexes (three complexes/sample) were used to determine the coupling index for each experimental group. Calculation of the percentage of radiolabeled uridine in oocytes internally controls for fluctuations in cumulus cell uptake of radiolabeled uridine induced by hormones or other substances (16). Unless otherwise stated, coupling indices were determined during the second hour of the inhibitor content protocol (see below), i.e., during incubation in IBMX-free medium containing a suboptimal concentration of dbcAMP.

Measurement of cAMP: cAMP was measured in denuded oocytes, COCs, or preovulatory antral follicles by radioimmunoassay as previously described (31). The cAMP antibody was the generous gift of Dr. Gary Brooker, Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia.

Iontophoretic Injection of Lucifer Yellow into Oocytes: COCs were attached to polylysine coated glass coverslips. The complexes were bathed in bicarbonate-free MEM/PVP containing either 0.2 mM IBMX or 0.2 mM IBMX and LH (1 μg/ml). Microelectrodes were filled with a 3% solution of Lucifer yellow (Sigma Chemical Co.). Iontophoresis, fluorescence visualization, and photographic procedures were performed as previously described (2).

Measurement of Maturation Inhibitory Factor: We have previously presented evidence for the existence of a cAMP-dependent inhibitor of oocyte maturation present in cumulus cells (17, 30). In the present study, inhibition of maturation was determined by a similar protocol: groups of COCs and denuded oocytes were incubated for 1 h in medium containing IBMX and suboptimal concentrations of the membrane permeable cAMP analog dibutyryl cAMP (dbcAMP). Both groups were then washed and transferred to IBMX-free medium containing another but not necessarily identical suboptimal concentration of dbcAMP (Fig. 1*A*). After 1.0 h, cumulus cell-enclosed oocytes were stripped of their cumulus cells and both groups transferred to IBMX- and dbcAMP-free medium. GVBD was scored with time by examining the oocytes with a Wild M5A microscope at 100-fold magnification. Results from such an experiment (Fig. 1*B*) invariably reveal that cumulus cell-enclosed oocytes matured later than denuded oocytes. The series of incubations shown in Fig. 1*A* will henceforth be called inhibitor content protocol. In this study, as in a previous study (17), the differences in extent of maturation at a given time are taken as evidence for the presence of a non-cAMP inhibitor of maturation in cumulus cells.

IBMX was included in the initial incubation to block GVBD while the oocytes were exposed to the initial suboptimal dbcAMP concentration. Both groups were kept for an additional hour in IBMX-free medium containing dbcAMP to generate the greatest possible difference in inhibitor content between denuded and cumulus cell-enclosed oocytes prior to scoring for GVBD with time. Complexes that did not appear compact were removed from the sample; only visually compact complexes were scored for GVBD with time. Further discussion of the rationale and validity of the assay and the role of cAMP in detecting the maturation inhibitor is presented elsewhere (17).

The experiments described in this report were carried out over a period of several months. During this time, the suboptimal concentrations of dbcAMP used in the inhibitor content protocol were changed on several occasions to maintain a constant difference in the mean times of maturation between the denuded oocytes and cumulus cell-enclosed oocytes. The concentrations of dbcAMP used in the inhibitor content protocol and the specific details for each experiment are given in the figure legends.

RESULTS

Treatment of Cumulus Cell-enclosed Oocytes with LH or Follicle-stimulating hormone Accelerates Oocyte Maturation

Cumulus cell-enclosed oocytes, initially incubated in medium containing 1 μg/ml LH and IBMX for 2 h and subsequently processed through the inhibitor content protocol (see Materials and Methods and Fig. 1*A* for experimental design and rationale), resumed meiosis prior to control complexes initially incubated in medium containing only IBMX (Fig. 1*B*). LH-induced acceleration of maturation was dependent on LH concentration (Fig. 2); under the experimental conditions, apparent maximal acceleration was obtained with an LH concentration of 10 ng/ml (0.32 nM). Similar results were obtained with highly purified LH (data not shown). Acceleration of maturation could be observed after a 1-h incubation in medium containing LH (10 ng/ml) (data not shown). In contrast, denuded oocytes (DOs) exposed or not exposed to LH (1 μg/ml) for 2 h and then processed through the inhibitor content protocol resumed meiosis with similar time courses (data not shown). This suggests that cumulus cells are the target for LH-mediated acceleration of maturation. It should be noted that the duration (2 h) and highest LH concentration (1 μg/ml) employed in the initial incubation are likely to approximate physiological conditions (3, 7, 25, 29).

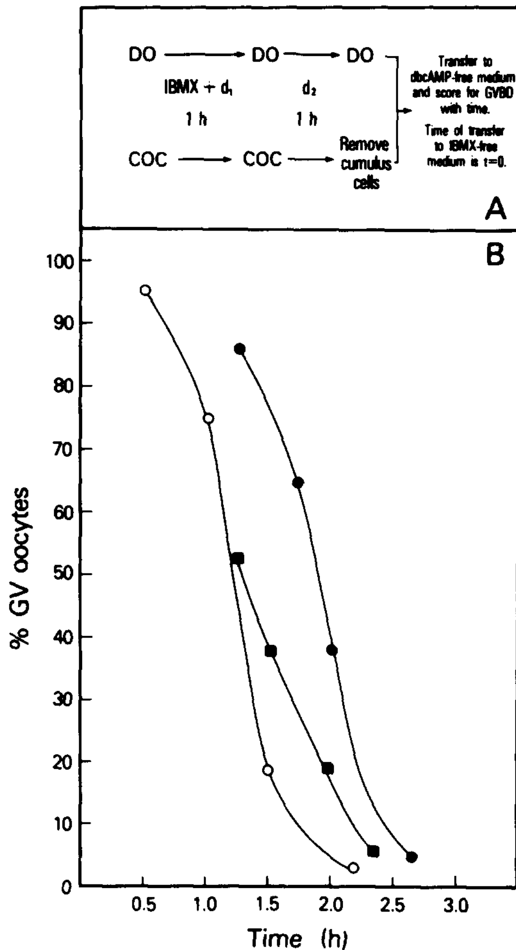

FIGURE 1 Assay for detecting maturation inhibitor. (*A*) Inhibitor content protocol. Groups of denuded oocytes (*DO*) and cumulus cell-enclosed oocytes (*COC*) were incubated for 1 h in medium containing IBMX and a suboptimal concentration of dbcAMP (d_1, values will be given in the figure legends). Both groups were then washed and transferred to IBMX-free medium containing another suboptimal concentration of dbcAMP (d_2, values will be given in the figure legends). After 1 h, cumulus cell-enclosed oocytes were stripped of their cumulus cells and both groups were washed, transferred to IBMX and dbcAMP-free medium, and scored for GVBD with time. (*B*) LH-mediated acceleration of maturation of cumulus cell-enclosed oocytes. Groups of cumulus cell-oocyte complexes were incubated for 2 h in medium containing IBMX (circles) or IBMX and LH (1 μg/ml) (squares). Both groups were then washed and processed through the inhibitor content protocol with d_1 = 75 μM dbcAMP and d_2 = 75 μM dbcAMP. At least 50 oocytes were included in each group. ○, denuded oocytes processed through the inhibitor content protocol; ●, oocytes obtained from complexes processed through the inhibitor content protocol; ■, oocytes obtained from LH-treated complexes processed through the inhibitor content protocol. Denuded oocytes from complexes initially incubated with IBMX and LH and then processed through the inhibitor content protocol matured with kinetics similar to those of the denuded oocytes shown in the figure (data not shown). *GV*, germinal vesicle.

