CYTOCHALASIN B-INDUCED PSEUDO-CLEAVAGE OF MOUSE OOCYTES IN VITRO: ASYMMETRIC LOCALIZATION OF MITOCHONDRIA AND MICROVILLI ASSOCIATED WITH A STAGE-SPECIFIC RESPONSE

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SUMMARY

Mouse oocytes are induced by cytochalasin B to undergo 'pseudo-cleavage' in vitro into 2 equally sized and separable compartments. This response to the drug is dependent upon the meiotic state of the oocytes, as well as upon the presence of an intact zona pellucida. The resulting 2 cellular compartments can be completely separated from one another and cultured in vitro.

Each of the compartments possesses characteristic structural features. The most pronounced structural differences include: (i) the presence of a nucleus (germinal vesicle) and nucleolus in one compartment; (ii) the presence of microvilli on the surface of the anucleate, but not the nucleate, compartment; and (iii) the localization (segregation) of mitochondria at the periphery of the anucleate, but not the nucleate, compartment. The results presented suggest that pseudo-cleavage induced by cytochalasin B arises as a consequence of a limited interaction of the drug with the oocyte surface and/or cortex and that it may represent a topographical dissociation of transporting and non-transporting regions of the membrane. These and other features of mouse oocytes treated with cytochalasin B are of interest in view of the involvement of the oocyte zona pellucida and plasma membrane during meiotic maturation, fertilization, and early embryogenesis.

INTRODUCTION

During 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 state and the nature of the stimulus which reinitiates meiosis are species-dependent (Baker, 1972a; Schuetz, 1974; Smith, 1975).

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

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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, 1976). 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 take 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, GV) 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. Cyclic AMP has been implicated in the regulation of this process by virtue of the ability of the dibutyryl derivative to inhibit reversibly spontaneous oocyte maturation in vitro (Stern & Wassarman, 1973, 1974; Cho, Stern & Biggers, 1974; Wassarman, 1974; Wassarman & Turner, 1976).

To define more accurately the nature, site, and mode of action of factors involved in the regulation of meiosis in the mammalian oocyte and egg, we have attempted to enucleate mouse oocytes using cytochalasin B, a fungal metabolite (Carter, 1972). In addition to its effects on cytokinesis and cell movement (Wessells et al., 1971; Spooner, 1973; Pollard & Weihering, 1974), this drug induces a variety of cultured mammalian cells to segregate the cell's nucleus into an evagination of the plasma membrane, which occasionally breaks from the cell body, yielding 'karyoplasts' (a nucleus enclosed by plasma membrane and surrounded by very little cytoplasm), and 'cytoplasts' (an enucleated part of a cell containing the bulk of cellular organelles) (Poste & Reeve, 1970; Wright & Hayflick, 1972; Prescott, Myerson & Wallace, 1972; Prescott & Kirkpatrick, 1973; Wright, 1973; Ladda, 1973; Follett, 1974; Croce, Tomasini & Koprowski, 1974). Experimentally, karyoplasts have proved very useful in studies of nucleocytoplasmic interactions in mitotic cells (Ladda & Estensen, 1970; Poste, 1973; Goldman & Pollack, 1974; Poste & Reeve, 1970). We report here that exposure of mouse oocytes, arrested in dictyate of the first meiotic prophase by dibutyryl cyclic AMP, to cytochalasin B in vitro, results in the formation of two equal-sized and separable compartments which possess distinct structural properties. The formation and nature of these compartments is of interest in relation to the involvement of the zona pellucida and the plasma membrane of the mammalian oocyte during meiotic maturation, fertilization, and early embryogenesis (Austin, 1961, 1965; Oikawa, Yanagimachi & Nicolson, 1973; Johnson, Eager, Muggleton-Harris & Grave, 1975; Nicolson, Yanagimachi & Yanagimachi, 1975).
MATERIALS AND METHODS

Oocytes were obtained by puncturing ovaries from adult (8–12 weeks of age) female Swiss mice (CD-1, Charles River Labs) (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. A stock solution of dibutyryl cyclic AMP (N°, O°'-dibutyryl adenosine 3',5'-cyclic monophosphate, dbcAMP) (Sigma) was made up in phosphate-buffered saline (10 mg/ml) and stored frozen. Cytochalasin B (CCB) (Calbiochem) was made up in dimethylsulphoxide (2 mg/ml) and stored frozen. A 0.5% solution of Pronase (Calbiochem, grade B) made up in phosphate-buffered saline was used to remove the oocyte zona pellucida.

