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The effect of PD98059 on MAPK regulation in cumulus-enclosed and cumulus-free mouse oocytes

Jaroslav Kalous, Michal Kubelka and Jan Motlík
Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburská 899, 277 21 Libečhov, Czech Republic

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Summary

The effect of the p42/44 mitogen-activated kinase (MAPK) inhibitor, PD98059, on MAPK activation and meiosis resumption in mouse oocytes was studied. When germinal vesicle (GV)-stage denuded oocytes (DOs) were cultured continuously in 50 μM PD98059, germinal vesicle breakdown (GVBD) was postponed for 2–3 h. MAPK phosphorylation and activation was delayed as well. However, PD98059 did not impair histone H1 kinase activation. After 14 h of culture there was no significant difference in the rate of DOs reaching metaphase II (MII) arrest in either control or experimental conditions. The effect of PD98059 on MAPK inhibition was further tested in epidermal growth factor (EGF)-treated oocyte–cumulus complexes (OCCs). Exposure of GV-stage OCCs for 5 min to EGF (10 ng/ml) induced a considerable increase in MAPK phosphorylation. After OCCs were further cultured in 50 μM PD98059 a rapid dephosphorylation of MAPK was induced. Already after 1 min of treatment the non-phosphorylated form of MAPK dominated, indicating the high effectivity of PD98059. This result indicates that short EGF/PD98059 treatment of OCCs induced MAPK phosphorylation/dephosphorylation in cumulus cells only. As only a transient delay in MAPK phosphorylation and activation was observed in PD98059-treated DOs we conclude that there is also another PD98059-nonsensitive pathway(s) leading to MAPK activation in mouse oocytes. The data obtained suggest that meiosis resumption in mouse oocytes is somehow influenced by the MEK/MAPK activation pathway.

Keywords: GVBD, Histone H1 kinase, MAP kinase, Meiosis, Metaphase II, Mouse oocyte

Introduction

Although the time sequence of meiotic maturation is species-specific (Motlík, 1989), oocyte maturation generally depends on the activation of a cytoplasmic maturation promoting factor (MPF), which is composed of a complex of protein kinase $p34^{cdc2}$ and cyclin B (Lohka *et al*., 1988; Motlík & Kubelka, 1990). Also p42/44 MAPK, named ERK (extracellular regulated kinase), is activated during oocyte maturation (Haccard *et al*., 1990; Verlhac *et al*., 1994). Phosphorylation catalysed by MPF and MAPK leads to germinal vesicle breakdown (GVBD), which is a complex event that includes condensation and individualisation of chromosomal bivalents, disassembly of the nuclear envelope and formation of a meiotic spindle (Calarco *et al*., 1972).

Activated MPF triggers meiosis resumption by phosphorylating different substrates, particularly nuclear lamins (Dessev *et al*., 1991) and histone H1 (Langan *et al*., 1989). MAPK in its active form phosphorylates a number of proteins, such as tubulin present in the meiotic spindle (Verde *et al*., 1990), p90rsk (Kaláb *et al*., 1996) and proteins associated with microtubule organising centres (Centonze & Borisy, 1990; Buendia *et al*., 1992). MAPK activation can be assayed by the phosphorylation shift of ERK 1,2 proteins and, with some reservations (Peter *et al*., 1992), by phosphorylation of myelin basic protein (MBP) in *in vitro* assay (Moos *et al*., 1995). MAPK is activated by MEK, a tyrosine/threonine kinase (Cobb *et al*., 1991). MEK itself is activated as the result of the activity of an upstream Raf-1 kinase. Raf-1 is not the only MEK activator as