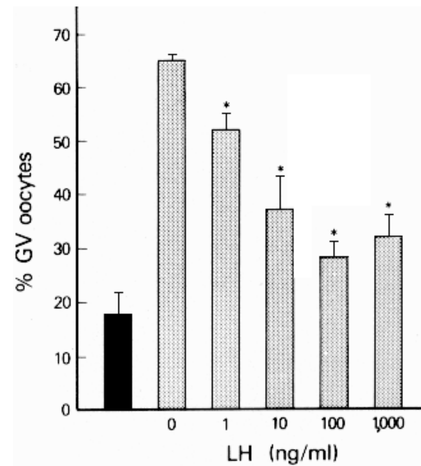

FIGURE 2 Concentration dependence of LH-mediated acceleration of maturation. Groups of cumulus cell-oocyte complexes were incubated for 2 h in medium containing IBMX or IBMX and 1, 10, 100, or 1,000 ng/ml of LH. All groups were then washed and processed through the inhibitor content protocol with d_1 = 75 μM dbcAMP and d_2 = 50 μM dbcAMP. The percentage of oocytes in each group possessing an intact germinal vesicle (*GV*) was determined 1.5 h after transfer to IBMX-free medium. The experiment was performed three times. The data were pooled and expressed as mean ± SEM. In each experiment at least 50 oocytes were included in each group. Solid bar, denuded oocytes; stippled bar, oocytes obtained from complexes treated with the indicated concentrations of LH. The differences between LH-treated and -untreated complexes are significant; $p < 0.01$ (*) (Student's t test). This test was used in all subsequent cases. In this and subsequent relevant experiments, each group of oocytes was monitored periodically until >90% had undergone GVBD as shown in Fig. 1 *B*. In addition, all groups of oocytes manifested equal proportions of polar body emission.

Since a decrease in oocyte cAMP has been correlated with resumption of meiosis (31, 35), it is also possible that LH-mediated acceleration of maturation could be due to an LH-induced decrease in oocyte cAMP levels. Results from the following experiment minimize the likelihood of this possibility. Oocytes obtained from complexes exposed or not exposed to LH (1 μg/ml) for 2 h and then processed through a modified inhibitor content protocol in which dbcAMP was omitted, resumed meiosis with similar time courses (data not shown). This result suggests that treatment of complexes with LH did not alter oocyte cAMP levels to an extent sufficient to cause an acceleration of maturation.

Another explanation for the observed LH-mediated acceleration of maturation might be an LH-induced reduction or termination of coupling between cumulus cells and the oocyte. This could result in reduction or termination of an inhibitory signal that might be transmitted from cumulus cells to the oocyte. To address this possibility, measurements of intercellular communication between the cumulus cells and oocytes in LH-treated and untreated complexes were carried out during (*a*) the second hour of the initial incubation in IBMX alone or IBMX plus LH (1 μg/ml) and (*b*) the second hour of the inhibitor content protocol, i.e., when both groups of complexes are incubated in medium containing a suboptimal concentration of dbcAMP alone (see Fig. 1*A*). In the first situation, coupling indices of 16.1 and 16.8% and in the second situation, coupling indices of 17.7 and 17.2% were obtained for LH-treated and untreated complexes, respectively. These results suggest that LH caused neither a transient, reversible uncoupling during the initial incubation in LH nor a more gradual uncoupling with time. Consistent with this interpretation are the results of experiments in which Lucifer yellow was iontophoretically injected into cumulus cell-enclosed oocytes initially incubated for 2 h in medium containing either IBMX or IBMX and LH (1 μg/ml) (Fig. 3). In both cases dye spread uniformly to all of the outermost cumulus

