

## Is cAMP decrease essential for resumption of meiosis in mouse oocytes?

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**Summary** — Resumption of meiosis was inhibited in mouse oocyte cumulus complexes (OCC) co-cultured with pig membrana granulosa (PMG). After 3 and 6 h of co-culture these oocytes possessed an intact nuclear envelope and their nucleolar surface was associated with granules  $\approx$  80 nm in diameter. Preincubation of OCCs for 30, 45, 60 or 90 min followed by co-culture with PMG for 2 h of either OCCs or denuded oocytes resulted in germinal vesicle breakdown (GVBD) in  $\approx$  0, 30, 70 and 100% mouse OCCs and in  $\approx$  30, 60, 80 and 100% denuded oocytes, respectively. It seems that the inhibitory contact between mouse oocytes and PMG was established during the first h of co-culture. After isolation from antral follicles the oocytes contained 2.75 fmol cyclic adenosine 3', 5'-monophosphate (cAMP). When OCCs were co-cultured for 1, 2 or 3 h with PMG, the amount of cAMP per oocyte was 1.34, 1.33 and 1.51 fmol, respectively. After culture of OCCs in control medium the amount of cAMP was 1.21, 1.39 and 2.16 fmol, respectively. The present results suggest that the inhibitory activity of PMG is not species-specific. Moreover, PMG prevented resumption of meiosis in mouse oocytes in spite of the cAMP drop in oocyte cytoplasm characteristic of the resumption of meiosis.

**mouse oocyte / meiosis / cAMP / pig membrane granulosa**

**Résumé** — La décroissance de l'AMPc est-elle essentielle pour la reprise de la méiose dans les ovocytes de souris ? La reprise de la méiose a été inhibée dans des complexes ovocyte-cumulus (OCC) de souris cocultivés avec de la «membrana granulosa» de porc (PMG). Ces ovocytes conservent une enveloppe nucléaire après 3 et 6 h de coculture et la surface nucléolaire est associée avec des granules de 80 nm de diamètre. La préincubation des OCCs pendant 30, 40, 60 ou 90 min puis la coculture avec la PMG d'OCCs ou d'ovocytes dénudés aboutit à la rupture de la vésicule germinative (GVBD) dans environ 0, 30, 70 et 100% des OCCs et environ 30, 60, 80 et 100% des ovocytes dénudés, respectivement. Il semble que l'inhibition par contact entre la PMG et les ovocytes s'établit pendant la première heure de coculture. Après isolement de follicules à l'antrum, les ovocytes contiennent 2,75 fmol d'adénosyl 3'5' monophosphate cyclique (AMPc). Quand les OCCs sont cocultivés pendant 1, 2 ou 3 h avec la PMG, la concentration d'AMPc par ovocyte est respectivement de 1,34; 1,33 et 1,51 fmol. Après culture des OCCs dans le milieu témoin, la concentration d'AMPc est alors de 1,21; 1,39 et 2,16 fmol. Les résultats présents suggèrent que l'activité inhibitrice de la PMG n'est pas limitée à l'espèce. De plus, la PMG empêche la reprise de la méiose d'ovocytes de souris en dépit de la chute d'AMPc, caractéristique de cette reprise, dans le cytoplasme ovocyttaire.

**ovocyte de souris / méiose / AMPc / membrana granulosa de porc**

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## INTRODUCTION

The hypothesis that the antral follicles inhibit the resumption of meiosis until the preovulatory gonadotropin surge was first put forward by Pincus and Enzman (1935). Further studies have confirmed that either intrafollicularly or under *in vitro* conditions, the membrana granulosa and cumulus granulosa cells are required for the prevention of oocyte maturation (Foote and Thibault, 1969; Tsafiri and Channing, 1975; Leibfried and First, 1980; Racowsky and Baldwin, 1989). In addition, pig oocytes surrounded by the cumulus with a part of membrana granulosa directly adjacent to the cumulus do not mature in gonadotropin-free medium (Mattioli *et al*, 1988; Motlik *et al*, 1991). We have recently found that under suitable culture conditions a piece of pig membrane granulosa (PMG) is able to prevent resumption of meiosis in cattle oocytes (Kalous *et al*, 1993). This heterologous effect of PMG was used in the present experiments when the mouse OCCs (OCC) were co-cultured with a piece of PMG.

