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Contents

Biochemical Transmitters Regulating the Arrest and Resumption of Meiosis in Oocytes

EIMEI SATO AND S. S KOIDE

Morphology and Cytochemistry of the Endocrine Epithelial System in the Lung

D W SCHEUERMANN

Intrinsic Nerve Plexus of Mammalian Heart: Morphological Basis of Cardiac Rhythmical Activity?

JOSEF MORAVEC AND MIREILLE MORAVEC

Structural and Functional Evolution of Gonadotropin-Releasing Hormone

ROBERT P MILLAR AND JUDY A KING

Excitons and Solitons in Molecular Systems

A S DAVYDOV

The Centrosome and Its Role in the Organization of Microtubules

I A VOROBIEV AND E S NADEZHDINA

Biochemical Transmitters Regulating the Arrest and Resumption of Meiosis in Oocytes[1]

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I. Introduction

Germ cells migrate to the genital ridge from the yolk sac region during early embryonic development. In the genital ridge, the female germ cells start to divide and differentiate into oogonia. They enter meiosis and become primary oocytes. Nuclear division progresses to the diplotene stage of the first meiotic prophase and is arrested. The chromosomes decondense and are distributed diffusely throughout the oocyte nucleus. Progression of meiosis to the diplotene stage occurs before or shortly after birth. The oocytes may remain arrested at the dictyate stage for a prolonged period Subsequently, a follicle develops enclosing the oocyte which contains a large clear nucleus designated as the germinal vesicle

It is generally accepted that the surge of luteinizing hormone (LH) during each ovarian cycle triggers the resumption of meiosis of the mature oocyte enclosed within Graafian follicles (Channing *et al.*, 1980, 1982a,b; Tsafriri, 1978b,c). The resumption of meiosis follows a sequence of programmed events while the oocytes are situated within the preovulatory follicles. The process is designated as oocyte maturation and is characterized by a series of biochemical, morphological, and functional changes that take place within the nucleus, highlighted by the following events: (1) dissolution of the nuclear membranes manifested as germinal vesicle breakdown (GVBD), (2) chromatin condensation and the formation of distinct chromosomes, (3) formation of the first meiotic spindle, (4) translocation of the spindle to the peripheral region, (5) formation and extrusion of the first polar body, (6) formation and positioning of the second meiotic division, (7) rearrest at the second metaphase.

To better understand the biochemical mechanisms involved in oocyte maturation, *in vitro* culture systems have been developed. Pincus and Enzmann (1935) were the first to demonstrate that rabbit oocytes removed

[1]The authors dedicate this paper to Dr Haruo Kanatani, pathfinder of starfish oocyte maturation and discoverer of 1-methyladenine

from follicles resume meiosis spontaneously and mature under *in vitro* culture without the addition of hormones. This phenomenon of spontaneous maturation has been observed in all mammalian species examined (Biggers, 1973). Oocytes with adhering cumulus cell complexes or denuded from cumulus cells are widely used as models to study oocyte maturation.

Studies with preovulatory follicles cultured *in vitro* demonstrate the interdependency of the various cells and fluid of the follicles and yield pertinent information on the resumption of meiosis triggered by LH added to the culture medium. The contrasting results obtained using denuded oocytes and follicle-enclosed oocytes suggest that maturation of mammalian oocytes is prevented by the follicular cells or factors in follicular fluid. By removing these factor(s) resumption of meiosis proceeds spontaneously. The involvement of the follicular cell–oocyte complex in the regulation of meiotic arrest was further investigated by coculturing isolated oocytes with follicular cells.

