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*Original Article*

# Interphase-like chromatin configuration induced by cycloheximide in maturing pig oocytes: effects of protein phosphatase inhibitors

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ABSTRACT **Embryo cloning methods could greatly benefit from the manipulation of cell cycle in oocytes from large domestic mammals. The present study was undertaken to examine the effects of the protein synthesis inhibitor cycloheximide and the inhibitors of protein phosphatases 1 and 2A okadaic acid and calyculin A on maturing pig oocytes. Cycloheximide treatment (10 μg/ml) induced an interphase-like chromatin configuration (ICC) in maturing oocytes. Up to 69% of the oocytes exhibited ICC when treated with cycloheximide after 24 h of *in vitro* culture. ICC starts to appear after a 4 h exposure to cycloheximide and the ICC percentage reached its plateau after 12 h of cycloheximide treatment. ICC is fully reversible. The addition of okadaic acid (0.5 μM) inhibited the ICC in cycloheximide-treated maturing oocytes and allowed the completion of maturation in 55% of them. In oocytes with ICC, the immunocytochemistry for tubulin revealed the rearrangement of microtubule into an interphase meshwork and these oocytes lost their ability to induce tubulin assembly, as shown after short-time taxol treatment. The addition of okadaic acid prevented this microtubule rearrangement and preserved a certain level of tubulin assembly. Calyculin appeared to be more effective than okadaic acid in the prevention of ICC. It is concluded that *de novo* protein synthesis is necessary during a certain period of meiotic maturation for the maintenance of metaphase chromatin configuration in pig oocytes. This protein (or proteins) acts through the inhibition of endogenous protein phosphatases, probably protein phosphatase of 2A type.**

KEY WORDS: *cycloheximide, protein phosphatases, interphase, chromatin, tubulin, maturing oocytes, pig*

## Introduction

A complex set of events occurs during meiotic maturation in mammalian oocytes. As a consequence of these events the immature oocytes develop into an unfertilized egg. The immature oocytes are arrested in the dictyate stage of the first meiotic prophase and the nuclei of these oocytes are called germinal vesicles (GV). From the point of view of the cell cycle, they are arrested at the late $G_2$ phase. The transition from $G_2$ to M phase is accompanied by breakdown of the nucleus envelope (germinal vesicle breakdown – GVBD), condensation of chromatin and formation of the meiotic spindle. Oocyte maturation is completed when the oocyte enters the metaphase II stage, where it is arrested until the egg is fertilized or activated parthenogenetically (Thibault *et al.*, 1987; Motlík and Kubelka, 1990; Parrish *et al.*, 1992).

Meiosis resumption in ungulate oocytes depends on *de novo* protein synthesis and the inhibitors of protein synthesis effectively inhibit GVBD in pig oocytes (Fulka, Jr. *et al.*, 1986; Mattioli *et al.*, 1991; Motlík *et al.*, 1991; Kubelka *et al.*, 1995). Further progression of oocyte maturation is also dependent on protein synthesis. When *de novo* protein synthesis is inhibited in oocytes at the metaphase I stage, chromatin is decondensed and enters the interphase-like configuration. This phenomenon was observed in the oocytes of mouse (Hashimoto and Kishimoto, 1988; Kang *et al.*, 1991), cattle (Milovanov and Sirard, 1994) and pig (Mattioli *et al.*, 1991; Ding *et al.*, 1992).

As the above studies reveal, the blocking of protein synthesis at a certain stage of meiotic maturation may allow the oocytes to return to interphase without activation. This possibility could benefit experiments directed towards embryo cloning. In such experiments, the cytoplasm of matured oocytes is used as a recipient of blastomeres at an unknown stage of the cell cycle. The compatibility of recipient cytoplasm with the transferred blastomere is essential for the success of cloning and inactivated interphase oocytes should represent an effective recipient milieu.

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The experiments involved in this study were therefore performed in order to determine the conditions in which the cycloheximide-treated pig oocyte can enter the interphase stage. The study also focused on the influence of protein phosphatase 1 and 2A inhibitors, okadaic acid and calyculin A (Cohen, 1989; Ishihara *et al.*, 1989), on the transition of pig oocytes to the interphase stage. Besides observing the chromatin configuration we investigated the arrangement of microtubules. The ability of oocyte cytoplasm to induce tubulin assembly was checked after short-term treatment of the oocytes with taxol, which is known to stabilize microtubules by shifting the equilibrium between the tubulin polymer and the dimer in favor of microtubule formation (Schiff *et al.*, 1979; Horwitz, 1992).