All correspondence to: Jaroslav Kalous, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburská 89, 277 21 Liběchov, Czech Republic. Tel: +420 305 639562. Fax: +420 305 697186. e-mail: Kalous@iapg.cas.cz

other protein kinases including Mos and MEKK can activate MEK (Campbell *et al*., 1995). The role of Mos and MAPK in meiosis resumption has been studied in various species. Translation of maternal mRNA encoding for the protein kinase Mos has an essential role during *Xenopus* oocyte maturation (Yew *et al*., 1992; Palmer & Nebreda, 2000). In *Xenopus* oocytes MAPK activity leads to GVBD and histone H1 kinase activation, suggesting that in frog oocytes MAPK is indispensable for meiotic resumption (Gotoh *et al*., 1995; Sagata, 1998). In oocytes of *Chaetopterus*, a marine invertebrate, MAPK activation was detected before GVBD but MAPK activity is not necessary to induce GVBD (Gould & Stephano, 1999). In starfish oocytes MAPK activity occurs after GVBD only (Abrieu *et al*., 1997) and is not involved in MPF activation (Okano-Uchida *et al*., 1998).

In mouse, Mos is not required for the initiation of oocyte maturation (Paules *et al*., 1989) and an increase in Mos synthesis is detected after GVBD only (Verlhac *et al*., 1996). MAPK activation occurs after GVBD and histone H1 kinase activation in mouse oocytes (Choi *et al*., 1996; Verlhac *et al*., 1996; Zernicka-Goetz *et al*., 1997). But GVBD can be induced in mouse fully grown oocytes as well as incompetent oocytes by okadaic acid under conditions when MAPK becomes activated while histone H1 kinase remains inactive (Verlhac *et al*., 1994; de Vantéry *et al*., 2000). This indicates that in mouse MAPK can substitute for histone H1 kinase in the induction of meiosis resumption. In cattle oocytes, MAPK activation occurs together with or slightly after the rise in histone H1 kinase activity and the activation of both kinases occurs at the time of GVBD (Fissore *et al*., 1996; Kubelka *et al*., 2000). In goat oocytes MAPK activity rises after GVBD and MPF activation, indicating that MAPK is not implicated in the early events of meiosis resumption in this species (Dedieu *et al*., 1996). MAPK becomes activated slightly before GVBD in porcine oocytes (Inoue *et al*., 1998; Kubelka *et al*., 2002).

Membrana granulosa cells and cumulus granulosa cells are important for intrafollicular regulation of meiosis (Thibault *et al*., 1975) and they also control resumption of meiosis under *in vitro* conditions (Racowsky & Baldwin, 1989; Motlík *et al*., 1996; Sršeň *et al*., 1998). In porcine granulosa cells, epidermal growth factor (EGF) is a major growth stimulatory factor (Gospodarowicz & Bialecki, 1979). EGF-receptor has been detected by immunostaining in porcine cumulus, granulosa and theca cells at all follicle stages (Singh *et al*., 1995). EGF has been shown to be a more potent activator of MAPK than gonadotropins (Cameron *et al*., 1996). Maximal phosphorylation of MAPK in porcine granulosa cells was induced with 5–10 ng/ml EGF (Keel & Davis, 1999). The MEK inhibitor PD98059 can prevent growth-factor-induced MAPK phosphorylation. The inhibitor binds to the inactive forms of MEK and inhibits its phosphorylation and activation by upstream activators such as MEK kinase and Raf-1 (Alessi *et al*., 1995; Dudley *et al*., 1995). Fifty micromolar PD98059 can effectively suppress MAPK activity in response to various growth factors including neural growth factor in PDO12 cells and EGF in Swiss 3T3 cells (Alessi *et al*., 1995). The effect of PD98059 on the inhibition of the MAPK pathway in oocytes was demonstrated in non-mammalian species and in mouse incompetent oocytes. In *Urechis caupo* oocytes MAPK activation was inhibited by PD98059 but GVBD still occurred (Gould & Stephano, 1999). Moreover, the metaphase II arrest induced by cytostatic factor (CSF) and the spindle assembly checkpoint were both abolished in the presence of this drug. This demonstrates that p42 MAPK is responsible for both CSF- and checkpoint-induced metaphase arrest in this species. In *Chaetopterus* oocytes the inhibition of MAPK phosphorylation by PD98059 blocked neither GVBD nor $p34^{cdc2}$ activation (Eckberg, 1997). In *Xenopus*, the Mos-activated MAPK pathway is sensitive to PD98059 and in the presence of the inhibitor only about 30% of oocytes underwent postponed GVBD (Cross & Smythe, 1988). But it has also been demonstrated that PD98059 does not prevent GVBD in *Xenopus* oocytes and it is sufficient only to retard temporarily GVBD and MAPK activation (Bitangcol *et al*., 1998). Incompetent mouse oocytes cultured for 2 days and then treated with okadaic acid (OA) resume meiosis with premature MAPK activation occurring before GVBD, whereas histone H1 kinase is not activated (Chesnel & Eppig, 1995). But when OA-treated incompetent mouse oocytes were exposed to PD98059, a transient inhibition of GVBD and MAPK activation occurred (de Vantéry *et al*., 2000).