Several review articles are available on various aspects of mammalian oocyte maturation. General and historical accounts have been covered by Donahue (1972), Tsafriri (1978b,c, 1984), and Masui and Clarke (1979). Technical problems relating to the *in vitro* culture of oocytes were discussed by Biggers (1973) and McGaughey (1978). Biochemical events involved in mammalian oocyte maturation have been presented by Mangia and Canipari (1977) and Wassarman *et al.* (1978). Morphological and ultrastructural changes were described by Albertini (1984). Informative review articles on the hormonal control and factors regulating oocyte maturation have been published (Lindner *et al.*, 1974, 1977, 1983; Schuetz, 1974; Channing and Tsafriri, 1977; Baker, 1979; Thibault, 1977; Channing *et al.*, 1978, 1980, 1981, 1982a,b; Tsafriri and Bar-Ami, 1982; Tsafriri *et al.*, 1982a; McGaughey, 1983; Eppig, 1980a). Cell-to-cell communication of cumulus–oocyte complexes has been discussed by Schuetz (1978), Moor (1983), and Dekel (1984). In the present article factors sustaining meiotic arrest and regulating resumption of meiosis in mammalian oocytes are discussed. A hypothesis of the sequence of events during oocyte maturation is proposed, based on our recent results.

II. Factors Sustaining Meiotic Arrest

Oogonia undergo the initial stages of the first meiotic division to reach the dictyate stage of prophase. The oocyte may remain in meiotic arrest for a prolonged period until activated shortly before ovulation or may undergo atretic degeneration. This suspended metabolic state of oocytes is an unusual phenomenon and has attracted the attention of many in-

vestigators. To identify the factors that sustain meiotic arrest, studies have been conducted with fully grown oocytes obtained from untreated follicles. Although the *in vitro* results may not fully reflect the physiological state, the findings are relevant and significant.

Isolated oocytes will resume meiosis spontaneously when placed in hormone-free media (Pincus and Enzmann, 1937; Edwards, 1965), while follicle-enclosed oocytes remain in the dictyate stage (Tsafriri, 1978b; Lindner *et al.*, 1983). These findings suggest that the follicular microenvironment plays a dominant role in the physiological stability of oocytes at the dictyate stage. To clarify the role of various structural elements sustaining meiotic arrest, it has been found that oocytes in contact with granulosa cells remain arrested in the dictyate stage (Foote and Thibault, 1969; Sato *et al.*, 1982). Also follicular fluid and extracts of granulosa cells suppressed the occurrence of spontaneous maturation (Tsafriri and Channing, 1975a,b; Tsafriri *et al.*, 1976, 1977; Tsafriri, 1978a; Hillensjö *et al.*, 1978; Stone *et al.*, 1978; Channing *et al.*, 1983; Eppig and Downs, 1984; Downs *et al.*, 1985). These preliminary studies indicate that granulosa cells produce factors that sustain meiotic arrest. The meiotic-arresting factors to be discussed are cyclic nucleotides, cyclic nucleotide-potentiating factor, and maturation inhibitory peptides or meiosis-arresting peptides.

A. Cyclic Adenosine 3′,5′-Monophosphate (cAMP)

There are substantial number of reports supporting the hypothesis that cAMP maintains meiotic arrest in oocytes. This contention is based on the finding that the derivatized cAMP, dibutyryl cAMP (db cAMP), blocks the spontaneous resumption of meiosis of isolated cumulus-enclosed and cumulus-free oocytes cultured *in vitro* (Cho *et al.*, 1974; Magnusson and Hillensjö, 1977; Dekel and Beers, 1978, 1980; Nekola and Smith, 1975; Ahren *et al.*, 1978). Also activators of adenylate cyclase and inhibitors of phosphodiesterase elevate intraoocyte cAMP and prevent GVBD (Nekola and Smith, 1975; Hillensjö, 1977; Hillensjö *et al.*, 1978; Dekel and Beers, 1978; Ekholm *et al.*, 1984; Hubbard and Terranova, 1982; Dekel *et al.*, 1984; Powers and Paleos, 1982; Olsiewski and Beers, 1983; Sato and Koide, 1984a). Unmodified cAMP added to the suspending medium of oocytes did not influence the occurrence of GVBD. The finding that unmodified cAMP does not influence GVBD while the derivatized cAMP is an effective inhibitor is attributed to the low uptake of the unmodified cAMP by the oocytes, its low plasma membrane permeability, instability, and rapid metabolism (Hillensjö *et al.*, 1978).

