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Original article

Control of germinal vesicle breakdown in bovine x murine hybrid oocytes

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Summary — Bovine oocytes cultured in control medium or in medium containing dibutyrylcyclic adenosine monophosphate (dbcAMP) or an inhibitor of cyclic nucleotide phosphodiesterase (3-isobutyl-1-methylxanthine, IBMX) undergo germinal vesicle breakdown (GVBD). On the other hand, mouse oocytes remain arrested at the germinal vesicle (GV) stage when dbcAMP or IBMX is present. When 1 bovine GV stage oocyte is fused to 1 GV stage mouse oocyte, dissolution of both species' GV occurred in dbcAMP-supplemented medium. Only when 4 to 5 GV stage mouse oocytes are fused to 1 GV stage bovine oocyte, and these giant cells are cultured in dbcAMP-medium, is maturation arrested with only GVs present in the cytoplasm. The inhibitory effect is more evident in IBMX-supplemented medium. Here nearly 50% of the fused cells exhibit GVs, both mouse and bovine, when 1 cattle GV oocyte is fused to 1 mouse GV oocyte and the fused cells are cultured for 24 h. Moreover, nearly all GVs are well preserved after fusion of 1 bovine oocyte to 2 or more mouse oocytes. When these hybrid cells after 24 h culture in IBMX are then washed and cultured in control medium for a further 24 h, GVBD occurred in all cells. We are of the opinion that this novel approach (*ie* mixing of sensitive and non-sensitive cytoplasm) may in the future better explain the mechanisms involved in the regulation of mammalian oocyte maturation.

meiosis / mammals / oocyte / cell fusion

Résumé — Contrôle de la rupture de la vésicule germinative dans des ovocytes hybrides (vache x souris). *Des ovocytes de vache cultivés dans un milieu témoin ou dans un milieu contenant du dibutyryl adénosine monophosphate cyclique (dbcAMP) ou un inhibiteur de la phosphodiestérase (3-isobutyl-1-méthylxanthine, IBMX) subissent la rupture de la vésicule germinative. Au contraire, des ovocytes de souris restent au stade de la vésicule germinative (VG) en présence de dbcAMP ou d'IBMX. Après fusion d'un ovocyte de vache et d'un ovocyte de souris, la rupture des VG se produit dans le milieu supplémenté en dbcAMP. C'est seulement quand 4 ou 5 ovocytes de souris sont fusionnés avec un ovocyte de vache que la maturation est bloquée au stade de la VG en présence de dbcAMP. L'effet inhibiteur de l'IBMX est supérieur : presque 50% des cellules restent au stade de la VG, après fusion d'un ovocyte de vache avec un ovocyte de souris et plus de 90% après fusion d'un ovocyte de vache avec 2 ovocytes de souris. Quand ces ovocytes hybrides cultivés 24 h avec IBMX sont lavés et cultivés dans le milieu témoin pendant 24 h, la rupture de la VG se produit dans tous les ovocytes. Cette nouvelle approche (mélange de cytoplasme sensible ou non) devrait être un outil pour étudier les mécanismes de la régulation de la maturation des ovocytes de Mammifères.*