## Results

### *Experiment 1: the sensitivity of maturing pig oocytes for ICC induction*

The stages of meiotic maturation attained by pig oocytes after 20, 22, 24, 26 and 28 h cultivation are shown in Table 1.

The interphase-like chromatin configuration (ICC) was induced in maturing pig oocytes exposed to cycloheximide. The highest percentage of oocytes with ICC (66%) was observed in oocytes cultured for 24 h *in vitro* before treatment with cycloheximide. Oocytes cultured *in vitro* for longer or shorter intervals were much more resistant to the ICC induction (Table 2). Only oocytes cultured for 24 h were used in further studies.

### *Experiment 2: the time course of ICC formation and reversibility*

In pig oocytes cultured 24 h *in vitro*, the first ICC appeared after 4 h of cycloheximide treatment. The percentage of oocytes with ICC further increased up to 10 h of cycloheximide treatment but it did not increase significantly after this interval (Table 3). Oocytes matured *in vitro* for 24 h and subsequently exposed to cycloheximide for 10 h exhibited 66% ICC. When these oocytes were cultured for a further 16 h in cycloheximide-free medium, 95% of them completed meiotic maturation reaching metaphase II stage and the remaining 5% oocytes were observed in metaphase I. No oocyte retained ICC.

### *Experiment 3: reversal of ICC by okadaic acid*

ICC did not form in oocytes first cultured *in vitro* for 24 h and then exposed for a further 24 h to the medium supplemented with both cycloheximide (10 μg/ml) and okadaic acid (1 μM). Twenty-eight percent of these oocytes reached the stage of metaphase II and the condensed chromatin in the remaining oocytes was dispersed within the oocyte cytoplasm. When after 24 h *in vitro* culture maturing oocytes were treated for another 24 h with a medium supplemented with cycloheximide (10 μg/ml) and decreased concentration of okadaic acid (0.5 μM), the increased percentage of oocytes completed meiotic maturation and reached the stage of metaphase II (55%). Oocytes with ICC were not observed and the remaining 45% of oocytes were observed in metaphase I with impaired configuration of chromosomes within the metaphase plate indicating defects of the meiotic spindle.

### *Experiment 4: the effect of ICC on the ability of oocyte cytoplasm to achieve tubulin assembly*

In oocytes cultured for 24 h in a culture medium, the immunocytochemistry for tubulin revealed the typical meiotic spindle and, after Hoechst staining, the chromosomes were observed to be arranged into a metaphase plate (Fig. 1A,B). After short-term taxol treatment, cytoplasmic asters were formed in the cytoplasm of these oocytes (Fig. 2A,B). After 24 h *in vitro* culture the oocytes exposed to cycloheximide exhibited ICC after Hoechst staining, and tubulin labeling revealed an interphase meshwork of microtubules (Fig. 3A,B). These oocytes did not respond to taxol treatment by the formation of cytoplasmic asters. When oocytes cultured *in vitro* for 24 h were further cultured in a medium supplemented by both cycloheximide and okadaic acid, a significant portion of them went on to mature to the metaphase II stage. Hoechst staining revealed condensed chromatin with the appropriate part excluded as a polar body. Immunocytochemistry for tubulin labeling revealed a weakened meiotic spindle, which was only weakly labeled or absent in many oocytes. The ability to achieve

TABLE 1

**MEIOTIC MATURATION IN PIG OOCYTES CULTURED *IN VITRO***

| Time of culture (h) | Stage of nuclear maturation | | | | | | |
|---|---|---|---|---|---|---|---|
| | n | LD | PM | M I | A I | T I | M II |
| 20 | 120 | 27 | 61 | 12 | | | |
| 22 | 120 | 23 | 60 | 17 | | | |
| 24 | 120 | 9 | 25 | 66 | | | |
| 26 | 120 | | | 67 | 33 | | |
| 28 | 120 | | | 66 | 34 | | |
| 30 | 120 | | | 19 | 25 | 19 | 37 |

LD, late diakinesis; PM, prometaphase; M I, metaphase I; A I, anaphase I; T I, telophase I; M II, metaphase II.

TABLE 2

**INDUCTION OF INTERPHASE-LIKE CHROMATIN CONFIGURATION (ICC) BY CYCLOHEXIMIDE (10 μg/ml) IN MATURING PIG OOCYTES**

| Time of culture (h) | n | ICC (%) |
|---|---|---|
| 20 | 120 | 12[a] |
| 22 | 120 | 48[b] |
| 24 | 120 | 69[c] |
| 26 | 120 | 40[b] |
| 28 | 120 | 20[a] |

[a,b,c] statistically significant differences.