To render credence to the hypothesis that cAMP is the regulator of

meiotic arrest, the level of this nucleotide in oocytes during the resting stage and following resumption of meiosis was determined. The cAMP content of resting oocytes was estimated to be 6.3 ± 0.7 fmol/oocyte (Moor and Heslop, 1981). Treatment with gonadotropin to induce maturation did not affect the cAMP level of oocytes. The addition of 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase activity, blocked the occurrence of spontaneous GVBD and induced a rise in the intracellular cAMP level of cumulus-free oocytes (Vivarelli *et al.*, 1983). In further support of this hypothesis, Schultz *et al.* (1983a,b) found that the level of oocyte cAMP decreased significantly during the period when the oocyte resumed meiosis. This fall in cAMP can be inhibited with IBMX at the same time preventing the occurrence of GVBD. This decrease in the oocyte's cAMP level precedes GVBD and occurs concomitantly with a paradoxical rise in cAMP of the follicular fluid and cumulus cells. These findings suggest that the resumption of meiosis in mammalian oocytes is triggered by a fall in oocyte cAMP level, similar to that observed with amphibian oocytes (Masui and Clarke, 1979). In the *Xenopus* oocyte progesterone probably acts by inhibiting adenylate cyclase (Sadler and Maller, 1985).

Cholera toxin, an activator of adenylate cyclase, inhibited spontaneous GVBD of cumulus-enclosed oocytes but not of cumulus-free oocytes (Dekel and Beers, 1980). The inability of cholera toxin to block GVBD of denuded oocytes is not clear in view of the fact that the oocytes are able to synthesize cAMP and that zona-free oocytes possess adenylate cyclase activity (Schultz *et al.*, 1983a,b; Urner *et al.*, 1983; Sato and Koide, 1984a; Bornslaeger and Schultz, 1985). The lack of response of denuded oocytes may be due to the lag period between the time of toxin exposure and the increase in cAMP level (Moss and Vaughan, 1979). The occurrence of a lag period before the resumption of meiosis is further supported by the observation that the fall in cAMP takes place earlier with denuded oocytes compared to cumulus-enclosed oocytes (Dekel and Beers, 1980). We have demonstrated that forskolin, an activator of adenylate cyclase, blocked GVBD of cumulus-free oocytes (Sato and Koide, 1984a). These findings using cholera toxin and forskolin suggest that oocytes do possess adenylate cyclase that lacks the stimulatory GTP-binding regulatory subunit. In this case the enzyme will not be affected by cholera toxin since adenosine diphosphoribosylation of the regulatory subunit will not take place (Gill, 1982).

During the initial period of oocyte maturation, protein synthesis takes place leading to GVBD. Richter and McGaughey (1981) reported that the oocytes synthesized stage-specific polypeptides during meiotic maturation and that db cAMP blocked the synthesis of some of these polypeptides.

The relationship of cAMP to protein synthesis is not clear. There are reports indicating that db cAMP did not alter the rate of protein synthesis nor the spectrum of proteins synthesized (Stern and Wassarman, 1974). Nonetheless the clearest evidence indicates that cAMP is involved in meiotic arrest. Determination of the level of cAMP in oocyte at the dictyate stage is a critical factor to test the validity of this hypothesis.

B. cAMP-Potentiating Factors

Intraoocyte level of cAMP may be the physiological factor sustaining meiotic arrest. In addition follicular fluid contains a cAMP-potentiating factor(s) (Eppig *et al.*, 1983; Eppig and Downs, 1984; Freter and Schultz, 1984; Downs *et al.*, 1985; Racowsky, 1983; Sato *et al.*, 1985). A factor was identified in porcine follicular fluid that blocks mouse oocyte maturation *in vitro* when combined with cAMP (Eppig and Downs, 1984). The substance was identified to be hypoxanthine (Fig. 1) (Downs *et al.*, 1985). Based on these findings it was proposed that the active factor is produced by a cAMP-dependent process in the granulosa–cumulus cells. The factor is transported to the oocyte through cytoplasmic channels that couple the cumulus cells to the oocyte. Alternatively, an inactive factor is taken up directly by the oocyte and activated by a cAMP-dependent process. The biochemical steps involved in the activation of the factor and the mech-