méiose / Mammifères / ovocytes / fusion

INTRODUCTION

The role of cyclic adenosine monophosphate (cAMP) in the maintenance of meiotic arrest has been well documented in mouse and rat oocytes (Dekel and Beers, 1978; Schultz, 1988). Oocytes of cattle, sheep and hamsters reportedly do not show similar responses to cAMP or its analogs or to an inhibitor of cyclic nucleotide phosphodiesterase, 3-isobutyl-1-methyl xanthine (IBMX) (Jagiello *et al,* 1981; Racowsky, 1985b; Moor, 1988). In cattle the process of germinal vesicle breakdown (GVBD) is transiently delayed, but after 24 h of culture, condensed chromosomes are observed in the cytoplasm even with doses of dbcAMP or IBMX that are high in comparison with those effective in blocking GVBD in mice (Sirard and First, 1988). Also in sheep, GVBD occurs *in vitro* even in the presence of a cAMP-elevating agent (Moor and Heslop, 1981; Crosby *et al,* 1985; Moor and Seamark, 1986). The drop in intracellular concentration of cAMP observed in mouse oocytes (Schultz, 1988) during commitment is not found in sheep oocytes. Here the levels of cAMP increase rather than decrease (Moor and Heslop, 1981; Crosby *et al,* 1985; Moor and Seamark, 1986) although gonadotrophins were included in the culture medium. This possibly indicates that different mechanisms are included in the initiation of GVBD in domestic animals. The second possibility may be that commitment of maturation occurs during collection of oocytes. When these oocytes are then exposed to dibutyrylcyclic adenosine monophosphate (dbcAMP) or IBMX, it is too late to inhibit GVBD.

The aim of the present study was to test whether cAMP is involved in germinal vesicle (GV) maintenance in cattle. The oocytes were directly exposed to dbcAMP or IBMX or they were fused with mouse immature GV oocytes in the presence of these meiotic inhibitors.

MATERIALS AND METHODS

Bovine oocytes were isolated from follicles (3–5 mm in diameter) of slaughtered animals. These primary oocytes, for the purpose of this work, are termed immature, implying that meiotic progression to metaphase II has not occurred. They were isolated in 2 ways. By the first method, follicles were dissected from the ovaries and transferred into medium containing inhibitor. The oocytes were released directly into the medium with inhibitor. Only healthy oocytes with compact cumulus were used. Some oocytes were fixed immediately (control) while others were washed 3 times in medium containing inhibitor, transferred into droplets of culture media (0.1 ml) and incubated for 24 h in an atmosphere of 5% CO_2 in air at 38°C.

Because follicular dissection was time consuming and only a limited number of oocytes could be collected, a second method was chosen for other experiments. The oocytes were aspirated from follicles and immediately released into medium containing inhibitor along with other follicular contents. The concentration of inhibitors was initially 10 mM dbcAMP and/or 2 mM IBMX. An equal volume of follicular fluid was added to the test tubes so that at the end of collection the concentrations of inhibitors were 5 mM dbcAMP or 1 mM IBMX. The oocytes were selected under a stereomicroscope. Healthy oocytes with compact cumulus were washed 3 times in medium (5 mM dbcAMP, 1 mM IBMX) and cultured under the conditions described above.

Mouse oocytes were released from large antral follicles of females (ICR) primed 44 h previously with 5 IU pregnant mare serum gonadotropin. The medium contained 5 mM dbcAMP or 1 mM IBMX.

Prior to fusion, cumulus cells of both species of oocytes were removed by repeatedly pipetting through a narrow bore pipette. Zonae pellucidae were dissolved by 0.5% pronase for mouse oocytes and 0.1% for cattle oocytes. Oocytes were then agglutinated in a solution of phytohemmaglutinin (PHA, 300 μg/ml) so that 1 bovine oocyte was agglutinated to 1–5 mouse

oocytes. Agglutinated cells were washed briefly in protein-free medium and transferred into polyethylene glycol (PEG, M_r 1000). PEG was dissolved into protein-free medium (0.9 g/ml) and the oocytes were incubated in it for 30–45 s (Fulka, 1985). They were then washed 5 times in medium and cultured. The composition of the medium was as follows: 9 ml TC199, 1 ml isotonic glucose solution, 0.2 mM pyruvic acid and 50 µg/ml gentamicin sulfate. The medium was supplemented with bovine serum albumin (4 mg/ml). For manipulations, HEPES-buffered medium (pH 7.6) was used. After collection (control) and at the end of culture the oocytes were fixed in acid alcohol (1:3) and evaluated under Nomarski optics. Each experiment was repeated at least 5 times. All chemicals used were purchased from Sigma.