TABLE 3

**TIME COURSE OF THE INTERPHASE CHROMATIN CONFIGURATION (ICC) IN PIG OOCYTES MATURED 24 h *IN VITRO* AND SUBSEQUENTLY TREATED WITH CYCLOHEXIMIDE (10 μg/ml)**

| Duration of cycloheximide treatment (h) | n | ICC (%) |
|---|---|---|
| 2 | 120 | 0[a] |
| 4 | 120 | 12[b] |
| 6 | 120 | 30[c] |
| 8 | 120 | 48[d] |
| 10 | 120 | 66[e] |
| 12 | 120 | 68[e] |
| 16 | 120 | 70[e] |

[a-e] statistically significant differences.

tubulin assembly was also reduced in these oocytes, as short-term taxol treatment only induced less distinct cytoplasmic asters (Fig. 4A,B). This contrasts with a marked ability to achieve tubulin assembly in oocytes reaching metaphase II stage during *in vitro* culture in a medium without cycloheximide and okadaic acid (Fig. 5A,B). On the other hand, this impaired microtubule arrangement resembled the microtubules observed in maturing oocytes at the metaphase I stage (i.e. oocytes after 24 h culture *in vitro*) treated with okadaic acid alone (Fig. 6A,B).

### *Experiment 5: effects of different protein phosphatase inhibitors 1 and 2A on the formation of ICC*

At a concentration of 250 nM okadaic acid is able to completely inhibit the formation of ICC in maturing pig oocytes treated with cycloheximide. A significant depression in the formation of ICC (ICC 47%) was still observed when okadaic acid was used in a concentration of 20 nM. When okadaic acid was used to reverse the effect of cycloheximide at a concentration of 10 nM, the percentage of ICC (ICC 66%) did not differ significantly from oocytes treated with cycloheximide alone (ICC 69%) (Table 4).

Calyculin A was also shown to inhibit the formation of ICC in maturing oocytes cultured in a medium with cycloheximide. The complete block of ICC was observed in a calyculin A concentration of 100 nM. A significant decrease of ICC was seen even when calyculin A was used in a concentration of 2 nM (ICC 52%). On the other hand, the percentage of ICC in a concentration of 1 nM (ICC 65%) did not differ from the ICC percentage in control experiments performed without calyculin A (ICC 69%).

## Discussion

Our experiments clearly showed that the inhibition of protein synthesis by cycloheximide can induce transition from metaphase to interphase in maturing pig oocytes. Similar results were reported by Mattioli *et al.* (1991) and Ding *et al.* (1992). The highest percentage of oocytes with ICC (66%) was observed when oocytes were precultured for 24 h before the cycloheximide treatment. Shorter or longer preincubation time caused a decreased percentage of ICC. The time course of *in vitro* maturation in pig oocytes