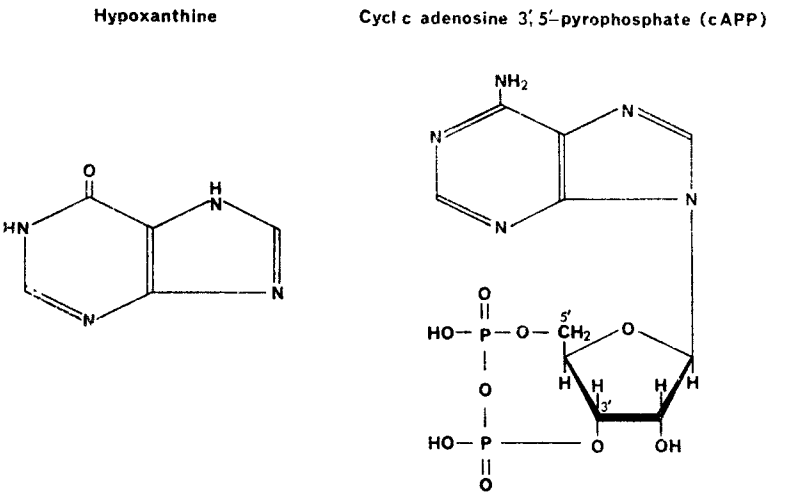

FIG. 1 Diagram of follicular substances that sustain meiotic arrest in mammalian oocytes

anism of its action are not clear. The presumptive control of oocyte maturation is that there is a decrease in the levels of both the follicular fluid factor and cAMP. A reduction of these factors may result from a fall in their production by the cumulus cells or alternatively a block in their transport from the cumulus cells to the oocyte. These events will trigger the resumption of meiosis.

We have recently purified a factor from bovine follicular fluid that inhibits mouse oocyte maturation in combination with cAMP (Sato *et al.*, 1985). The factor was purified by extraction with 70% ethanol, chromatography on a Dowex 1-X8 column, and reversed-phase high-performance liquid chromatography. The physicochemical properties of the follicular fluid substance are similar to that of cyclic adenosine 3′,5′-pyrophosphate (cAPP) (Fig. 1). Both the follicular fluid factor and cAPP in combination with db cAMP blocked mouse oocyte maturation in combination with cAMP and inhibited protein kinase activity.

17β-Estradiol inhibits maturation of denuded porcine oocytes (McGaughey, 1977). This steroid is effective when used in a chemically defined medium containing bovine serum albumin (BSA) or dextran (Richter and McGaughey, 1979), but not in a BSA-free medium (Racowsky and McGaughey, 1982). The inhibition is reversible (Richter and McGaughey, 1979). Testosterone can potentiate the maturation-arresting activity of db cAMP (Richter and McGaughey, 1981; Racowsky, 1983). Androgens, however, may modulate cAMP-induced meiotic arrest *in vitro* by being converted enzymatically to 17β-estradiol via the aromatase system (Racowsky, 1983). This conversion to 17β-estradiol as the mediator of the meiotic arrest is supported by the observations that follicle-stimulating hormone (FSH) and cAMP stimulate aromatase activity (Lacroix *et al.*, 1974; Moon *et al.*, 1975; Armstrong *et al.*, 1979; Lindsey and Channing, 1979; Anderson *et al.*, 1979).

C. Oocyte Maturation Inhibitor (OMI)

Inhibition of spontaneous maturation of isolated rabbit oocytes by follicular fluid was first described by Chang (1955). Tsafriri and Channing (1975a,b) demonstrated a similar factor in porcine follicular fluid designated as oocyte maturation inhibitor (OMI). Other investigators claim that follicular fluid does not influence oocyte maturation (Liebfried and First, 1980a,b; Racowsky and McGaughey, 1982; Fleming *et al.*, 1983). This controversy has not been resolved. An explanation was offered by Channing *et al.* (1982a) for the apparent contradictory results. They suggested

that follicular fluid contains an inhibitory factor and a maturation-inducing factor, and varying content of these two factors can account for the different results reported. The inhibitor and inducer were separated by chromatography on CM-Sephadex column (Channing *et al.*, 1982a). The inducer has not been characterized.