Light microscopy was performed on isolated, unfixed oocytes 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 ethanol. The specimens were then infiltrated with epoxypropane and embedded in Epon 812. Thin sections were stained with methanolic uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963) and were examined in a Philips 200 electron microscope.

RESULTS

Meiotic maturation takes place spontaneously when mouse oocytes are physically released from their ovarian follicles into a suitable culture medium. This process, which takes about 12 h to complete in vitro, is characterized by dissolution of the GV 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. Under the experimental conditions used in this study, approximately 85% of the mouse oocytes placed in culture underwent GV breakdown within 3 h and, of these, approximately 80% subsequently emitted polar bodies. On the other hand, in the presence of dbcAMP (100 μg/ml) less than 5% of the oocytes placed in culture resumed meiosis, as evidenced by the retention of an intact GV and diffuse chromatin, even after 16 h. The inhibitory effect of dbcAMP was completely reversible even after several hours of culture.

Meiotic maturation is initiated in mouse oocytes exposed to cytochalasin B (CCB) (5 μg/ml) alone; meiosis proceeds in a normal manner as far as metaphase I and arrests (Wassarman, Letourneau & Josefowicz, unpublished results). However, oocytes cultured in the presence of both dbcAMP and CCB slowly undergo a process which we term 'pseudo-cleavage'. This process involves the division of the oocyte into 2 approximately equal-sized compartments, one of which retains an intact GV and nucleolus (Figs. 1, 2). The 2 compartments appear to be always connected, albeit in some instances by a very fine ‘thread’ of material. However, the compartments can be separated into nucleate and anucleate portions by careful manipulation of the treated oocyte with a micropipette and both portions reseal after separation from each other (Fig. 3). In the absence of dbcAMP approximately 15% of the oocytes cultured in vitro underwent CCB-induced pseudo-cleavage, whereas, in its presence this value
Fig. 1. Photomicrographs of mouse oocytes during culture in vitro using Nomarski differential-interference optics. Shown are isolated oocytes in dictyate of the first meiotic prophase (A) and after culture for 5 h in the presence of dbcAMP (100 μg/ml) and CCB (5 μg/ml) (B). gp, germinal vesicle; n, nucleolus; zp, zona pellucida. × 730 approx.

Fig. 2. Photomicrographs of CCB induced pseudo-cleavage of mouse oocytes in vitro using Nomarski differential-interference optics. Shown are typical results following culture of isolated mouse oocytes in dbcAMP (100 μg/ml) and CCB (5 μg/ml). × 530 approx.

Fig. 3. Photomicrographs of mouse oocytes, after various treatments, using Nomarski differential-interference optics. A, oocytes with and without zona pellucida after culture in the presence of dbcAMP (100 μg/ml) and CCB (5 μg/ml) for 1 h. Note the blebbing on the oocyte lacking a zona pellucida. B, oocyte which has undergone pseudo-cleavage and has subsequently had its zona pellucida removed with Pronase. C, the GV-containing compartment which has been separated from an oocyte which had undergone pseudo-cleavage. Note that the ratio of the diameter of the nucleolus to that of the compartment is approximately 0.13. A, B, C, × 330, 700, and 950, approx.
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increased to more than 80% (Fig. 4). The retention of an intact GV (dictyate stage of meiosis) appears to be essential for pseudo-cleavage to occur to any great extent and we attribute the low level of pseudo-cleavage observed in the absence of dbcAMP to the small percentage of oocytes which normally fail to resume meiosis (undergo GV dissolution) in culture. The rate of pseudo-cleavage is dependent upon the dose of CCB present in the culture medium (Fig. 5). Exposure of zona pellucida-free oocytes to CCB, in the presence of dbcAMP, results in a high incidence of 'blebbing' rather than in a true pseudo-cleavage (Fig. 3); a similar response could be obtained with oocytes surrounded by a zona pellucida, but as a rule, only at very high CCB concentrations.