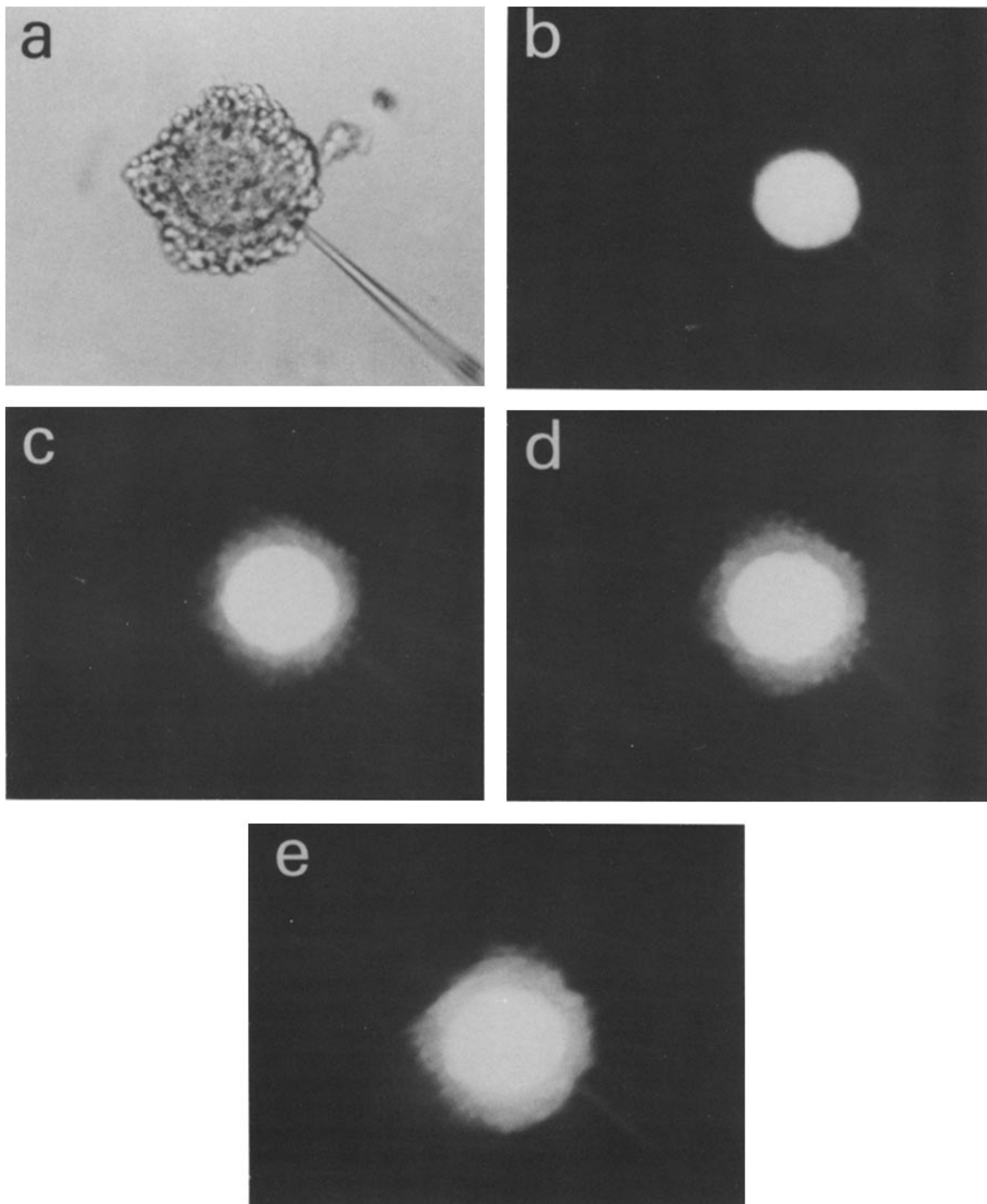

FIGURE 3 Transfer of iontophoretically injected Lucifer yellow from oocytes to cumulus cells. Cumulus cell-oocyte complexes were incubated in medium containing IBMX or IBMX and LH (1 μg/ml) for 2 h. Complexes were then attached to coverslips and Lucifer yellow iontophoretically injected as described in Materials and Methods. About 10 LH-treated and untreated complexes were examined. Shown is a representative example of an LH-treated complex. For both groups of complexes it took ~15 min for the dye to reach the peripheral cumulus cells. (*a*) Phase contrast photomicrograph of a complex; (*b*–*e*) Photomicrographs of complexes 1, 7, 20, and 40 min, respectively, after Lucifer yellow was injected into the oocyte.

cells and the time courses for dye spread were similar. Thus, it is unlikely that the observed LH-mediated acceleration of maturation was due to uncoupling, but rather reflected a change in the nature or amount of an inhibitory signal involved in regulation of oocyte maturation.

The effect of FSH on acceleration of cumulus cell-enclosed oocyte maturation was tested by initially incubating complexes in medium containing IBMX and FSH at either 10 or 20 ng/ml for 2 h followed by processing through the inhibitor content protocol. FSH-promoted acceleration of oocyte maturation was concentration-dependent. (Fig. 4). The magnitudes of FSH-mediated acceleration of maturation were similar to those elicited by LH. Furthermore, oocytes obtained from complexes exposed or not exposed to FSH (20 ng/ml) for 2 h and then processed through a modified inhibitor content protocol in which dbcAMP was omitted resumed

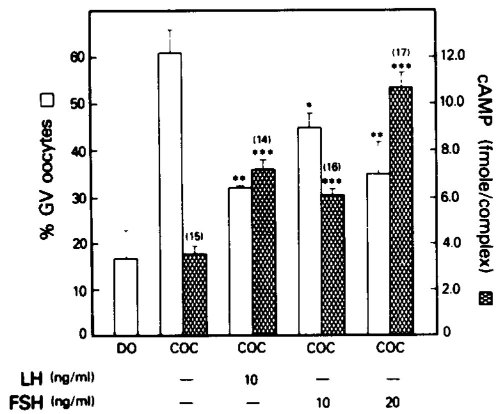

FIGURE 4 Relationship between gonadotropin-induced acceleration of maturation and elevation of cAMP levels in complexes. Groups of cumulus cell-oocyte complexes (COC) were incubated for 2 h in medium containing IBMX, IBMX and LH (10 ng/ml), or IBMX and FSH (10 or 20 ng/ml). All groups were then washed and either processed through the inhibitor content protocol, with d_1 = 75 μM dbcAMP and d_2 = 60 μM dbcAMP, or assayed immediately for cAMP content. The percentage of oocytes in each group possessing an intact GV (open bar) was determined 1.25 h after transfer to IBMX-free medium. The experiment was performed four times. The data were pooled and results are expressed as mean ± SEM. In each experiment that determined extent of maturation, at least 50 oocytes were included in each group. 10–15 complexes were used for each cAMP determination (cross-hatched bar; femtomoles per complex). The number of cAMP determinations per group is given in parentheses. The differences between treated and untreated complexes are significant; $p < 0.05$ (*); $p < 0.01$ (**); and $p < 0.001$ (***).

meiosis with similar kinetics (data not shown). This result suggests that, as in the case of LH, FSH did not alter oocyte cAMP levels to an extent sufficient to cause the observed FSH-mediated acceleration of maturation.

Associated with either LH- or FSH-induced acceleration of maturation was an LH- or FSH-induced elevation of the cAMP levels of complexes (Fig. 4). It should be noted that results from a previous study (30) indicated that this cAMP increase occurs in the cumulus cells and not the oocyte. In addition, the coupling indices of FSH-treated and control complexes were similar (21.6 and 22.2%, respectively).

Acceleration of Cumulus Cell-enclosed Oocyte Maturation is cAMP-dependent

The observation that LH and FSH elicited acceleration of maturation and elevated cAMP levels in COCs suggested a possible role for cAMP in this process. If LH and FSH mediate acceleration of maturation via a cAMP-dependent mechanism, then complexes initially incubated in dbcAMP should exhibit accelerated maturation relative to complexes initially incubated in dbcAMP-free medium. Oocytes obtained from complexes initially incubated for 2 h in medium containing IBMX and 10 to 100 μm dbcAMP resumed meiosis prior to untreated controls (Fig. 5). Maximal acceleration was obtained with an initial incubation in 10 μm dbcAMP. The accelerations of cumulus cell-enclosed oocyte maturation obtained after incubations in either dbcAMP, LH, or FSH were of similar magnitude. These results present an apparent paradox; whereas detecting the inhibitor requires elevating cAMP levels in complexes (17, 30), elevating cAMP in complexes can also accelerate resumption of meiosis. A resolution to this paradox will be presented in the Discussion.

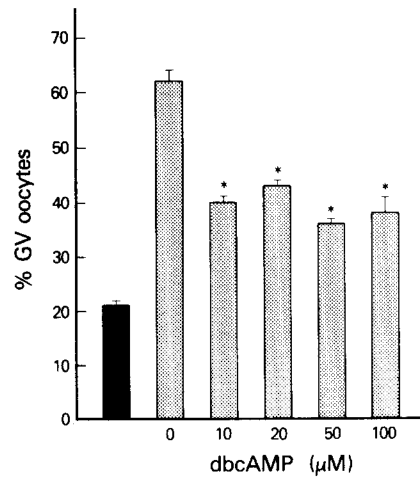

FIGURE 5 Concentration dependence of dbcAMP-mediated acceleration of maturation. Groups of cumulus cell-oocyte complexes were incubated for 2 h in medium containing IBMX or IBMX and 10, 20, 50, or 100 μM dbcAMP. All groups were then washed and processed through the inhibitor content protocol with d_1 = 75 μM dbcAMP and d_2 = 60 μM dbcAMP. The percentage of oocytes in each group possessing an intact GV was determined 1.25 h after transfer to IBMX-free medium. The experiment was performed three times. The data were pooled and results expressed as mean ± SEM. In each experiment, at least 50 oocytes were used in each group. Solid bar, denuded oocytes; stippled bar, oocytes obtained from complexes treated with the indicated concentrations of dbcAMP. The differences between treated and untreated complexes are significant; $p < 0.01$ (*).

