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Regulation of Mouse Oocyte Maturation: Effect of Elevating Cumulus Cell cAMP on Oocyte cAMP Levels[1]

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ABSTRACT

We have reexamined the possibility that cumulus cell cAMP can enter the oocyte via the gap junctions connecting the two cell types (Schultz et al., 1983a). Since our recent results indicate that the mouse oocyte possesses a very active cyclic nucleotide phosphodiesterase (PDE) (Bornslaeger et al., 1984), we have altered our experimental protocol to ensure that mouse oocyte PDE activity is inhibited throughout the duration of an experiment. Our results demonstrate the apparent transfer of cAMP from cumulus cells to the oocyte; these results are discussed in terms of current models for regulation of mammalian oocyte maturation.

INTRODUCTION

A substantial body of evidence indicates that cAMP is involved in maintenance of meiotic arrest of mammalian oocytes (Cho et al., 1974 and Wassarman et al., 1976 for mice; Hillensjo, 1977 and Dekel and Beers, 1978 for rats; Racowsky and McGaughey, 1981 for pigs) and that a drop in oocyte cAMP is involved in resumption of meiosis (Schultz et al., 1983b; Vivarelli et al., 1983). We recently presented evidence that oocyte cyclic nucleotide phosphodiesterase (PDE) is involved in the maturation-associated decrease in mouse oocyte cAMP (Bornslaeger et al., 1984) that occurs during the period of time in which oocytes become committed to resume meiosis (Schultz et al., 1983b). During the course of our last study (Bornslaeger et al., 1984), it became apparent that the mouse oocyte possesses a very active PDE. For example, oocyte cAMP levels can be elevated 5–10-fold with forskolin, a reversible activator of adenylate cyclase (Seamon et al., 1981), in the presence of isobutylmethylxanthine, an inhibitor of mouse oocyte PDE (Bornslaeger et al., 1984). cAMP levels fall to initial levels within a couple of minutes in oocytes subsequently transferred to medium containing neither of these agents.

Results of these studies have led us to reexamine the possibility that cAMP generated in cumulus cells may enter the oocyte via the heterologous gap junctions connecting the cumulus cells and the oocyte. Previously, we reported that we could not detect significantly more cAMP in oocytes derived from cumulus cell-oocyte complexes (COCs) exposed to either follicle-stimulating hormone (FSH) or cholera toxin compared to similarly exposed denuded oocytes (oocytes freed of attached cumulus cells) (Schultz et al., 1983a). We have altered our experimental protocol (see *Results*) to ensure that oocyte PDE is inhibited at all times during the course of an experiment and can now demonstrate the apparent transfer of cumulus cell cAMP to the oocyte. The results of these experiments are discussed in terms of current models for regulation of mammalian oocyte maturation.

MATERIALS AND METHODS

Oocyte Collection and Culture

Cumulus cell-oocyte complexes and denuded oocytes were obtained from 24-day-old randomly bred Swiss albino mice (Swiss Webster, Ace Animals Inc., Boyertown, PA) that were primed 48 h previously with 7.5 IU pregnant mare's serum gonadotropin (Sigma Chemical Co., St. Louis, MO) as previously described (Schultz et al., 1983a). The culture medium used was Eagle's minimum essential medium (MEM, GIBCO Laboratories, Grand Island, NY) containing Earle's salts, pyruvate (100 μg/ml), 10 mM HEPES (pH 7.2), polyvinylpyrrolidone (3 mg/ml), and gentamicin (10 μg/ml) (MEM/PVP).

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Cumulus cell-oocyte complexes were collected in MEM/PVP containing 0.2 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma). This concentration of IBMX reversibly inhibits maturation and inhibits PDE activity in oocyte extracts >98% (Bornslaeger et al., 1984). Complexes or denuded oocytes were incubated in MEM/PVP at 37°C in agarose-coated petri dishes (Falcon Labware, Los Angeles, CA) in a humidified atmosphere of 5% CO_2 in air (the exact incubation conditions are described in the figure legends). Germinal vesicle breakdown (GVBD) was scored by examining the oocytes with a Wild M5A microscope at 100-fold magnification.

