MEIOTIC MATURATION OF MOUSE OOCYTES IN VITRO: INHIBITION OF MATURATION AT SPECIFIC STAGES OF NUCLEAR PROGRESSION

P. M. WASSARMAN*, W. J. JOSEFOWICZ AND G. E. LETOURNEAU

The Department of Biological Chemistry and Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115, U.S.A.

SUMMARY

In vitro studies of meiotic maturation of mouse oocytes have been carried out in the presence of several drugs. The individual steps of nuclear progression, including dissolution of the nuclear (germinal vesicle) membrane, condensation of dictyate chromatin into compact bivalents, formation of the first metaphase spindle, and extrusion of the first polar body, are each susceptible to one or more of these drugs.

Germinal vesicle breakdown, the initial morphological feature characteristic of meiotic maturation, is inhibited by dibutyryl cyclic AMP. However, even in the presence of dibutyryl cyclic AMP, the nuclear membrane becomes extremely convoluted and condensation of chromatin is initiated but aborts at a stage short of compact bivalents. Germinal vesicle breakdown and chromatin condensation take place in an apparently normal manner in the presence of puromycin, Colcemid, or cytochalasin B. Nuclear progression is blocked at the circular bivalent stage when oocytes are cultured continuously in the presence of puromycin or Colcemid, whereas oocytes cultured in the presence of cytochalasin B proceed to the first meiotic metaphase, form an apparently normal spindle, and arrest. Emission of a polar body is inhibited by all of these drugs. The inhibitory effects of these drugs on meiotic maturation are reversible to varying degrees dependent upon the duration of exposure to the drug and upon the nature of the drug.

These studies suggest that dissolution of the mouse oocyte's germinal vesicle and condensation of chromatin are not dependent upon concomitant protein synthesis or upon microtubules. On the other hand, the complete condensation of chromatin into compact bivalents apparently requires breakdown of the germinal vesicle. Failure of homologous chromosomes to separate after normal alignment on the meiotic spindle in the presence of cytochalasin B suggest that microfilaments may be involved in nuclear progression at this stage of maturation. Cytokinesis, in the form of polar body formation, is blocked when any one of the earlier events of maturation fails to take place.

INTRODUCTION

During the process of oogenesis, oocytes of many animal species undergo meiotic arrest prior to the completion of chromosomal reduction and it is in this state that they undergo tremendous growth. The length of time that oocytes remain in this arrested

* To whom enquiries and reprint requests should be directed.
P. M. Wassarman, W. J. Joselewicz and G. E. Letourneau

state and the nature of the stimulus which reinitiates meiosis are species-dependent (Baker, 1972a; Schuetz, 1974; Smith, 1973).

In the mouse, nearly all oocytes have arrested at the diplotene (dictyate) stage of prophase of the first meiotic division by 5 days post partum and they remain in dictyate until just prior to ovulation, a period extending from several weeks to more than a year. The resumption of meiosis can be mediated by a hormonal stimulus in vivo (Baker, 1972b) or by the release of oocytes from their ovarian follicles into a suitable culture medium in vitro (Biggers, Whittingham & Donahue, 1967; Donahue, 1968; Sorensen, 1973; Wassarman & Letourneau, 1976a). The oocytes undergo nuclear progression from dictyate to metaphase II and remain at this stage of meiosis in the oviduct, or in culture, until fertilization or parthenogenetic activation takes place. The period of time during which meiosis progresses from dictyate to metaphase II is termed the period of 'meiotic maturation'. Mouse oocytes matured and fertilized in vitro have produced viable foetuses following transplantation to the uteri of foster mothers (Cross & Brinster, 1970).

During the period of meiotic maturation, mouse oocytes undergo a series of well defined morphological and physiological changes in preparation for fertilization and embryogenesis. This process which takes approximately 12 h to complete in vitro, is characterized by dissolution of the nuclear (germinal vesicle) membrane, condensation of diffuse dictyate chromatin into distinct bivalents, separation of homologous chromosomes and emission of the first polar body, and arrest of meiotic progression at metaphase II.

We have examined the effects of several drugs on meiotic maturation of mouse oocytes in vitro in an attempt to block reversibly the process at discrete stages prior to the second meiotic metaphase. It is the purpose of this paper to provide a descriptive account of meiotic maturation of mouse oocytes in vitro in the presence of dibutylryl cyclic AMP, puromycin, Colcemid, or cytochalasin B.

