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# Spontaneous Maturation In Vitro of Cumulus-Enclosed Rat Oocytes Is Inhibited by Forskolin

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## ABSTRACT

We have recently reported that the adenylate cyclase activator, forskolin, induces in the rat ovarian follicle both cAMP accumulation and oocyte maturation. We demonstrate here, on the other hand, that the spontaneous maturation in vitro of isolated rat cumulus-enclosed oocytes is inhibited by forskolin. The inhibitory effect of forskolin is dose dependent with an $ED_{50}$ at 15 $\mu$M. Forskolin inhibition decreases gradually with time, being completely relieved by 20 h of culture. Methylisobutylxanthine significantly prolongs the duration of the inhibitory action of forskolin. In addition to its inhibitory effect on oocyte maturation, forskolin triggers the cumulus-oocyte complex to generate cAMP. Cyclic AMP accumulation is maximally stimulated by 100 $\mu$M of forskolin with an $ED_{50}$ at 60 $\mu$M. The potency of the cumulus-oocyte complex to respond to forskolin in terms of cAMP accumulation decreases with time. The pattern of the decrease in the potency of the cumulus-oocyte complexes to generate cAMP corresponds with the relief of its inhibitory influence on the oocyte. These results indicate that inhibition of maturation of the cumulus-enclosed oocyte may be coupled to elevation of cAMP levels in the cumulus-oocyte complex. As isolated cumulus-free oocytes are not inhibited by forskolin, we suggest that in the cumulus-enclosed oocyte system, cAMP generated by the cumulus cells is apparently transferred to the oocyte and maintains it in a meiotically arrested state. Maturation in this system occurs upon relief of inhibition which results from cessation of cAMP generation by the cumulus cells.

## INTRODUCTION

Luteinizing hormone (LH) triggers the rat oocyte to resume meiosis both in vivo (Ayalon et al., 1972) and in vitro, in culture of intact follicles (Tsafriri et al., 1972; Hillensjö, 1976). Membrane-permeable cAMP derivatives block LH-induced maturation of follicle-enclosed oocytes (Hillensjö et al., 1978; Dekel et al., 1981) as well as the spontaneous maturation of isolated oocytes (Cho et al., 1974). The apparent antagonism between the stimulatory action of LH and the inhibitory influence of cAMP on the female gamete seems to be contradictory since a predominant effect of LH is to elevate cAMP levels in the ovary (Lindner et al., 1974).

The hypothesis that cAMP may serve as the physiological inhibitor responsible for meiotic arrest, has been raised in an attempt to resolve this paradox (Lindner et al., 1974; Anderson and Albertini, 1976; Dekel and Beers, 1978; Dekel et al., 1981). According to this hypothesis, maturation results from cessation of cAMP transfer to the oocyte following LH action which terminates cell-to-cell communication in the cumulus-oocyte complex. The question whether uncoupling between the oocyte and the cumulus cells is a prerequisite for meiosis resumption, has been extensively studied (Moor et la., 1980; Dekel et al., 1981; Eppig, 1982; Eppig and Ward-Bailey, 1982). On the other hand, the presence of cAMP transfer from the cumulus to the oocyte, which is as crucial for the validity of this theory, has not been examined until very recently (Schultz et al., 1983). Our present study was designed to challenge the suggested hypothesis from this latter point of view. A possible inhibitory effect of forskolin, if obtained in cumulus-enclosed but not in cumulus-free oocytes, could indicate that cAMP from the cumulus cells is transferred to the oocytes. Our experiments were designed primarily to test this possibility. Following confirmation of this primary condition, we have extended our study to characterization of forskolin action on the oocyte as related to cAMP accumulation by the cumulus-oocyte complex.

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## MATERIALS AND METHODS

Sexually immature Wistar female rats (26 days old) from our departmental colony were injected subcutaneously with 15 IU of pregnant mare's serum gonadotropin (PMSG; Gestyl, Organon, Oss, Netherlands) in 0.1 ml of 0.9% NaCl. The rats were killed by cervical dislocation 48 h after the injection. The ovaries were removed and placed in Leibovitz's L-15 tissue culture medium (GIBCO, Grand Island, NY), supplemented with 10% fetal bovine serum (Sera-Lab, England), penicillin (100 U/ml) and streptomycin (100 μg/ml; GIBCO). This composition is referred to as control medium throughout this paper.

