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Original Article

Full activation of the rat oocyte by protein synthesis inhibition requires protein phosphatase activity

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ABSTRACT The rat oocyte provides an interesting system in which to dissect the control mechanisms involved in the transition between a meiotic M phase and a mitotic interphase. In this study, we show that in rat oocytes activated parthenogenetically by puromycin, okadaic acid (a potent inhibitor of protein phosphatases 1 and 2A) induced an increase in histone H1 kinase activity suggesting that MPF was reactivated. However, the inhibition of phosphatases 1 and 2A shortly after second polar body extrusion did not allow the formation of a metaphase-like spindle, although microtubule polymerization was not inhibited. Instead, the chromatin remained condensed as a single mass and a large aster formed around it.

KEY WORDS: *rat egg, spindle, microtubules, activation, cell cycle*

Introduction

During activation, the rat oocyte provides an interesting model to study the regulation of the transition from the meiotic to the mitotic control of the cell cycle, when major changes in both chromatin and microtubule organization take place. In contrast to ovulated oocytes of other vertebrates, the rat oocyte does not remain arrested in metaphase II (M II), but undergoes spontaneous activation during *in vitro* culture (Keefer and Schuetz, 1982; Zernicka-Goetz, 1991). This leads to the extrusion of the second polar body, but the activated oocyte does not stay in interphase. Instead, it enters a new metaphase-like arrest, metaphase III (M III) where distinct chromosomes become associated to spindle-like structures (Zernicka-Goetz *et al.*, 1993). A similar transition can be induced in mouse oocytes (Kubiak, 1989) where it has been shown that, after activation, maturation promoting factor (MPF) activity drops to the level characteristic for interphase and then increases when a new spindle forms (Kubiak *et al.*, 1992). MPF activity is maintained during the M II arrest by an activity called cytostatic factor (CSF; Masui and Markert, 1971; Masui, 1991). Following fertilization or parthenogenetic activation, the *c-mos* gene product, the catalytic subunit of CSF, is not destroyed immediately (Lorca *et al.*, 1991; Watanabe *et al.*, 1991; Weber *et al.*, 1991). Thus, the presence of CSF in oocytes where MPF is reactivated after second polar body extrusion allows the existence of a subsequent metaphase arrest, M III. Experiments using protein synthesis inhibitors have demonstrated that protein synthesis is required to reactivate MPF during the transition between M II and M III (Zernicka-Goetz, 1991; Kubiak *et al.*, 1992). It appears that inhibition of protein synthesis (by puromycin) during a 3 h period is sufficient to activate parthenogenetically the rat oocyte leading to a full activation response and, after second polar body extrusion, to the formation of a pronucleus (Zernicka-Goetz, 1991; this study).

As with most other cellular processes, both activation and inactivation of MPF are regulated by cascades of protein phosphorylation and dephosphorylation events (for review see Norbury and Nurse, 1992). To study the role of phosphatases during the period following activation of the rat oocyte, we have used okadaic acid (OA), a specific inhibitor of protein phosphatases 1 and 2A (Bialojan and Takai, 1988; Cohen *et al.*, 1990).

Results

OA interferes with pronuclear formation and microtubule distribution

Puromycin is known to induce parthenogenetic activation of rat oocytes (Zernicka-Goetz, 1991) and in this study, we observed that more than 90% of oocytes (152/168) underwent activation after a 3 h puromycin treatment, whereas all the oocytes in the control group were in metaphase (76% (66/87) remained in metaphase II and 24% (21/87) activated spontaneously and went into metaphase III). After 3 h of culture in puromycin, oocytes had extruded the second polar body and contained a single mass of condensed chromatin in the ooplasm (Fig. 1A). The only microtubules found were those within the midbody, between the oocyte and the second polar body. When such activated eggs were cultured for 2 h in control medium, a pronucleus and an interphase network of microtubules developed (Fig. 1B). A further hour of culture revealed a dense microtubule network, typical for interphase (Fig. 1C). Similar trans-

