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Dissociation between the inhibitory and the stimulatory action of cAMP on maturation of rat oocytes

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Key words: Cyclic AMP; Oocyte maturation

Summary

The possible mediatory role of cAMP in the induction of oocyte maturation by luteinizing hormone (LH) is not yet clear since evidence for both inhibitory and stimulatory actions of the nucleotide on the oocyte has been provided. To elucidate the role of cAMP in regulation of oocyte meiosis we tried in the present study to dissociate between the inhibitory and stimulatory action of this nucleotide on oocyte maturation. To induce maturation, oocytes enclosed by their follicles were transiently exposed to either dibutyryl cAMP (dbcAMP) or to the phosphodiesterase inhibitor methylisobutylxanthine (MIX). Inhibition of maturation was obtained by the addition of the above agents to either follicle-enclosed oocytes incubated in the presence of LH or isolated cumulus-free oocytes that mature spontaneously in vitro. We found that inhibition of oocyte maturation is obtained by a relatively low dose of either dbcAMP or MIX while higher concentrations of these agents are required to induce oocyte maturation. Coupling of the oocyte to the cumulus cells, as expressed by the fraction of labeled uridine transferred from the cumulus cells to the oocyte following exposure of the follicle-enclosed cumulus–oocyte complex to MIX, was also determined. We found that uncoupling of the oocyte from the cumulus cells corresponded with the induction, but not inhibition of oocyte maturation, both by its concentration dependence and time-course. We suggest that cAMP has a dual role in regulation of oocyte maturation. Lower levels of the nucleotide act to maintain meiotic arrest, while elevated levels of cAMP mediate LH action to induce meiosis resumption.

Introduction

Both oocyte maturation and luteinization are included among the responses of the rat ovary to LH (Baker, 1972). While it is clearly evident that luteinization is a cAMP-mediated response (Channing and Seymour, 1970), the role of cAMP in the induction of meiosis resumption has not been fully elucidated. Moreover, the available data related to the possible involvement of cAMP in regulation of oocyte maturation are contradictory, since they provide evidence for both inhibitory and stimulatory actions of the nucleotide. Tsafriri et al. (1972) have demonstrated that injection of a cAMP derivative into isolated rat follicles could mimic the effect of LH and induce oocyte maturation. In a later study, however, the presence of the

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cyclic nucleotide phosphodiesterase inhibitor, theophylline, was found to antagonize LH action on follicle-enclosed oocytes (Lindner et al., 1974). Moreover, other studies demonstrate that either membrane-permeable derivatives of cAMP or cyclic nucleotide phosphodiesterase inhibitors completely block the spontaneous maturation in vitro of isolated oocytes (Cho et al., 1974; Dekel and Beers, 1978, 1980) as well as LH-induced maturation of follicle-enclosed oocytes (Hillensjo et al., 1978; Dekel et al., 1981). These later reports demonstrate, however, that it is the continuous presence of cAMP modulators that blocks LH-induced oocyte maturation, while a transient exposure to elevated levels of the nucleotide will, by itself, induce meiosis resumption. On the other hand, we have recently found that the continuous presence of forskolin, which is a potent activator of adenylate cyclase (Seamon and Daly, 1981), does not interfere with its action as an inducer of oocyte maturation (Dekel and Sherizly, 1983). Is cAMP an inducer or an inhibitor of oocyte maturation? Considering that in vivo, following the preovulatory LH surge, both oocyte maturation and cAMP elevation are concomitantly stimulated, this question becomes even more relevant.

In an attempt to resolve this puzzle we have tried, in our present study, to dissociate the inhibitory from the stimulatory effect of cAMP on rat oocyte maturation. Specifically, experiments were designed to determine whether maturation is induced by cAMP levels which are different from that required to block this process. In addition, the timing and dose-dependency of cAMP-induced uncoupling of the oocyte from the cumulus cells were analyzed and their correspondence to either induction or inhibition of oocyte maturation tested.