In contrast to cumulus cell-enclosed oocytes, denuded oocytes, after a 2-h initial incubation in either IBMX alone or IBMX and 10 or 100 μm dbcAMP followed by processing through the inhibitor content protocol, resumed meiosis with similar time courses (data not shown). This suggests that cumulus cells are the target of dbcAMP-mediated acceleration of maturation.

Coupling indices in complexes treated with 100 μM dbcAMP and untreated complexes were determined during (*a*) the second hour of the initial incubation in IBMX alone or IBMX and 100 μm dbcAMP or, (*b*) the second hour of the inhibitor content protocol (as before with the LH-mediated accelerations). In the first situation, coupling indices of 18.0 and 16.8% and in the second situation, coupling indices of 19.5 and 22.2% were obtained for dbcAMP-treated and -untreated complexes, respectively. It is unlikely, therefore, that dbcAMP-mediated acceleration of maturation is due to uncoupling of oocytes from their respective cumulus cells.

If LH mediates acceleration of oocyte maturation by elevating cumulus cell cAMP levels, then blocking this increase should result in a loss of LH-induced acceleration of maturation. $N\alpha$-p-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma Chemical Co.) has been reported to inhibit gonadotropin-induced elevation of cAMP in granulosa cells (21). Oocytes obtained from complexes, initially incubated in 0.25 mM TLCK for 30 min, followed by a 2-h incubation in LH (10 ng/ml), and then processed through the inhibitor content protocol, did not exhibit accelerated maturation relative to untreated controls and also failed to exhibit the LH-mediated increase in cumulus cell cAMP levels (Fig. 6). TLCK had no direct effect on oocytes; treatment of denuded oocytes with 0.25 mM TLCK did not alter (*a*) the kinetics of maturation or (*b*) the extent of polar body emission, relative to untreated denuded oocytes (data not shown).

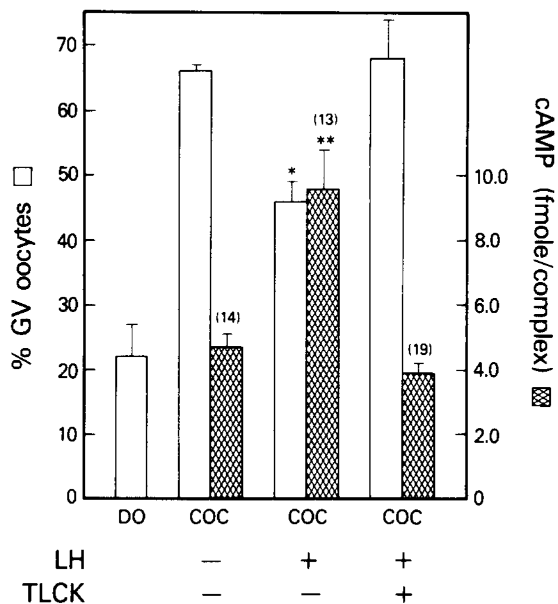

FIGURE 6 TLCK inhibition of LH-mediated increase in cumulus cell cAMP levels and acceleration of maturation. Groups of cumulus cell-oocyte complexes were incubated for 30 min in medium containing IBMX or IBMX and 0.25 mM TLCK. TLCK-treated complexes were washed and incubated for 2 h in medium containing IBMX and LH (10 ng/ml). Complexes not treated with TLCK were incubated for 2 h in medium containing IBMX or IBMX and LH (10 ng/ml). All groups were then washed and either processed through the inhibitor content protocol, with d_1 = 75 μM dbcAMP and d_2 = 60 μM dbcAMP, or assayed immediately for cAMP content (cross-hatched bar; femtomoles per complex). The percentage of oocytes in each group possessing an intact GV (*open bar*) was determined 1.25 h after transfer to IBMX-free medium. The experiment was performed three times. The data were pooled and results expressed as mean ± SEM. For each experiment measuring extent of maturation, at least 50 oocytes were used for each group. 10–15 complexes were used for each cAMP determination. The number of cAMP determinations per group is given in parentheses. The differences between LH-treated and untreated complexes are significant; $p < 0.01$ (*); $p < 0.001$ (**). The differences between LH and TLCK treated and untreated complexes are not significant.

To determine the site of TLCK inhibition of LH-induced cAMP elevation, complexes were incubated for 30 min in medium containing IBMX and 0.25 mM TLCK and then incubated for 1 h in IBMX-containing medium supplemented with either cholera toxin (CT) (2 μg/ml), which activates adenylate cyclase via ADP-ribosylation of the regulatory subunit (18) or forskolin (20 μg/ml), which appears to activate adenylate cyclase via the catalytic subunit (32) (although this point is controversial, see reference 8). cAMP levels were then determined by radioimmunoassay. TLCK treatment of complexes did not inhibit either CT- or forskolin-induced elevation of cAMP levels relative to complexes not treated with TLCK (CT, 73 ± 17; CT + TLCK, 154 ± 51; forskolin, 130 ± 35; forskolin + TLCK, 156 ± 36. Units are femtomoles of cAMP/complex ± standard deviation; in each case the results of at least eight cAMP determinations were pooled). This suggests that TLCK-induced inhibition of LH-mediated cAMP elevation is not at the level of the cyclase but rather is at the level of hormone-receptor binding or signal transduction to the cyclase.

Several attempts were made to "bypass" TLCK inhibition of LH-mediated acceleration of maturation by adding dbcAMP to mimic the normal cumulus cell response to LH. Complexes were incubated in medium containing IBMX and 0.25 mM TLCK for 30 min and then incubated for 2 h in medium containing IBMX and 100 μm dbcAMP before processing through the inhibitor content protocol. Using this protocol exposure to dbcAMP did not accelerate maturation; the reason(s) for this failure is unknown at this time.