The effect of forskolin was studied in cumulus-enclosed oocytes and compared to that on cumulus-free oocytes. Cumulus-oocyte complexes were isolated from the large antral follicles (Dekel and Beers, 1978) into control medium in the presence or absence of forskolin (7β-acetoxy-8, 13-epoxy-1α,6β,9α-trihydroxy-Labd-14-en-11-one; Calbiochem-Behring Corp., La Jolla, CA), methylisobutylxanthine (MIX; Sigma Chemical Co., St. Louis, MO) or a combination of these agents. To prepare cumulus-free oocytes, the cumulus cells were removed as described earlier (Dekel and Beers, 1980). Following transfer into a fresh drop of medium, either cumulus-oocyte complexes or cumulus-free oocytes were incubated in 35-mm Petri dishes at 37°C in air at a relative humidity of 100%. At the end of the incubation times, both the cumulus-free oocytes and the cumulus-enclosed oocytes were examined by Nomarski Interference Contrast Microscopy. In the presence of the germinal vesicle (GV), oocytes were classified as meiotically arrested. Resumption of meiosis was indicated by the absence of the GV in the individual oocytes. For each study, the data of several individual experiments were combined and the results are reported as the fraction of GV oocytes.

Cyclic AMP determinations were performed by the competitive protein binding assay (Gilman, 1970) as modified by Lamprecht et al. (1973). Since measurable levels of cAMP could be determined in samples of 50 cumulus-oocyte complexes, and since cAMP accumulation was found to be linearly correlated with increasing cell number, 100 cumulus-oocyte complexes were incubated for each determination point. The data represent accumulation in the tissue and the medium separately following 1 h of incubation.

## RESULTS

By 20 h of incubation, over 90% of all the isolated oocytes examined lost their GV whether forskolin (100 μM) was present or absent in the culture medium. However, a fine analysis of the time course of GV breakdown revealed that forskolin delayed the process in cumulus-enclosed oocytes (Fig. 1). When isolated into control medium, 50% of the cumulus-enclosed oocytes lost their GV by 90 min of incubation, while a period of about 5 h was required for the same fraction of oocytes to resume meiotic maturation in the presence of forskolin. In the absence of the cumulus cells, however, forskolin (up to 300 μM) failed to inhibit oocyte maturation

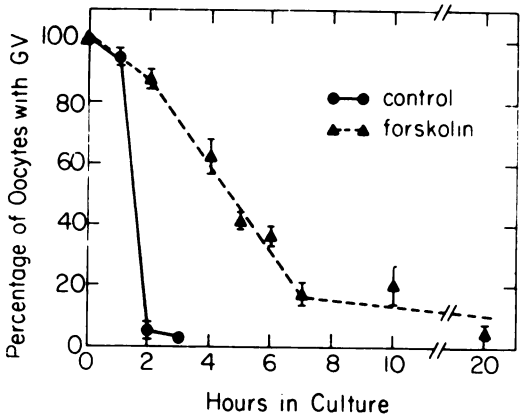


FIG. 1. Effect of forskolin on the time course of GV breakdown in cumulus-enclosed oocytes. Cumulus-oocyte complexes were isolated and incubated in the presence (▲---▲) or absence (●—●) of 100 μM of forskolin. The presence of GV in the oocytes was analyzed at the indicated time points as described in *Materials and Methods*. The mean ± SEM of the results of three individual experiments are presented and a total number of at least 110 oocytes were examined at each time point.

(Fig. 2). In fact, the time course of GV breakdown in cumulus-free oocytes isolated either in the presence or the absence of forskolin was indistinguishable.

As seen in Fig. 1, the maximal inhibitory effect of forskolin was obtained by 2 h after isolation. While all but 5% of the cumulus-enclosed oocytes isolated into forskolin-free medium had lost their GV following 2 h of incubation, 87% of the oocytes were kept meiotically arrested by forskolin at this time point. Concentration-dependence analysis, assessed therefore at this time point, revealed that maximal inhibition was obtained with 100 μM of forskolin and that the $ED_{50}$ value was 15 μM (Fig. 3).