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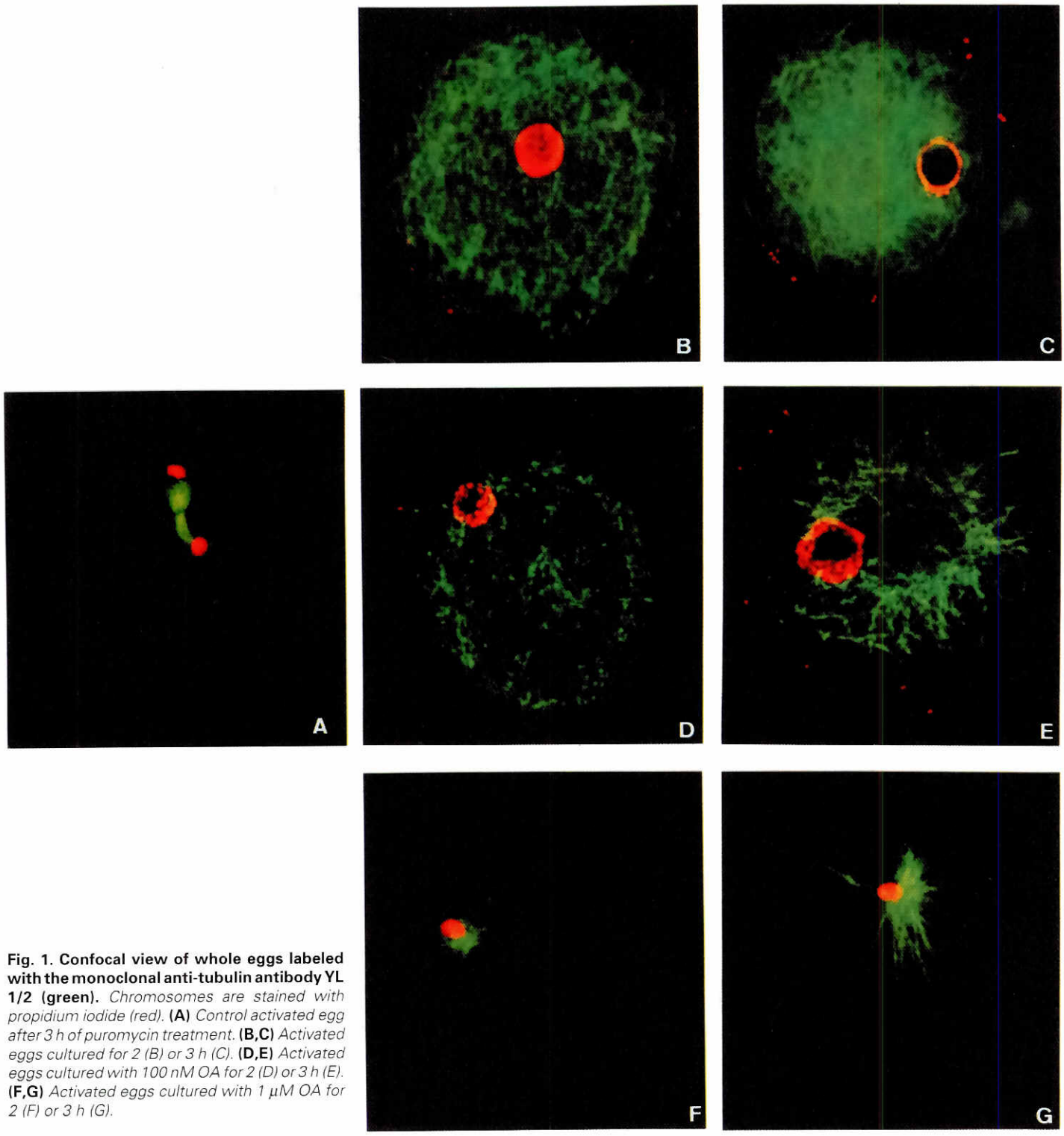