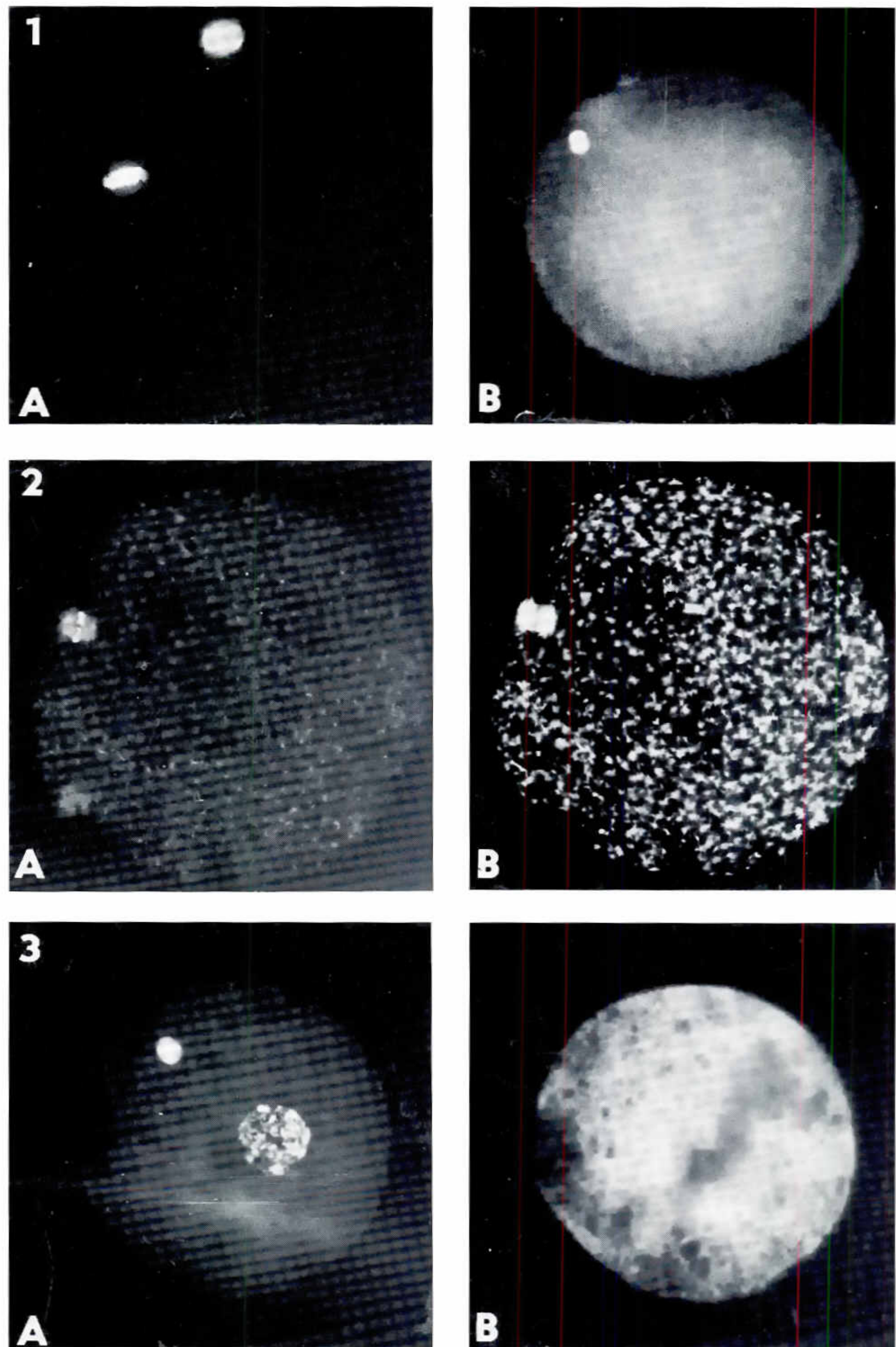


**Fig. 1. Pig oocyte at metaphase I stage after the Hoechst staining exhibited condensed chromosomes within a metaphase plate (A).** *Tubulin labeling* **(B)** *revealed a meiotic spindle. Magnification, x450.*

**Fig. 2. Chromatin configuration (A) and tubulin arrangement (B) in metaphase I pig oocyte after taxol treatment.** *Numerous cytoplasmic asters are induced by short-time exposure to taxol. Magnification, x450.*

**Fig. 3. Cycloheximide-treated maturing pig oocyte formed an interphase-like chromatin configuration (ICC) as revealed by Hoechst staining (A).** *After tubulin labeling* **(B)**, *the microtubules were rearranged into an interphase meshwork. Taxol treatment did not induce the formation of cytoplasmic asters* **(B)**. *Magnification, x450.*