Several factors with maturation inhibitory activity are present in the follicular fluid. OMI is a peptide with an estimated molecular weight of 2000 (Tsafriri *et al.*, 1976; Stone *et al.*, 1978). A similar factor was extracted from granulosa cells (Centola *et al.*, 1981). The granulosa cell factor when added to the culture medium prevented oocyte maturation (Tsafriri, 1978b), suggesting that granulosa cells produced OMI. Also it was found that an extract prepared from granulosa cells of small follicles was more potent than those obtained from large follicles, indicating that its content decreases as the follicles mature, paralleling the physiological state of the follicles (Channing *et al.*, 1982a; Tsafriri *et al.*, 1982a,b; Tsafriri and Bar-Ami, 1982). Another oocyte maturation inhibitory factor was discovered in follicular fluid. Its properties differ from that of OMI (Chari *et al.*, 1983). It apparently potentiates the inhibitory potency of cAMP (Eppig and Downs, 1984) and is identified as hypoxanthine (Downs *et al.*, 1985).

A third inhibitory factor is an immunoreactive prolactin-like substance that cross-reacts with anti-prolactin antiserum (Baker and Hunter, 1978; Channing *et al.*, 1982a). When anti-prolactin antiserum is added to the medium containing follicle-enclosed porcine oocytes, maturation is accelerated (Baker and Hunter, 1978), suggesting that prolactin might induce oocyte maturation. It has been further suggested that this hormone may act indirectly on the oocyte by stimulating the granulosa cells to synthesize OMI (Channing *et al.*, 1982a,b). The production of OMI is blocked by testosterone and dihydrotestosterone (Channing *et al.*, 1982a). Since androgens or estrogens fail to influence oocyte maturation directly, follicular androgens probably act by decreasing OMI production by the granulosa cells (Channing *et al.*, 1982a). OMI acts on cumulus cells instead of directly on the oocytes since denuded oocytes will undergo spontaneous maturation in the presence of OMI (Hillensjò *et al.*, 1979). There are multiple effects attributed to OMI on the cumulus cells. It inhibits the spontaneous maturation of cumulus-enclosed porcine oocytes, prevents morphological differentiation of cumulus cells, and blocks progesterone secretion by cumulus cells (Schaerf *et al.*, 1982). These data suggest that cumulus cells take up OMI and transport it to the oocyte where it can sustain meiotic arrest. An alternative possibility is that OMI promotes the production of yet another inhibitor, e.g., cAMP. It may also act by preventing the formation of an oocyte maturation inducer.

D. Granulosa Cell Factor (GCF)

When isolated porcine oocytes are in contact with porcine granulosa cells, maturation is inhibited (Foote and Thibault, 1969; Sato *et al.*, 1977). Isolated oocytes will remain in the dictyate stage when juxtaposed to a layer of granulosa cells. The oocytes will undergo maturation when detached from these cells. Granulosa cells obtained from small follicles were more potent in preventing the spontaneous maturation of isolated oocytes than cells from Graafian follicles (Tsafriri and Channing, 1975a), suggesting that GCF is the active agent within the follicles. Other investigators claim that granulosa cells did not affect the occurrence of spontaneous maturation of isolated oocytes (Liebfried and First, 1980a,b). Nonetheless they found that segments of follicular wall attached to the oocytes can prevent the occurrence of GVBD. The addition of LH to hemisectioned follicles induced resumption of meiosis of the oocytes. The inhibitory potency of granulosa cells can be demonstrated providing the cells are in contact with each other (Sato *et al.*, 1977, 1980, 1982, 1984b, 1986). The mere coculturing of oocytes with a granulosa cell layer (about 10^7 cells) obtained from medium-sized (2–5 mm) follicles did not prevent the occurrence of spontaneous GVBD. Inhibition was observed only when the oocytes were in direct contact with the granulosa cells. Maturation block can be induced with cumulus-enclosed oocytes by having a portion of the granulosa cell layer in contact with the cumulus cells, whereas to induce maturation block of denuded oocytes the entire surface has to be enclosed by the granulosa cells (Sato *et al.*, 1982), indicating that meiotic arrest is dependent upon cell-to-cell communication between the cumulus–oocyte complex and the granulosa cells. These findings further suggest that the inhibitory factor is located on the surface of the granulosa cells or may be a component of the extracellular matrix of the granulosa cell layer.