MATERIALS AND METHODS

Oocytes were obtained by puncturing ovaries from adult (8–12 weeks of age) female Swiss mice (CD-1, Charles River Laboratories) (Donahue, 1968). Oocytes containing an intact germinal vesicle and free of cumulus cells were harvested and washed in culture medium (Biggers, 1971). Cell culture was carried out in plastic dishes (Falcon) in 50- or 100-μl drops of medium under paraffin oil at 37 °C in an atmosphere of 5% CO₂ in air. Stock solutions of dibutyryl cyclic AMP (N^6, O^6′-dibutyryl adenosine 3’,5’-cyclic monophosphate, dbcAMP) (Sigma), puromycin (Sigma), and Colcemid (Calbiochem) were stored frozen. Cytochalasin B (CCB) (Calbiochem) was made up in dimethylsulphoxide and stored frozen.

Air-dried chromosome spreads were prepared essentially by Tarkowski's (1966) procedure. Chromosomes were stained with Giemsa (Harleco no. 620) at 1:50 dilution with 0.1 M phosphate buffer, pH 6.8, for 30 min. Light microscopy was performed on isolated, unfixed oocytes and on fixed preparations using a Zeiss Photomicroscope II equipped with Nomarski differential-interference optics.

For electron microscopy, oocytes were fixed in 3 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h at 4 °C and then postfixed in 1 % osmium tetroxide in the same buffer for 1 h at 4 °C. The oocytes were rinsed in buffer, transferred to 30 % ethanol for 5 min, stained with 3 % uranyl acetate (in 30 % ethanol) for 15 min, and dehydrated through a series
RESULTS

Meiotic maturation in vitro

Meiotic maturation takes place spontaneously when mouse oocytes are released from their ovarian follicles into a suitable culture medium. This process, which takes approximately 12 h to complete in vitro, is characterized by dissolution of the nuclear (germinal vesicle, GV) membrane, condensation of diffuse dictyate chromatin into distinct bivalents, separation of the homologous chromosomes and emission of the first polar body, and arrest of meiotic progression at metaphase II. Some of these events are shown in Fig. 1. Under the experimental conditions used in this study, approximately 85% of the oocytes placed in culture underwent GV breakdown within 4 h and, of these, approximately 80% subsequently emitted polar bodies (Fig. 2).
Effect of dibutyryl cyclic AMP on maturation in vitro

Dibutyryl cyclic AMP, at a concentration of 100 μg/ml, is an effective inhibitor of GV breakdown in mouse oocytes. Less than 5% of the oocytes placed in culture resume meiosis, as evidenced by the retention of an intact GV, even after 16 h (Fig. 2). The inhibitory effect of dbcAMP is reversible although the degree of reversibility decreases over extended periods of culture (Fig. 3). Light-microscopic examination of Giemsa-stained chromosome spreads prepared from mouse oocytes cultured in the presence of dbcAMP shows that chromosome condensation is initiated in these oocytes, but aborts prior to the formation of compact bivalents. The stage at which chromosome condensation aborts is seen transiently during nuclear progression of oocytes cultured in medium without the drug (Fig. 4). Furthermore, electron-microscopic examination of fixed and sectioned oocytes cultured in the presence of dbcAMP showed that the nuclear envelope becomes very convoluted, which is usually the first morphological indication of the onset of meiotic maturation, but still retains many nuclear pores (Fig. 5).
Inhibition of meiotic maturation

Fig. 3. Analysis of the degree of reversibility of the inhibition of meiotic maturation of mouse oocytes in vitro by several drugs. Oocytes were cultured for 5 h in the presence of dbcAMP (100 μg/ml), puromycin (10 μg/ml), Colcemid (10 μg/ml), or CCB (5 μg/ml), respectively, and were then transferred to medium containing the same drug (closed bar) or to medium alone (open bar) for an additional 12 h of culture. The percentage of polar body formation was determined at the end of the culture period and compared with oocytes cultured continuously in medium alone (A). The data shown for each treatment represent the average of a minimum of 2 replicates, with a total of 60 or more oocytes.

