Effect of cycloheximide on nuclear maturation of pig and mouse oocytes

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Summary. Culture of mouse oocytes in medium with 1 or 100 µg cycloheximide/ml did not prevent germinal vesicle breakdown (GVBD). In contrast, GVBD in pig oocytes was absolutely blocked at concentrations of 1, 5, 10, 50 and 100 µg cycloheximide/ml, respectively. The inhibition of GVBD was not influenced by the presence or absence of cumulus cells and it was fully reversible. When cycloheximide treatment (5 µg/ml) was given after preincubation for 6, 12 and 16 h, GVBD occurred in 15, 46 and 75% of oocytes, respectively. It is concluded that proteins important for GVBD of pig oocytes were present in sufficient amounts at about 12 h of culture.

The fusion of pig oocytes in metaphase I to oocytes with an intact germinal vesicle revealed that cycloheximide did not inhibit GVBD induced by maturing ooplasm. Therefore, induction of prematurely condensed chromosomes by the maturing ooplasm did not require protein synthesis. However, continuous protein synthesis was necessary to maintain metaphase I and prematurely condensed chromosomes in a typical configuration.

Introduction

Protein synthesis plays an important role in the maturation of mammalian oocytes. Germinal vesicle breakdown (GVBD) is accompanied by qualitative and quantitative changes in the polypeptide pattern (McGaughey & Van Blerkom, 1977; Warnes, Moor & Johnson, 1977; Van Blerkom & McGaughey, 1978). Mouse oocytes cultured in a medium with puromycin or cycloheximide cannot proceed beyond the circularly arranged bivalent stage (for review, see Mangia & Canipari, 1977; Schultz & Wassarman, 1977). It means that GVBD in this species occurs independently of protein synthesis. Rat oocytes behave similarly, but when they are cultured in the presence of dbcAMP and puromycin and then in the medium with puromycin alone, GVBD is prevented (Ekholm & Magnusson, 1979). These results indicate the existence of short-lived proteins necessary for the initiation of meiosis. If protein synthesis related to the resumption of meiosis is required for the first 9 h in sheep oocytes (R. M. Moor & C. Polge, unpublished results, cited by Osborn & Moor, 1983), then there may be differences between mammalian species in sensitivity of GVBD to inhibitors of protein synthesis.

In the present experiments, we have compared the effect of cycloheximide on maturation of mouse oocytes, which need only 2 h for GVBD (Donahue, 1968), and pig oocytes, in which GVBD takes 16–20 h (Motlík & Fulka, 1976). The influence of cycloheximide on the appearance of prematurely condensed chromosomes after fusion was also tested.

Materials and Methods

Pig oocytes were obtained from ovaries of slaughtered animals by aspiration of antral follicles of about 5 mm in diameter. Only oocytes surrounded by compact cumuli were chosen for further

experiments. Mouse oocytes were isolated from late antral follicles of sexually mature females (strain A) and only those containing germinal vesicles were used.

Pig and mouse oocytes were cultured in 0.1 ml culture medium as droplets under paraffin oil at 38°C under 5% CO₂ in air according to the experimental scheme (see Tables 1 and 2). The culture medium contained 72 ml isotonic TC 199 medium (Usol, Prague, Czechoslovakia), 18 ml 1.45% NaHCO₃ + 0.002% phenol red, 10 ml 5.5% (v/v) glucose solution, 4 mg sodium pyruvate, 10 mg freeze-dried calf serum growth proteins (Usol)/ml, and 50 i.u. penicillin and 5 mg streptomycin/ml. This medium was supplemented by cycloheximide (Serva, Heidelberg, FRG) at concentrations 1, 5, 10, 50 or 100 µg/ml.

For fusion of pig oocytes (see Table 3) cumuli oophori were removed mechanically and zonae pellucidae were dissolved by pronase treatment (0.5%). Pairs of these oocytes were agglutinated in the culture medium supplemented with phytohaemagglutinin (100 µg/ml; Burroughs Wellcome, U.K.). The contact between oocytes was achieved by means of a fine glass micropipette. The agglutinated cells were washed in protein-free medium and transferred to polyethylene glycol (Lachema, Brno, Czechoslovakia) of molecular weight 6000 in a 50% (w/v) concentration for 45–60 sec. After repeated washing in the culture medium, the fused cells were cultured in medium containing cycloheximide (25 µg/ml) as described above.

At the end of all culture intervals oocytes and fused cells were mounted on slides, fixed in acetic alcohol (1:3, v/v) for at least 24 h, stained with 1% orcein and examined under the phase-contrast microscope.