The inhibitory factor can be extracted from the surface of granulosa cells with a buffer containing 1 *M* urea and 5 m*M* ethylenediaminetetraacetate (EDTA) (Sato and Koide, 1984b; Sato *et al.*, 1986). This buffer was used to dissociate sea urchin embryo (Kondo and Sakai, 1971) to extract surface components from cultured fibroblasts (Igarashi and Yaoi, 1975) and sperm-aggregating factor from *Spisula* oocytes (Sato *et al.*, 1983). Cells treated with the urea–EDTA solution recover without any deleterious effect. The maturation inhibitory factor was extracted from bovine granulosa cells with a buffer containing 1 *M* urea and 5 m*M* EDTA and purified by gel filtration on Sephadex G-25. Two protein peaks were obtained (Fig. 2). The material in the second (minor) peak at a concentration of 400 μg (dry weight)/ml of culture medium completely prevented spontaneous maturation of isolated mouse oocytes. At a lower concentration (50 μg/ml),

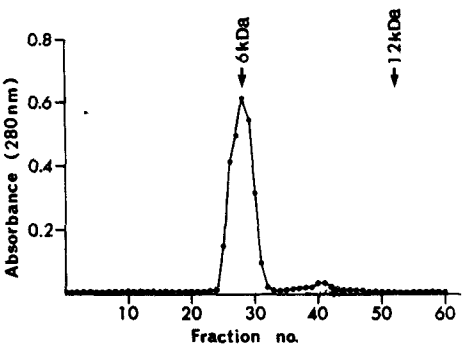

FIG 2 Gel filtration of peptides extracted from bovine granulosa cells on Sephadex G-25 column Column size. 1.5 × 75 cm Arrows indicate position of reference markers insulin, 6 kDa, bradykinin, 1 2 kDa. Fraction 1 (tube numbers 24–32) and fraction 2 (tube numbers 33–49) were pooled. Fraction 2 possessed oocyte maturation-preventing activity. Effective concentration was 400 μg/ml.

it blocked GVBD by 58% (Fig. 3). Peak 1 (major) possessed slight inhibitory activity. Inhibition was 58 and 6% at concentrations of 4000 and 500 μg/ml, respectively. The factor in peak 2, designated as granulosa cell factor (GCF), was further purified by affinity chromatography on Con A–Sepharose 4B column. The unabsorbed fraction contained the maturation-preventing activity showing that the factor is probably devoid of sugar moieties. The inhibitory effect of GCF was found to be reversible at lower concentrations. At a high concentration (400 μg/ml), the oocytes remain in meiotic arrest even after washing and transfer to the control medium. At a concentration of 200 μg/ml, approximately 10% of the oocytes have undergone GVBD. The remainder of the oocytes resumes meiosis after washing. GCF at a concentration of 50 μg/ml permits GVBD in 18% of the oocytes after 2 hours of incubation which increased gradually to 35% by the end of 6 hours. During this period, 86% of control oocytes have undergone GVBD. When oocytes are cultured in medium containing GCF for 3 hours and transferred to control medium, 76% of oocytes undergo GVBD compared to 35% of unwashed oocytes. The possibility of contaminating EDTA or urea to account for the inhibitory effect was excluded by experimental design (Sato *et al.*, 1984b, 1986).

GCF is a peptide since it is destroyed by Pronase but not by DNase, RNase, or glycosidase. Its estimated molecular weight has been determined to be less than 6000 by gel filtration on Sephadex G-25. Thus, GCF and OMI are related compounds possessing common properties.