Fig. 1. Confocal view of whole eggs labeled with the monoclonal anti-tubulin antibody YL 1/2 (green). *Chromosomes are stained with propidium iodide (red).* **(A)** *Control activated egg after 3 h of puromycin treatment.* **(B,C)** *Activated eggs cultured for 2 (B) or 3 h (C).* **(D,E)** *Activated eggs cultured with 100 nM OA for 2 (D) or 3 h (E).* **(F,G)** *Activated eggs cultured with 1 μM OA for 2 (F) or 3 h (G).*

formations occurred in activated eggs cultured with 100 nM OA, although we observed a less dense interphase network (Fig. 1D,E). However, treatment of the eggs with 1 μM OA resulted in an inhibition of these developmental events (Fig. 1F,G). First, the chromatin remained condensed as one mass, and the pronucleus never formed. Immunostaining with an anti-lamin antibody was negative, demonstrating that the nuclear lamina did not form in 1 μM OA-treated eggs, while it was present in control and 100 nM OA-treated eggs (not shown). Second, OA at a concentration of 1 μM also inhibited the formation of the interphase microtubule network.

Instead, microtubule polymerization occurred only near the chromatin: short microtubules were first observed after 2 h of OA treatment (Fig. 1F) and one large microtubule aster formed around the chromatin mass after 3 h of treatment (Fig. 1G).

Effects of OA on protein phosphorylation

We checked the effects of OA on phosphate incorporation into proteins during the period following activation. In control eggs [^{32}P] incorporation was still high during the first hour (Fig. 2, lane 1), but it decreased rapidly during the following hour reaching the interphase level (Fig. 2, lanes 2 and 3). Treatment with 100 nM OA resulted in a steady level of [^{32}P] incorporation (Fig. 2, lanes 4 and 5), but the normal decrease did not take place. 1 μM OA induced a dramatic change in the pattern of [^{32}P] incorporation (Fig. 2, lanes 6 and 7). The changes concerned hyperphosphorylation of many proteins with Mr of about 150, 95, 89, 71, 66, 54, 35 and 33x10^3. In addition, two new phosphoproteins (50 and 48x10^3 Mr) appeared, which were not detected in control or 100 nM OA-treated eggs. These transformations were observed already after 2 h of culture with 1 μM OA. This pattern was similar to the one observed in metaphase II arrested oocytes treated with 1 μM OA for 3 h (Fig. 2, compare lane 9 with lane 7). It was not possible to perform chase experiments since they proved to be unreliable in our system, probably because there is a large pool of labeled ATP within the egg.

Effects of OA on histone H1 kinase activity

Since 1 μM OA inhibited the formation of pronuclei in activated eggs and led to a phosphorylation pattern similar to the one observed in M-phase oocytes treated with OA, we analyzed MPF activity in OA-treated eggs by measuring the histone H1 kinase activity. An increase in histone H1 kinase activity was observed after 3 h of incubation with 1 μM OA, while it remained low in control and 100 nM OA-treated eggs (Fig. 3). These results show that OA is able to reactivate histone H1 kinase during the G1 phase of the cell cycle in rat eggs.