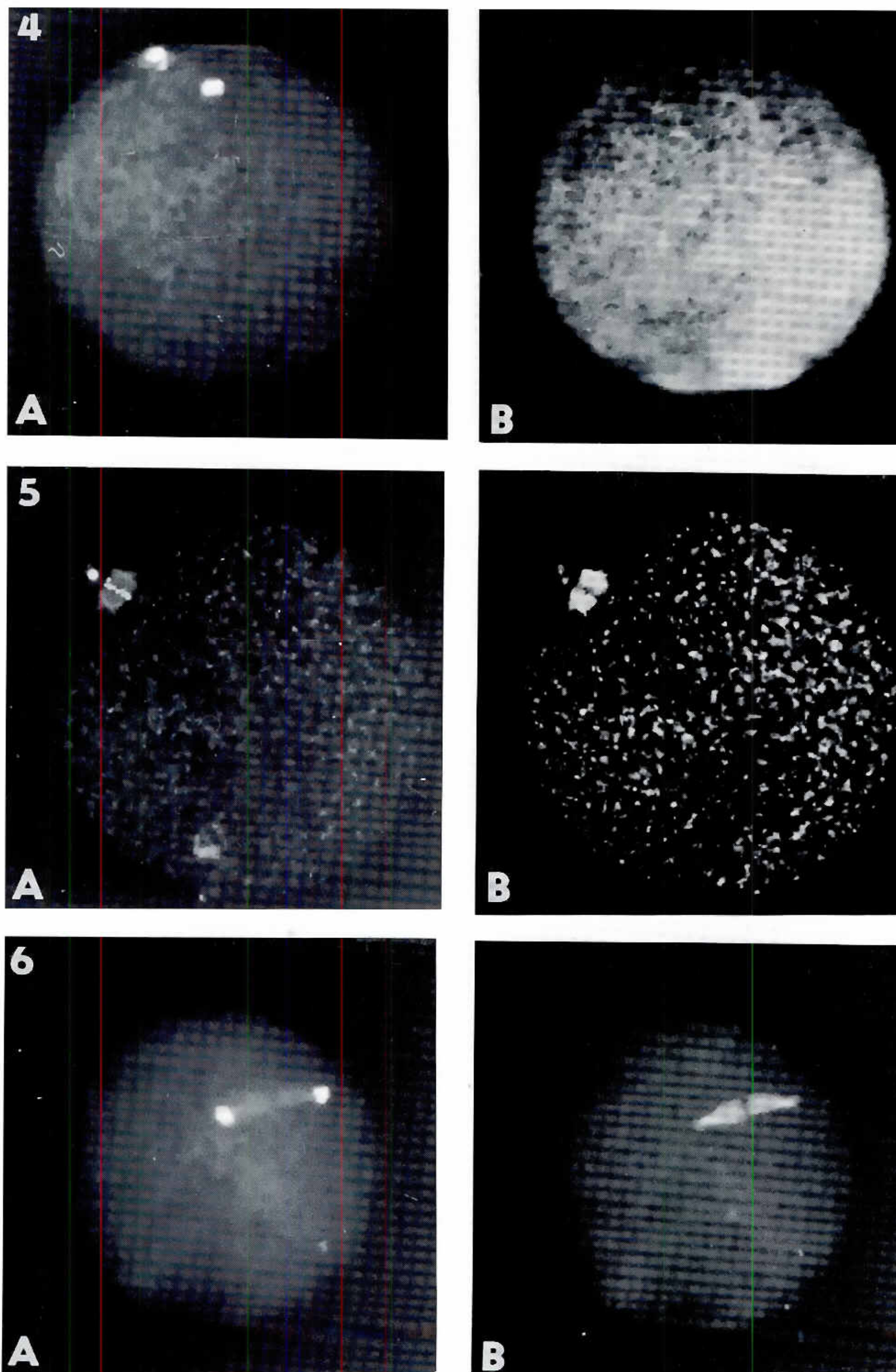


**Fig. 4. Maturing oocyte treated with both cycloheximide and okadaic acid maintained condensed chromatin and extruded the first polar body (A).** *Tubulin labeling* **(B)** *revealed absence of meiotic spindle and absence of cytoplasmic asters after taxol treatment. Magnification, x450.*

**Fig. 5. Matured pig oocyte at metaphase II stage.** *Condensed chromosomes are arranged in a metaphase plate and the first polar body is extruded from the oocyte* **(A)**. *Meiotic spindle is formed and taxol treatment induced the formation of cytoplasmic asters* **(B)**. *Magnification, x450.*

**Fig. 6. Maturing pig oocyte treated with okadaic acid.** *Chromatin remained condensed* **(A)** *but the meiotic spindle and the induction of cytoplasmic asters by taxol is impaired* **(B)**. *Magnification, x450.*

is very variable (Motlík and Fulka, 1976, this study), nevertheless, on the basis of our data on the dynamics of nuclear maturation during the culture of oocytes for 20 up to 28 h we can conclude that oocytes at early metaphase I and probably also oocytes spontaneously entering this stage shortly after the beginning of cycloheximide treatment are especially sensitive to the formation of ICC. On the contrary, a profound decrease in ICC in oocytes precultured for 28 h indicates that oocytes at late metaphase I or oocytes entering anaphase I shortly after the beginning of the cycloheximide treatment are more resistant to the formation of ICC. The retention of the metaphase chromatin configuration in pig oocytes at the late stages of meiotic maturation treated with a protein synthesis inhibitor was also reported by Ding *et al.* (1992). In this respect, pig oocytes differ from cattle oocytes in that ICC can be induced in cattle oocytes at stages from metaphase I up to metaphase II (Milovanov and Sirard, 1994). Our results indicate that the pig oocyte passes through a period during which the maintenance of the metaphase chromatin configuration is dependent on protein synthesis. Evidence of independence of chromatin condensation in pig oocytes of the protein synthesis besides this period is provided by the situation observed during the inhibition of maturation by protein synthesis inhibitors. The nuclear membrane is well preserved in these oocytes, but the condensation of chromatin is adequate to the metaphase stage (Kubelka *et al.*, 1988; Mattioli *et al.*, 1991; Rozinek *et al.*, 1995).