Removal of Zonae Pellucidae

Zonae were removed by low pH treatment as previously described (Bornslaeger and Schultz, 1985).

cAMP Radioimmunoassay

Radioimmunoassays (RIAs) were performed as previously described (Bornslaeger and Schultz, 1985). Briefly, oocytes in about 2–3 μl of IBMX-containing medium were transferred directly into 100 μl of 0.1 N HCl containing 50 μM IBMX; the final concentration of IBMX in the RIA was 10 μM. A similar volume of IBMX-containing medium was used as the blank. The standard curve was constructed in the presence of 10 μM IBMX, and in no case did the blank produce detectable displacement of the radiolabeled ligand. The antibody to cAMP was the generous gift of Dr. Gary Brooker.

RESULTS

Our recent studies on mouse oocyte PDE revealed that the enzyme is very active in the oocyte (Bornslaeger et al., 1984). In an earlier study designed to detect movement of cumulus cell cAMP to the oocyte, denuded oocytes or COCs were incubated in medium containing both IBMX and either FSH or cholera toxin (Schultz et al., 1983a). Isobutylmethylxanthine was included to inhibit the presumed oocyte PDE (it had not yet been demonstrated that mouse oocytes possess this enzyme) in order to maximize the possibility of detecting transfer to the oocyte of cumulus cell cAMP. All incubations were conducted in the presence of IBMX until the last washes of the denuded oocytes or oocytes derived from COCs; IBMX was omitted from the medium at this point since it is cross-reactive in our cAMP RIA and produces a significant blank value. Our recent results (Bornslaeger et al., 1984), however, indicate that the time the oocytes were washed in this IBMX-free medium could be sufficient for the oocyte PDE to reduce, in oocytes derived from COCs, initially elevated cAMP to the "basal" level, i.e., the amount present in denuded oocytes. Therefore, we have accordingly performed similar experiments, but have instead omitted the wash in IBMX-free medium, and have altered the conditions of our cAMP RIA to account for the displacement caused by IBMX (see *Materials and Methods*).

Using three agents that elevate cumulus cell cAMP by activating adenylate cyclase through different mechanisms (i.e., forskolin,[3] cholera toxin, and FSH activate the catalytic subunit, regulatory subunit, and hormone receptor, respectively), oocytes derived from complexes always contained more cAMP than similarly treated denuded oocytes (Table 1). Thus, it is likely that cAMP was transferred from cumulus cells to the oocyte. A similar result has been reported for oocytes derived from rat complexes that were exposed to forskolin (concentration range 0.2–100 μM) (Racowsky, 1984). The increased amount of cAMP we observed in mouse oocytes derived from stimulated complexes was not due to increased cAMP levels in cumulus cell remnants adhering to such oocytes, since cAMP levels were not diminished significantly by removing zonae pellucidae (Table 2). Zona removal leaves oocytes nearly free of cumulus cell remnants (Bornslaeger and Schultz, 1985).

The fold increase in cAMP (Table 1, fourth column), which reflects the extent of apparent cAMP transfer, was about 2 in complexes whose cAMP levels were increased to less than 200 fmol. When complex cAMP levels were increased to greater than 200 fmol, however, disproportionately greater increases in cAMP transfer were observed (Fig. 1). A possible explanation for the biphasic relationship between the cAMP transfer ratio and level of cAMP in complexes is that the cAMP-binding capacity of cumulus cells is titrated with increasing cAMP. Below 200 fmol/complex, little cAMP appears free to move, whereas above 200 fmol/complex, more cAMP can apparently move to the oocyte. Another possible contributing factor is cAMP-induced gap junction formation between cumulus cells and the oocyte when cumulus cAMP levels are greater than 200 fmol. cAMP-induced gap junction formation has been well documented (see, for example, Flagg-Newton et al., 1981; Radu et al., 1982; Azarnia and Russell, 1985).

[3] There is evidence that a functional G subunit is necessary for forskolin activation of adenylate cyclase (Darfler et al., 1982).