Fig. 4. Analysis of the extent of pseudo-cleavage of mouse oocytes cultured in vitro under various conditions. Oocytes were cultured; A, in plain medium; B, in the presence of dbcAMP (100 ng/ml); C, dbcAMP (100 ng/ml) and dimethylsulphoxide (1%); D, CCB (5 ng/ml); E, dbcAMP (100 ng/ml) and CCB (5 ng/ml); for 16 h and the % pseudo-cleavage was determined. The data shown for each treatment represent the average of a minimum of 4 replicates, with a total of 100 or more oocytes.

The process of CCB-induced pseudo-cleavage of mouse oocytes has been characterized using light and electron microscopy of fixed and sectioned material. Examination of stained thick sections revealed that each of the compartments possessed distinctive ultrastructural features (Fig. 6). An obvious feature is the presence of a large number of microvilli at the surface of the anucleate compartment ('new compartment') and the virtual absence of microvilli surrounding the nucleate compartment ('old compartment') (Figs. 6, 7). Since isolated oocytes, cultured in vitro in the presence or absence of dbcAMP, possess many microvilli, the induction of pseudo-cleavage appears to result in an asymmetric loss of microvilli. An additional ultrastructural feature which always accompanies pseudo-cleavage is the pronounced localization of mitochondria around the periphery of the anucleate compartment (in apparent association with the microvilli), as compared to the random distribution of mitochondria in the cytoplasm of the nucleate compartment (Figs. 6, 7).
Fig. 5. The rate of CCB-induced pseudo-cleavage of mouse oocytes in vitro. Oocytes were cultured in the absence of CCB (○) and in the presence of 1 (●), 3 (●), 5 (△) or 10 (▲) μg/ml of CCB and the % pseudo-cleavage was determined at various intervals. The data shown represent the average of 2 replicates at each CCB concentration, with a total of 40 or more oocytes at each concentration.

**DISCUSSION**

The results of this study verify earlier reports that dbcAMP prevents the spontaneous meiotic maturation of mouse oocytes cultured in vitro (Stern & Wassarman, 1973, 1974; Cho et al. 1974; Wassarman, 1974; Wassarman & Turner, 1976). Moreover we have found that concomitant treatment of meiotically arrested oocytes with CCB induces the oocytes to undergo a process which we term 'pseudo-cleavage'. The CCB effect is reversible and is dependent on the meiotic state of the oocytes; oocytes allowed to resume meiosis (undergo GV breakdown) do not undergo pseudo-cleavage. Pseudo-cleavage is quite distinguishable from the cytochalasin-induced processes of 'blebbing' (Schroeder, 1970; Perry & Snow, 1975) and 'zeiosis' (Godman, Miranda, Deitch & Tannenbaum, 1975) already described.

Although cautioned by recent controversy concerning the site of action of CCB within cells, we suggest that CCB-induced pseudo-cleavage of mouse oocytes occurs as a consequence of the interaction between the drug and the oocyte surface and/or cortex. CCB was originally thought to interact specifically with contractile components of cell cytoplasm, impairing various aspects of cellular mobility; however, this interpretation has been criticized (Estensen, Rosenberg & Sheridan, 1971; Forer, Emmersen & Behnke, 1972; Pollard & Weihing, 1974). Certainly, the results of several studies of
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Fig. 6. Light micrographs illustrating the ultrastructural changes which accompany CCB-induced pseudo-cleavage of mouse oocytes in vitro. Shown is a toluidene blue-stained thick section from a mouse oocyte which has undergone pseudo-cleavage. A and B were printed under different conditions from the same negative so as to emphasize several of the ultrastructural features associated with pseudo-cleavage. Note in A the band of mitochondria associated with the surface of the anucleate compartment and in B the microvillous surface of the anucleate as compared to the nucleate, surface. 
gv, germinal vesicle; mt, mitochondria; mv, microvilli; n, nucleolus; zp, zona pellucida.