An interphase-like chromatin configuration starts to appear in some oocytes already after 4 h of cycloheximide treatment, but in the majority of oocytes it is established after 10 h of cycloheximide treatment. When oocytes with ICC are removed from the cycloheximide-supplemented medium, their chromatin regains the metaphase configuration. These observations should be interpreted as evidence that the cytoplasmic conditions which maintained the metaphase chromatin configuration depend on the continuous synthesis of short-lived proteins. Depletion of these proteins induces a transition from the metaphase to the interphase stage, and conversely, their new synthesis after

TABLE 4

**COMPARISON OF EFFECTS OF OKADAIC ACID AND CALYCULIN A ON THE REVERSION OF ICC INDUCED IN MATURING PIG OOCYTES BY TREATMENT WITH CYCLOHEXIMIDE (10 μg/ml)**

| Concentration of protein phosphatase inhibitors (nM) | Calyculin A | | Okadaic acid | |
|---|---|---|---|---|
| | n | % of ICC | n | % of ICC |
| 0 | 100 | 69 | 100 | 69 |
| 1 | 120 | 65 | | |
| 2 | 120 | 52 | | |
| 5 | 120 | 35 | | |
| 10 | 120 | 30[a] | 120 | 66[b] |
| 25 | 120 | 21[a] | 120 | 47[b] |
| 50 | 120 | 5[a] | 120 | 22[b] |
| 100 | 120 | 0 | 120 | 8 |
| 250 | | | 120 | 0 |

[a,b]Statistically significant differences between the percentages of ICC in oocytes treated with identical concentrations of okadaic acid and calyculin A.

washing out the protein synthesis inhibitor induces a return from interphase to metaphase.

We showed that the inhibitors of protein phosphatases 1 and 2A, okadaic acid and calyculin A, are able to protect the formation of ICC induced by the inhibition of protein synthesis. While the higher concentrations of okadaic acid (1 μM) allow completion of meiotic maturation and entry to the metaphase II stage in a very low percentage of oocytes (M II 28%), the lower concentrations of okadaic acid (0.5 μM) allowed complete maturation to a high percentage of oocytes (M II 55%). The higher incidence of oocytes with meiotic spindle defects when treated with higher concentration of okadaic acid indicates that incomplete maturation is due to the adverse effects of okadaic acid on the microtubule of the meiotic spindle. Defects during the formation of the spindle or its complete absence were reported in oocytes of different species in which maturation was induced by okadaic acid (starfish: Picard *et al.*, 1989; frog: Rime *et al.*, 1990; mouse: Alexandre *et al.*, 1991; Gavin *et al.*, 1991). Kalous *et al.* (1993) reported different effects of two concentrations of okadaic acid (0.5 and 2.5 μM) on the morphology of the meiotic spindle after the induction of meiosis in cattle and pig oocytes. It can be concluded from studies on the meiotic spindle in rat metaphase II oocytes, that okadaic acid induces hyperphosphorylation of some proteins associated with the spindle microtubule. These proteins are released from the spindle and as a consequence, the microtubules are destabilized (Zernicka-Goetz and Maro, 1993).