An initial 1-h incubation in medium containing dbcAMP accelerates maturation of cumulus cell-enclosed oocytes relative to that of oocytes in non-dbcAMP-treated complexes. To determine the persistence of the dbcAMP-induced effect(s) in the cumulus cells, the experiment shown in Fig. 7 was performed. Oocytes obtained from (*a*) complexes initially incubated in medium containing IBMX and 100 μm dbcAMP for 1 h and then incubated for a further 2 h in medium containing IBMX only and (*b*) complexes initially incubated in medium containing only IBMX for 2 h and then incubated an additional 1 h in medium containing IBMX and 100 μM dbcAMP matured with similar time courses after processing through the inhibitor content protocol. The three groups of complexes shown in Fig. 7 had similar coupling indices (untreated, 14.7%; dbcAMP and IBMX to IBMX, 14.3%; IBMX to dbcAMP and IBMX, 13.8%). Hence, over the 2-h chase period, i.e., in dbcAMP-free medium, no decrease was observed in the magnitude of dbcAMP-mediated acceleration of oocyte maturation.

Properties of the Cumulus Cell Inhibitor of Oocyte Maturation

To examine the persistence of maturation inhibitor in cumulus cells, groups of complexes were processed through the inhibitor content protocol either (*a*) immediately after collection or (*b*) after a 4-h initial incubation in medium containing IBMX only (Fig. 8). In both cases, inhibitor was present as evidenced by the significant delays in maturation of cumulus cell-enclosed oocytes compared with that of denuded oocytes. It was also apparent, however, that the de-

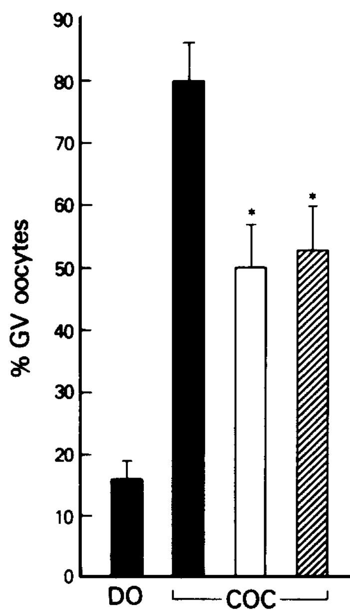

FIGURE 7 Effect of a subsequent incubation in dbcAMP-free medium on dbcAMP-mediated acceleration of maturation. Groups of cumulus cell-oocyte complexes were incubated in medium containing IBMX for 3 h (solid bar), IBMX for 2 h followed by transfer to medium containing IBMX and 100 μM dbcAMP for 1 h (open bar), or IBMX and 100 μM dbcAMP for 1 h followed by washing and transfer to medium containing IBMX for 2 h (slashed bar). All groups were then washed and processed through the inhibitor content protocol with d_1 = 90 μM dbcAMP and d_2 = 85 μM dbcAMP. The percentage of oocytes in each group possessing an intact GV was determined 1.25 h after transfer to IBMX-free medium. The experiment was performed three times. The data were pooled and expressed as mean ± SEM. For each experiment at least 50 oocytes were included in each group. The differences between the treated and untreated complexes are significant; $p < 0.05$ (*).

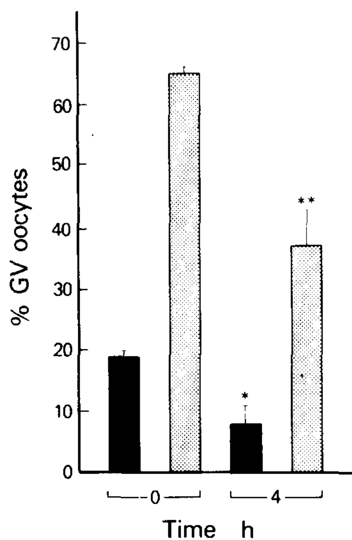

FIGURE 8 Maturation of cumulus cell-enclosed and denuded oocytes after various times in culture. Groups of cumulus cell-oocyte complexes were processed immediately after collection or after a 4-h initial incubation in IBMX-containing medium, through the inhibitor content protocol with $d_1 = 75$ μM dbcAMP and $d_2 = 60$ μM dbcAMP. The percentage of oocytes in each group possessing an intact GV was determined 1.25 h after transfer to IBMX-free medium. The experiment was performed three times. The data were pooled and expressed as mean ± SEM. For each experiment at least 50 oocytes were included in each group. The differences between denuded oocytes (solid bar) at 0 and 4 h and between cumulus cell-enclosed oocytes (stippled bar) at 0 and 4 h are significant; $p < 0.05$ (*) and $p < 0.01$ (**), respectively.

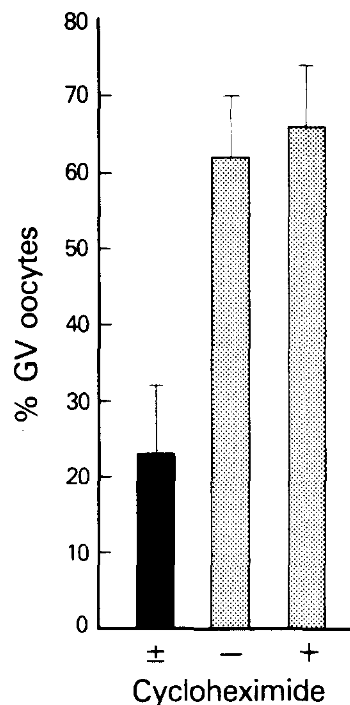

FIGURE 9 Effect of CHM on maturation of cumulus cell-enclosed oocytes. Groups of cumulus cell-oocyte complexes were incubated for 2 h in medium containing IBMX or IBMX and CHM (10 μg/ml). All groups were then washed and processed through the inhibitor content protocol with $d_1 = 75$ μM dbcAMP and $d_2 = 60$ μM dbcAMP. The group that initially had been exposed to CHM continued to be exposed to CHM (10 μg/ml) while being processed through the inhibitor content protocol. The percentage of oocytes in each group possessing an intact GV was determined 1.25 h after transfer to IBMX-free medium. The experiment was performed three times. The data were pooled and results expressed as mean ± SEM. For each experiment at least 50 oocytes were included in each group. Solid bar, denuded oocytes; stippled bar, oocytes obtained from complexes. There is no significant difference between the CHM-treated and -untreated complexes.

nuded oocytes and oocytes obtained from complexes initially incubated in IBMX for 4 h resumed meiosis significantly faster than their respective counterparts that were processed through the inhibitor content protocol immediately after collection. This suggests a decrease with time in the amount of inhibitor present in complexes.

Since a decrease in oocyte cAMP has been correlated with resumption of meiosis (31, 35), an alternative explanation for the above result is that cumulus cell-enclosed oocytes may have developed decreased levels of cAMP with time. cAMP levels were determined in denuded oocytes (*a*) liberated from complexes immediately after collection or (*b*) liberated from complexes after a 4 h-incubation in IBMX-containing medium. No significant difference in the amount of cAMP was observed between the two groups; values of 0.19 and 0.22 femtomoles of cAMP/oocyte (average of two determinations, 250 oocytes were used per determination) were obtained after 0 and 4 h of incubation, respectively. This renders the alternative explanation unlikely.