\( \times 1100 \text{ approx.} \)

cytokinesis in marine, amphibian, and mammalian eggs indicate that CCB interferes with normal cleavage; however, while in some instances the effect 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). The present work on CCB-treated mouse oocytes has not revealed significant ultrastructural alterations in the disposition of the oocyte microfilaments. On the other hand, the compartmentalization of CCB-treated oocytes into microvillar and amicrovillar segments suggests to us that the drug may exert its effect primarily at the oocyte surface and/or cortex. Such a proposal is consistent with recent evidence dealing with the effects of CCB on the transport of various small molecules and the relationship of these effects to the binding of CCB to plasma membrane (Kletzien, Perdue & Springer, 1972; Kletzien & Perdue, 1973; Lin, Santi & Spudich, 1974; Lin & Spudich, 1974). Furthermore, recent reports demonstrating an interaction between radioactively labelled CCB and lipid monolayers (Mayhew et al. 1974) strengthen the idea that cellular membranes may be directly affected by this drug. Since an oocyte freed of its zona pellucida responds to CCB treatment with blebbing at a large number
Fig. 7. Electron micrographs illustrating the ultrastructural changes which accompany CCB induced pseudo-cleavage of mouse oocytes in vitro. A, the surfaces of the nucleate (top) and anucleate (bottom) compartments at the 'furrow' between the 2 compartments. B, surface of the nucleate compartment displaying a smooth (amicrovillar) profile. C, surface of the anucleate compartment displaying a microvillar profile with an accompanying band of mitochondria. (Note that the mitochondrial morphology is that described by Wischnitzer (1967) for mouse oocytes.) D, surface of the anucleate compartment at higher magnification than in C. E, surface of an oocyte cultured for 16 h in the presence of dbcAMP (100 μg/ml) alone. F, surface of an oocyte cultured for 16 h in the presence of dbcAMP (100 μg/ml) alone, at higher magnification than in E.

A, B, C, D, E, F, x 11,800, 10,000, 10,000, 20,000, 7,500, 20,000, respectively.

of sites on the oocyte surface, it is possible that pseudo-cleavage reflects a very limited interaction of the drug with the mouse oocyte. Following the initial interaction at a particular region of the surface, which leads ultimately to pseudo-cleavage, subsequent interactions are ineffectual; such a situation would be analogous to the block to polyspermy following fertilization which is thought to involve the zona pellucida (Austin, 1965).

Because mitochondria and cytoplasmic vacuoles are preferentially localized to the microvillous compartment produced by CCB, we are encouraged to speculate that the oolemma may be segregated into transporting and non-transporting regions and that
CCB induces a topographical dissociation of these regions. In this context, it is not uncommon to find mitochondria associated with the microvillar surface of transporting epithelial cells which carry out secretory work against a gradient, such transport being energy-dependent (Berridge & Oschman, 1972). It is well known that within differentiated cells mitochondria are frequently localized to that area of the cell in which the energy demand is maximal, e.g. in muscle as rings around the T-band of the contractile myofibrils, in nerves at the synapse, in sperm at the neck of the cell where the head joins the tail, and in the renal tubule in the infoldings of the absorbing plasma membrane (Novikoff, 1961; De Robertis, Nowinski & Saez, 1970). Although in most cells the mitochondria are, in general, uniformly distributed throughout the cytoplasm, in some cases, they actually localize around the nucleus or in the peripheral cytoplasm. For example, it has been shown that during both mitosis and meiosis mitochondria are concentrated in the area of the spindle, once again emphasizing the intimate relationship between the cellular distribution of mitochondria and the energy demands of the cell. The localization of mitochondria at the periphery of the anucleate compartment of CCB-treated oocytes suggests that factors exist within the oocyte which are concerned with the establishment of compartmentation based upon membrane transport and/or other membrane functions. This hypothesis should be testable experimentally using the individual compartments described in this report.