The effect of okadaic acid on the meiotic spindle and on tubulin assembly was confirmed by our immunocytochemistry study. We observed a typical chromatin configuration and metaphase microtubule arrangement with a distinct meiotic spindle in maturing oocytes at metaphase I stage (similarly to Maro *et al.*, 1990; Mattson and Albertini, 1990; Van Blerkom, 1991). In these oocytes, taxol treatment induced the formation of cytoplasmic asters. This formation is known to be a typical attribute of the cytoplasm of metaphase oocytes (Rime *et al.*, 1987; Van Cauwenberge and Alexandre, 1990, 1992). After incubation of maturing oocytes with cycloheximide, the formation of ICC is accompanied by a rearrangement of the microtubule into an interphase meshwork and the cytoplasm loses its ability to respond to short-time taxol treatment through the formation of cytoplasmic asters. This status is typical for the interphase stage and was also observed in oocytes before germinal vesicle breakdown (Rime *et al.*, 1987; Van Cauwenberge and Alexandre, 1990, 1992). A maturing oocyte exposed to cycloheximide and okadaic acid exhibited condensation of the chromatin but the microtubules were not arranged into a typical metaphase pattern. The weakly formed and in some cases absent meiotic spindle and its reduced ability to induce cytoplasmic asters by taxol should be attributed to the effects of okadaic acid. This is proved by our experiments in which maturing oocytes were treated with okadaic acid and by observations published by Zernicka-Goetz and Maro (1993) and de Pennart *et al.* (1993). On the other hand, the microtubule arrangement in oocytes treated with cycloheximide and okadaic acid exhibit some signs of interphase network. So we cannot exclude the possibility that okadaic acid is unable to fully convert microtubules into the metaphase status.

Kalous *et al.* (1993) report that okadaic acid is able to bypass the inhibition of spontaneous maturation in pig and cattle oocytes induced by cycloheximide. These authors suppose that the resumption of meiosis needs *de novo* synthesis of a protein which induces this process through inhibition of protein phosphatase 2A. This phosphatase is known to control meiosis resumption in *Xenopus* oocytes through dephosphorylation of phosphatase cdc25 (Clarke *et al.*, 1993). Our data do not permit a definite decision about the nature of the proteins responsible for the maintenance of metaphase chromatin configuration but we cannot exclude the identity of these protein(s) with those involved in the regulation of meiosis resumption.

The formation of ICC can be reversed by protein inhibitors phosphatases 1 and 2A, okadaic acid and calyculin A. This indicates that short-lived protein(s) responsible for the maintenance of metaphase chromatin configuration perform their role during oocyte maturation through the inhibition of endogenous protein phosphatases 1 and 2A. As okadaic acid and calyculin A inhibit both protein phosphatases 1 and 2A, it is difficult to decide, whether the effects described are due to the inhibition of protein phosphatase 1, protein phosphatase 2A or the inhibition of phosphatases of both types. However, the results of our experiments, during which the effects of okadaic acid on the reversion of ICC were comparable to the effects of a two-fold lower concentration of calyculin A, should be interpreted as proof of the key role of protein phosphatase 2A in this process. Calyculin A has a much more profound effect on protein phosphatase 1 than okadaic acid. The $ID_{50}$ for calyculin A is about 1-2 nM while the $ID_{50}$ for okadaic acid is 60-500 nM (Ishihara *et al.*, 1989). The $ID_{50}$ for okadaic acid and calyculin A for protein phosphatase 2A is almost the same and fluctuates in the range of 0.5-1 nM (Ishihara *et al.*, 1989). The differences in the effects of okadaic acid and calyculin A on the reversion of ICC therefore can be explained as a result of the variable effect of individual inhibitors on phosphatase 2A. Calyculin A should have reversed ICC at much lower concentrations than okadaic acid, when protein phosphatase 1 should have been the target enzyme. Such a ratio of effective concentrations of okadaic acid and calyculin A was described when protein phosphatase 1 was involved in processes in somatic cells (Downey *et al.*, 1993; Gurland and Gundersen, 1993; Koch and Lutz-Bucher, 1994).

We can conclude that maturing pig oocytes pass through a period during which their metaphase chromatin configuration is

dependent on *de novo* protein synthesis. When oocytes were treated with a protein synthesis inhibitor during this sensitive period, they formed ICC. The inhibitors of protein phosphatases 1 and 2A are able to reverse the formation of ICC induced by cycloheximide. This indicates, that *de novo* synthesized protein(s) maintain the metaphase chromatin configuration through the inhibition of endogenous protein phosphatases. The protein phosphatase 2A is probably a target enzyme for this protein or proteins.

## Materials and Methods

***Oocyte collection and culture***

Porcine ovaries were obtained from a local slaughterhouse. The ovaries were transported to the laboratory in a saline solution at 39°C. Oocytes were aspirated from follicles that were 2 to 5 mm in diameter with a 20-gauge needle syringe. Only oocytes with compact cumuli were used for culture. Before culture, oocytes were washed 3 times in a culture medium. An E199 medium (USOL, Prague, Czech Republic) supplemented with 0.039 ml 7.5% solution of sodium bicarbonate per millilitre, calcium lactate (0.6 mg per ml of medium), sodium pyruvate (0.2 mg/ml), gentamycin (0.025 mg/ml), HEPES (1.5 mg/ml) and with 10% of bovine serum (ZD Hustopeče, Czech Republic) was used.