TABLE 1. Effect of elevating complex cAMP levels on the amount of cAMP in complex-derived oocytes.[a]

Agent	Amount cAMP (fmol/complex or oocyte)			Fold increase[b]	Percent equilibration[c]
	Complex	Complex-derived oocyte	Oocyte		
FSH	190 ± 20	4.4 ± 0.4	0.35 ± 0.01[d]	13	2.3
	230 ± 40	4.1 ± 0.6	0.4 ± 0.1[d]	10	1.8
	250 ± 20	3.3	0.36 ± 0.02[d]	9	1.3
	400 ± 30	21.5 ± 1.6	0.36 ± 0.03[d]	60	5.4
Cholera toxin	90 ± 10	0.58 ± 0.10[d]	0.41 ± 0.01[d]	1.4	0.6
	60 ± 10	0.69 ± 0.08[d]	0.50 ± 0.00[d]	1.4	1.2
	200 ± 10	0.8 ± 0.2	0.3 ± 0.1[d]	2.7	0.4
	110 ± 10	0.65 ± 0.02	0.51 ± 0.01[d]	1.3	0.6
Forskolin					
75 μg/ml	460 ± 30	43 ± 3	1.3 ± 0.1	33	9.3
	1100 ± 100[e]	110 ± 10	1.0 ± 0.1	110	10.0
25 μg/ml	430 ± 20	23 ± 1	0.8 ± 0.1[f]	29	5.3
15 μg/ml	320 ± 40	16 ± 2	1.3 ± 0.1[d]	12	5.0
	400 ± 40	45 ± 6	1.6 ± 0.1[d]	28	11.3
	160 ± 20	2.7 ± 0.2	1.3 ± 0.1	2.1	1.7
10 μg/ml	80 ± 10	1.3 ± 0.1	1.1 ± 0.1[d,f]	1.2	1.6
	70 ± 10	1.6 ± 0.2	1.1 ± 0.2[d,f]	1.5	2.3
7.5 μg/ml	80 ± 10	1.3 ± 0.1	1.0	1.3	1.6

[a]COCs or denuded oocytes were incubated in MEM/PVP plus 0.2 mM IBMX containing either: 1) 2 μg/ml FSH for 1.5 h; 2) 2 μg/ml cholera toxin for 2.0 h; or 3) the indicated concentrations of forskolin for 1.0 h or as indicated (see below). At the end of the incubation, cumulus cells were removed as previously described (Schultz et al., 1983b). The cells were then transferred through several washes of MEM/PVP containing IBMX, and cAMP was determined by radioimmunoassay as described under *Materials and Methods*. cAMP levels were also determined in intact complexes, using appropriate dilutions to ensure that measurements were made on the sensitive portion of the curve, i.e., 10–100 fmol. Data are expressed as mean ± SEM, unless otherwise indicated (see below). An unstimulated complex contains about 2.0 fmol of cAMP.

[b]Amount cAMP in complex-derived oocyte/amount cAMP in oocyte, i.e., column 2/column 3.

[c]Amount cAMP in complex-derived oocyte/amount cAMP in COC, i.e., (column 2/column 1) ×100.

[d]Range of duplicate determinations.

[e]These data are not plotted in Fig. 1.

[f]Incubated in forskolin for 1.5 h.

TABLE 2. Effect of elevating complex cAMP levels on the amount of cAMP in complex-derived oocytes with or without zonae.[a]

	Amount cAMP (fmol/complex or oocyte)		
	Complex	Complex-derived oocyte + zona	Complex-derived oocyte −zona
Exp. 1	850 ± 40	56 ± 3	56 ± 2
Exp. 2	550 ± 30	25 ± 2	27 ± 1

[a]COCs were incubated for 1 h in MEM/PVP containing 0.2 mM IBMX and 50 μg/ml of forskolin. At the end of the incubation, cAMP was determined in some of the COCs. Cumulus cells were removed from the remaining COCs. The denuded oocytes were split into two groups. Zonae were removed from one group as described under *Materials and Methods*. The amount of cAMP in both groups was then determined as described in the legend to Table 1. Data are expressed as mean ± SEM, n=2–4.