For the developmental stages of germinal vesicle in the process of maturation the criteria published by Motlik & Fulka (1976) were used. In their criteria GV I represents an intact germinal vesicle with distinct nuclear membrane, finely granulated nucleoplasm and chromatin which is stained in the form of a ring only around a visible nucleolus. GV II is typical, with the appearance of orcein-positive zones localized on the nuclear membrane, and a discernible nucleolus. In GV III the nucleoplasm loses its granulation and, after staining, only the nucleolar area can be distinguished. The chromatin is distributed in well-stained clumps or filamentous bivalents always appear. GV IV is characterized by the chromatin occurring as more condensed individual bivalents, the nuclear membrane becoming less distinct and the nucleolus is completely absent.

Results

The present results confirmed that cycloheximide did not influence resumption of meiosis in mouse oocytes (Table 1). Concentrations of 1 or 100 µg/ml did not prevent GVBD. In contrast, pig oocytes were influenced by cycloheximide at a very low concentration. While about 90% of oocytes underwent GVBD in the control medium after 24 h of culture, all concentrations of cycloheximide (from 1 to 100 µg/ml) completely blocked GVBD. The inhibition of GVBD was not influenced by the presence or absence of cumulus cells. To check that these results were not caused by a toxic effect of cycloheximide, the reversibility of the meiotic block was tested. After 24 h of culture in medium with 5 µg cycloheximide/ml, oocytes were thoroughly washed in the culture medium and cultured for the following 24 h without cycloheximide. GVBD occurred in 112 of 117 cultured oocytes (95%).

To test the time of in-vitro maturation when protein synthesis important for GVBD of pig oocytes occurred, pig oocytes were preincubated for 6, 12 and 16 h in culture medium and then cultured in the presence of 5 µg cycloheximide/ml for an additional 18, 12 and 8 h, respectively. All oocytes fixed after 6 h of preincubation were in GV I. After 18 h of culture in medium with 5 µg cycloheximide/ml only 15.3% of oocytes underwent GVBD (Table 2). After 12 h of preincubation oocytes at GV II predominated, and only 6% did not possess a germinal vesicle. Subsequent culture for 12 h in cycloheximide led to GVBD in 46% of oocytes. After 16 h of preincubation, oocytes were mainly in GV III and GV IV and about 20% exhibited GVBD. Further culture for 8 h

Table 1. Effect of cycloheximide on nuclear maturation of mouse and pig oocytes

Species	Time of culture	Type of culture*	Conc. cycloheximide (µg/ml)	No. of oocytes	No. of oocytes:		
					With GV	With GVBD	Degenerated†
Mouse	6 h	C+	0	40	3	37	
		C+	1	40	5	35	
		C+	100	40	8	52	
Pig	24 h	C+	0	44	5	39	
		C+	1	71	66	1	4
		C-	1	64	61		3
		C+	5	61	60		1
		C+	10	66	63		3
		C+	50	59	58		1
		C+	100	56	55		1

* Oocytes were cultured with a compact cumulus (C+) or the cumulus was removed mechanically before culture (C-).

† Oocytes in which the stage of maturation could not be recognized by phase-contrast microscopy.

Table 2. Effect of oocyte preincubation on cycloheximide block of nuclear maturation in pig

Time of culture (h) in		No. of oocytes	No. of oocytes:			
Control medium	Medium with 5 µg cycloheximide/ml		With GV	With GVBD	Degenerated*	% of GVBD
6	0	28	27	1	0	3.5
6	18	183	143	28	12	15.3
12	0	29	27	2	0	6.8
12	12	285	139	131	15	46.0
16	0	43	34	9	0	20.9
16	8	209	40	157	12	75.1

* Oocytes in which the stage of maturation could not be recognized by phase-contrast microscopy.

Table 3. Fusion and culture of pig oocytes in medium with cycloheximide

Type of fusion	Medium with cycloheximide (25 µg/ml)	Culture after fusion (h)	No. of fused cells	No. of cells with:			
				2 M I	M I+PCC	2 clumps of chromatin	1 clump of chromatin +1 GV 2 GV's
M I+GV	-	3	18		16		
	+	3	31			26	5
M I+MI	-	3	14	14			
	+	3	24			22	2

GV, oocytes fused immediately after isolation from follicles; M I, oocytes cultured for 24 h before fusion; PCC, prematurely condensed chromosomes.

with cycloheximide led to GVBD in 75% of oocytes. All preincubated oocytes that did not resume meiosis in the presence of cycloheximide contained germinal vesicles at stages I or II.