For further characterization of the response of the cumulus-oocyte complex to forskolin, determinations of cAMP accumulation were performed. Like all the other tissues tested previously (Seamon and Daly, 1981), cumulus-oocyte complexes were stimulated by forskolin to generate cAMP. The maximal effective dose of forskolin-induced elevation of cAMP was 100 μM, with an $ED_{50}$ at 60 μM (Fig. 4). The potency of the cumulus-oocyte complexes to accumulate cAMP in response to forskolin decreased with time. After 2 h of incubation, the complexes could generate only half of the levels of cAMP generated by freshly isolated

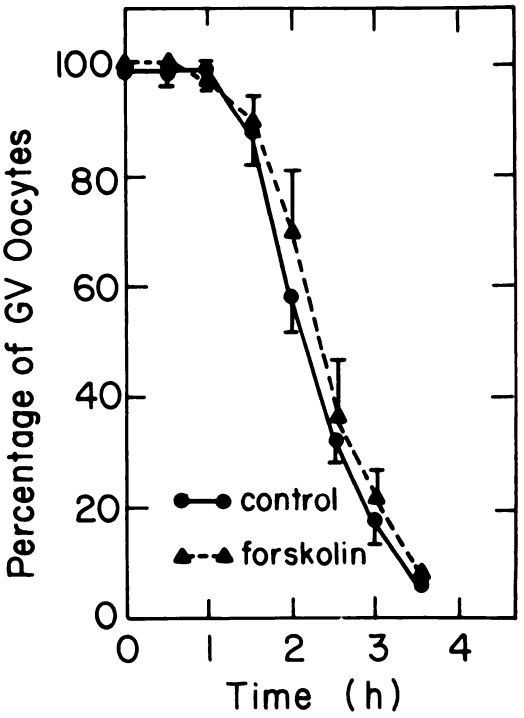


FIG. 2. Effect of forskolin on the time course of GV breakdown in cumulus-free oocytes prepared as described in *Materials and Methods* and incubated in the presence (▲---▲) or the absence (●—●) of 300 μM of forskolin. The presence of GV in the oocytes was analyzed at the indicated time points. The mean ± SEM of the results of three individual experiments are presented and a total number of at least 110 oocytes were examined for each time point.

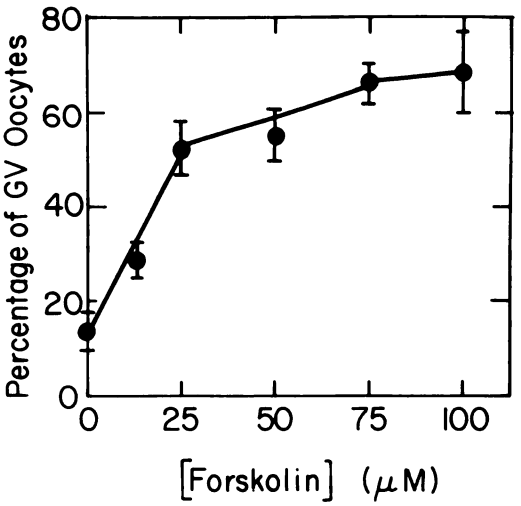


FIG. 3. Dose dependency of the inhibitory effect of forskolin on the spontaneous maturation of cumulus-enclosed oocytes in vitro. Cumulus-enclosed oocytes were isolated in the presence of the indicated concentrations of forskolin. After 2 h of incubation the oocytes were examined for the presence of GV as described in *Materials and Methods*. The mean ± SEM of the results of 4 individual experiments are presented and at least 130 oocytes were examined for each experimental point.

complexes stimulated by the same dose of forskolin (Fig. 5). By 5 h of culture the potency of the complexes to respond to forskolin was completely lost. The decrease in potency of the cumulus cells to generate cAMP corresponded with the relief of forskolin-induced inhibition of cumulus-enclosed oocyte maturation (Fig. 6). The linear relation between these two variables with a correlation coefficient $r$=0.92, suggests that a possible association between these two responses cannot be excluded. The potentiation of forskolin action obtained by the use of the cyclic nucleotide phosphodiesterase inhibitor, MIX (Fig. 7) provided additional evidence for the involvement of cAMP in the forskolin-induced inhibition of the spontaneous meiotic maturation.