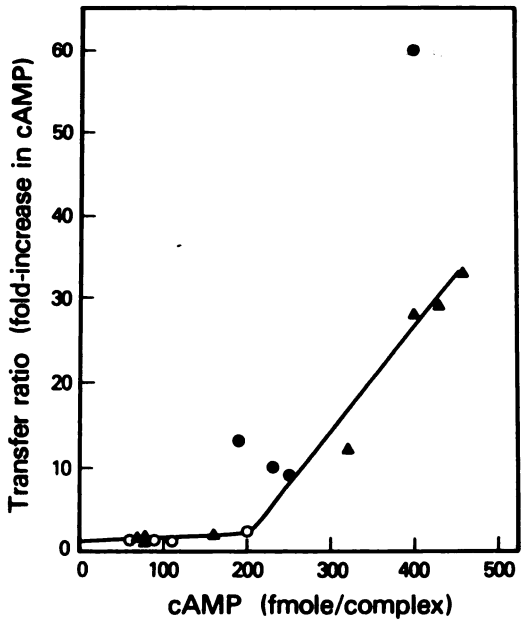

FIG. 1. Relationship between transfer ratio (fold increase in oocyte cAMP) and complex cAMP level. The data in the first and fourth columns in Table 1 have been plotted. (○), cholera toxin; (●), FSH; (▲), forskolin.

cAMP did not appear to equilibrate under the conditions employed in our experiments (Table 1, last column), since the ratio of cAMP in an oocyte derived from a stimulated complex to the total amount of cAMP in that stimulated complex was less than the fractional volume (i.e., about 15%) that the oocyte occupies in the complex. This is in contrast to results obtained for other metabolites (Schultz et al., 1983a).

In the absence of attached cumulus cells, the increased amount of cAMP present in oocytes derived from FSH- or cholera toxin-treated complexes was not sufficient to inhibit maturation (Fig. 2); oocytes derived from complexes exposed to either FSH or cholera toxin matured with kinetics similar to oocytes derived from untreated complexes. In addition, oocytes incubated in medium containing 1.0 mM IBMX for 20 min, conditions that elevate their cAMP level about 3-fold (Bornslaeger and Schultz, 1985), and then transferred to IBMX-free medium resumed meiosis with kinetics similar to control oocytes, i.e., oocytes collected in medium containing 0.2 mM IBMX and then transferred to IBMX-free medium; after 1.0, 1.25, and 1.5 h in IBMX-free medium, percent GVBD in experimental and control groups (70 oocytes per group) was 49 and 43, 16 and 14, and 11 and 11, respectively. This lack of

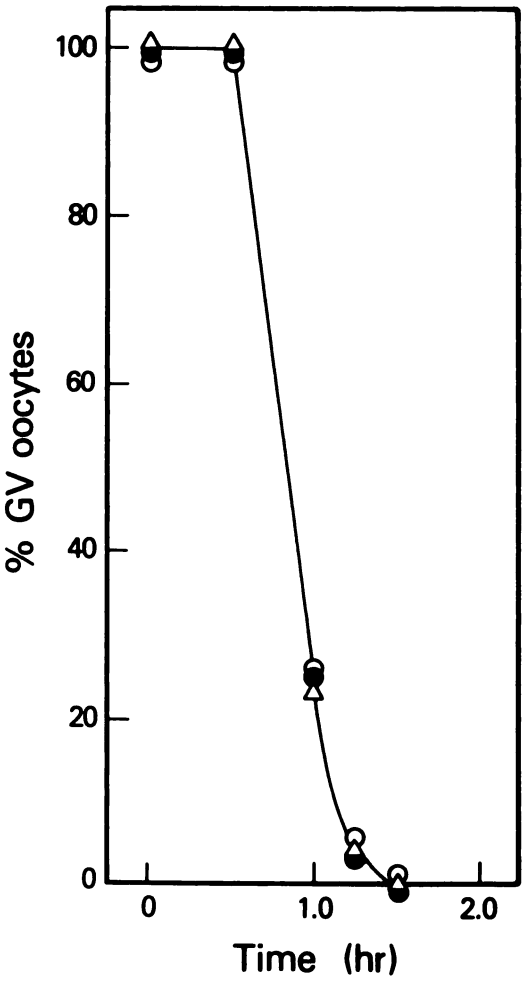

FIG. 2. Kinetics of maturation of oocytes obtained from complexes stimulated with either FSH or cholera toxin. Complexes were incubated in MEM/PVP containing 0.2 mM IBMX (control) (●) or MEM/PVP containing 0.2 mM IBMX and either FSH (○) or cholera toxin (△), each at a concentration of 2 μg/ml. Incubations in FSH or cholera toxin were for 1.5 or 2.0 h, respectively; the control groups were incubated in IBMX-containing medium for the corresponding lengths of time prior to transfer to IBMX-free medium. At the end of the incubation, the cumulus cells were stripped from the oocytes, which were then washed and cultured in IBMX-free medium. The percentage of oocytes with intact germinal vesicles (*%GV*) was then scored with time, time 0 being the time at which the oocytes were transferred to IBMX-free medium. At least 50 oocytes were in each group, and the experiment was performed two times. Similar results were obtained in each case and the data have been pooled.

inhibition of maturation was not surprising, since oocyte PDE can, in a few minutes, return cAMP elevated 5–10-fold to basal levels (Bornslaeger et al., 1984).