Under *in vitro* conditions, analogs of cAMP and phosphodiesterase inhibitors prevent spontaneous meiotic resumption in mouse (Cho *et al*, 1974; Wassarman *et al*, 1976) and rat oocytes (Magnusson and Hillensjo, 1977; Dekel and Beers, 1978). These findings imply that a drop in the cAMP levels in rodent oocytes is related to germinal vesicle breakdown (GVBD). In fact, a decrease in oocyte cAMP precedes GVBD in mouse (Schultz *et al*, 1983a, b; Vivarelli *et al*, 1983) and rat oocytes (Racowsky, 1984; Aberdam *et al*, 1987).

The present experiments were undertaken to answer 2 questions: first, does PMG prevent resumption of meiosis in oocytes with a rapid time sequence of GVBD? Second, does co-culture with PMG influence cAMP levels in the cytoplasm of the mouse oocytes?

## MATERIALS AND METHODS

### *Oocytes and granulosa cells*

Mouse oocytes were isolated from large antral follicles of sexually mature females (strain A) primed 48 h before with 5 IU pregnant mare serum gonadotropin. Only oocytes surrounded by compact cumulus were chosen for these experiments. Membrana granulosa was isolated from ovaries of slaughtered prepubertal gilts. Only large antral follicles (7–10 mm in diameter) with a transparent, vascularized follicular wall were dissected. With the help of 2 preparation needles, large pieces of granulosa layer were carefully separated from the theca. Only pieces of the PMG containing  $\approx 1-3 \times 10^5$  granulosa cells were used. A previous ultrastructural study (Kalous *et al*, 1993) revealed that PMG was always isolated with the basement membrane.

### *Co-culture of mouse oocytes with pig membrana granulosa*

In the present experiments modified Parker's culture medium (M-199, Sevac, Prague) supplemented with 2.92 mM Ca-lactate, 2 mM Na-pyruvate, 33.9 mM Na-bicarbonate, 4.43 mM Hepes buffer, 50 IU/ml penicillin, 50 mg/ml streptomycin sulfate, and 10% heat-treated bovine serum (BOS, Sevac, Prague) was used (Pavlok *et al*, 1988).

About 15 mouse OCCs were placed on the basement membrane (concave side) of each piece of PMG and cultured in 0.5 ml Parker's medium under paraffin oil at 38°C under 5% CO<sub>2</sub> in air for 1, 2, 3, or 6 h. In preincubation experiments,  $\approx 10$  OCCs were cultured in 0.1 ml of the medium under the above-described conditions for 30, 45, 60 and 90 min before either oocytes with compact cumulus or oocytes with mechanically removed cumulus (cumulus-free oocytes) were co-cultured for 2 h with PMG.

As a control, mouse OCCs were cultured in the control medium (control I) or in the medium with several pieces of PMG but without direct contact with them (control II). In further control experiments, the PMG basement membrane was covered with a nitrocellulose membrane. Both the adhered membranes and OCCs were

situated on the nitrocellulose membrane and were cultured for 3 h.

At the end of the culture period, OCCs were removed from contact with PMG and used either for light and electron microscopic evaluation or for cAMP determination.

### **Determination of cAMP by radioimmunoassay (RIA)**

After isolation (0 h) or incubation (1, 2 or 3 h) the OCCs were transferred to medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) to minimize cAMP hydrolysis within the cells. Cumulus cells were mechanically removed from the oocytes. Denuded oocytes were washed 3 times in the medium with IBMX and then transferred into 10  $\mu$ l 0.5% sodium dodecyl sulfate (SDS, w/v; Racowsky, 1984). When lysis was completed, the samples were capped and stored at  $-80^{\circ}\text{C}$ . Before extraction with 100  $\mu$ l 6% trichloroacetic acid (TCA) at  $4^{\circ}\text{C}$ , 10  $\mu$ l bovine serum albumin (10 mg/ml) was added to each microcap to act as carrier protein. The extracted samples were centrifuged at 2 000  $g$  for 15 min and supernatants were removed and lyophilized. TCA extracts were diluted and assayed after acetylation. Cyclic AMP was measured by radioimmunoassay using kits (UVVVR, Prague) (Procházka *et al.*, 1991). The sensitivity of the assay was 4 fmol/tube. The intra- and inter-assay coefficients of variation in this RIA were 7.8 and 10.2%. The significance of the differences in cAMP concentrations between experimental groups was assessed using the Student's *t*-test.