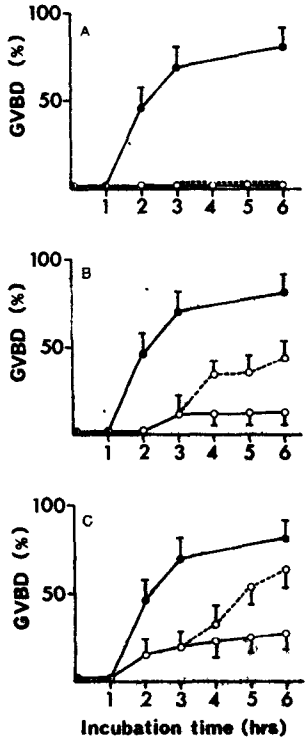

FIG. 3. Effect of bovine granulosa cell factor (GCF) on the time course of spontaneous GVBD of isolated mouse oocytes. Fraction purified by gel filtration on Sephadex G-25 was used. •——•, Control medium; o——o, GCF in the medium throughout the experiment; o--o, GCF in medium for 3 hours, oocytes washed three times, and resuspended in control medium. Concentrations tested were 400 μg of GCF/ml of medium (A), 200 μg/ml (B), 50 μg/ml (C). Values are mean ± SD (n = 5).

E. CALCIUM

Plasma membranes of many cells are usually impermeable to Ca^{2+}. When the cells are stimulated or activated they become sensitive to exogenous Ca^{2+}. These findings suggest that the mechanism of activation of mammalian oocytes and other cells may involve influx of exogenous calcium. External Ca^{2+} is essential in maintaining mouse oocytes viable in the culture medium (Paleos and Powers, 1981; De Felici and Siracusa, 1982). Small meiotically incompetent oocytes and early embryos do not require exogenous Ca^{2+} in the medium for survival, indicating that the

Ca^{2+} requirement is restricted to specific stages in the growth and development of oocytes (De Felici and Siracusa, 1982).

Various hypotheses have been proposed to account for the Ca^{2+} requirement of the dictyate oocytes. One possibility is that there is an activation of the membrane calcium pumps and internal calcium buffering systems in the oocytes upon release from the ovary. In the absence of extracellular calcium, the intraoocyte calcium level will fall below that required to sustain metabolic activities. Another reason is that calcium may be necessary for the repair of membrane injury sustained during the mechanical release of the oocytes from the follicles (Okamoto *et al.*, 1977).

The effect of calcium was studied by using the calcium ionophore A21387. Although the ionophore does not influence the spontaneous maturation of isolated rat oocytes, it can induce GVBD in follicle-enclosed rat oocytes (Tsafriri and Bar-Ami, 1978), suggesting that calcium may trigger the resumption of meiosis. This thesis is supported by the report that the total calcium concentration of cumulus-enclosed rat oocytes increases paralleling the serum LH level (Batta and Knudsen, 1980). The rise in oocyte calcium and serum LH levels occurs during the time maturation is initiated. Unfortunately the oocyte calcium level was not determined with the onset of GVBD. Although lowering calcium or magnesium content of the medium appears not to influence the resumption of meiosis of isolated bovine oocytes with adherent cumulus cells, oocyte maturation was blocked when cultured in calcium- and magnesium-free medium (Liebfried and First, 1979). We have demonstrated that there is a dramatic decrease in the incidence of GVBD of oocytes incubated in a Ca^{2+}- and Mg^{2+}-free medium (Sato *et al.*, 1980). This finding supports the thesis that calcium and magnesium ions are essential ingredients for the occurrence of GVBD.

It is interesting that db cAMP-induced meiotic arrest in mouse oocytes can be overcomed by elevating the extracellular calcium level; although at a higher concentration of db cAMP (0.2 m*M*), the block cannot be reversed with calcium (Paleos and Powers, 1981). Moreover, the ionophore is able to induced GVBD in oocytes treated with db cAMP (0.1 m*M*) (Powers and Paleos, 1982). These findings suggest that calcium and cAMP may regulate oocyte maturation by influencing a common mechanism. The proposed mechanism is based on the premise that the intracellular reservoir of calcium is sufficient to promote spontaneous GVBD *in vitro*. The addition of db cAMP to the medium may create a need for exogenous calcium (Powers and Paleos, 1982). It is postulated that db cAMP reduces cytoplasmic Ca^{2+} level by stimulating the calcium pumps of the membrane (Berridge, 1975) as found in other cell systems. To elucidate the role of intracellular Ca^{2+} in the resumption of meiosis of oocytes, further technical