DISCUSSION

In our previous experiments, we found that exposure of cumulus cell-oocyte complexes to FSH or cholera toxin elevated complex cAMP levels to 100–200 fmol. However, we did not detect more cAMP in oocytes derived from such stimulated complexes relative to denuded oocytes (Schultz et al., 1983a). Results of experiments presented here indicate that oocytes derived from FSH- or cholera toxin-stimulated complexes (in which cAMP levels were elevated to 100–200 fmol) do, in fact, contain more cAMP than similarly treated denuded oocytes. In the absence of IBMX, the oocyte PDE could presumably abolish this difference within 1–2 min. Our previous protocol included such an exposure to IBMX-free medium; this most likely explains our initial failure to detect a higher level of cAMP in oocytes obtained from complexes with elevated cAMP levels.

Transfer of cAMP from cumulus cells to the oocyte could theoretically be demonstrated directly by following movement of radiolabeled cAMP using a pulse-chase protocol. Results of such an experiment would be equivocal, however, since the only way to radiolabel cumulus cell cAMP in an entire complex is by metabolic labeling. Since both radiolabeled cAMP and ATP could be transferred from cumulus cells to the oocyte, an increase in radiolabeled cAMP in the oocyte could be due to either transfer of cAMP or de novo synthesis in the oocyte of cAMP from transferred ATP. Thus, results of the experiments described here, although indirect, are consistent with movement into the oocyte of cumulus cell cAMP made in response to agents that activate cumulus cell adenylate cyclase. It should be noted, however, that the conditions we used to detect the apparent movement of cAMP are nonphysiologic; IBMX was present to inhibit oocyte PDE, and cumulus cell cAMP was elevated to a much greater extent than occurs in vivo (Schultz et al., 1983b).

It is not surprising that maturation was not delayed in oocytes removed from stimulated complexes, compared to oocytes from unstimulated complexes (Fig. 2), given that the oocyte PDE could rapidly reduce any differences in cAMP levels in these two groups of oocytes. Thus, both groups would undergo the maturation–associated decrease in oocyte cAMP and subsequent commitment to resume meiosis (Schultz et al., 1983) at approximately the same time.

We previously observed transient inhibition of maturation, however, for oocytes within stimulated complexes relative to oocytes within unstimulated complexes (see Fig. 2; Eppig et al., 1983). In those experiments, one group of complexes was treated with FSH and IBMX for 1 h and then exposed to FSH for 1.5 h (experimental group). A control group of complexes was exposed to IBMX alone for 1 h and then cultured for 1.5 h in IBMX-free medium. After the 1.5-h incubation, oocytes in both groups were liberated from their surrounding cumulus cells and GVBD was scored with time. A transient inhibition of maturation was observed for oocytes in the experimental group. A likely explanation for this result is that, in the experimental group, the continuous presence of cumulus cells allowed cAMP influx into oocytes during the incubation in IBMX-free medium at a time when cAMP levels in oocytes within unstimulated complexes were decreasing. Thus, after removal of the cumulus cells, the maturation-associated decrease in oocyte cAMP and subsequent commitment to resume meiosis would occur later for oocytes in the experimental group and a transient inhibition of maturation would be observed.

It has been proposed that, under basal conditions in the follicle, in vivo, a portion of the oocyte cAMP is derived from the surrounding follicle cells (Albertini and Anderson, 1974; Dekel and Beers, 1978; Gilula et al., 1978). However, there are no data directly supporting this premise. Likewise, our demonstration of apparent cAMP movement in vitro under nonphysiologic conditions does not provide direct evidence for cAMP movement in the unstimulated follicle, but suggests that cAMP movement under basal conditions is possible; the active oocyte PDE may drive the flux of cAMP from follicle cells to the oocyte by continuous cAMP hydrolysis. If such movement does occur, its level is likely to be low, since elevating cumulus cell cAMP by 50–100-fold [which is much greater than the increase observed in vivo (Schultz et al., 1983a)] results in only a 2-fold increase in oocyte cAMP. Thus, the follicle cell contribution of cAMP to the oocyte, although potentially significant in main-

taining meiotic arrest, may be quite small under basal conditions. It is necessary, therefore, to determine the relative contributions of oocyte adenylate cyclase and incoming follicle cell cAMP to the pool of oocyte cAMP in the unstimulated follicle in order to assess the relative contribution of exogenous cAMP in maintenance of meiotic arrest.