### **Light microscopy**

To prepare the OCCs for light microscopy cumulus cells were mechanically removed. Oocytes were mounted on slides, fixed in an acetic acid/alcohol (1:3) mixture for 24 h, stained with orcein and examined under a phase-contrast microscope. Frequency of GVBD in I and II was calculated from data pooled from 3 replicate experiments and the frequency of GVBD between groups was compared by  $\chi^2$  analysis.

### **Electron microscopy**

Samples were fixed in a mixture of 0.6% paraformaldehyde and 2.5% glutaraldehyde in 0.2 cacodylate buffer (pH 7.4) for 90 min, washed, postfixed in 1%  $\text{OsO}_4$  in cacodylate buffer for 1 h and dehydrated in ascending ethanol series. After infiltration in a mixture composed of propylene oxide and Epon, the samples were embedded in Epon 812. The blocks were cut in Reichert–Jung Ultracut, mounted on grids, contrasted with uranyl acetate and lead citrate, and examined in a Jeol 1200 EX electron microscope.

## **RESULTS**

### **Co-culture**

When the freshly isolated mouse OCCs were cultured on PMG, the OCCs did not resume meiosis during the 3-h (table I) and 6-h (data not shown) co-culture period. The mouse denuded oocytes were also blocked at the GV stage by PMG. In contrast, mouse OCCs cultured in the same drop of medium but without direct contact with PMG matured spontaneously. A sheet of nitrocellulose membrane between PMG and OCCs abolished the inhibitory effect of PMG upon mouse oocytes. It was concluded that the direct contact of the mouse denuded or cumulus-enclosed oocytes with PMG was essential to prevent resumption of meiosis.

Fifty-six percent of the mouse oocytes denuded after the 45-min preincubation period underwent GVBD during the subsequent 2-h co-culture with PMG (table II). The cumulus-enclosed oocytes required 60-min preincubation to undergo GVBD (66%) during the co-culture period. These data indicate that the inhibitory effect of PMG upon GVBD was effective in the commitment period only.

**Table I.** GVBD in mouse OCCs co-cultured with PMG and nitrocellulose membrane.

Type of culture	No of oocytes	Stage of meiosis		GVBD (%)
		GV	LD/MI	
Control I	54	2	53	96 <sup>a</sup>
Control II	59	3	47	95 <sup>a</sup>
OCCs and PMG	46	46	–	0 <sup>b</sup>
OCCs and NM	51	1	50	98 <sup>a</sup>
OCCs and PMG + NM	47	2	45	96 <sup>a</sup>

Mouse OCCs were cultured for 3 h in control medium (control I), in the presence of PMG without contact with PMG (control II), in contact with PMG (OCCs and PMG), in contact with nitrocellulose membrane (OCCs and NM) and in contact with nitrocellulose membrane adhering to PMG (OCCs and PMG + NM). Values with different superscripts are significantly different ( $P < 0.05$ ).

### cAMP levels

In the control medium, all oocytes underwent GVBD during 3 h of culture. The alterations in intraoocyte cAMP content in oocytes during culture are shown in table III and figure 1. The oocyte intracellular cAMP content was  $2.75 \pm 0.24$  fmol/oocyte before incubation (0 h). The significant drop in intraoocyte cAMP content, which was detected after 1 h of incubation ( $1.21$

$\pm 0.07$  fmol/oocyte), was followed by GVBD. After co-culture with PMG, the resumption of meiosis was prevented during 1, 2 or 3 h of incubation, but surprisingly the significant cAMP drop was detected in the mouse oocytes ( $1.34 \pm 0.12$  fmol/oocyte) after 1 h of co-culture. No significant difference was observed in the intraoocyte cAMP content after 1 h incubation when the control and PMG groups were compared.