In the present study we have investigated the effect of the MEK inhibitor PD98059 on regulation of the MAPK pathway in mouse DOs and oocyte–cumulus complexes (OCCs). In DOs, PD98059 exhibited only a transient inhibitory effect upon meiosis resumption and MAPK activation whereas histone H1 kinase activation was not affected. We further report that PD98059 substantially accelerated dephosphorylation of MAPK in cumulus cells of EGF-treated OCCs.

Materials and methods

Source of oocytes

Mouse ovaries were obtained from 3- to 4-week-old PMGS-treated ICR female mice (Velaz Praha, Czech Republic). Ovaries were transferred to pre-warmed (37 °C) Parker's medium supplemented with 3 mg/ml bovine serum albumin (BSA, Sigma) and 5 mM IBMX (Sigma) to prevent GVBD during isolation of oocytes. Antral follicles were punctured and fully

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grown oocytes surrounded by regular cumulus layer (OCCs) were isolated. In experiments with cumulus-free oocytes cumulus cells were mechanically removed either before (denuded oocytes; DOs) or after culture procedure (stripped OCCs).

Oocyte culture

Modified Parker's medium (M199, Sevac, Prague), supplemented with 2.92 mM calcium lactate, 2 mM sodium pyruvate, 33.9 mM sodium bicarbonate, 4.43 mM Hepes buffer, pH 7.2, 50 IU/ml penicillin, 50 µg/ml streptomycin sulfate and 3 mg/ml BSA (Sigma) was used as a basic medium (BM). To prepare control medium 5 µl of dimethylsulfoxide (DMSO) was added to 1 ml of BM. In order to analyse the effect of EGF and PD98059, BM was supplemented with either EGF (10 ng/ml) or PD98059 (50 µM). Ten millimolar PD98059 diluted in DMSO was used as a stock solution. Due to a precipitate occurring in media it was impossible to prepare higher concentrations than 50 µM PD98059. Groups of 50 OCCs or DOs were cultured in 500 µl of appropriate medium under paraffin oil at 38 °C in a 5% CO_2 atmosphere. GV-stage OCCs were cultured for 5 min in EGF (10 ng/ml)-supplemented BM. Subsequently, OCCs were washed in EGF-free BM and cultured further in either control medium or BM supplemented with 50 µM PD98059. After culture, OCCs were washed in protein-free medium (Parker's medium + 30 mg/ml polyvinyl alcohol (PVA), Sigma) and a proportion of OCCs was mechanically freed of cumulus cells. Washed oocytes were frozen in Eppendorf tubes at –80 °C (5 or 50 OCCs deprived of cumulus after culture or 5 whole OCCs were stored in each tube).

To test the effect of PD98059 on meiosis progression and kinase activation, mouse DOs were cultured for up to 14 h in control medium or in BM supplemented with 50 µM PD98059. After culture, DOs were washed in protein-free medium and either used for morphological analysis or stored in Eppendorf tubes at –80 °C (10 DOs in each tube).

