Maintenance of Meiotic Arrest in Isolated Rat Oocytes by the Invasive Adenylate Cyclase of Bordetella Pertussis

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ABSTRACT

Rat oocytes resume meiosis spontaneously in vitro within 3 h after their isolation from the ovarian follicles. We report here that the spontaneous maturation of isolated rat oocytes is preceded by a drop in intracellular levels of cyclic adenosine 3',5'-monophosphate (cAMP). Further experiments were carried out to examine the possible correlation between the meiotic status and cAMP levels within the oocyte. To challenge rat cumulus-free oocytes to generate cAMP, bypassing their own adenylate cyclase, a preparation of an invasive adenylate cyclase from Bordetella pertussis was used. We found a dose-dependent elevation of cAMP levels within these oocytes that corresponded to inhibition of their spontaneous maturation. Persistent inhibition of meiosis was obtained with the continuous presence of the enzymatic preparation, whereas its removal resulted in a transient inhibition associated with a drop in cAMP. We suggest that the presence of elevated cAMP levels in the oocyte is directly responsible for the maintenance of meiotic arrest.

INTRODUCTION

Meiosis of the mammalian oocyte, initiated during fetal life, proceeds up to the diplotene stage of the first prophase and is arrested at birth. Reinitiation of meiosis in vivo is clearly dependent upon the preovulatory surge of luteinizing hormone (LH) (Lindner et al., 1974). The observation that meiotically arrested oocytes removed from the ovarian follicle resume meiosis spontaneously (Pincus and Enzmann, 1935) led to the generally held conclusion that the follicle inhibits oocyte meiosis. The nature of the ovarian factor responsible for meiotic arrest remained one of the major puzzles of reproductive biology for many years. Shortly after Cho et al. (1974) had demonstrated that the spontaneous maturation in vitro of mouse oocytes is blocked by dibutyryl cyclic adenosine 3',5'-monophosphate, the idea that this cyclic nucleotide could serve as an intracellular inhibitor of oocyte maturation was raised (Lindner et al., 1974; Anderson and Albertini, 1976; Schultz and Wassarman, 1977; Dekel and Beers, 1978). The fact that not only the spontaneous maturation but also LH-induced meiosis resumption in follicle-enclosed oocytes was found to be blocked by inhibitors of cyclic adenosine 3',5'-monophosphate (cAMP) degradation (Lindner et al., 1974; Hillensjö et al., 1978; Dekel et al., 1981) provided support for this notion.

Arrest of meiotic maturation by cAMP could possibly be obtained by maintaining a certain threshold level of the nucleotide within the oocyte. Alternatively, it was suggested (Eppig et al., 1983) that a cAMP-dependent inhibitory mediator, which is not cAMP itself, is transferred from the follicle to the oocyte to maintain meiotic arrest. The objective of this study was to look for a direct correlation between elevated levels of cAMP within the oocyte and the maintenance of meiotic arrest. To elevate cAMP levels within the oocyte, we used a highly active adenylate cyclase preparation, which is produced by the bacteria of the genus Bordetella and can be internalized by mammalian cells (Confer and Eaton, 1982). This invasive adenylate cyclase triggers the host cell to generate high amounts of intracellular cAMP by using its own pool of adenosine 5'-triphosphate (ATP) (Hanski and Farfel, 1985). We examined the possible inhibition of spontaneous maturation of cumulus-free oocytes subjected to the invasive adenylate cyclase and tested its correspondence to intraoocyte levels of cAMP.
MATERIALS AND METHODS
Sexually immature Wistar female rats (26 days old), from our departmental colony, were injected s.c. 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. As mammalian oocytes apparently possess an active cyclic nucleotide phosphodiesterase (Bornsleaeg et al., 1984), the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical Co., St. Louis, MO) was included in the culture medium throughout the entire course of all the experiments performed unless otherwise indicated.