**Table II.** The effect of PMG upon GVBD in preincubated mouse OCCs.

	Cumulus-enclosed oocytes					Cumulus-free oocytes				
	0	30	45	60	90	0	30	45	60	90
Min	0	30	45	60	90	0	30	45	60	90
GVBD (%)	0 <sup>a</sup>	0 <sup>a</sup>	31 <sup>b</sup>	66 <sup>c</sup>	100 <sup>d</sup>	0 <sup>a</sup>	34 <sup>b</sup>	56 <sup>c</sup>	77 <sup>d</sup>	100 <sup>e</sup>

Mouse OCCs were preincubated for 30, 45, 60 and 90 minutes in control medium and subsequently OCCs (cumulus-enclosed oocytes) and oocytes after mechanical removing of cumulus (cumulus-free oocytes) were co-cultured with PMG for 2 h. Each interval was repeated 3 times and at least 50 oocytes were used. Values with different superscripts are significantly different ( $P < 0.05$ ).

**Table III.** Cyclic AMP levels in oocytes incubated without PMG.

	Group			
	C-		OCC	
Time (h)	0	1	2	3
Mean	2.75*	1.21	1.39	2.16
SEM	0.24	0.07	0.24	0.23

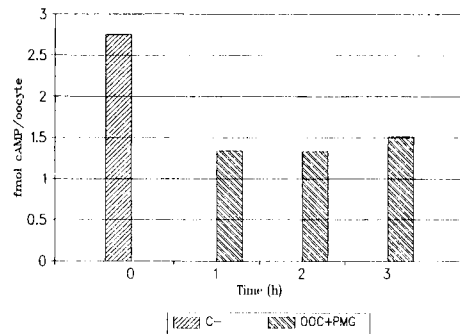
The mouse oocyte cumulus complexes (OCC) were cultured for 1, 2, and 3 h in control medium. The oocytes were denuded (C-) before (time zero) or after incubation in control medium (time 1, 2 and 3 h). Values represent the mean fmol cAMP per oocyte and the SEM from at least 3 independent experiments. \* The time zero is significantly different from that of other groups ( $P < 0.05$ ).

### Electron microscopy

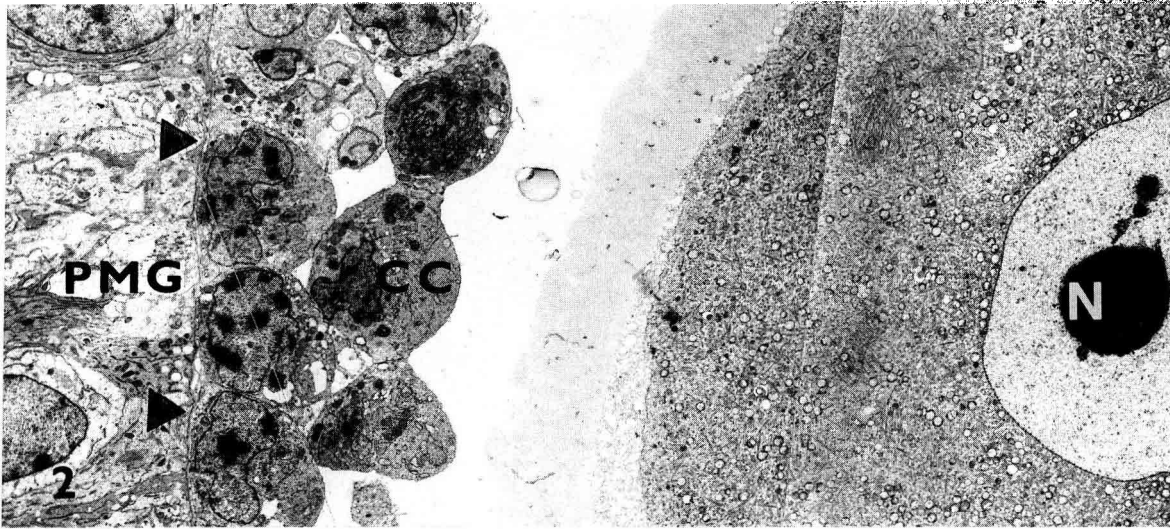
The co-culture of mouse OCCs with PMG resulted in the close attachment of mouse cumulus cells (CC) to the pig basement membrane (BM) (fig 2). The cells in the mouse cumulus were interconnected by extensive network of gap junctions (GJs) at all time points (0, 3, 6 h of culture) (fig 3A). Similarly, a large number of GJs coupled PMG cells during co-culture (fig 3B). Finally, the cytoplasmic projections of mouse corona radiata cells remained in contact with the oocyte after 6 h co-culture with PMG (fig 4). The direct contact between cytoplasmic membranes of PMG cells and mouse CC was prevented by the presence of the continuous pig BM (fig 5). In no case did the cells from the pig or mouse compartment of the co-culture system penetrate across the BM. In areas where the space between mouse cumulus and pig MG was enlarged, the BM was differentiated into 2 distinguishable layers (lamina rara densa and lamina lucida) (fig 5).