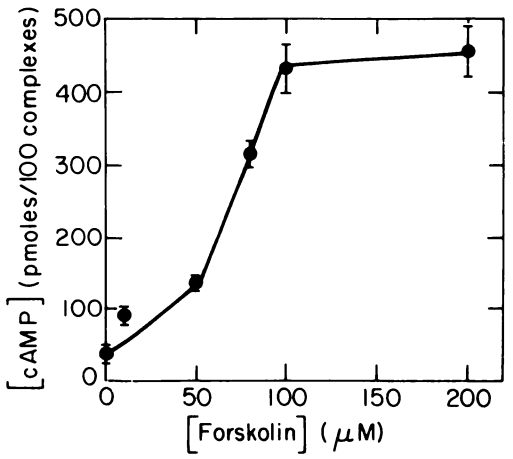


FIG. 4. Concentration dependency of forskolin-induced cAMP accumulation by cumulus-oocyte complexes. Cyclic AMP determinations were performed in samples of 100 cumulus-oocyte complexes following 1 h of incubation in the presence of the indicated concentrations of forskolin. The mean ± SEM of the results of three individual experiments are presented.

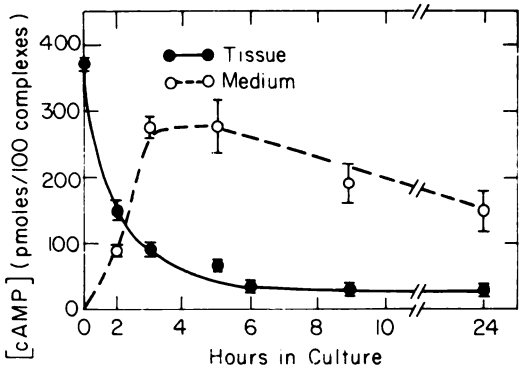


FIG. 5. Decrease in responsiveness of cumulus-oocyte complexes to forskolin during culture. Cumulus-oocyte complexes were incubated in the presence of forskolin (100 μM). At the indicated time points the incubation medium was removed and kept for cAMP determination. The complexes were then re-incubated in fresh forskolin containing medium. Cyclic AMP levels were determined in the cells following 1 h of further incubation. One hundred cumulus-oocyte complexes were used for each determination. The mean ± SEM of the results of three individual experiments are presented.

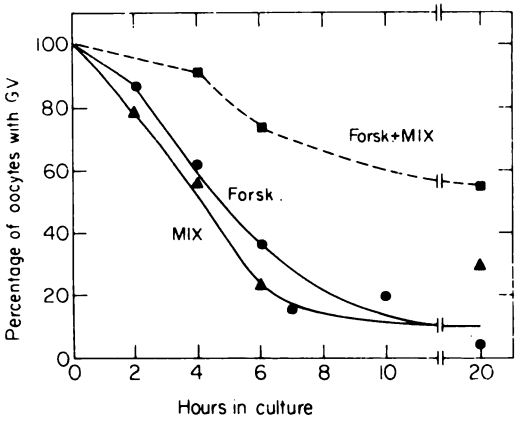


FIG. 7. Potentiation of forskolin-induced inhibition of the spontaneous maturation of cumulus-enclosed oocytes by MIX. Isolated cumulus-enclosed oocytes were incubated in the presence of either forskolin (100 μM), MIX (10 μM) or a combination of these agents. At the indicated time points oocytes were examined for the presence of GV as described in *Materials and Methods*. The combined results of 3 individual experiments are presented and over 100 oocytes were examined for each time point.