Materials and methods

Sexually immature Wistar female rats (25 days old) from our departmental colony were injected subcutaneously with 15 IU of pregnant mare's serum gonadotropin (PMSG, Gestyl, Organon, Oss, The 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, U.K.), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Gibco). This composition is referred to as control medium throughout this paper.

The ovarian follicles were dissected under a stereoscopic microscope, and placed in control medium with or without either dibutyryl cAMP (dbcAMP, Sigma Chemical Co., St. Louis, MO), methylisobutylxanthine (MIX, Sigma Chemical Co.), ovine LH (oLH, NIH LH S-20) or their combination, in 25 ml flasks, gassed with 50% O_2 and 50% N_2. Incubations were carried out at 37°C in an oscillating water bath. At the end of the incubation times the follicles were incised and the oocytes were removed for microscopic examination. Cumulus-free oocytes were prepared as described earlier (Dekel and Beers, 1980) and incubated in control medium with or without MIX in 35 mm Petri dishes at 37°C in air at a relative humidity of 100%.

Oocytes were analyzed for maturation by Nomarski Interference Contrast microscopy (Dekel et al., 1981). Resumption of meiosis was indicated by the absence of the germinal vesicle (GV) in the individual oocytes. For each study, the data of several individual experiments were combined and the results are reported as the means ± SE of the fraction of oocytes with GV breakdown (GVB).

Maturation of the follicle-enclosed oocytes was induced by a transient exposure to either dbcAMP or MIX as described earlier (Dekel et al., 1981). Briefly, follicles exposed for the indicated times to various dbcAMP or MIX concentrations were rinsed and further incubated for 5 h in control medium. Resumption of oocyte maturation was inhibited by the addition of the indicated concentrations of MIX or dbcAMP to cultures of either follicle-enclosed oocytes incubated in the presence of 5 μg/ml of oLH, or cumulus-free oocytes incubated in control medium.

Coupling in cumulus–oocyte complexes was determined following their exposure, enclosed by their follicles, to various dbcAMP or MIX concentrations for the indicated times. Analysis of the level of coupling in the cumulus–oocyte complex is based on the assumption that the amount of uridine transferred to the cumulus-enclosed

oocytes is a function of the extent of communication between the oocyte and the cumulus cells since in the absence of cumulus cells, oocytes incorporate a negligible amount of uridine (Moor et al., 1980; Eppig, 1982; Salustri and Siracusa, 1983). The follicles were incised and the recovered cumulus–oocyte complexes were incubated for 1 h in 10 μCi/ml of [5.6-^{3}H]uridine (KMG, Israel, spec. act. 40 Ci/mmol) followed by an extensive rinse of the labeled marker. Part of the cumulus–oocyte complexes were transferred directly to scintillation vials (ten complexes/vial) dissolved in 1 N NaOH, acidified by 1 N HCl and counted to obtain a value for total uptake of radioactivity by the cumulus–oocyte complexes. Another group of complexes was mechanically treated to remove the cumulus cells (Dekel and Beers, 1980). The denuded, cumulus-free, oocytes were then transferred to scintillation vials (ten oocytes/vial), dissolved as described above and counted to obtain a value for the amount of radioactive marker transferred to the oocytes. The coupling factor is expressed as the amount of uridine transferred to cumulus-enclosed oocytes as a fraction of total cumulus uptake and calculated by the following equation:

$$\frac{\text{cpm in denuded oocytes} \times 100}{\text{cpm in cumulus–oocyte complex} - \text{cpm in denuded oocyte}}$$

cAMP determinations were performed on cumulus–oocyte complexes recovered from the follicles following incubation with different MIX concentrations. The tissue was tested by the competitive protein binding assay (Gilman, 1970) as modified by Zor et al. (1973). Our assay detects between 5 and 50 pmol of cAMP. The data represents accumulation as determined in samples of 25 complexes each.