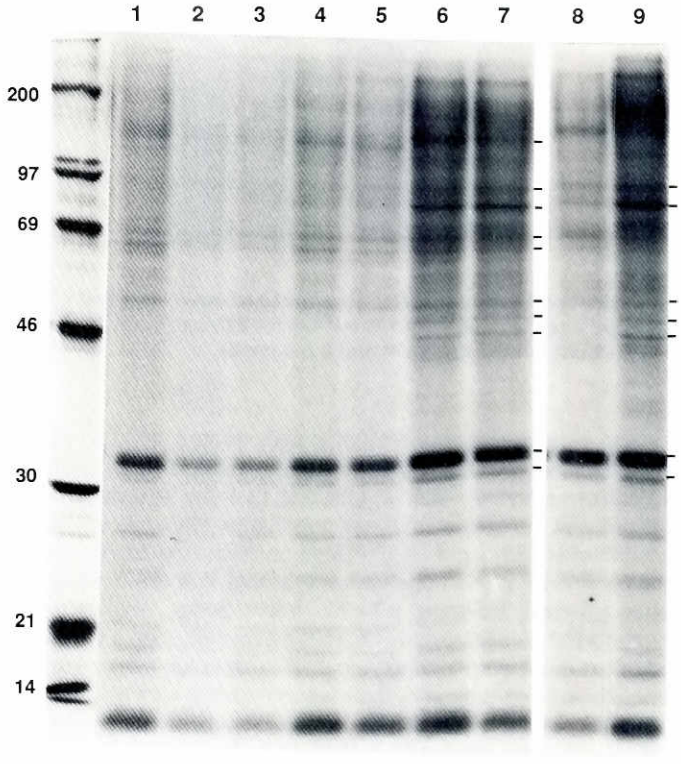

Fig. 2. One-dimensional gel electrophoresis of [^{32}P]-orthophosphate labeled proteins synthesized in activated rat eggs. (Lanes 1-3) *Control activated eggs (lane 1) and after 2 (lane 2) and 3 h (lane 3) of culture in control medium.* **(Lanes 4-7)** *Activated eggs treated with 100 nM OA for 2 (lane 4) and 3 h (lane 5) or with 1 μM OA for 2 (lane 6) and 3 h (lane 7).* **(Lanes 8-9)** *Metaphase II arrested rat oocytes treated with 1 μM OA for 2 (lane 8) and 3 h (lane 9). Lines indicate the changes described in the text. Each lane corresponds to 64 eggs or oocytes.*

Discussion

One peculiarity of the rat oocyte is that activation takes place very easily and that MPF is also very easily reactivated: spontaneous activation leads to the formation of the so-called metaphase III arrest (Zernicka-Goetz, 1991; Zernicka-Goetz *et al.*, 1993). In our experimental system, it is clear that two conditions are required to allow a full activation response leading to interphase; first, inhibition of protein synthesis (Zernicka-Goetz, 1991) and second, active phosphatases (this paper). It is also possible to induce a full activation response in rat oocyte with the anesthetic chloral hydrate (Zernicka-Goetz, 1991), which probably induces a dramatic rise in intracellular calcium. In mouse oocytes, cyclin turns over during the metaphase II arrest, being both destroyed and synthesized (Kubiak *et al.*, 1993), and the more important cyclin destruction taking place after activation seems to be controlled by a Ca^{++}-calmodulin kinase (Lorca *et al.*, 1991). In addition, calcium is able to modulate the activation response of the mouse oocyte: at low doses, it induces a transition to metaphase III, while at high doses it induces a transition to interphase (Vincent *et al.*, 1992), possibly by changing the amount of residual cyclin within the activated oocyte. Thus, it is likely that the protein that should not be synthesized to induce full activation of the rat oocyte might be cyclin.

The transition from interphase to metaphase is characterized by the breakdown of the nuclear envelope, condensation of chromatin, disassembly of the cytoplasmic microtubule network and formation of spindle structure, all these events being regulated by the p34^{cdc2} protein kinase, the catalytic subunit of MPF (for review see Karsenti, 1991; Norbury and Nurse, 1992). Similarly, dephosphorylation of many proteins takes place after the metaphase/anaphase transition, leading to the decondensation of chromosomes and the formation of the nuclear envelope and of an interphase network of microtubules.

OA has been shown to induce activation of MPF and both nuclear envelope breakdown and chromatin condensation in starfish (Pondaven and Mejier, 1986; Picard *et al.*, 1989), frog (Rime *et al.*, 1990) and mouse oocytes (Rime and Ozon, 1990; Gavin *et al.*, 1991; Schwartz and Schultz, 1991). However, spindle formation is inhibited after OA treatment in starfish and frog oocytes (Picard *et al.*, 1989; Rime *et al.*, 1990), whereas contradictory results have been reported in mouse oocytes: no spindle (Alexandre *et al.*, 1991) or formation of abnormal spindles (Gavin *et al.*, 1991).