Cumulus-oocyte complexes were isolated from the large antral follicles (Dekel and Beers, 1978) into control medium containing 0.1 mM IBMX. The cumulus cells were removed as described earlier (Dekel and Beers, 1980) to prepare cumulus-free oocytes. The oocytes were incubated in 35-mm Petri dishes at 37°C in air at a relative humidity of 100% in the presence of 0.1 mM IBMX with or without the indicated concentrations of Bordetella Pertussis dialyzed urea extract (BPUE). BPUE was prepared as previously described (Hanski and Farfel, 1985).

At the end of the incubation times, the oocytes were examined by Nomarski interference contrast microscopy. Oocytes were classified as meiotically arrested if the germinal vesicles (GV) were present. 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 oocytes with GV.

Intraoocyte cAMP was determined by the use of cAMP [125]I-radioimmunoassay kit (New England Nuclear, Boston, MA). At the end of the incubation with or without the indicated concentrations of BPUE, the oocytes were extensively washed (4 times) and transferred into polypropylene tubes in a minimum volume of control medium containing 0.1 mM IBMX. Repeated freezing and thawing (3 times) of the oocytes in liquid nitrogen was used to break the membranes and release the cAMP content into the medium. A similar volume of control IBMX-containing medium of the last wash was similarly treated to obtain blank values for intraoocyte cAMP determinations. Each sample was lyophilized and kept at −70°C. The samples were reconstituted with 200 μl of sodium acetate buffer, pH 6.2. The number of oocytes used to determine cAMP at the different experimental points varied to fit into the sensitivity range of the cAMP assay (0.1 to 4.0 pmoles/ml).

RESULTS
Intracellular levels of cAMP in oocytes isolated from the ovarian follicles were 1.24 ± 0.1 fmoles/oocyte. After a 1-h incubation in the absence of IBMX in the culture medium, cAMP levels within the oocytes decreased to 0.72 ± 0.06 fmoles/oocyte (Fig. 1). The drop in intraoocyte cAMP levels was followed by germinal vesicle breakdown (Fig. 1). Incubation of oocytes with BPUE led to an increase in intracellular levels of cAMP that was time- and dose-dependent.

![Graph showing intracellular cAMP levels](image-url)

FIG. 1. Levels of cAMP in isolated oocytes undergoing spontaneous maturation in vitro. Values for cAMP levels at Time zero of isolation were obtained by cAMP determination in oocytes isolated into 0.2 mM IBMX-containing medium. To obtain the values for the later time points, cAMP was determined in oocytes isolated in IBMX-free medium and incubated for the indicated time interval. Thirty oocytes were used for Time zero determinations, and 60 oocytes were required to determine cAMP at each of the later time points. The results of one representative experiment are presented. A separate group of oocytes was analyzed for the presence of GV at the indicated time points, as described in Materials and Methods.
Accumulation of cAMP could be detected at the earliest time assayed (15 min after exposure to BPUE) and reached a plateau within 60 min (Fig. 2). The maximal production of cAMP reached a level of 221 ± 28 fmoles/oocyte (Fig. 3). This response was obtained by 1.2 mg/ml of BPUE with an ED$_{50}$ at 0.6 mg/ml. Intracellular levels of cAMP in oocytes incubated with BPUE increased in a linear correlation with cell number (Table 1).

Oocytes incubated in control medium resumed meiotic maturation within 2 h, while addition of BPUE resulted in total inhibition of meiosis resumption (Fig. 4). IBMX, which was included in the BPUE-containing medium, exhibited by itself only a transient inhibitory effect (Fig. 4). The inhibitory effect of BPUE was dose-dependent and corresponded to the elevation of cAMP levels within the oocytes (Fig. 5). The BPUE preparation contains pertussis toxin. This toxin acts on the inhibitory guanine nucleotide regulatory protein of the adenylate cyclase system to uncouple it from the hormone, leading to an increase in the intracellular levels of cAMP (Ui et al., 1984). Incubation of oocytes with purified pertussis toxin did not delay the time course of their spontaneous maturation (data not shown).