The nuclear ultrastructure of mouse oocytes underwent substantial changes after

co-culture with PMG (fig 6). Whereas the nuclear envelope remained intact, the compact nucleoli were surrounded by a conspicuous rim composed of nucleolus-associated chromatin and large granules  $\approx 80$  nm in diameter. The same granules formed small clusters in the nucleoplasm.



**Fig 1.** Effect of pig membrana granulosa on the content of cAMP in mouse oocytes. The mouse oocyte cumulus complexes (OCC) were cultured for 1, 2 and 3 h upon pig membrana granulosa (OCC + PMG). The oocytes were denuded of cumulus cells (C-) before (time zero) or after (time 1, 2 and 3 h) the experimental treatment. Bars represent mean fmol cAMP per oocyte and the standard error of the mean from at least 3 independent experiments. The time zero is significantly different from other groups ( $P < 0.05$ ).



**Fig 2.** Composition of 3 serial micrographs showing the experimental model used in this study. Large compact nucleolus (N) is visible inside the intact GV of mouse oocyte cultured for 6 h with pig membrana granulosa (PMG). Mouse cumulus cells (CC) are attached to continuous pig basement membrane (arrowheads) (x 3 000).

In addition, round-shaped clumps of chromatin granules  $\approx 20$  nm in diameter and numerous nucleolus-like bodies (NLB), were scattered throughout the nucleoplasm. These 3 structures were often associated with the nuclear envelope.

## DISCUSSION

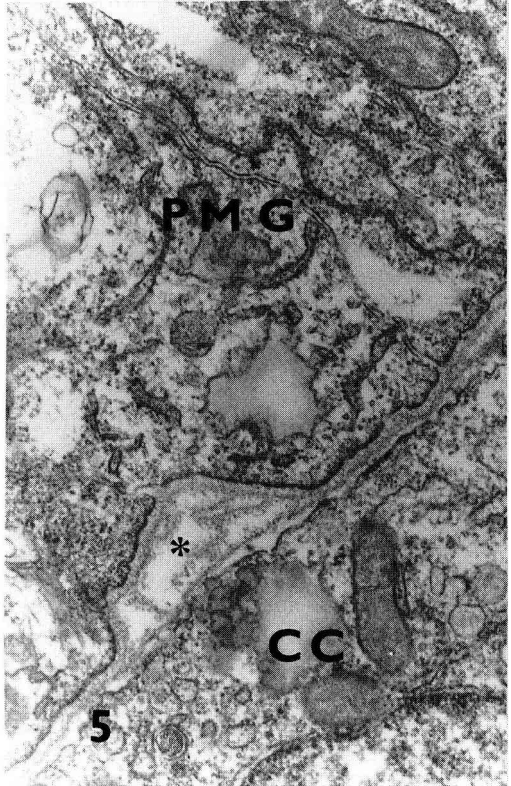
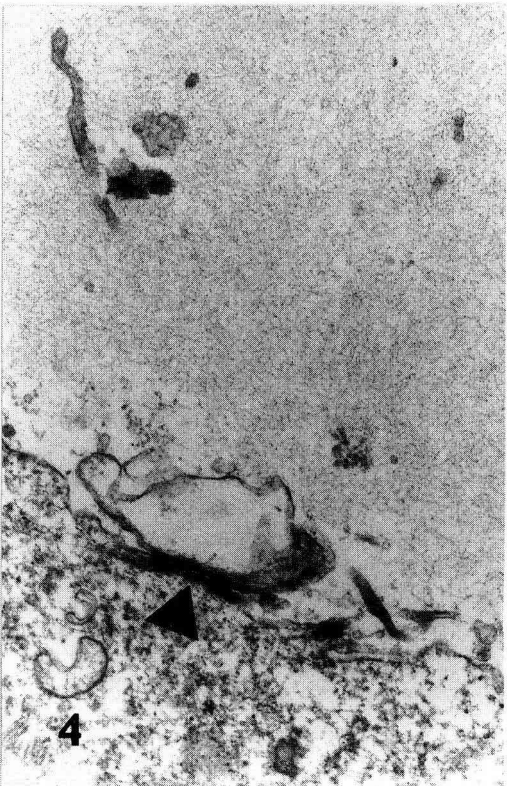
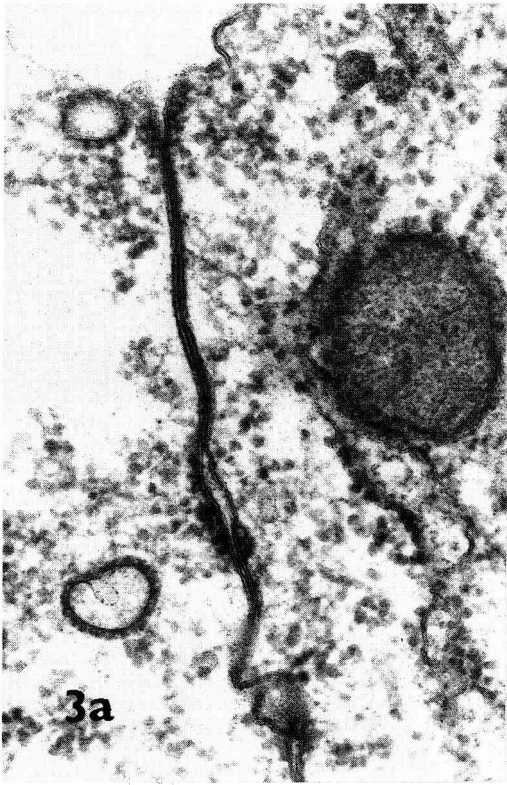
The pig cumulus-enclosed oocytes isolated with attached piece of membrana granulosa did not resume meiosis *in vitro* (Mot-