In vitro, spontaneous resumption of meiosis by an oocyte removed from its follicle may result, in part, from termination of basal transfer of follicle cell cAMP to the oocyte. Although mouse oocytes possess an adenylate cyclase [zona-free mouse oocytes, which are nearly free of contaminating cumulus cell remnants, respond to forskolin to an extent similar to intact denuded oocytes (Bornslaeger and Schultz, 1985)],[4] the amount of this enzyme activity may not be adequate to compensate for the highly active oocyte PDE. If this were the case, cAMP originating in follicle cells would then be an important factor in maintaining the steady-state level of cAMP in the oocyte required for meiotic arrest. In the absence of this source of cAMP, the oocyte PDE would elicit a decrease in oocyte cAMP, and resumption of meiosis would ensue.

The aforementioned explanation for spontaneous meiotic maturation in vitro is consistent with the proposal that gonadotropin-induced reduction of cummunication between cumulus cells and the oocyte triggers resumption of meiosis in vivo (Dekel and Beers, 1978). An apparent paradox arises, however, since the maturation-associated decrease in oocyte cAMP occurs during a period of time when both follicle and cumulus cell cAMP are increasing (Schultz et al., 1983b) and intercellular communication in the complex is apparently not reduced (Eppig, 1982; Salustri and Siracusa, 1983; Freter and Schultz, 1984). As previously pointed out (Eppig, 1982; Freter and Schultz, 1984), however, the coupling assay for intercellular communication probably detects mainly the transfer of metabolites between the inner layers of cumulus cells and the oocyte; these cumulus cells are the last to undergo the mucification reaction and become uncoupled from one another and the oocyte. Thus, even though a reduction in the extent of intercellular communication could occur between the outer cumulus cells of the complex, this decrease would not be detected by the communication assay, but would effectively isolate the oocyte from communicating with the entire follicle cell mass. Thus, if transfer of follicle cell cAMP to the oocyte is vital for maintenance of meiotic arrest (see above), such a reduction in communication between the outer cumulus cells could result in the maturation-associated decrease in oocyte cAMP at a time when follicle cell cAMP levels are increasing. Consistent with this idea are results of a quantitative morphometric study revealing that a marked reduction in the extent of gap junctional surface area between rat cumulus cells occurs during resumption of meiosis in vivo, and that this decrease correlates well with resumption of meiosis (Larsen et al., 1984).

It should be pointed out that, although a decreased flux of cAMP from the follicle cells to the oocyte may be involved in resumption of meiosis, reduced flux of other follicular factors, possibly regulating oocyte cAMP metabolism, may also be involved (Freter and Schultz, 1984). Lastly, it should be noted that even if a reduction in intercellular communication precedes or is concurrent with GVBD, uncoupling need not initiate maturation, since a commitment event, e.g., inhibition of oocyte adenylate cyclase or activation of oocyte PDE, could occur prior to the reduction in communication.

[4] Other investigators have also shown that denuded mouse oocytes respond to forskolin (100 μM) by elevating cAMP (Urner et al., 1983) and that GVBD is inhibited in these oocytes (Urner et al., 1983; Sato and Koide, 1984). The situation in the rat is not as unified; this is likely the result of different protocols. Racowsky (1984) reported that forskolin (0.2–100 μM) did not inhibit GVBD in oocytes examined after 4 h of culture or significantly increase oocyte cAMP. Dekel et al. (1984) did not observe any inhibition of maturation by 300 μM forskolin, whereas Ekholm et al. (1984) reported a very transient inhibition induced by 100 μM forskolin. Likewise, Olsiewski and Beers (1983) reported that, after 2 h of culture, 300 μM forskolin inhibited GVBD and stimulated cAMP synthesis.

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