The effect(s) of cycloheximide (CHM) on the amount of inhibitor present in cumulus cells was examined. Complexes initially incubated in medium containing IBMX or IBMX and CHM (10 μg/ml) for 2 h were then processed through the inhibitor content protocol. The experimental group was exposed to CHM in all incubations. Oocytes obtained from complexes treated or not treated with CHM matured with similar kinetics (Fig. 9). CHM (10 μg/ml) (*a*) did not affect the time or rate of maturation of denuded oocytes and (*b*) reduced incorporation of [^{35}S]methionine into acid-insoluble radioactive material by >95% in treated complexes compared with untreated complexes (data not shown). These results suggest that continuous protein synthesis is not required for the persistence of detectable amounts of inhibitor in cumulus cells.

Cholera Toxin-induced Maturation of Follicle-enclosed Oocytes

Treatment of follicles with LH, FSH, prostaglandin E_2 or dbcAMP initiates resumption of meiosis in vitro (13, 34). In these studies, however, GVBD was assessed after 18 h of exposure of the follicles to the maturation-inducing substance. All of these substances raise follicular cAMP which induces cumulus cell mucification within 18 h (11, 15, 20). Mucification results in physical disruption of coupling between cumulus cells and the oocyte (16, 19). Thus, during the 18-h incubation, cAMP-induced cumulus cell mucification would terminate influx to the oocyte of a maturation inhibitor and permit resumption of meiosis. In contrast, on the basis of the data presented here, one may predict that any agent capable of elevating follicle cAMP should induce maturation during a time when (*a*) follicle cAMP levels are still elevated and (*b*) cumulus cells remain fully coupled to the oocyte as is the case during gonadotropin-induced maturation in vivo (16, 31).

Follicle-enclosed oocytes resumed meiosis when cultured in medium containing CT (Fig. 10). A positive correlation exists between CT-induced elevation of follicle cAMP content and resumption of meiosis. In contrast, follicle-enclosed oocytes incubated in medium without cholera toxin did not resume meiosis (Fig. 10). Under these conditions, follicle cAMP content did not increase (data not shown). Complexes, expressed from follicles after 2 h of incubation in medium containing or not containing CT and assayed immediately for metabolic cooperativity had similar coupling indices (12.4 and 11.9%, respectively).

DISCUSSION

Although it has been known for some time that exposure of follicles to LH and other agents that raise follicle cAMP levels can result in resumption of meiosis, the sequence of events linking elevation of cAMP and resumption of meiosis is not clear. In addition, the relationship between gonadotropin-induced maturation and a non-cAMP inhibitor of maturation

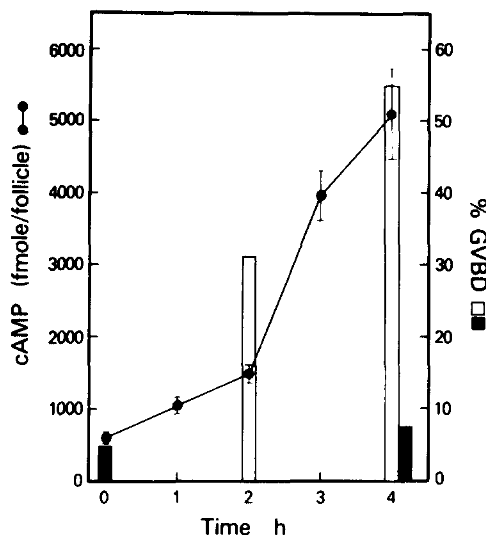

FIGURE 10 Effect of cholera toxin on follicle cAMP levels and oocyte maturation. Preovulatory antral follicles (~30/group) were incubated in medium containing or not containing cholera toxin (CT, 10 μg/ml). At the indicated times cumulus cell-oocyte complexes were removed from their follicles and the oocyte scored for GVBD or follicles were assayed for cAMP content. Open bar, % GVBD in CT-treated follicles; solid bar, % GVBD in non-CT-treated follicles. The experiment was performed three times. The total number of oocytes scored for GVBD was ~80-90 in each case. Duplicate measurements of cAMP content were made on at least six follicles at each time; results are expressed as mean ± SEM.

(17, 30) is also not clear. Using an assay (inhibitor content protocol) that can detect relative changes in the amount of a non-cAMP inhibitor of oocyte maturation, results of the experiments described here suggest a relationship between elevation of cAMP and a subsequent decrease in the amount of inhibitor.

Cumulus cells possess LH receptors (4, 22). Decreased amounts of a maturation inhibitor present in COCs after LH treatment would be expected to result in an acceleration of maturation relative to untreated COCs when both groups are processed through the inhibitor content protocol. Two types of control experiments, however, were always performed before ascribing an acceleration of maturation to decreased levels of inhibitor. The first control (no dbcAMP present during the inhibitor content protocol) demonstrated that whatever changes may have occurred in the levels of cAMP in treated relative to untreated cumulus cell-enclosed oocytes could not account for an acceleration of maturation. The second control (measuring the coupling index) demonstrated that the initial treatment did not disrupt intercellular communication between the cumulus cells and the oocyte. Further justification of the assay is presented elsewhere (17). Using the above criteria, LH accelerated maturation of cumulus cell-enclosed oocytes by apparently promoting a decrease in the amount of maturation inhibitor.

Acceleration of maturation, i.e., detection of a reduced amount of maturation inhibitor, appeared to be mediated by cAMP. Both LH and FSH accelerated oocyte maturation and elevated cumulus cell cAMP levels. Inhibiting LH-induced elevation of cAMP levels with TLCK, inhibited LH-induced acceleration of oocyte maturation. A role for cAMP in acceleration of maturation was also suggested by the observation that an initial incubation in medium containing the cAMP analog dbcAMP promoted acceleration of maturation and that elevating follicle cAMP levels with cholera toxin induced maturation when cumulus cell-oocyte coupling was still fully maintained. Consistent with these results is the recent observation that forskolin can induce maturation of rat follicle-enclosed oocytes during a time when cumulus cell-oocyte coupling was presumably still fully maintained (12). Taken together, results from our experiments suggest that cAMP-induced acceleration of maturation was due to reducing the amount of a maturation inhibitor and not due to uncoupling of cumulus cells from the oocyte. This conclusion, however, raises the previously mentioned paradox: whereas gonadotropins apparently decrease the amount of a maturation inhibitor in a cAMP-dependent manner, the inhibitor is only detected in the presence of cAMP. A resolution to this paradox is presented below.

Two alternative possibilities for the basis of LH-induced relief of inhibition of maturation are (*a*) uncoupling the outer cumulus cells from the inner cumulus cells or (*b*) decreasing the molecular weight cut-off of the heterologous gap junctions present between cumulus cells and the oocyte. LH-induced uncoupling of outer cumulus cells from the inner cumulus cells could result in accelerated maturation by decreasing the influx of inhibitor to the oocyte. The likelihood of this possibility is minimized by the observation that LH did not affect the spread of Lucifer yellow throughout the cumulus cell mass of complexes, together with the coupling assay using radiolabeled uridine, strongly suggests that a complete "outer-uncoupling" did not occur. Alternatively, LH may mediate a reduction in the number of gap junctions between cumulus cells. Although the time courses of dye movement to the periphery of the cumulus cell mass were similar in LH-treated and untreated complexes, the level of sensitivity in these measurements could not exclude this possibility.