Continuous presence of the invasive enzyme is absolutely required to maintain a constant level of intracellular cAMP in lymphocytes (Hanski et al., 1986). Similarly, removal of BPUE from the medium resulted in a drop of cAMP levels within the oocytes (Fig. 6). The inhibition of oocyte maturation was also reversible upon removal of PBUE. Maturation of oocytes following an initial incubation with BPUE for 2 h was detected by 4 h after the removal of the enzyme, with 60% maturation at 9 h (Fig. 7). Complete recovery of BPUE-induced inhibition was observed after 24 h. At this time, 90% of the oocytes examined underwent meiotic maturation (n=112). The recovery rate following preincubation in IBMX alone was much faster, with 93% of the oocytes undergoing maturation 5 h after removal of IBMX from the medium.

![FIG. 2. Time course of BPUE-induced cAMP accumulation by cumulus-free oocytes. Groups of 10 or 20 oocytes were isolated and incubated in control medium containing 0.1 mM IBMX and 1.2 mg/ml BPUE. At the indicated time points, the oocytes were extensively washed in 0.1 mM IBMX-containing medium. After appropriate dilutions, cAMP determinations were performed as indicated in Materials and Methods. The means ± SEM of the results of 5 individual experiments are presented.](image)

![FIG. 3. Concentration dependency of BPUE-induced cAMP accumulation by oocytes. Groups of 10 oocytes were isolated and incubated for 2 h in 0.1 mM IBMX-containing control medium in the presence of the indicated concentrations of BPUE. After extensive washing and appropriate dilutions in 0.1 mM IBMX-containing control medium, cAMP determinations were performed as described in Materials and Methods. The means ± SEM of the results of 5 individual experiments are presented.](image)
TABLE 1. Linearity of BPUE-induced cAMP accumulation in relation to the number of oocytes.*

<table>
<thead>
<tr>
<th>Number of oocytes</th>
<th>cAMP (fmoles)</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>131.4</td>
</tr>
<tr>
<td>1.0</td>
<td>250.4</td>
</tr>
<tr>
<td>2.0</td>
<td>408.8</td>
</tr>
<tr>
<td>3.0</td>
<td>688.7</td>
</tr>
</tbody>
</table>

*Samples of 10 oocytes were isolated and incubated for 2 h in control medium containing 0.1 mM IBMX and 1.2 mg/ml BPUE. After extensive washing and appropriate dilution in 0.1 mM IBMX-containing medium, cAMP determinations were performed as described in Materials and Methods. The results of one representative experiment are presented.

DISCUSSION

The results reported in this study strongly support the idea that intraoocyte levels of cAMP are involved in regulating oocyte maturation. In addition to our initial demonstration that the spontaneous maturation in vitro was preceded by a sharp drop in intraoocyte cAMP, the use of an invasive adenylate cyclase enabled us to demonstrate that a dose-dependent elevation of cAMP within the oocytes corresponded with inhibition of their spontaneous maturation. Moreover, persistent inhibition was obtained upon continuous presence of the enzyme in the medium, whereas its removal resulted in a drop of intraoocyte cAMP that was associated with resumption of meiosis. These results clearly indicate that elevated levels of intraoocyte cAMP are directly responsible for the maintenance of meiotic arrest.

Our findings in the rat are consistent with the results of earlier studies in the mouse in which the levels of cAMP within the oocytes were assayed before, during, and after maturation. Schultz et al. (1983a) showed that the levels of cAMP in mouse oocytes drop shortly before meiosis resumption both in vivo and in vitro, whereas no decrease in cAMP levels is observed in oocytes maintained meiotically arrested. Similarly, Vivarelli et al. (1983) have demonstrated that spontaneous oocyte maturation is associated with a drop in cAMP content within the

![Figure 4](image1.png)

**FIG. 4.** The inhibitory effect of BPUE on the spontaneous maturation of oocytes in vitro. Oocytes were isolated and incubated in control medium (○—○) or in 0.1 mM IBMX-containing control medium in the presence (■—■) or absence (△—△) of 1.2 mg/ml BPUE. The oocytes were analyzed for the presence of GV at the indicated time points. The results of one representative experiment are presented. At least 50 oocytes were examined at each time point.