The fusion of pig oocytes in metaphase I with one oocyte at the germinal vesicle stage revealed that cycloheximide did not inhibit GVBD induced by maturing ooplasm (Table 3). However, the

behaviour of metaphase and prematurely condensed chromosomes was quite different in control experiments and in experiments with cycloheximide. After 3 h of culture in the control medium, the fused cells had perfect groups of metaphase I and prematurely condensed chromosomes. Induction of prematurely condensed chromosomes occurred in all fused cells. However, after 3 h in medium with cycloheximide, individual bivalents had a tendency to aggregate together and form one or two clumps of condensed chromatin. To confirm this effect of cycloheximide on metaphase I chromosomes, 2 oocytes in metaphase I were used and cultured with or without cycloheximide. While the metaphase I configuration in the fused cells was quite typical in the culture medium, the presence of cycloheximide induced, 3 h after fusion, formation of chromatin clumps as described above.

In four instances two germinal vesicles were found in giant cells. These particular cases were apparently caused by fusion of two oocytes both containing germinal vesicles instead of being at metaphase I. The probability that the oocytes chosen for fusion had remained in the germinal vesicle stage is very low, because 80–90% of them progressed after 24 h of culture to late diakinesis or metaphase I. However, in our experiments obviously oocytes that did not progress beyond the germinal vesicle stage were taken.

Discussion

The present results have revealed a high sensitivity of pig oocytes to cycloheximide since GVBD was prevented even at a concentration 1 µg cycloheximide/ml. Under the same culture conditions, GVBD of mouse oocytes was insensitive to this inhibitor of protein synthesis. These experiments indicate the substantial species differences in the requirement of protein synthesis for GVBD. The results of Ekholm & Magnusson (1979) show that this difference may be related to the time sequence of GVBD. When maturation of rat oocytes is prevented by dibutyl cAMP and protein synthesis is blocked concomitantly, subsequent culture with puromycin alone inhibits GVBD. These results indicate the existence of short-lived proteins in fully grown rodent oocytes which are necessary for rapid GVBD (Donahue, 1968; Zeilmaker, Vermeiden, Verhame & van Vliet, 1974). The continuous presence of puromycin leads to the depletion of these proteins which may be analogous to the tyrosine-rich short-lived proteins synthesized during the first 3 h of maturation in mouse oocytes (Mangia & Canipari, 1977). The high sensitivity of pig oocytes to cycloheximide indicates that specific proteins necessary for GVBD are not present in the ooplasm of fully grown immature pig oocytes. When cycloheximide treatment was given after preincubation for 6, 12 or 16 h, GVBD was observed in 15, 46 and 75% of pig oocytes, respectively, indicating that active protein synthesis in the period preceding chromatin condensation (10–14 h of culture) is necessary for the resumption of nuclear maturation. Significant changes in the pattern of protein synthesis before GVBD in pig oocytes support this conclusion (McGaughey & Van Blerkom, 1977). Pig oocytes with more or less condensed bivalents in the nucleoplasm (GV III and GV IV) were not blocked by cycloheximide. Similarly, in progesterone-treated toad (*Xenopus*) oocytes, the later cycloheximide treatment is initiated, the higher the number of oocytes that undergo GVBD (Wasserman & Masui, 1975). The resistance of toad oocytes to cycloheximide coincides with the time at which the maturation promoting activity rapidly increases. This observation indicates that only the initial phase of induction of this activity may be inhibited by cycloheximide, but further amplification of the maturation-inhibiting activity is resistant to the inhibitor. Maturation-inhibiting activity also occurs in the cytoplasm of growing pig oocytes because nuclear maturation of fully grown oocytes is blocked after homologous fusion (Fulka, Motlík, Fulka & Crozet, 1985). This inhibiting activity can be gradually overcome by preincubation of fully grown oocytes. The length of the preincubation interval leading to the abolition of maturation-inhibiting activity well coincides with the sensitive period of pig oocytes to cycloheximide observed in the present experiments. It is possible that the oocytes preincubated for 12 h possessed the active molecules of maturation

promoting factor and their further amplification cannot be prevented by cycloheximide or by the maturation inhibiting activity of growing oocytes.

In accordance with observations of toad oocytes (Wasserman & Masui, 1975), maturation promoting activity of maturing ooplasm induced prematurely condensed chromosomes in the fused oocytes, even in the presence of cycloheximide. However, after 3 h in medium with cycloheximide individual bivalents had a tendency to aggregate and to form one or two clumps of condensed chromatin. We have concluded that the induction of prematurely condensed chromosomes by maturing ooplasm does not require protein synthesis, but that continuous protein synthesis is necessary to maintain metaphase I and prematurely condensed chromosomes in a typical configuration. In contrast, Clarke & Masui (1983, 1985) treated mouse oocytes with puromycin and observed formation of the first polar body and transformation of chromosomes to interphase nuclei. The opposite behaviour of chromosomes in our experiments with pig oocytes might have been caused by a qualitative difference in proteins synthesized at the time of inhibitor application.

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