Histone H1 and MAP kinase assay

Eppendorf tubes with frozen oocytes were placed on ice and 4 µl of buffer A (15 mM para-nitrophenyl phosphate, 60 mM b-glycerophosphate, 15 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.25 mM Na_3VO_4, 60 µg/ml leupeptin and 60 µg/ml aprotinin) added. The tubes were briefly vortexed, centrifuged at 10 000 *g* for 15 s, and then the kinase reaction was initiated by addition of 5 µl of buffer B (25 mM Hepes, pH 7.2, 5 mM EDTA, 0.1 mM EGTA, 20 M cAMP-dependent protein kinase inhibitor (Sigma), 60 µg/ml leupeptin, 60 µg/ml aprotinin, 600 µM ATP, 2 mg/ml histone H1 (Boehringer) and 3 mg/ml MBP (Sigma) and 500 Ci/ml (γ-^{32}P]ATP (Amersham). The reaction was allowed to proceed for 30 min at 30 °C and was stopped by the addition of 15 µl, of double-concentrated sample buffer (Laemmli, 1970). Samples were boiled for 3 min and separated on 15% SDS PAGE gel. Gels were stained with Coomassie Blue R250 (Serva), destained overnight, dried and subjected to autoradiography.

Immunoblotting

Electrophoresis and immunoblotting conditions used were essentially as described in Verlhac *et al.* (1994) with minor modifications. OCCs and DOs stored in Eppendorf tubes at –80 °C were removed from the freezer and lysed in 10 µl of sample buffer for SDS PAGE (Laemmli, 1970). After heating at 100 °C for 3 min, samples were separated on 10.5% 'shift' polyacrylamide gel (ratio acrylamide/bis-acrylamide 99:1) the concentration of stacking gel being 3% (ratio acrylamide:bisacrylamide 36.5:1). Lauryl sulfate (SDS) from Sigma (L-4509) was used in all SDS electrophoresis buffers and freshly prepared ammonium persulfate (Bio Rad) was used to polymerise the gels. After electrophoresis, proteins were electrically transferred to PVDV membrane (Immobilon-P, Millipore) using a wet blotting system. Blots were incubated for 1 h in 10% teleostein gelatin (Sigma) in Tris-buffered saline (20 mM Tris, pH 7.4, 150 mM NaCl) with 0.05% Tween 20 (TTBS). TTBS was used for all following incubations and washes as well. To detect MAPK, after 1 h of incubation with rabbit anti-ERK 1 antibody (Santa Cruz, diluted 1:1000), blots were washed (3 times 5 min) in TTBS and incubated 1 h with secondary antibody (horseradish peroxidase linked donkey anti-rabbit Ig, Amersham, 1:5000). After extensive washing (5 times 5 min), enhanced chemiluminescence detection was used to reveal the reaction of antibodies (ECL, Amersham).

Phase-contrast microscopy

To evaluate the rate of GVBD the DOs were mounted on slides, fixed for 24 h in acetic acid: ethanol (1:3, v/v), stained with orcein and then examined using a phase-contrast microscope.

Statistical analysis

Each experiment of DO maturation was performed three times and the mean percentage of GVBD ± SEM were calculated. The data were subjected to paired *t*-test analysis.

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Results

PD98059 affects the induction of GVBD in mouse DOs

To study the effect of the MEK inhibitor PD98059 on the kinetics of meiosis resumption, mouse GV-stage DOs were cultured in BM supplemented with 50 μM PD98059. GVBD occurred later in PD98059-treated DOs than in those cultured in control conditions. During 2 h of culture 80% of control DOs underwent GVBD but only 20% of DOs cultured in 50 μM PD98059 resumed meiosis at this time interval. The significant delay in GVBD was evident also after 3 and 4 h of culture (Fig. 1). But when the culture interval was prolonged up to 5–14 h, more than 95% of DOs in both control and experimental groups underwent GVBD. After 14 h, 98% of control and PD98059-treated DOs were arrested at meiosis II (MII) stage. Also MII arrest was not abolished when control MII-stage DOs were treated with PD98059 (data not shown). These results indicate that PD98059 postponed the onset of meiotic maturation in mouse DOs. The data obtained further suggest that the effect of PD98059 on meiosis resumption is only transient and neither the meiotic competence nor MII arrest are impaired. When the rate of GVBD was scored in PD98059-treated OCCs similar results were obtained as for DOs (data not shown).