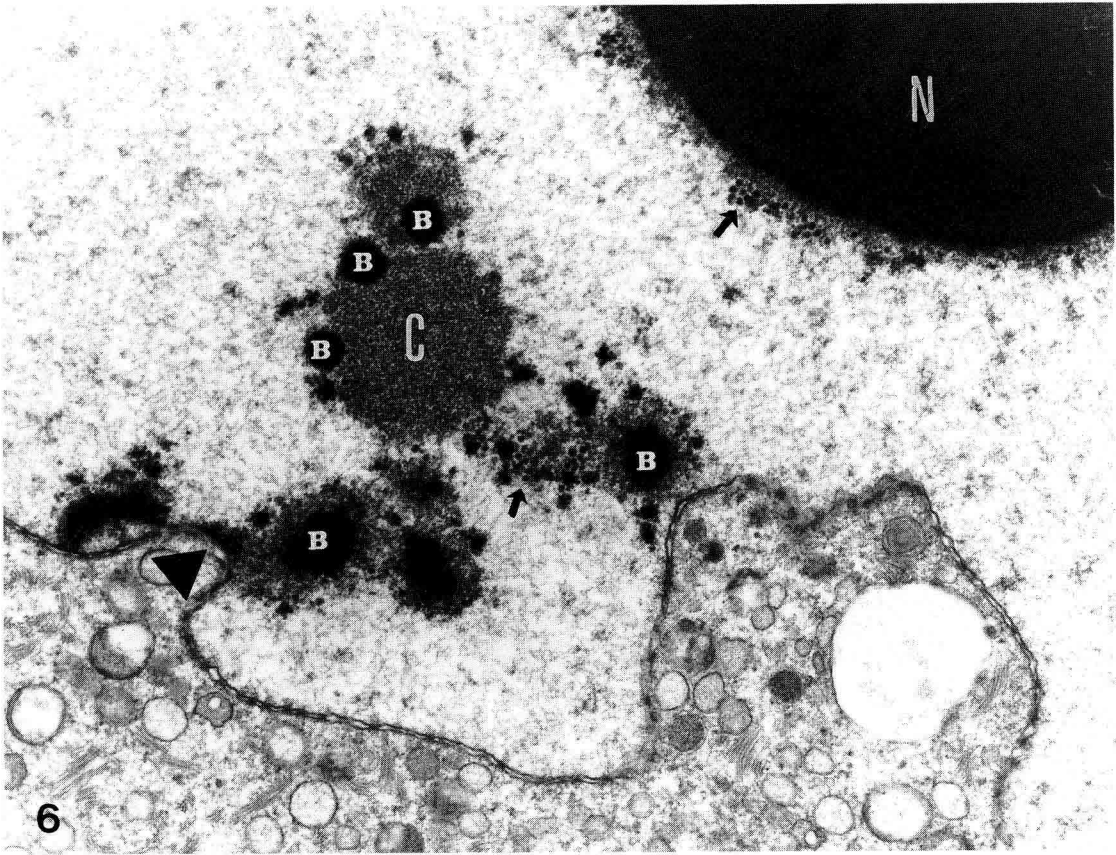
lik *et al*, 1991). The isolated pig membrana granulosa (PMG) effectively and reversibly prevented GVBD in heterologous (cattle) oocytes (Kalous *et al*, 1993). The present data demonstrate that PMG prevented resumption of meiosis in oocytes, also with a rapid GVBD time sequence. Nearly all mouse oocytes retained the GV stage after the 3- and 6-h co-culture. The preincubation of the mouse denuded and cumulus-enclosed oocytes for 45 and 60 min, respectively, significantly abolished the inhibitory effect of PMG. The data suggest that the inhibitory activity of PMG, similarly to