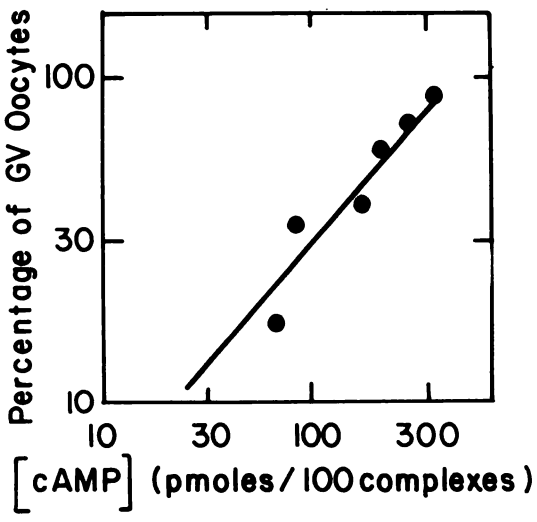


FIG. 6. Correlation between forskolin-induced inhibition of cumulus-enclosed oocyte maturation and cAMP production by the cumulus-oocyte complexes. Inhibition of cumulus-enclosed oocyte maturation was analyzed after 0, 1, 2, 3, 4 and 5 h of incubation in the presence of forskolin (100 μM). The potency of the cumulus-oocyte complex to generate cAMP was determined at these same time points. At least three determinations were performed for each variable at each time point. The linear correlation coefficient $r$=0.92.

## DISCUSSION

We have demonstrated in the present study that the spontaneous maturation in vitro of the rat oocyte can be inhibited by forskolin. Forskolin-induced inhibition of rat oocyte maturation can be demonstrated, however, only in the presence of the cumulus cells. These findings suggest that the inhibitory mediator generated in response to forskolin originates in the cumulus cells. To elicit its inhibitory action, this factor should apparently be communicated to the oocyte. Established cell-to-cell communication in the cumulus-oocyte complex has been demonstrated by both ionic coupling and transmission of either small molecules or fluorescent dyes (Gilula et al., 1978; Moor et al., 1980; Dekel et al., 1981; Brower and Schultz, 1982; Eppig, 1982). The gap junctions which are present in the regions of contact of the oocyte with the cumulus cells serve apparently as the communicative channels between these two cell types (Anderson and Albertini, 1976; Amsterdam et al., 1976; Gilula et al., 1978).

Forskolin is known as a potent activator of cAMP-generating systems (Seamon and Daly, 1981). In the present study we have shown that cAMP accumulation in response to forskolin can also be demonstrated by the cumulus cells.

As administration of cAMP to the oocyte has already been shown to inhibit the spontaneous maturation in vitro (Cho et al., 1974; Dekel et al., 1978) it seems likely that the forskolin-induced inhibitory mediator generated by the cumulus cells and transferred to the oocyte is cAMP. Being a small molecule, cAMP can easily pass through gap junctions. The fact that the inhibitory action of forskolin decreased with time (Fig. 1) in parallel to the reduction in potency of the cumulus to make cAMP (Fig. 5), suggests that high levels of cAMP do correlate with the presence of inhibition of oocyte maturation. Moreover, the prolongation of the inhibitory action of forskolin obtained when the phosphodiesterase inhibitor MIX was included in the culture medium provides additional support for this idea.

Similar results to those demonstrated in the present study were obtained earlier by the use of cholera toxin (CT) (Dekel and Beers, 1980). This other activator of the cyclase system effectively maintained meiotic arrest in cumulus-enclosed oocytes failing to inhibit maturation in cumulus-free oocytes. Using forskolin in our present study, we confirmed the CT data. Moreover, forskolin is a preferable tool to test our hypothesis, being a more potent activator of adenylate cyclase. As opposed to CT stimulation, which is characterized by a lag period of at least 60 min, accumulation of cAMP in response to forskolin is immediate. The rapid action of forskolin is of great importance when inhibition of the spontaneous maturation is studied, since it has been demonstrated that at 45 min after isolation into inhibitor-free medium, the oocytes are irreversibly committed to undergo maturation (Dekel and Beers, 1980). Thus, the failure of CT to maintain meiotic arrest in cumulus-free oocytes could possibly be due to its inability to cause generation of sufficient cAMP to inhibit maturation during the first hour after isolation from the follicle. In fact, even in cumulus-enclosed oocytes the inclusion of MIX (in low concentrations) was necessary to observe the CT-dependent arrest.