RESULTS

All control bovine oocytes isolated from ovaries contained intact GVs (32 oocytes examined). When cultured for 24 h in the inhibitor-free medium, GVBD occurred in all oocytes (table I) and ≈ 80% of these oocytes reached second metaphase. Germinal vesicles in mouse oocytes were observed under the stereomicroscope, so that only immature oocytes were used for fusion. No difference was observed when bovine oocytes were released from follicles directly into inhibitor-supplemented medium or when they were aspirated and then quickly released into this medium. The data are thus pooled (table I). It is apparent that GVBD was not blocked when bovine oocytes were incubated in medium containing inhibitors at the relatively high concentrations used. However, most of the oocytes cultured in dbcAMP or IBMX-supplemented medium were observed at late diakinesis or metaphase I. These data indicate that commitment to meiotic maturation probably did not occur during collection of oocytes. Low concentrations of inhibitors which block mouse oocyte maturation (dbcAMP 0.4–0.5 mM, IBMX 0.3 mM) had no effect upon maturation (data not shown).

In the next experiment, we fused 1 bovine oocyte with 1–5 immature mouse oocytes. The purpose of this study was to introduce cAMP-sensitive cytoplasm into insensitive cells. Moreover after fusion, the membrane permeable to dbcAMP or IBMX becomes part of a giant cell, so the possibility that the bovine oocyte membrane is impermeable to those drugs can be ruled out. As is seen in table II, meiotic maturation was not influenced in the hybrid cells when 1 GV-stage bovine oocyte was fused to 1–3 GV-stage murine oocytes and these cells were cultured in dbcAMP-supplemented medium. In these cells

Table I. Meiotic maturation of bovine oocytes in control, dbcAMP (5 mM) or IBMX (1 mM) supplemented medium.

Inhibitor	*Maturation stage**					
	N	*GV*	*LD–MI*	*AI–TI*	*MII + IPB*	*DEG*
Control	61	0	9	4	47	1
dbcAMP	103	3	80	5	12	3
IBMX	131	2	117	5	6	1

* GV: germinal vesicle; LD: late diakinesis; MI: metaphase I; AI: anaphase I; TI: telophase I; MII + IBP: metaphase II with the first polar body.

Table II. Effect of dbcAMP (5 mM) and IBMX (1 mM) upon bovine x mouse hybrid oocytes.

Inhibitor used	*Stage of maturation / type of fusion (bovine/mouse)**, **									
	1:1		*1:2*		*1:3*		*1:4*		*1:5*	
	GV	*GVBD*	*GV*	*GVBD*	*GV*	*GVBD*	*GV*	*GVBD*	*GV*	*GVBD*
dbcAMP (24 h)	0	22	0	10	2	9	8	4	10	1
IBMX (24 h)	23	21	27	2	11	0	6	0	11	0
IBMX (48 h)	7	5	9	1	16	0				

* One bovine oocyte was fused to the number of murine oocytes indicated by 1:1, 1:2, etc; ** GV: germinal vesicle; GVBD: germinal vesicle breakdown.

GVBD occurred in most cases with individually condensed groups of chromosomes seen in the cytoplasm.

Quite a different situation was seen when 4 or 5 mouse oocytes were fused to 1 bovine oocyte. Here, after 24 h of culture all GVs were well preserved. In unfused bovine oocytes GVBD occurred in all cases (36/36) and maturation proceeded up to metaphase I, whereas nearly all mouse oocytes arrested at the GV phase (72/78).

The inhibitory effect was much more evident when fused cells were incubated in IBMX-supplement medium. Here, nearly 50% of the fused cells contained 2 GVs when 1 immature mouse (GV) oocyte was fused to 1 bovine GV-stage oocyte (fig 1). In the rest, both GVs broke down and in the cytoplasm condensed chromosomes were visible (fig 2). Nearly complete inhibition, *ie* intact GVs (table II) was observed when more than 1 mouse GV-stage oocytes were fused to 1 bovine oocyte (fig 3).