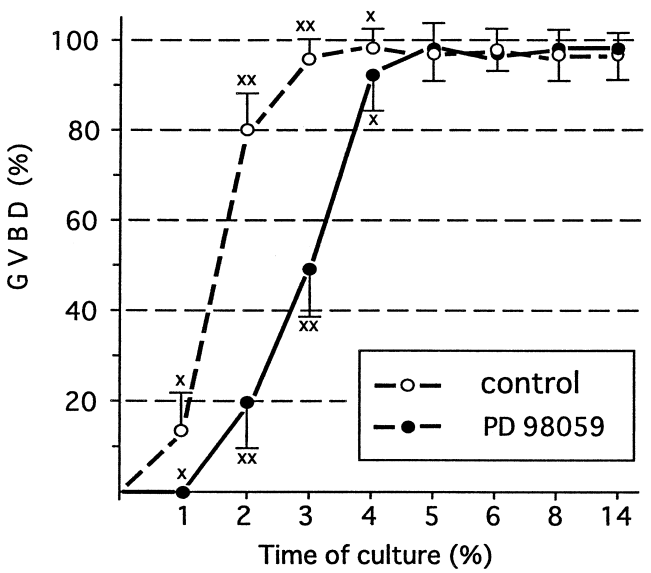

Figure 1 The effect of PD98059 on resumption of meiosis in mouse DOs (oocytes deprived of cumulus before culture). DOs were cultured up to 14 h either in DMSO (5 μl/ml) or PD98059 (50 μM)-supplemented BM. The percentage of GVBD was scored at various time points of culture. Results were pooled from three replicated experiments. The differences between control and PD98059-treated groups were analysed by pair *t*-test. Standard deviation bars are depicted; significantly different values are marked XX ($p < 0.01$) and X ($p < 0.05$). A total of 966 oocytes was evaluated.

The effect of PD98059 on histone H1 kinase and MAPK activation in DOs

Immunoblot analysis (Fig. 2*A*) and kinase assay (Fig. 2*B*) revealed that MAPK phosphorylation and activation in PD98059-treated mouse DOs was temporarily suppressed. In control DOs, MAPK phosphorylation shift (Fig. 2*A*, lane 2) and activation (Fig. 2*B*, lane 3) were detected after 2h of culture (e.g. 30 min after GVBD). But these both events were delayed in PD98059-treated DOs, where MAPK phosphorylation shift (Fig. 2*A*, lane 8) and activation (Fig. 2*B*, lane 13) were detected only after 4 and 6 h of culture, respectively. At 6 h of culture in PD98059-supplemented medium a full expression of MAPK phosphorylation was parallel to the increase of MAPK activity (Fig. 2*A*, lane 9 and Fig. 2*B*, lane 13). The dynamics of histone H1 kinase activation in PD98059-treated DOs (Fig. 2*B*, lanes 8–14) was similar to that detected in the control