Transmission of cAMP between communicating cells to regulate hormonal stimulation has already been suggested by Lawrence et al. (1978). These investigators demonstrated that exposure of coupled cocultures to a hormone specific for one cell type causes the heterologous cells to respond. They suggest that this cross-stimulation results from the intercellular communication of a mediator that is common to both cell types. The most likely candidate of such a communicated mediator according to these investigators is cAMP.

Even though indirect, ours and the other studies mentioned seem to provide some strong indications that cAMP could be communicated in the cumulus-oocyte complex. However, very recently Schultz et al. (1983) failed to demonstrate transfer of cAMP from the cumulus cells to the oocyte. These investigators did get cumulus cell-dependent inhibition of oocyte maturation by cAMP modulators. They also demonstrated that the inhibitory effect correlates with an increase in cAMP levels in the cumulus-oocyte complex. However, no differences in cAMP levels were found in oocytes derived from either stimulated or unstimulated complexes. These investigators suggest, therefore, that the inhibitory effect is directly mediated by an agent other than cAMP, although cAMP generation is required for its action. The fact that the cumulus cells mediate the inhibition is clear in both our and Schultz's study. The presence of a cAMP-dependent inhibitory mediator which is not cAMP itself can also fit in the case of forskolin-induced oocyte maturation inhibition shown in our present study. However, before the possibility that cAMP is the inhibitory signal is ruled out, it should be mentioned that in the amphibia it is no more than 10% difference in the oocyte content of cAMP which is responsible for alternation between arrest and resumption of meiosis (Schorderet-Saltkine and Baulieu, 1982). As it is possible that the major fraction of cAMP produced by the cumulus cells is protein-bound, only a small number of free molecules of the nucleotide can freely pass the gap junctions. As in the amphibian oocyte, these low amounts of cAMP available for transfer can maintain meiotic arrest although being undetectable by the available techniques for cAMP determination.

As administration of high cAMP levels to the oocyte has been shown to inhibit maturation (Cho et al., 1974), the inability of both cyclase activators to affect the naked oocyte may indicate that the rat oolemma lacks the adenylate cyclase system. The definitive answer to this intriguing possibility can be provided only by direct biochemical analyses. These studies, however, indicate that in response to both CT and forskolin the rat oocyte cannot generate sufficient levels of cAMP required for maintenance of meiotic arrest.

The inhibitory influence of forskolin described in our present study seems to contradict our earlier report in which a stimulatory action of forskolin on oocyte maturation has been demonstrated (Dekel and Sherizly, 1983). The apparent conflicting results can be explained assuming that cAMP plays a dual role in regulation of oocyte maturation. When communicated from the follicular cells, it maintains meiotic arrest. However, elevated levels of the nucleotide terminate communication, stop the transfer of the nucleotide to the oocyte and allow meiosis resumption. This hypothesis has originally been suggested by Lindner et al. (1974) in an attempt to overcome the apparent paradox between the antagonistic effects of cAMP and LH on the oocyte, and the fact that LH is known to act via cAMP as a mediator. Later studies demonstrating that elevated cAMP does terminate communication in the cumulus-oocyte complex and that this event is associated with oocyte maturation (Dekel et al., 1981) provided supportive evidence to this theory. Based on this model and considering that the levels of cAMP generated by the isolated cumulus are obviously lower than that produced by the entire follicle, the following explanation to the contradiction is suggested. The isolated cumulus-oocyte complex generates cAMP in levels (3 pmol per cumulus as shown here) which are presumably not high enough to interfere with communication. It is the inhibitory action of cAMP transferred to the oocyte which is manifested, therefore, under these conditions. On the other hand, when the entire follicle is studied (Dekel and Sherizly, 1983), stimulation of oocyte maturation rather than inhibition is obtained, since cAMP levels produced by the whole follicle (300 pmol/follicle) are in the range that does interrupt communication, terminates the nucleotide transfer and relieves the oocyte from the inhibitory influence. Communication in the cumulus-oocyte complex following exposure of the follicle to forskolin is being subjected to further investigation in our laboratory.

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## REFERENCES

Amsterdam, A., Josephs, R., Lieberman, M. E. and Lindner, H. R. (1976). Organization of intermembrane particles in freeze-cleaved gap junctions of rat graafian follicles: optical diffraction analysis. J. Cell. Sci. 21:93–105.