For *in vitro* culture, oocytes were placed in 35 mm Petri dishes (Nunc, Roskilde, Denmark) in 3 ml of culture medium for an appropriate time at 39°C in air with 5% of $CO_2$. At the end of culture, oocytes were mounted on slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h and stained with 1% orcein. Oocytes were examined under a phase-contrast microscope to check their nuclear maturation.

***Experiment 1***

This experiment was set up to determine the sensitivity of maturing pig oocytes to the induction of an interphase-like chromatin configuration (ICC). Oocytes were cultured *in vitro* for 20, 22, 24, 26 or 28 h and then they were transferred to the medium supplemented with cycloheximide (10 μg/ml) and cultured for a further 24 h.

***Experiment 2***

The aim of this experiment was to follow the time course of ICC formation in maturing pig oocytes. Oocytes were cultured *in vitro* for 24 h and then exposed to cycloheximide for 2, 4, 6, 8, 12 and 16 h. The reversibility of ICC was tested as follows. Oocytes were matured in the culture medium for 24 h and then they were exposed to a medium with cycloheximide (10 μg/ml) for 10 h. After this interval, oocytes were carefully washed and cultured in a cycloheximide-free medium for an additional 16 h.

***Experiment 3***

The reversal of ICC formation using okadaic acid, an inhibitor of protein phosphatases 1 and 2A, was tested in this experiment. Oocytes were cultured for 24 h in a culture medium and then they were exposed to a medium supplemented with cycloheximide (10 μg/ml) and okadaic acid (0.5 or 1 mM) for an additional 24 h.

***Experiment 4***

This experiment, designed to establish immunocytochemistry for tubulin, was performed on maturing oocytes cultured 24 h *in vitro*, on maturing oocytes exposed to cycloheximide (10 μg/ml) or okadaic acid (0.5 mM) and on maturing oocytes exposed both to cycloheximide (10 μg/ml) and okadaic acid (0.5 mM). Zona-free oocytes were washed in a HEPES buffer and fixed for 60 min at room temperature in a HEPES buffer containing 2.5 % formaldehyde, 2.5 mM MgCl, and 2.5 mM EGTA and 1 mM taxol. After careful washing in the PBS buffer with 0.1% Triton X and 0.1% human serum albumin (HSA), oocytes were incubated with a mouse monoclonal antibody to α-tubulin (Sigma) diluted in PBS supplemented with 0.1 % HSA and 0.01 % Tween 20 (antibody dilution 1:200) for 13-16 h at 4°C. Following 3 washes in PBS the oocytes were incubated with an ovine antibody to mouse IgG (Sigma) conjugated with FITC diluted in PBS supplemented with 0.1% HSA and 0.01% Tween 20 (antibody dilution 1:50). After careful washing oocytes were stained with Hoechst 33 258 (10 mg/ml PBS buffer) for 10 min at room temperature. After washing in a PBS buffer with 0.1% HSA the oocytes were mounted in glycerine on slides coated with poly-L-lysine and covered with a cover glass with Tris buffer pH 8.5 (1:1 v/v).

Labeled oocytes were analyzed on a Jenalumar fluorescence microscope (510 nm for tubulin labeled with fluorescein and 410 nm for Hoechst).

To evaluate the nucleating ability of cytoplasmic microtubule, oocytes were incubated in a culture medium with 10 mM taxol (Sigma) for 20 min at 38.5°C and immediately fixed for immunofluorescent analysis.

***Experiment 5***

The effects of two inhibitors of protein phosphatases 1 and 2A, okadaic acid and calyculin A, on the formation of ICC were compared in this experiment. The oocytes were cultured 24 h *in vitro* and were then cultured for an additional 24 h in the presence of both cycloheximide (10 μg/ml) and a protein phosphatases inhibitor. Okadaic acid was used in concentrations of 0, 10, 20, 50, 100 and 250 nM, calyculin A was used in concentrations of 0, 5, 10, 20, 50 and 100 nM.

***Statistical analyses***

Each experiment was carried out 4 times. The results were pooled for presentation and evaluated by the Chi-square analysis (Snedecor and Cochran, 1980). The mean percentage of the respective stage of nuclear status for the 4 trials did not vary from the pooled percentage by more than 2.5%. A P value of less than 0.05 was considered significant.

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