Results

Follicle-enclosed oocytes incubated in control medium remain meiotically arrested for at least 24 h. Continuous presence of MIX in the culture medium is uneffective while a transient exposure to this phosphodiesterase inhibitor followed by incubation in control medium induces oocyte maturation (Dekel et al., 1981). To accurately assess the concentration dependence of the induction of maturation in follicle-enclosed oocytes it was first necessary to determine the time-course of the action of MIX in this system. We found that induction of maturation was initially detected in follicle-enclosed oocytes expressed to MIX for 4 h and examined after an additional 5 h of incubation in control medium (Fig. 1). After a transient exposure of 8 h 50% of the oocytes underwent maturation with a full response demonstrated in oocytes transiently exposed to MIX for 20 h (Fig. 1).

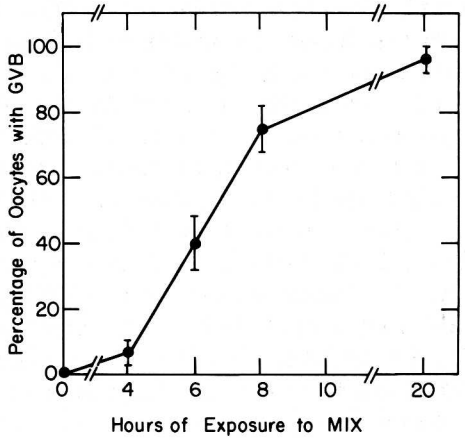

Fig. 1. Time-course of induction of maturation of follicle-enclosed oocyte maturation by MIX. Follicles were incubated in the presence of 0.2 mM of MIX. After the indicated times the follicles were rinsed and further incubated for 5 h in MIX-free medium. The oocytes were recovered and examined for maturation as described in Materials and Methods. The means ±SE of three individual observations for each experimental point are presented. Over 100 oocytes were examined for each experimental point.

The concentration dependence of the induction of maturation by MIX was therefore assessed in follicle-enclosed oocytes exposed to the phosphodiesterase inhibitor for 20 h and further incubated in control medium for 5 h. We found that MIX at a concentration of 0.2 mM was fully effective in the induction of maturation of follicle-enclosed oocytes. The ED_{50} for MIX stimulatory action was 0.13 mM (Fig. 2).

Follicle-enclosed oocytes are induced to mature in vitro by LH (Tsafriri et al., 1972). In the presence of phosphodiesterase inhibitors LH-induced oocyte maturation in this system is inhibited (Lindner et al., 1974; Dekel et al., 1981). As seen in Fig. 3, maximal inhibition of LH-in-

oocytes is a function of the extent of communication between the oocyte and the cumulus cells since in the absence of cumulus cells, oocytes incorporate a negligible amount of uridine (Moor et al., 1980; Eppig, 1982; Salustri and Siracusa, 1983). The follicles were incised and the recovered cumulus–oocyte complexes were incubated for 1 h in 10 μCi/ml of [5.6-^{3}H]uridine (KMG, Israel, spec. act. 40 Ci/mmol) followed by an extensive rinse of the labeled marker. Part of the cumulus–oocyte complexes were transferred directly to scintillation vials (ten complexes/vial) dissolved in 1 N NaOH, acidified by 1 N HCl and counted to obtain a value for total uptake of radioactivity by the cumulus–oocyte complexes. Another group of complexes was mechanically treated to remove the cumulus cells (Dekel and Beers, 1980). The denuded, cumulus-free, oocytes were then transferred to scintillation vials (ten oocytes/vial), dissolved as described above and counted to obtain a value for the amount of radioactive marker transferred to the oocytes. The coupling factor is expressed as the amount of uridine transferred to cumulus-enclosed oocytes as a fraction of total cumulus uptake and calculated by the following equation:

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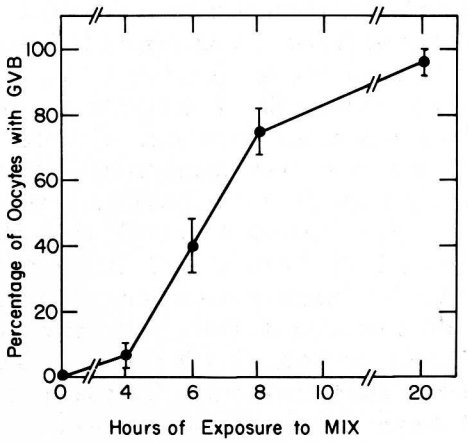

Fig. 1. Time-course of induction of maturation of follicle-enclosed oocyte maturation by MIX. Follicles were incubated in the presence of 0.2 mM of MIX. After the indicated times the follicles were rinsed and further incubated for 5 h in MIX-free medium. The oocytes were recovered and examined for maturation as described in Materials and Methods. The means ±SE of three individual observations for each experimental point are presented. Over 100 oocytes were examined for each experimental point.

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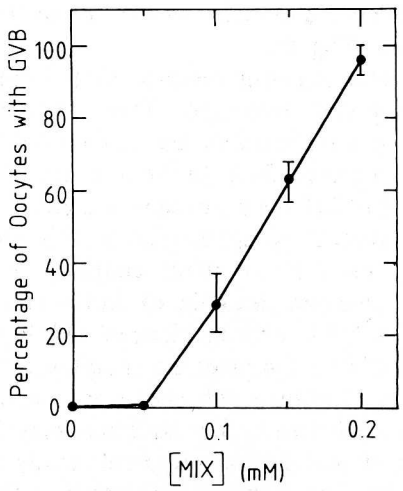

Fig. 2. Dose-response of the induction of maturation of follicle-enclosed oocyte maturation by MIX. Follicles were incubated in the presence of the indicated concentrations of MIX. After 20 h the follicles were rinsed and further incubated for 5 h in MIX-free medium. The oocytes were recovered and examined for maturation as described in Materials and Methods. The means ± SE of three individual experiments are presented. Over 100 oocytes were examined for each experimental point.

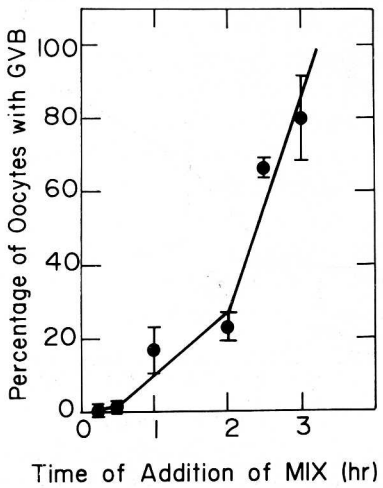

Fig. 3. Timing of inhibition of LH-induced maturation in follicle-enclosed oocytes by MIX. Follicles were incubated in the presence of 5 μg/ml of oLH. At the indicated times 0.2 mM of MIX were added to the culture medium. After 20 h of further incubation oocytes were recovered and examined for maturation as described in Materials and Methods. The means ± SE of three individual experiments are presented. Over 100 oocytes were examined for each experimental point.

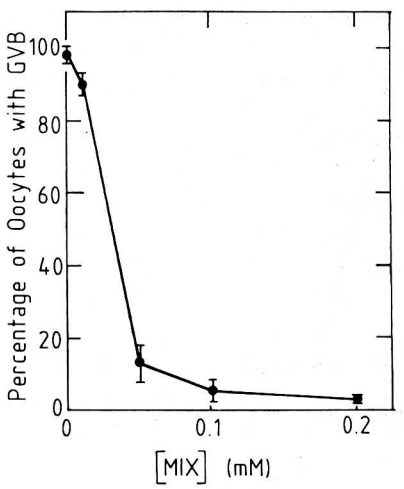

Fig. 4. Dose-response of the inhibitory action of MIX on LH-induced maturation in follicle-enclosed oocytes. Follicles were incubated in the presence of 5 μg/ml of oLH with or without the indicated concentrations of MIX. After 20 h of incubation oocytes were recovered and examined for maturation as described in Materials and Methods. The means ± SE of four individual experiments are presented. Over 100 oocytes were examined for each experimental point.