Anderson, E. and Albertini, D. F. (1976). Gap junctions between the oocyte and the companion follicle cells in the mammalain ovary. J. Cell Biol. 71:680–686.

Ayalon, D., Tsafriri, A., Lindner, H. R., Cordova, T. and Harell, A. (1972). Serum gonadotrophin levels in pro-estrous rats in relation to the resumption of meiosis by the oocytes. J. Reprod. Fertil. 31:51–58.

Brower, P. T. and Schultz, R. M. (1982). Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. Dev. Biol. 90:144–153.

Cho, W. K., Stern, S. and Biggers, J.D. (1974). Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. Exp. Zool. 187:383–386.

Dekel, N. and Beers, W. H. (1978). Rat oocyte maturation in vitro: relief of cyclic AMP inhibition by gonadotropins. Proc. Natl. Acad. Sci. USA 75:4369–4373.

Dekel, N. and Beers, W. H. (1980). Development of the rat oocyte in vitro: inhibition and induction of maturation in presence or absence of the cumulus oophorus. Dev. Biol. 75:247–254.

Dekel, N. and Sherizly, I. (1983). Induction of maturation in rat follicle-enclosed oocyte by forskolin. FEBS (Fed. Eur. Biochem. Soc.) Lett. 151: 153–155.

Dekel, N., Lawrence, T. S., Guilula, N. B. and Beers, W. H. (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.

Eppig, J. J. (1982). The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation and cumulus expansion. Dev. Biol. 89:268–272.

Eppig, J. J. and Ward-Bailey, P. F. (1982). The mechanism of cumulus cell-oocyte uncoupling: evidence for the participation of both cumulus cells and oocytes. Gamete Res. 6:145–154.

Gilman, A. G. (1970). A protein binding assay for adenosine 3′,5′-cyclic monophosphate. Proc. Natl. Acad. Sci. USA 67: 305–312.

Gilula, N. B., Epstein, M. C. and Beers, W. H. (1978). Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. J. Cell Biol. 78:58–75.

Hillensjo, T. (1976). Oocyte maturation and glycolysis in isolated preovulatory follicles of PMS-injected immature rats. Acta Endocrinol. 82:809–830.

Lamprecht, S. A., Zor, U., Tsafriri, A. and Lindner, H. R. (1973). Action of prostaglandin $E_2$ and of luteinizing hormone on ovarian adenylate cyclase, protein kinase and ornithine decarboxylase activity during postnatal development and maturity in the rat. J. Endocrinol. 57:217–233.

Lawrence, T. S., Beers, W. H. and Gilula, N. B. (1978). Transmission of hormonal stimulation by cell-to-cell communication. Nature 272:501–506.

Lindner, H. R., Tsafriri, A., Lieberman, M. E., Zor, U.,

Koch, Y., Bauminger, S. and Barnea, A. (1974). Gonadotropin action on cultured Graafian follicles: induction of maturation division of the mammalian oocyte and differentation of the luteal cell. Recent Prog. Horm. Res. 30:79–138.

Hillensjo, T., Ekholm, C. and Ahren, K. (1978). Role of cAMP in oocyte maturation and glycolysis in the preovulatory rat follicle. Acta Endocrinol. 87:377–388.

Moor, R. M., Smith, M. W. and Dawson, R.M.C. (1980). Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. Exp. Cell Res. 126:15–29.

Schorderet-Slatkine, S. and Baulieu, E. (1982). Forskolin increases cAMP and inhibits progesterone induced meiosis reinitiation in xenopus laevis oocytes. Endocrinology 111:1385–1387.

Schultz, R. M., Montgomery, R. R., Ward-Bailey, P. F. and Eppig, J. J. (1983). Regulation of oocyte maturation in the mouse: possible roles of intercellular communication, cAMP and testosterone. Dev. Biol. 95:294–304.

Seamon, K. and Daly, W. J. (1981). Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. 7:201–224.

Tsafriri, A., Lindner, H. R. and Zor, U. (1972) In-vitro induction of meiotic division in follicle-enclosed rat oocytes by LH, c-AMP and $PGE_2$. J. Reprod. Fertil. 31:39–50.