Previous results have suggested that passage of the maturation inhibitor to the oocyte is via the gap junctions (17). This would require the molecular weight of the maturation inhibitor to be <1,000 daltons (24). An LH-mediated decrease in gap junction diameter could reduce the junctional cutoff to a molecular weight less than that of the maturation inhibitor but still greater than that of radiolabeled uridine and its metabolites. Although there are no data to rule out this possibility, to our knowledge, no examples of a cAMP-mediated reduction in gap junction permeability are documented.

Although the molecular mechanism by which LH, or any other agent capable of raising cumulus cell cAMP levels, decreases the amount of maturation inhibitor is not known, several points can be made. CHM had no effect on levels of maturation inhibitor in complexes. Thus, continuous protein synthesis is not required for maintenance of detectable levels of inhibitor. Furthermore, although maturation inhibitor was present after a 4-h incubation of complexes in IBMX-containing medium, denuded oocytes from this group resumed meiosis significantly earlier than denuded oocytes assayed immediately after collection. This suggests that oocytes catabolize and/or secrete the inhibitor.

The following model extends our previous model for maintenance of meiotic arrest (17) and accounts for gonadotropin-induced resumption of meiosis in vivo. A steady state level of a maturation inhibitor is produced predominantly by granulosa cells from an inactive precursor via a cAMP-dependent mechanism (Fig. 11). Follicle cells and/or the oocyte can secrete and/or catabolize the inhibitor. Passage of the inhibitor through the follicle cell syncytium to the oocyte results in maintenance of meiotic arrest. The in vivo LH surge elevates

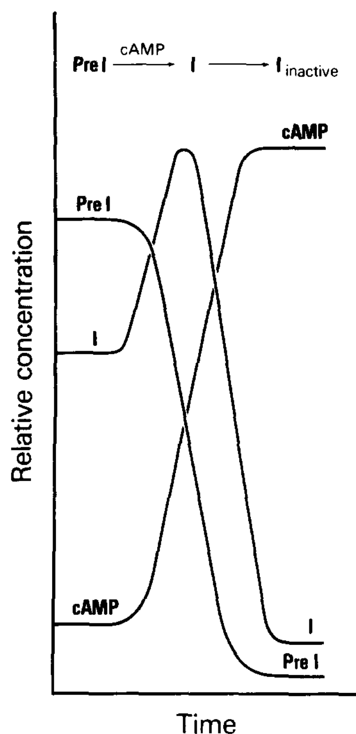

FIGURE 11 Proposed temporal relationships between cAMP, a precursor of the maturation inhibitor, and a maturation inhibitor. See text for discussion. *PreI*, precursor for a maturation inhibitor; *I*, a maturation inhibitor. A decrease in the concentration of *I* below the initial steady-state concentration results in resumption of meiosis. The depicted changes in the relative concentrations are arbitrary and should not be construed as being based on experimentally determined values. According to the results presented here, the time scale is on the order of 2 h. Note that at early times after elevating cAMP, the concentration of *preI* has decreased but that of *I* has increased. At later times subsequent to elevating cAMP, the concentration of both *preI* and *I* has decreased. Thus, using the inhibitor content protocol, less *I* can be generated at these later times.

granulosa/cumulus cell levels of cAMP. This increases production of the inhibitor and results in depleting the pool of inactive precursor. Increased levels of inhibitor are transient since the inhibitor is catabolized/secreted. Inhibitor catabolism/secretion results ultimately in reducing the level of inhibitor in the oocyte below a level necessary for maintenance of meiotic arrest.

This model resolves the apparent paradox previously mentioned. The inhibitor content protocol detects the putative inhibitor by determining differences in the extent of maturation at a given time after an experimental elevation of cumulus cell cAMP with dbcAMP. Increased cAMP would initially generate higher levels of inhibitor in cumulus cell-enclosed oocytes relative to denuded oocytes. Thus, inhibition of maturation of cumulus cell-enclosed oocytes relative to denuded oocytes would be observed and correlated with an initial elevation of cumulus cell cAMP (17). In the experiments described here, initial elevation of cumulus cell cAMP levels with LH, FSH, or dbcAMP would also generate higher levels of inhibitor. By the time the complexes are processed through the inhibitor content protocol, however, the combined effects of catabolism and/or secretion and reduction in pool size of the inactive precursor would result in decreased levels of inhibitor in treated complexes relative to untreated complexes (Fig. 11). Hence, an acceleration of maturation would be observed.

A low molecular weight inhibitor of oocyte maturation present in follicular fluid (oocyte maturation inhibitor) and possibly synthesized by granulosa cells has been reported (5). In the absence of follicular fluid, however, oocytes grafted to granulosa cells do not resume meiosis (23, 33). This clouds the issue regarding the physiological role of oocyte maturation inhibitor.

We are currently examining the effect of microinjecting mouse oocytes with extracts of granulosa cells to determine if a maturation inhibitory factor exists and if so, using this approach as an assay for monitoring the purification of the maturation inhibitor and determining its relationship, if any, to oocyte maturation inhibitor.

The authors would like to thank John Eppig, Nicola Neff, Rolf Freter, and William Telfer for helpful discussion and criticism, Naomi Buklad for assistance with the experiments concerning maturation and cAMP levels in follicles, Gordon Ellis for what is becoming a permanent loan of one of his micromanipulators, Bill Telfer for permitting us to use his fluorescent microscope, and Richard Woodruff for kindly providing instruction in use of his iontophoretic microinjection set-up. Biological grade LH (bovine), biological grade FSH (rat), and highly purified LH (bovine) were provided by the National Pituitary Agency hormone distribution program.

This research was supported by grants from the National Science Foundation (PCM-82-07322) and The Rockefeller Foundation to R. M. Schultz. R. R. Freter is supported by a Medical Scientist training grant from the National Institutes of Health (5-T-32-GM-07170); portions of this work are being submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of Pennsylvania.

Received for publication 26 July 1983, and in revised form 11 October 1983.