Effect of puromycin on maturation in vitro

Puromycin, at a concentration of 10 μg/ml, is an effective inhibitor of meiotic maturation of mouse oocytes (Fig. 2) and dramatically reduces protein synthesis in these oocytes (Fig. 6). However, GV breakdown and chromosome condensation take place in a normal manner, despite the effect on protein synthesis, with meiotic arrest occurring at the circular bivalent stage (Fig. 7). The inhibitory effect of puromycin is completely reversible, even after exposure of oocytes to the drug for 5 h in culture (Fig. 3).

Effect of Colcemid on maturation in vitro

Colcemid, at a concentration of 10 μg/ml, a dose sufficient to prevent formation of a metaphase I spindle, inhibits meiotic maturation at the circular bivalent stage (Fig. 2). As with puromycin, GV breakdown and chromosome condensation take place in a normal manner (Fig. 7) and the inhibitory effect is reversible (Fig. 3).
Inhibition of meiotic maturation

Effect of cytochalasin B on maturation in vitro

Eighty per cent or more of mouse oocytes cultured in the presence of CCB (5 μg/ml) resume meiosis, undergo nuclear progression to metaphase I with the formation of a spindle, and then arrest without emitting the first polar body (Fig. 2). Light- and electron-microscopic examination of CCB-treated eggs shows that meiosis aborts at a stage which is seen transiently with oocytes cultured in medium without the drug (Fig. 8). The metaphase I spindle appears to be quite normal in configuration and is completely surrounded by a dense area composed of mitochondria, vacuoles and granules.

DISCUSSION

The process of meiotic maturation of mouse oocytes consists of a series of well-defined morphological changes, beginning with GV breakdown and chromosome condensation and ending with alignment of chromosomes on the second metaphase spindle (Donahue, 1968; Sorensen, 1973). However, the question of the regulation of mammalian oocyte maturation is quite perplexing in so far as oocytes released from large ovarian follicles undergo meiotic maturation ‘spontaneously’ when cultured in vitro in a suitable medium. This may be compared with in vitro oocyte maturation in the starfish and in the frog which requires the interaction of a diffusible molecule (1-methyladenine and progesterone, respectively), probably produced by the follicle cells, with the oocyte’s plasmalemma (Smith & Ecker, 1969; Schuetz, 1974; Smith, 1975; Kanatani, 1975). Recent studies in our laboratory have shown that, in the juvenile mouse (up to 21 days post partum), there is a direct relationship between the size of isolated oocytes and their ability to undergo meiotic maturation in vitro (Sorensen & Wassarman, 1976). These results suggest that the ability to resume meiosis (‘meiotic competence’) is acquired at a specific stage of oocyte growth in the juvenile mouse and that, perhaps, the acquisition of meiotic competence is associated with a change in the oocyte’s biochemical capabilities. It is in this context that we have begun to study the sequence of events which constitutes meiotic maturation in the mammalian oocyte.

The results reported here have confirmed and extended previous observations on the effect of dbcAMP on meiotic maturation of mouse oocytes in vitro (Stern & Wassarman, 1973, 1974; Cho, Stern & Biggers, 1974; Wassarman & Turner, 1976). The fact that dbcAMP reversibly inhibits the onset of nuclear progression in isolated mouse oocytes may be related to the proposed regulatory role for cAMP during the mitotic cell cycle (Burger, Bombik, Breckenridge & Sheppard, 1972; Willingham, Johnson & Pastan, 1972). In this regard it has been shown that elevated cAMP levels inhibit cell multiplication (Johnson & Pastan, 1972; Bombik & Burger, 1973), that

Fig. 4A–C. Giemsa-stained chromosome spreads from mouse oocytes cultured in vitro. Chromosome spreads were prepared from oocytes immediately after isolation (A), after 1 h of culture in medium alone (B), or after 16 h of culture in medium containing dbcAMP (100 μg/ml) (C). ×830 approx.
5A

P. M. Wassarman, W. J. Josefowicz and G. E. Letourneau
Inhibition of meiotic maturation

Fig. 6. The effect of puromycin on protein synthesis in mouse oocytes during culture in vitro. Incorporation of [H]valine into TCA-insoluble material was examined in the presence (○—○) and absence (■—■) of 10 μg/ml puromycin as previously described (Stern & Wassarman, 1974). The data points shown represent the mean values of 3 replicates.