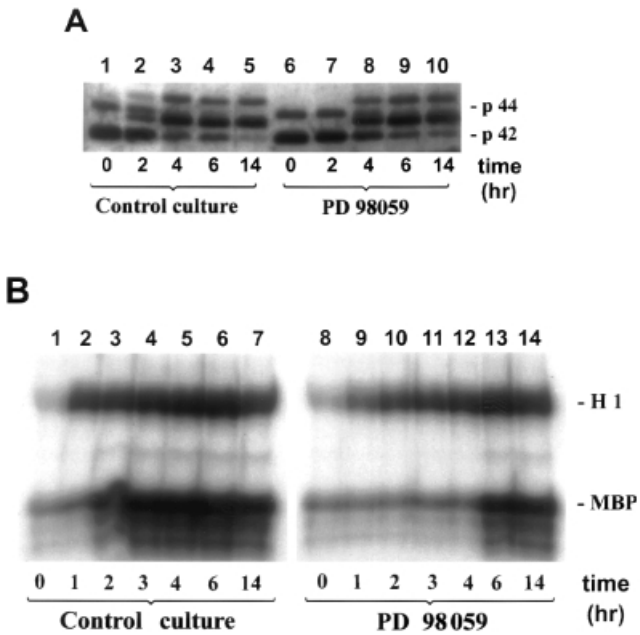

Figure 2 Histone H1 kinase and MAPK activities in control and PD98059-treated mouse DOs (oocytes deprived of cumulus before culture). (*A*) MAPK immunoblotting of DOs cultured for 14 h in BM supplemented with either DMSO (5 μl/ml) (lanes 1–5) or PD98059 (50 μM) (lanes 6–10). The dynamics of MAPK phosphorylation is documented by mobility shift of p42/44 MAPK. (*B*) Histone H1 kinase and MAPK activation in control and PD98059-treated DOs. Histone H1 and MBP double-kinase assay was performed in lysates of oocytes cultured for 0–14 h in BM supplemented with either DMSO (5 μl/ml) (lanes 1–7) or PD98059 (50 μM) (lanes 8–14).

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(Fig. 2*B*, lanes 1–7). MAPK activation in PD98059-treated DOs was more delayed relative to histone H1 kinase activation than it was in control DOs (Fig. 2*B*). After 14 h, both control and PD98059-treated DOs were arrested at MII stage exhibiting high histone H1 kinase and MAPK activities (Fig. 2*B*, lanes 7 and 14). MAPK phosphorylation shift was detected at this culture interval as well (Fig. 2*A*, lanes 5 and 10).

PD98059 promotes MAPK inactivation in EGF-treated OCCs

As PD98059 inhibited only transiently GVBD and MAPK activation in DOs, the effect of PD98089 was further tested on EGF-treated OCCs. MAPK immunoblotting revealed that in GV-stage OCCs exposed to EGF (5 min, 10 ng/ml) phosphorylation of both p42/44 MAPK isoforms was stimulated (Fig. 3*B*, lanes 2 and 9). After EGF-treated OCCs were further cultured in control medium the intensity of MAPK phosphorylation shift decreased slowly and reached its initial level (before EGF treatment) in 30 min (Fig. 3*B*, lanes 3–8). When EGF-treated OCCs were exposed to 50 μM PD98059, the MAPK phosphorylation shift disappeared rapidly (Fig. 3*B*, lanes 10–13). These results proved that PD98059 effectively suppressed MAPK phosphorylation in EGF-treated OCCs. The stimulating effect of EGF (10 ng/ml, 5 min) on MAPK phosphorylation was not observed in OCCs deprived of cumulus after the culture procedure (Fig. 3*A*, lanes 5 and 8). The observed changes in MAPK phosphorylation in whole EGF/PD98059-treated OCCs represent the general situation in cumulus cells, as the amount of MAPK protein in samples of 5 OCCs denuded after culture (Fig. 3*A*, lanes 4 and 5) is negligible compared with that detected in samples of 5 whole OCCs (Fig. 3*A*, lanes 1–3).