duced oocyte maturation could be obtained only when the phosphodiesterase inhibitor and the hormone were simultaneously added to the culture medium. The dose of MIX required to block LH action, under these conditions, is shown in Fig. 4. To fully inhibit LH-induced maturation in follicle-enclosed oocytes, 0.1 mM of MIX were required. The ED_{50} for the inhibitory action of MIX was 0.03 mM. A similar dose-response curve is obtained for the inhibitory action of MIX on the spontaneous maturation of isolated cumulus-free oocytes (Fig. 5). The maximal effective dose of MIX in this system is 0.1 mM and the ED_{50} is 0.025 mM.

In an earlier study we demonstrated that cAMP interrupts communication in the cumulus–oocyte complex (Dekel et al., 1981). In the present study the timing and dose of MIX that uncouple the oocyte from the cumulus cells were analyzed. We found that follicle-enclosed oocytes remained fully coupled to their cumulus cells following 20 h of incubation in control medium (Fig. 6). The dose of 0.05 mM of MIX failed to affect the coupling in the cumulus–oocyte complex. However, in the presence of 0.2 mM of MIX coupling of the

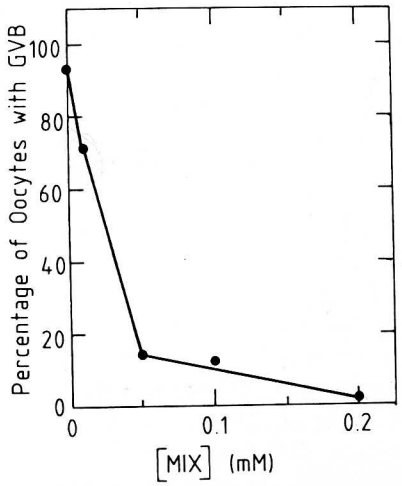

Fig. 5. Dose-response of the inhibitory action of MIX on the spontaneous maturation of isolated cumulus-free oocytes. Isolated cumulus-free oocytes were incubated in the presence of the indicated concentrations of MIX. Oocytes were examined for maturation after 4 h of incubation. The means ± SE of three individual experiments are presented. Over 100 oocytes were examined for each experimental point.

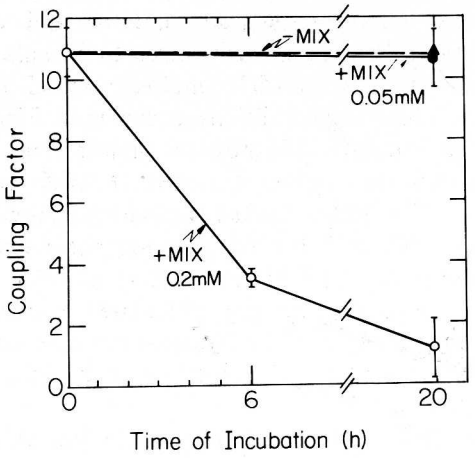

Fig. 6. Uncoupling of the cumulus–oocyte complex by MIX. Follicles were incubated in the absence or the presence of the indicated MIX concentrations. At the indicated times cumulus–oocyte complexes were recovered and transferred to [^{3}H]uridine containing control medium for further incubation of 1 h. The percentage of coupling was determined as described in Materials and Methods. Four groups of ten, either cumulus-enclosed or cumulus-free, oocytes each were used for each experiment. The means ± SE of three individual experiments are presented.

oocyte to the cumulus cells was reduced to 10% of its initial level (Fig. 6).