the concentration of cAMP is lower in fast-growing than in slow-growing cells (Sheppard, 1971), that lowered cAMP levels occur during G₁ and mitosis relative to S-phase (Willingham et al. 1972; Burger et al. 1972) and that exogenously supplied dbcAMP results in termination of the cell cycle in G₁-phase (Zimmerman & Raska, 1972; Willingham et al. 1972; Kaukel, Fuhrmann & Hilz, 1972). It is tempting to suggest that fluctuations in cAMP levels may play an analogous role in the mammalian oocyte, with elevated intracellular levels of cAMP maintaining mouse oocytes in diacyte of the first meiotic prophase from several weeks to more than a year prior to undergoing ovulation or atresia. Since cAMP is believed to influence cellular processes by activation of protein kinase(s) (Bitensky & Gorman, 1973) it is of interest to note that the appearance of a new cAMP-dependent protein kinase has been detected following progesterone-induced meiotic maturation of amphibian oocytes (Wiblet, 1974). On the other hand, Smith (1975) has reported that neither cAMP, its dibutyryl analogue, nor theophylline, either singly or in combination, mimicked the effects of progesterone on amphibian oocytes, regardless of whether they were injected into the oocytes or were present in the culture medium. Although the molecular basis of the inhibitory effect of dbcAMP on meiotic maturation of mouse oocytes is not clear at
present, it appears likely that it interferes primarily with one or more of the events leading to GV dissolution, e.g. activation of (a) specific enzyme (s) involved in degrading the GV membrane. Our observations indicate that the nuclear membrane becomes convoluted and that chromosome condensation is initiated in oocytes even in the presence of dbcAMP; however, condensation aborts prematurely. This phenomenon is probably a direct consequence of the failure of the GV to break down. It has been reported previously that much of the chromatin condensation during meiotic

maturation of mouse oocytes takes place along the nuclear envelope, suggesting that the chromosomes are actually attached to it (Calarco, Donahue & Szollosi, 1972; Szollosi, Calarco & Donahue, 1972; Calarco, 1972). Accordingly, it may be inferred from these observations that nuclear progression, in the form of chromosome condensation, is initiated in the presence of dbcAMP but is not completed due to the maintenance of an intact nuclear envelope.

It is well documented that protein synthesis is required for the successful completion of meiotic maturation of oocytes from a variety of species, including echinoderms (Brachet & Steinert, 1967; Zampetti-Bosseler, Huez & Brachet, 1973; Houk & Epel, 1974), amphibians (Dettlaff, Nikitina & Stroeva, 1964; Dettlaff, 1966; Brachet, 1967; Schuetz, 1967; Smith & Ecker, 1969; Merriam, 1972; Brachet et al. 1974), and mammals (Jagiello, 1968; Stern, Rayyis & Kennedy, 1972; Wassarman & Letourneau, 1976). Extensive in vitro studies carried out by Smith & Ecker (1969), using oocytes isolated from Rana pipiens, show that there are changes in both the rate of protein synthesis and the nature of proteins synthesized during progesterone-induced meiotic maturation (see also Smith & Ecker, 1969; Smith, 1975). Furthermore, in the

Fig. 7A–C. Giemsa-stained chromosome squashes prepared from mouse oocytes cultured in vitro in the presence or absence of drugs. Shown are chromosomes from oocytes cultured for 16 h in the presence of puromycin (10 µg/ml) (A) or Colcemid (10 µg/ml) (B) and for 6 h in medium alone (C).
Inhibition of meiotic maturation

Fig. 8A–C. Light and electron micrographs of mouse oocytes cultured in vitro in the presence of CCB. Shown are photomicrographs of mouse oocytes cultured for 16 h in the presence of CCB (5 μg/ml) using Nomarski differential-interference optics (A, B) and an electron micrograph of a thin section from an oocyte cultured under the same conditions (C). A, × 550; B, × 1100; C, × 5500, approx.