REFERENCES

1. Amsterdam, A., R. Josephs, M. E. Lieberman, and H. R. Lindner. 1976. Organization of intramembrane particles in freeze-cleaved gap junctions of rat Graafian follicles: optical-diffraction analysis. *J. Cell Sci.* 21:93–105.
2. Brower, P. T., and R. M. Schultz. 1982. Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. *Dev. Biol.* 90:144–153.
3. Butcher, R. L., W. E. Collings, and N. W. Fugo. 1974. Plasma concentration of LH, FSH, prolactin, progesterone, and estradiol-17β throughout the 4-day estrous cycle of the rat. *Endocrinology.* 94:1704–1708.
4. Channing, C. P., I. Bae, S. L. Stone, L. D. Anderson, and S. C. Fowler. 1981. Porcine granulosa and cumulus cell properties: LH/hCG receptors, ability to secrete progesterone and ability to respond to LH. *Mol. and Cell. Endocrin.* 22:359–370.
5. Channing, C. P., L. D. Anderson, D. J. Hoover, J. Kolena, K. G. Osteen, S. H. Pomerantz, and K. Tanabie. 1982. The role of nonsteroidal regulators in control of oocyte and follicular maturation. *Recent Prog. Horm. Res.* 38:331–408.
6. Cho, W., S. Stern, and J. D. Biggers. 1974. Inhibitory effect of dibutyryl cyclic AMP on mouse oocyte maturation *in vitro. J. Exp. Zool.* 187:183–186.
7. Daane, T. A., and A. F. Parlow. 1971. Periovulatory patterns of rat serum follicle stimulating hormone and luteinizing hormone during the normal estrous cycle: effects of pentobarbitol. *Endocrinology.* 88:653–663.
8. Darfler, F. J., L. C. Mahan, A. M. Koachman, and P. A. Insel. 1982. Stimulation by forskolin of intact S49 lymphoma cells involves the nucleotide regulatory protein of adenylate cyclase. *J. Biol. Chem.* 257:11901–11907.
9. Dekel, N., and W. H. Beers. 1978. Rat oocyte maturation *in vitro*: Relief of cAMP inhibition by gonadotropins. *Proc. Natl. Acad. Sci. USA.* 75:4369–4373.
10. Dekel, N., and W. H. Beers. 1980. Development of the rat oocyte *in vitro*: inhibition and induction of maturation in the presence or absence of the cumulus oophorus. *Dev. Biol.* 75:247–254.
11. Dekel, N., and P. F. Kraicer. 1978. Induction *in vitro* of mucification of rat cumulus oophorus by gonadotropins and adenosine 3',5'-monophosphate. *Endocrinology.* 102:1797–1802.
12. Dekel, N., and I. Sherizly. 1983. Induction of maturation in rat follicle-enclosed oocyte by forskolin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 151:153–155.
13. Dekel, N., T. S. Lawrence, N. B. Gilula, and W. H. Beers. 1981. Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH. *Dev. Biol.* 86:356–362.
14. Edwards, R. G. 1965. Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature (Lond.)* 208:349–351.
15. Eppig, J. J. 1979. Gonadotropin stimulation of expansion of cumuli oophori isolated from mice: general conditions for expansion *in vitro. J. Exp. Zool.* 208:111–120.
16. Eppig, J. J. 1982. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. *Dev. Biol.* 89:268–272.
17. Eppig, J. J., R. R. Freter, P. F. Ward-Bailey, and R. M. Schultz. 1983. Inhibition of oocyte maturation in the mouse: participation of cAMP, steroids, and a putative maturation inhibitory factor. *Dev. Biol.* 100:39–49.
18. Gill, D. M. 1977. Mechanism of action of cholera toxin. *Adv. Cyclic Nucleotide Res.* 8:85–118.
19. Gilula, N. B., M. L. Epstein, and W. H. Beers. 1978. Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. *J. Cell Biol.* 78:58–75.
20. Hillensjo, T. 1977. Dissociation of preovulatory maturational events in rat oocytes and cumuli in the presence of dibutyryl cAMP. *Acta Physiol. Scand.* 100:261–263.
21. LaBarbera, A. R., E. R. Bergert, and R. J. Ryan. 1982. The effects of hormones, cholera toxin and N-α-p-tosyl-L-lysine chloromethyl ketone on adenylate cyclase in viable granulosa cells. *Endocrinology.* 111:1897–1903.
22. Lawrence, T. S., N. Dekel, and W. H. Beers. 1980. Binding of human chorionic gonadotropin by rat cumuli oophori and granulosa cells: a comparative study. *Endocrinology.* 106:1114–1118.
23. Leibfried, L., and N. L. First. 1980. Follicular control of meiosis in the porcine oocyte. *Biol. Reprod.* 23:705–709.
24. Loewenstein, W. R. 1979. Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta.* 560:1–65.

25. Meeuwsen, K., and R. R. Seeley. 1979. The effects of indomethacin on plasma LH levels in female and male deermice (Peromyscus maniculatus). *Prostaglandins.* 18:639–645.
26. Moor, R. M., M. W. Smith, and R. M. C. Dawson. 1980. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. *Exp. Cell Res.* 126:15–29.
27. Moor, R. M., J. C. Osborn, D. G. Cran, and D. E. Walters. 1981. Selective effect of gonadotropins on cell coupling, nuclear maturation and protein synthesis in mammalian oocytes. *J. Embryol. Exp. Morphol.* 61:347–365.
28. Pincus, G., and E. V. Enzmann. 1935. The comparitive behavior of mammalian eggs *in vivo* and *in vitro*. I. The activation of ovarian eggs. *J. Exp. Med.* 62:665–675.
29. Rush, M. A., A. A. Oladopo, H. Lipner, A. T. Williams, C. McRae, and C. A. Blake. 1981. The actions of porcine follicular fluid and estradiol on periovulatory secretion of gonadotrophic hormones in rats. *Endocrinology.* 108:2316–2323.
30. Schultz, R. M., R. R. Montgomery, P. F. Ward-Bailey, and J. J. Eppig. 1983. Regulation of oocyte maturation in the mouse: possible roles of intercellular communication, cAMP, and testosterone. *Dev. Biol.* 95:294–304.
31. Schultz, R. M., R. R. Montgomery, and J. R. Belanoff. 1983. Regulation of mouse oocyte meiotic maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. *Dev. Biol.* 97:264–273.
32. Seamon, K. B., W. Padgett, and J. W. Daly. 1981. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. USA.* 78:3363–3367.
33. Thibault, C. G. 1972. Final stages of mammalian oocyte maturation. *In* Oogenesis. J. D. Biggers, and A. W. Schuetz, editors. University Park Press, Baltimore. 397–411.
34. Tsafriri, A., H. R. Lindner, U. Zor, and S. A. Lamprecht. 1972. *In vitro* induction of meiotic division in follicle-enclosed rat oocytes by LH, cyclic AMP and prostaglandin E_2. *J. Reprod. Fertil.* 31:39–50.
35. Vivarelli, E., M. Conti, M. DeFelici, and G. Siracusa. 1983. Meiotic resumption and intracellular cAMP levels in mouse oocytes treated with compounds which act on cAMP metabolism. *Cell Differ.* 12:271–276.
36. Wassarman, P. M., W. J. Josefowicz, and G. E. Letourneau. 1976. Meiotic maturation of mouse oocytes *in vitro*: inhibition of maturation at specific stages of nuclear progression. *J. Cell Sci.* 22:531–545.