cAMP determinations revealed that cumulus–oocyte complexes recovered from follicles incubated in control medium accumulate 4.8 ± 1.6 pmol/25 complexes/24 h. In the presence of 0.05 mM of MIX cAMP concentrations are elevated to 18.3 ± 5.5 pmol/25 complexes/24 h. This level is apparently too low to affect coupling in the cumulus–oocyte complex (Fig. 6). In the presence of 0.2 mM of MIX concentrations of cAMP reach the level of 28.04 ± 2.4 pmol/25 complexes/24 h. Under these conditions the oocyte is uncoupled from the cumulus cells. As xanthines may have some other effects that do not result solely from inhibition of phosphodiesterase activities (Wells and Kramer, 1981), we have also tested the effect of increasing concentrations of dbcAMP on both induction and inhibition of oocyte maturation. Induction of oocyte maturation by a transient exposure to dbcAMP was dose dependent with an ED_{50} at 25 mM (Fig. 7). The ED_{50} for the inhibitory action of dbcAMP on LH-induced maturation was 5.0 mM (Fig. 8). Similar to MIX, the

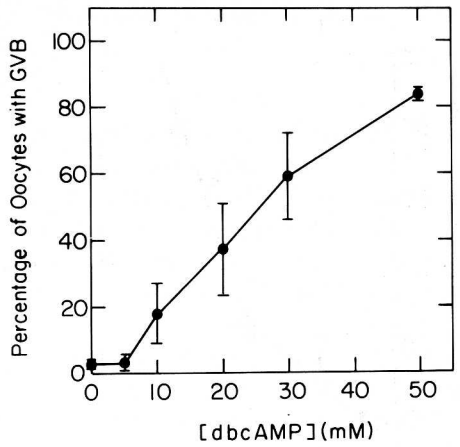

Fig. 7. Dose-response of the induction of maturation of follicle-enclosed oocyte maturation by dbcAMP. Follicles were incubated in the presence of the indicated concentrations of dbcAMP. After 20 h the follicles were rinsed (×4) and further incubated for 5 h in dbcAMP-free medium. The oocytes were recovered and examined for maturation as described in Materials and Methods. The means ± SE of three individual experiments are presented. Over 100 oocytes were examined for each experimental point.

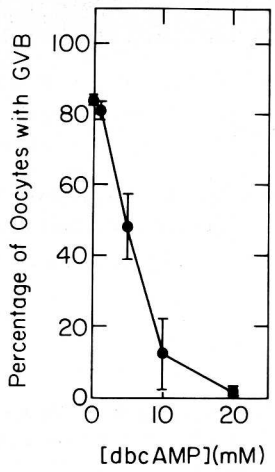

Fig. 8. Dose-response of the inhibitory action of dbcAMP on LH-induced maturation in follicle-enclosed oocytes. Follicles were incubated in the presence of 5 μg/ml of oLH with or without the indicated concentrations of dbcAMP. After 20 h of incubation oocytes were recovered and examined for maturation as described in Materials and Methods. The means ± SE of four individual experiments are presented. Over 100 oocytes were examined for each experimental point.

half-maximal concentration of dbcAMP that inhibits oocyte maturation is 5-fold lower than that required to stimulate this process in Fig. 8.

Discussion

It is very well established that LH elicits ovarian functions by elevation of cAMP concentrations. The possible role of cAMP as a mediator of LH in the induction of oocyte maturation was studied by us earlier (Dekel and Sherizly, 1983). In that study we demonstrated that induction of oocyte maturation by forskolin was associated with elevation of cAMP in the follicle. We suggested, therefore, that LH-induced oocyte maturation is a cAMP-mediated response. This conclusion seems to present an apparent paradox. If cAMP inhibits meiosis resumption (Dekel and Beers, 1978, 1980), what mechanism allows the oocyte to mature under conditions of LH-stimulated elevated cAMP? As both inhibition and induction of oocyte maturation could be elicited by cAMP we assumed that each of these reponses should be stimulated by a different dose of the nucleotide. To test this assumption in the present study we compared the dose dependency of cAMP-mediated inhibition and induction of oocyte maturation.