Inhibition of meiotic maturation

amphibian oocyte, both GV breakdown and the appearance of 'maturation-promoting factor' during meiotic maturation are dependent upon continued protein synthesis (Schuetz, 1967; Smith & Ecker, 1969; Wasserman & Masui, 1976). In this connexion, Ziegler & Masui (1973) have shown that puromycin blocks chromosome condensation in brain nuclei injected into amphibian oocytes arrested at metaphase I. On the other hand, GV breakdown and chromosome condensation during maturation of the mouse oocyte do not appear to be dependent upon concomitant protein synthesis (or
RNA synthesis; Wassarman & Letourneau, 1976a and unpublished results). The presence of puromycin (or cycloheximide) has no effect on the rate of resumption of meiosis, only on the extent of maturation, since nuclear progression stops at the circular bivalent stage. These results suggest that, unlike amphibian oocytes, the fully-grown mouse oocyte is poised to resume meiosis, such that no new proteins need be synthesized for GV breakdown and chromosome condensation to take place spontaneously in vitro. Previous studies have shown that dbcAMP has no effect on the apparent rate of protein synthesis or on the spectrum of proteins synthesized by the mouse oocyte during culture in vitro (Stern & Wassarman, 1973, 1974). In this context, it should be pointed out that Lindner et al. (1974) have reported that de novo protein synthesis is required in isolated rat follicles for the initiation of oocyte maturation during the first 2 h of LH action. It is not clear whether the protein(s) essential for LH-induced meiosis in follicle-enclosed oocytes is synthesized by the oocyte itself or by some other follicular component. These results can be reconciled with those presented in this report if it is assumed that protein synthesis is required for an event which is obviated by physical removal of the oocyte from its surrounding follicle.

![Diagram of the inhibitory effects of drugs on meiotic maturation of mouse oocytes](image)

The first morphological indication of incipient oocyte maturation appears to be the increased undulatory activity of the nuclear envelope which precedes GV breakdown (Sorensen, 1973). During this period a number of microtubules are located around the oocyte's GV and some actually project perpendicularly toward the nuclear envelope as it becomes convoluted (Szollosi et al. 1972). Since in some instances, folding of the nuclear envelope during mitosis has been directly attributed to impinging microtubules (Bajer & Mole-Bajer, 1969) it was of interest to find that GV dissolution and chromosome condensation took place in a normal manner in the presence of Colcemid, a drug which disaggregates spindle microtubules, at concentrations sufficient to prevent spindle formation. These observations suggest that microtubules do not play an essential role in the initial phases of meiotic maturation of mouse oocytes.

Cytochalasin B, a fungal alkaloid first described by Carter (1967), has many diverse effects on cultured cells, ranging from inhibition of morphogenetic and movement processes to interference with hormone-mediated cellular responses (Carter, 1972). In addition, CCB induces a variety of cultured mammalian cells to segregate the cell's nucleus into an evagination of the plasma membrane which occasionally breaks away from the cell body (Poste, 1973). CCB was originally thought to interact specifically with contractile components of the cell, affecting various aspects of cellular motility;
Inhibition of meiotic maturation

However, this interpretation has been the subject of some controversy (Estensen, Rosenberg & Sheridan, 1971; Forer, Emmerens & Behnke, 1972; Pollard & Weihing, 1974). The results of several studies of cytokinesis in marine, amphibian and mammalian eggs indicate that CCB interferes with normal cleavage; however, while in certain instances it can be correlated with disruption or disorganization of arrays of microfilaments associated with the contractile ring, in others it cannot (Schroeder, 1970, 1972; Bluemink, 1971a, b; Snow, 1973). In a study of the effects of CCB on morphogenetic events in the fertilized egg from the surf clam, Spisula solidissima, Longo (1972) found that the drug prevented the formation of a polar body; this was attributed to the failure of a cleavage furrow to form in the presence of CCB. On the other hand, CCB did not interfere with migration of the metaphase spindle from its central position to the cell's cortex. In the experiments reported here, CCB appeared to prevent not only polar body formation, but migration of the meiotic spindle of the mouse oocyte to a position in the cell's cortex as well. These results suggest that the cytoskeletal apparatus of the mouse oocyte is involved in movement of the spindle from its central position to the cortex.

The effects of dbcAMP, puromycin, Colcemid, and CCB on meiotic maturation of mouse oocytes in vitro are summarized in Fig. 9. The results presented here have shed light on the molecular basis of meiotic maturation in the mammal and indicate that these reversible inhibitors will prove useful in further biochemical studies of this process.

This research was supported by grants awarded to P.M.W. by The National Institute of Child Health and Human Development (HD-06916) and The National Science Foundation (GB-43528).

A preliminary account of some of this work was reported at the Annual Meeting of the Tissue Culture Association as part of a Symposium on Differentiation of The Haploid Cell, Montreal, Canada, June 1975 (Wassarman, 1974).

REFERENCES


Inhibition of meiotic maturation


(Received 18 May 1976)