In unfused bovine oocytes GVBD occurred in all cases (37/37), whereas mouse oocytes were arrested at the GV stage (78/82) after 24 h of culture (fig 4). Morphology of the chromatin in bovine oocytes was not different from intact oocytes. No inhibition was observed when fused cells were cultured in low concentrations of dbcAMP (0.5 mM or IBMX 0.3 mM).

In order to exclude the possibility that fusion treatment negatively influences bovine oocyte maturation, 2 immature bovine oocytes were fused and cultured in dbcAMP or IBMX-supplemented medium or in medium without inhibitor. Under these conditions maturation in fused cells was not different from control oocytes (IBMX GV:GVBD = 2:26; dbcAMP GV:GVBD = 2:40; no inhibitor GV:GVBD = 0:15). Because the inhibition was more evident with IBMX, this drug was used in further experiments. At first, reversibility of inhibition was tested. Cells were fused (bovine/mouse 1:2) and cultured for 24 h in IBMX. Some cells were then fixed. The remaining cells were washed in control medium and cultured for the next 24 h. A typical inhibitory pattern was observed in the control cells, while those cultured for an additional 24 h in control medium underwent GVBD in all cases (40 cells evaluated). This indicates that inhibition was fully reversible. The possibility that maturation is delayed only in IBMX medium was tested in the last part of the experiment. Fused cells were cultured for 48 h. The pattern of inhibition was not different in these cells compared to the cells cultured for only 24 h (table II).

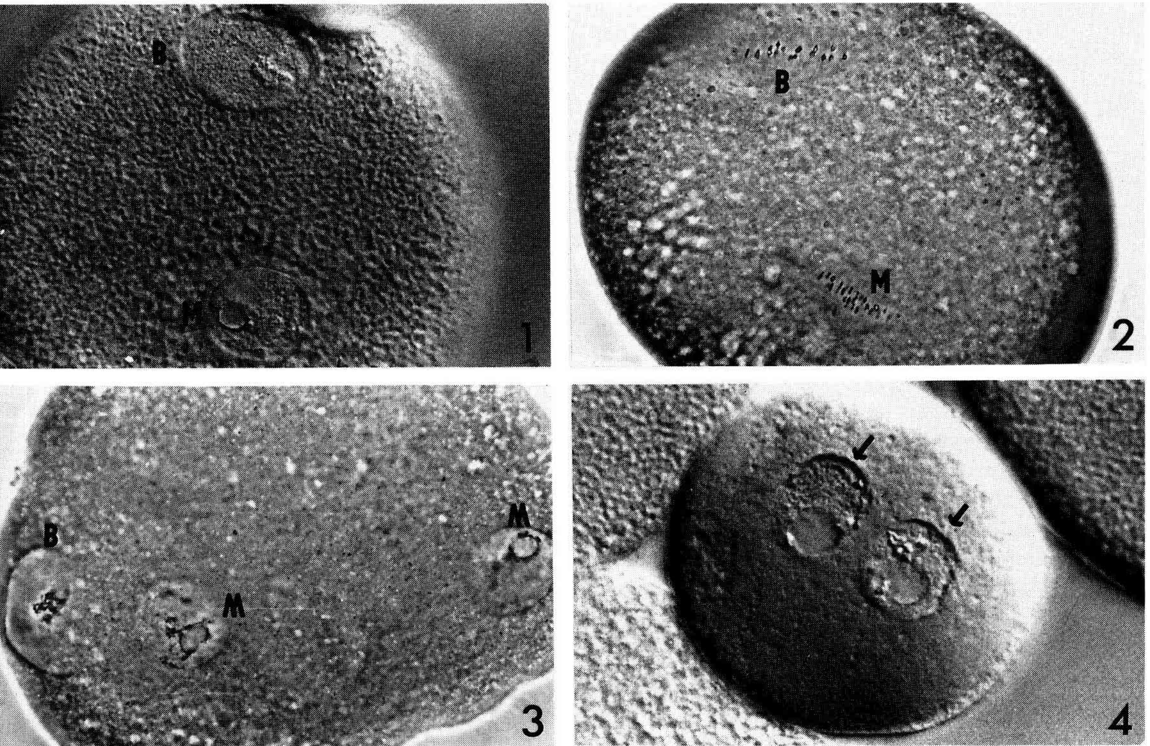

Fig 1. One bovine and one mouse immature oocyte were fused and cultured for 24 h in IBMX-supplemented medium. Germinal vesicles (GVs) of both species are well conserved in half the hybrid cells created and treated in this manner. Nomarski optics; x 200; M: mouse GV; B: bovine GV.