Our present results demonstrate that the concentrations of either dbcAMP or MIX, which are needed for induction of meiosis resumption, are substantially higher than those required for inhibition of oocyte maturation. cAMP determinations in follicle-enclosed cumulus–oocytes exposed to the different doses of MIX confirmed this conclusion. We therefore suggest that cAMP plays a dual role in regulation of oocyte maturation. Basal, sustained levels of cAMP result in maintenance of meiotic arrest, while transiently elevated, LH-stimulated levels of the nucleotide mediate the induction of oocyte maturation.

It is not only the changes in concentrations of cAMP but also the different target cells for the nucleotide action that generate the opposite responses. The follicle represents a heterologous system composed of two entirely different cell populations, the somatic follicular cells and the oocyte. The possibility that responses to hormones and other agents are limited to one cell type and absent in the other, should be taken into consideration. Indeed, dissociation of the follicle into its cellular components reveals that both dbcAMP and MIX differ from forskolin in their action on the isolated cumulus-free oocyte. While both dbcAMP and MIX block maturation of isolated cumulus-free oocytes (Dekel and Beers, 1980), forskolin failed to elicit any inhibitory response (Dekel et al., 1984). The failure of forskolin to inhibit maturation of isolated denuded oocytes suggests that, in terms of cAMP accumulation, the oocyte is probably not a target cell for forskolin action. Forskolin-induced cAMP elevation, in the follicle (Dekel and Sherizly, 1983), reflects therefore the response of the somatic cells. Thus, induction of follicle-enclosed oocyte maturation by forskolin is probably mediated through the somatic cells of the follicle. The absence of LH receptors on the oocyte (Lawrence et al., 1980) and the lack of response of the isolated cumulus-free oocyte to this gonadotropin (Dekel and Beers, 1980) suggest that LH action, like forskolin action to induce oocyte maturation, is also mediated through the somatic follicular cells. As both dbcAMP and MIX elicit a direct inhibitory effect on the isolated oocyte (Dekel and Beers, 1980) it seems quite

obvious that in the presence of these cAMP modulators maturation does not occur. The positive trigger that these agents provide by elevating cAMP levels in the follicular cells, could therefore be demonstrated only upon their removal. Thus, induction of oocyte maturation could be obtained only upon a transient exposure to these agents (Dekel et al., 1981).

Collectively, our earlier findings and present results suggest that when the oocyte is the target for cAMP, lower levels of the nucleotide are sufficient to induce inhibitory action. At higher cAMP concentration it is the response of the somatic cells to the nucleotide which leads to oocyte maturation.

What is the mechanism by which the follicular cells mediate cAMP action to induce oocyte maturation? Our earlier studies indicate that maintenance of meiotic arrest depends on the integrity of the cumulus–oocyte complex which probably permits continuous transfer of the inhibitory cAMP to the oocyte (Dekel et al., 1984). It is therefore possible that uncoupling of the oocyte from the cumulus cells will decrease the flow of the nucleotide under the threshold level required to maintain meiotic arrest and lead to meiosis resumption. We have earlier demonstrated that cAMP indeed interrupts communication in the cumulus–oocyte complex (Dekel et al., 1981). The results obtained in the present study indicate that the concentrations of cAMP that interfere with coupling in the cumulus–oocyte complex correspond with those required to induce maturation of the oocyte. We therefore suggest that cAMP is a key molecule in regulation of oocyte maturation. Prior to the LH surge tonic levels of cAMP are continuously transferred to the oocytes to maintain meiotic arrest. In the preovulatory follicle, in response to LH, follicular cAMP levels are elevated. These increased concentrations of the nucleotide affect the cumulus cells and interrupt communication in the cumulus–oocyte complex. Under these conditions the flow of cAMP to the oocyte declines, inhibition is relieved and meiosis is resumed.

Acknowledgements

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