In two other studies of the effect of CCB on oocyte maturation, morphological changes such as those described in this report, i.e. pseudo-cleavage and its associated characteristics, were not observed. Brachet et al. (1974) found that CCB, over a wide range of concentrations, had no effect on the progesterone-induced meiotic maturation of Xenopus laevis oocytes, as evidenced by GV breakdown. However, on the basis of certain morphological observations of amphibian oocytes in the presence of CCB, these investigators proposed that microfilaments may be important for maintaining the oocyte's polarity necessary for proper migration of the spindle and chromosomes towards the animal pole. Similarly, Longo (1972) reported that GV breakdown and formation of the first meiotic spindle took place normally in CCB-treated oocytes from the surf clam, Spisula solidissima; on the other hand, the drug did prevent the development of a cleavage furrow and, hence, the emission of a polar body. However, we have shown in this report that CCB-induced pseudo-cleavage is very dependent upon the meiotic state of the oocytes and that once the initial events of meiotic maturation (i.e. GV breakdown and chromosome condensation) have occurred, CCB does not induce pseudo-cleavage; instead, in the mouse, it inhibits meiotic progression at metaphase I (Wassarman, Josefowicz & Letourneau, unpublished results). Others have noted that the effect of CCB on mitotic cells is often dependent upon the cell cycle stage of the cells being treated (Miranda, Godman, Deitch & Tannenbaum, 1974; Porter, Prescott & Frye, 1973; Perry & Snow, 1975). Such dependency is probably related to changes in the surface architecture of mitotic cells during the cell cycle (Scott, Carter & Kidwell, 1971; Fox, Sheppard & Burger, 1971; Porter et al. 1973; Knutton, Sumner & Pasternak, 1975). In this context it is possible that changes in the architecture of the oocyte surface and/or cortex accompany the onset of meiotic maturation and that these changes make the oocyte refractile to CCB-induced pseudo-cleavage.
Indeed, it has been reported by several investigators that the permeability of amphibian oocytes to a variety of molecules changes during the period of progesterone-induced meiotic maturation (Morrill & Watson, 1966; Morrill, Rosenthal & Watson, 1967; Ecker & Smith, 1971; Pennequin, Schorderet-Slatkine, Drury & Baulieu, 1975).

The precise role of dbcAMP in these studies is not clear. The fact that dbcAMP prevents meiotic maturation of mouse oocytes is not surprising in view of several reports that elevated endogenous cAMP levels are associated with more stringent growth properties in normal and virally transformed cultured cells (Otten, Johnson & Pastan, 1971, 1972; Sheppard, 1972; Johnson & Pastan, 1972; Bombik & Burger, 1973). Since cAMP is known to affect various cellular parameters, such as cellular motility (Johnson, Morgan & Pastan, 1972a, b), transport (Hauschka, Everhart & Rubin, 1972), and morphology (Hsie & Puck, 1971; Hsie, Jones & Puck, 1971; Johnson & Pastan, 1972; Willingham & Pastan, 1975), it is difficult to assign the exact role of dbcAMP in these studies of CCB-induced pseudo-cleavage. It has been postulated that cAMP influences the organization of intracellular microfilamentous and microtubular systems, as well as the microstructure of the cell surface (Porter et al., 1973; Porter, Puck, Hsie & Kelley, 1974). This proposal has been defined further by Willingham & Pastan (1975) who suggested that the actin of microfilaments interacts in some way with the plasma membrane of the cell and that high levels of cAMP inhibit microfilament-dependent membrane functions (i.e. retraction of processes). If such be the case, it is possible that dbcAMP prevents those changes in the architecture of the surface and/or cortex of the mouse oocyte which accompany meiotic maturation and this in turn allows CCB induced pseudo-cleavage to take place.

While the mechanism by which CCB induces pseudo-cleavage of meiotically arrested mouse oocytes remains obscured, these observations strongly suggest that there is 'communication' between the oocyte's surface and GV during meiotic maturation. It is likely that the system described here will permit a more detailed analysis of those factors which regulate meiosis and the manner by which information received at the oocyte surface is transduced to the nucleus. This in turn should lead to a deeper understanding of cell surface-nuclear events that occur during cytokinesis in mitotic cells.

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