Fig 2. In the second half of the population of hybrid oocytes treated as in fig 1 both GVs broke down. In the cytoplasm 2 groups of chromosomes were observed; x 200; B: bovine; M: mouse chromosomes.

Fig 3. After fusion of 1 bovine (GV) oocyte to 2 mouse (GV) oocytes and culture in IBMX-medium, the complete inhibition of GVBDs was observed in nearly all cases and in the cytoplasm 3 GVs are clearly visible. B: bovine; M: mouse; x 200.

Fig 4. Mouse giant oocyte developed after occasional fusion of 2 immature oocytes and culture for 24 h in IBMX medium. Both GVs are perfectly visible; x 200.

DISCUSSION

Our data clearly show an inability of cAMP analogs or IBMX to cause more than a transient inhibition of GVBD in intact or zona-free bovine oocytes. Similar results were obtained by Sirard and First (1988) and Jagiello *et al* (1981), although Homa (1988) reported IBMX-inhibited meiotic resumption in bovine oocytes for a 24-h period. In most of these studies a transient delay of maturational events was also observed using agents recognized as meiotic inhibitors in mice. A more interesting situation was observed when immature bovine oocytes were fused to ≥ 1 immature

mouse oocytes. Here GVBD was prevented under certain conditions. The more potent inhibitor of this arrest was IBMX. One possible explanation may be that the bovine vitelline membrane has low penetrability to dbcAMP and IBMX. This penetrability is enhanced when the mouse vitelline membrane becomes part of the giant oocyte after fusion. We are not certain of the plausibility of this hypothesis. In sheep oocytes the levels of cAMP increase rather than decrease when *in vitro* maturation begins in the presence of gonadotropins (Moor, 1988), but time points selected for measurement were late in relation to initial commitment events. Also, in pigs, cattle and hamsters, cAMP analogs alone do not block GVBD (Racowsky, 1985a, b; Hubbard, 1986; Homa, 1988). This may mean that only mouse and rat oocytes belong to the category in which intracellular cAMP concentrations alone are sufficient to regulate meiotic state (Dekel and Beers, 1978; Schultz, 1988). In light of the fact that the bovine oocyte can be maintained in meiotic arrest (GV) by an invasive adenylate cyclase (Aktas *et al,* 1990), this may not be a tenable hypothesis.

Although we do not know the cause of the GV arrest induced in bovine oocytes in the presence of meiotic inhibitors when fused with an immature mouse oocyte, it is certainly possible that the bovine oocyte is responding to murine cytoplasmic regulatory factors. Conversely, the murine oocytes responded to maturational signals within the bovine cytoplasm under conditions which would normally prevent GVBD in murine oocytes. Whether the bovine oocyte is just less responsive to artificial agents that attempt to maintain increased cAMP levels or whether upstream signals for GV arrest differ slightly, it appears that the regulatory cascade needed for maintenance of meiotic arrest eventually coincide between the 2 species. The biochemical cascade leading to GVBD in mouse includes the drop of cAMP levels, thus influencing the phosphorylation status of so-called "X" protein. Its dephosphorylation leads then to GVBD (Schultz, 1988). However we must keep in mind the other modulators of this process (PKC, Ca^{2+}). In order to explain the results in this paper we have to study the mechanisms leading to GVBD in cattle oocytes in more detail. These processes were partially clarified only in the mouse, but not studied yet in bovine. The speculation of how the cytoplasm coming from mouse oocytes influences bovine GVBD and *vice versa* is at present very risky.

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