Our results show that 1 μM OA interferes with the formation of a pronucleus and of an interphase network of microtubules in activated rat oocytes whereas 100 nM does not. Although histone H1 kinase activity is induced by the treatment of activated rat eggs with 1 μM OA, it does not lead to all changes characteristic for the interphase/metaphase transition. OA clearly induced some changes in the protein phosphorylation pattern of activated rat eggs. [^{32}P]

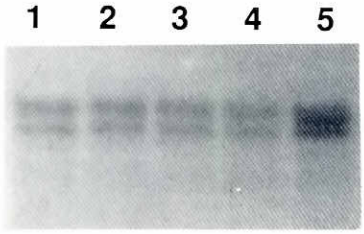

Fig. 3. Histone H1 kinase assay of control activated eggs (lane 1) and activated eggs treated with 100 nM OA for 2 (lane 2) and 3 h (lane 3) or with 1 μM OA for 2 (lane 4) and 3 h (lane 5).

labeling experiments showed that in eggs treated with 100 nM OA, phosphate incorporation remained steady instead of decreasing as in control activated eggs, suggesting that the activity of some kinases was maintained in these conditions. When phosphatases were inhibited with 1 μM OA, [^{32}P] incorporation increased and some new phosphoproteins appeared suggesting that the activation of some kinases, including p34^{cdc2}, might occur. The increased phosphorylation of proteins with a Mr of about 66 and 71x10^3 might represent the hyperphosphorylation of nuclear lamins, which were depolymerized as judged by the immunostaining with an anti-lamin B antibody after 1 μM OA treatment. The hyperphosphorylated protein with a Mr of 35x10^3 is likely to correspond to protein with a Mr of 32x10^3, which is phosphorylated during M phase in early mouse embryos (Howlett, 1986). The hyperphosphorylation of a protein with a Mr of 94x10^3 has been observed in OA-treated mouse oocytes (Rime and Ozon, 1990; de Pennart *et al.*, 1993), but there is no evidence concerning its role. Finally, we must point out that most of these changes were observed after 2 h of OA treatment, while the rise in histone H1 kinase activity was observed after 3 h of OA treatment, suggesting that p34^{cdc2} is not involved in these phosphorylation events.

Inhibition of phosphatases after activation by protein synthesis inhibitor was not sufficient to form spindle-like structures as in metaphase III oocytes, despite the fact that histone H1 kinase activity was high. The chromatin remained condensed as a single mass and the microtubules formed a large aster around it. The absence of spindle-like structures after OA treatment may be related to the following observations. First, it has been shown that kinetochores fail to develop in okadaic acid-induced premature mitosis in HeLa cells (Ghosh *et al.*, 1992) and that the interaction between microtubules and kinetochores is lost in OA-treated mouse oocytes (de Pennart *et al.*, 1993) where kinetochores were already formed and functional when OA was applied. Second, in M-phase, chromatin induces microtubule polymerization in *Xenopus* (Karsenti *et al.*, 1984) and mouse (Maro *et al.*, 1986) oocytes. Since chromatin remained condensed as a single mass after OA treatment, this would lead to the formation of an aster around it.

Materials and Methods

Recovery of oocytes

Rat oocytes were recovered from 25-30 day old immature females of the Wistar strain (IFFA-CREDO, France) injected with 15 IU of pregnant mares' serum gonadotrophin (PMSG, Intervet) and 48-53 h later with 15 IU of human chorionic gonadotrophin (hCG, Intervet). The rats were sacrificed between 14 and 16 h post hCG and ovulated oocytes were collected from the ampullae of the oviducts. Cumulus cells were removed with 250 IU/ml hyaluronidase (Sigma), followed by a rinse in Medium 2 (Fulton and Whittingham, 1978) containing 4 mg/ml of bovine serum albumin (M2+BSA). Oocytes were cultured in M2+BSA at 37°C under 5% CO_2 in air.