![Figure 5](image2.png)

**FIG. 5.** Concentration of dependence of the effect of BPUE on the oocytes. Oocytes were isolated and incubated in 0.1 mM IBMX with the indicated concentrations of BPUE for 11 h. Cyclic AMP was determined by RIA as described in Materials and Methods. Each group of oocytes was analyzed individually for the presence of GV, and the results are presented as the fraction of GV oocytes out of the total examined. The means ± SEM of the results of at least 3 individual experiments are presented.
FIG. 6. Cyclic AMP content in oocytes preincubated in BPUE after removal of the enzyme. Groups of 10 or 15 oocytes were incubated for 24 h in 0.1 mM IBMX-containing control medium in the presence of 1.2 mg/ml BPUE. The oocytes were washed extensively (4 times) and then incubated in fresh control medium. Cyclic AMP determination was performed at the indicated time points after transfer to control medium. The means ± SEM of the results of 5 individual experiments are presented.

oocyte, whereas either maintenance or elevation of cAMP levels results in a substantial delay in meiosis resumption. In mouse oocytes, cAMP levels drop within 45 min after isolation, and meiosis resumption is observed only 2 h later (Schultz et al., 1983a). In the present study, a similar time interval between the drop in intraoocyte cAMP levels and meiosis resumption is observed in rat oocytes transiently exposed to BPUE or undergoing spontaneous maturation in vitro.

The role for cAMP in maintaining meiotic arrest is not unique to the mammalian oocyte. A decrease in oocyte cAMP observed in progesterone-stimulated oocyte of *Xenopus laevis* and *Rana Pipiens* has been correlated with resumption of meiosis (Maller et al., 1979; Maller and Krebs, 1980; Morrill et al., 1981). In the amphibian, it is clear that the origin of the inhibitory cAMP is in the oocyte itself (Schorderet-Slatkine et al., 1978); however, the question whether or not mammalian oocytes can generate cAMP is still under debate. Inhibition of maturation in both rat and mouse cumulus-free oocytes could not be demonstrated by cholera toxin, which interacts with the stimulatory guanosine 5'-triphosphate-binding regulatory component of the adenylate cyclase system (Dekel and Beers, 1980; Schultz et al., 1983b). Forskolin, which interacts with the catalytic moiety of the adenylate cyclase system, did stimulate cAMP generation in denuded mouse oocytes (Schultz et al., 1983a; Urner et al., 1983) and in the rat, as reported by Olsiewski and Beers (1983); however, forskolin failed to affect rat oocytes in our (Dekel et al., 1984) and Racowsky's (1984) experiments. As our rat oocytes failed to respond to both activators of the adenylate cyclase system, the invasive bacterial adenylate cyclase provided us with a unique tool to induce generation of cAMP within the oocytes bypassing their own adenylate cyclase system. By the use of this means, we were able to demonstrate that high levels of cAMP within the oocyte are directly responsible for maintenance of meiotic arrest, whereas decreasing levels of this nucleotide allow the oocytes to resume their meiotic maturation.

FIG. 7. Time course of maturation in vitro of oocytes after removal of BPUE. Oocytes were isolated and incubated for 2 h in 0.1 mM IBMX-containing control medium, in the presence of 1.2 mg/ml BPUE. The oocytes were washed extensively (4 times) and then incubated in fresh control medium with (●—●) or without (■—■) 1.2 mg/ml BPUE. At the indicated time points of reincubation, the oocytes were examined for the presence of GV. The means ± SEM of the results of 3 individual experiments are presented. At least 100 oocytes were examined at each experimental point.
ACKNOWLEDGMENTS

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REFERENCES