**Fig 3a,b.** Homologous gap junctions remaining in mouse cumulus cells (A) and pig granulosa cells (B) co-cultured for 6 h (a x 90 000; B x 80 000).

**Fig 4.** Heterologous contacts (arrowhead) between corona radiata projections and oocyte in mouse OCCs conserved until the end of co-culture (x 27 000).

**Fig 5.** Pig basement membrane separating the mouse (CC) and pig (PMG) compartment of experimental model. The basement membrane is separated into 2 layers in site of gap between the pig and the mouse cells (asterisk) (x 28 500).





**Fig 6.** Nuclear ultrastructure of mouse oocyte co-cultured with pig membrana granulosa cells. Large compact nucleolus (N) and small nucleolus-like bodies (B) are surrounded by the rim of chromatin, containing clumps of 80-nm granules (arrows). Note the round-shaped clump of chromatin (C) and the association of nucleolus-like bodies with nuclear membrane (arrowhead) (x 17 500).

the effect of IBMX (Eppig *et al*, 1983), cannot be exerted after irreversible commitment of mouse oocytes to GVBD.

FSH and cholera toxin significantly increase the cumulus-cell cAMP levels. In spite of the fact that it does not result in any detectable rise in oocyte cAMP, resumption of meiosis in mouse oocytes is postponed (Schultz *et al*, 1983b). If cAMP is not transmitted from cumulus cells to oo-

cytes then some factor other than cAMP may be transferred from cumulus cells to the oocyte to inhibit oocyte maturation. The action of FSH in delaying maturation is mediated by the cumulus cells and it is only transient while all oocytes passed GVBD within 4 h (Eppig *et al*, 1983).

The action of FSH, cholera toxin and suboptimal concentrations of dbcAMP in inhibiting mouse oocyte GVBD depends on

an intact coupling pathway between the cumulus cells and oocytes (Eppig *et al*, 1983; Schultz *et al*, 1983b). In the present experiments, the pig granulosa cells as well as the mouse cumulus cells and corona radiata cells with the oolemma were tightly coupled by numerous gap junctions. However, both compartments were strictly separated by the basement membrane. This means that a putative maturation inhibiting factor was secreted by the PMG through the basement membrane and was transported in both cumulus-enclosed and denuded oocytes.

The main characteristic of the commitment period in the mouse oocytes is a decrease in oocyte cAMP levels (Schultz *et al*, 1983a; Vivarelli *et al*, 1983; present results). Surprisingly, our experiments also documented this drop in oocytes blocked at the GV stage by PMG. These data could help to answer a question raised by Thibault *et al* (1987): "Is the decrease in the cAMP level a prerequisite to meiosis resumption?" If cAMP dropped similarly in the ooplasm of committed and inhibited oocytes, this significant decrease could be caused by liberation of oocytes from follicular environment to the culture conditions and a putative intrafollicular inhibitor of meiosis could act downstream of the step which is sensitive to the cAMP level.

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