Parthenogenetic activation

Oocytes were cultured in M2+BSA containing 10 μg/ml of puromycin (prepared from a stock solution, 1 mg/ml in dimethylsulfoxide, stored at -20°C), at 37°C under 5% CO_2 in air. After 3 h of culture, activated oocytes were rinsed twice in M2+BSA and either used immediately as controls or cultured for 2 or 3 more h in the presence or absence of OA.

Okadaic acid treatment

Okadaic acid (Moana Bioproducts Inc., Hawaii, USA) was used at concentrations of 10, 100 nM and 1 μM in M2 medium and was freshly prepared for each experiment from a 125 μM stock solution in dimethylformamide (DMF) stored at 4°C. We checked that DMF had no effect on the oocyte and on the microtubule network at the doses used.

Radiolabeling of proteins

[^{32}P]-orthophosphate (Amersham) was used at a final specific activity of 500 μCi/ml. Oocytes were incubated with or without OA in M2+BSA for various times prior to labeling with 500 μCi [^{32}P] in M2+BSA without phosphate (with or without OA) for 1 hour. Then, oocytes were washed three times in M2 supplemented with 6 mg/ml polyvinyl-pyrrolidone, lysed in twice concentrated Laemmli buffer and prepared for gel electrophoresis (Laemmli, 1970).

Histone H1 kinase assay

Histone H1 kinase activity was determined as described by Félix *et al.* (1989) in HK buffer (80 mM ß-glycerophosphate, 20 mM EGTA pH 7.3, 15 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin) using exogenous histone H1 (HIII-S from calf thymus, Sigma) as a substrate. Samples of 40 oocytes in 5 μl of water were lysed by freezing and thawing three times, diluted twice in two times concentrated HK buffer and incubated 15 min at 20°C in the presence of 3.3 mg/ml histone H1, 1 mM ATP and 0.25 mCi/ml [^{32}P]-ATP. The reaction was stopped by the addition of a similar volume of twice concentrated Laemmli buffer (Laemmli, 1970) and incubation for 2 min at 90°C.

Electrophoresis

Samples were analyzed using a 10% SDS-polyacrylamide gel (Laemmli, 1970) in a BioRad Protean IIxi vertical slab cell system and autoradiographed using Amersham ß-MAX films. Gels were soaked in Amersham Amplify for 20 min prior to exposure.

Immunofluorescence

The zonae pellucidae of oocytes were removed by a brief exposure to acid Tyrode's solution (Nicolson *et al.*, 1975) followed by three washes in M2+BSA. Oocytes were placed in specially designed chambers as previously described (Maro *et al.*, 1984) except that the chambers were coated with 0.1 mg/ml concanavalin A (Sigma). The samples were centrifuged at 450 g for 10 min at 37°C, fixed and extracted with 0.2% glutaraldehyde, 1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min at 30°C, then permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature and neutralized with 2 mg/ml $NaBH_4$ in PBS for three times 10 min. Immunofluorescence staining was performed as previously described (Maro *et al.*, 1984). For tubulin staining, the rat monoclonal YL1/2 antibody (Kilmartin *et al.*, 1982) was used and fluorescein labeled anti-rat antibodies (Biosys) as second layer. The other antibody used was a human serum directed against lamin B (Guilly *et al.*, 1987). To visualize chromatin, propidium iodide (5 μg/ml) was added to the second layer.

Photomicroscopy and confocal scanning microscopy

After removing the coverslips from the chambers, the samples were mounted in «Citifluor» (City University, London) and viewed under a Leitz Diaplan microscope. Confocal laser scanning microscopy was performed as described by Zernicka-Goetz *et al.* (1993) using a BioRad MRC-600,

mounted on an Optiphot II Nikon microscope equipped with a 60x objective (plan apo; NA 1.4). Black and white pictures were taken on Kodak T-Max 100 using a Nikon F-301 camera mounted on a high resolution monitor.

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