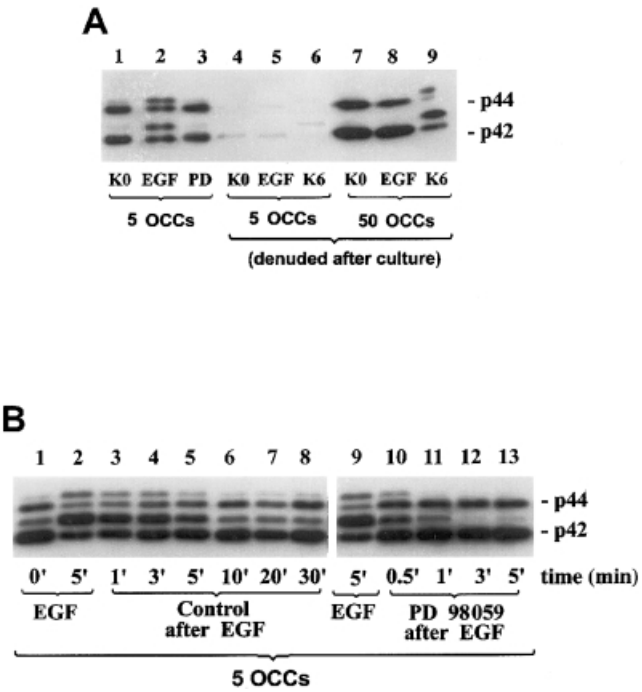

Figure 3 Immunoblot analysis of MAPK phosphorylation in EGF- and PD98059-treated oocytes. (*A*) The effect of EGF on MAPK phosphorylation in whole OCCs and OCCs denuded after culture. Lane 1, non-treated GV-stage whole OCCs; lane 2, EGF (5 min, 10 ng/ml)-treated GV-stage whole OCCs; lane 3, EGF (5 min, 10 ng/ml)+PD98059 (5 min, 50 μM)-treated GV-stage whole OCCs; lanes 4 and 7, untreated GV-stage denuded OCCs; lanes 5 and 8, EGF (5 min, 10 ng/ml)-treated GV-stage denuded OCCs; lanes 6 and 9, OCCs denuded after 6 h of control culture. Lysates of 5 whole OCCs (lanes 1–3), 5 (lanes 4–6) and 50 denuded OCCs (lanes 7–9) were loaded into the appropriate lane. (*B*) The effect of PD98059 on MAPK phosphorylation in EGF-treated GV-stage whole OCCs. Lane 1, untreated OCCs; lanes 2 and 9, EGF (5 min, 10 ng/ml)-treated OCCs; lanes 3–8, EGF-treated OCCs cultured subsequently in control conditions (i.e. in DMSO (5 μl/ml)-supplemented BM); lanes 10–13, EGF (5 min, 10 ng/ml)-treated OCCs cultured subsequently in PD98059 (50 μM)-supplemented BM. Lysates of 5 whole OCCs were loaded onto each lane.

Discussion

This work was performed to analyse the role of MAPK activation during meiotic maturation of mouse oocytes.

In fully grown *in vitro* cultured mouse oocytes MAPK is activated only after GVBD and histone H1 kinase activation is induced (Zernicka-Goetz *et al.*, 1997). Also it has been revealed that MAPK activation does not play a role in GVBD induction and histone H1 kinase activation in oocytes from $mos^{-/-}$ mice (Verlhac *et al.*, 1996). In our experiments the MEK inhibitor PD98059 induced a transient inhibition of GVBD and MAPK activation in mouse DOs. As it has been reported that PD98059 specifically inhibits MEK/MAPK activation by MEK kinase and Raf1 (Dudley *et al.*, 1995; Alessi *et al.*, 1995), we conclude that other PD98059-insensitive pathway(s) also play role in MAPK activation during meiosis resumption in mouse oocytes. This idea is supported by the fact that synthesis of Mos, a MEK-activating kinase, is needed for MAPK activation in mouse oocytes (Verlhac *et al.*, 1996) and there are no data indicating that PD98059 prevents MEK activation by Mos. Alternatively, PD98059 might not fully inhibit MEK activation and a small proportion of activated MEK might be sufficient to induce gradual phosphorylation and activation of MAPK.

It has been documented that MAPK participates in microtubule, chromosome and nuclear envelope changes leading to GVBD in mouse oocytes, but these MAPK activities are not essential for GVBD (Choi *et al.*,

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1996). Moreover, in mos$^{-/-}$ mouse oocytes activation of histone H1 kinase is followed by GVBD while MAPK activity remains inactive (Verlhac *et al*., 1996). This means that histone H1 kinase activity alone is sufficient to induce meiotic resumption in mouse oocytes under conditions when MAPK is prevented from contributing to this event. Our data reveal that PD98059 did not affect events leading to histone H1 kinase activation in mouse DOs. But the delay in MAPK activation was accompanied by a delay in GVBD, indicating that the events leading to MAPK activation are possibly involved in GVBD induction. This idea is supported by the fact that MAPK, rather than MPF, can induce GVBD in competent okadaic-acid-treated mouse oocytes (Gavin *et al*., 1994; Chesnel & Eppig, 1995). Also, when incompetent mouse oocytes were exposed to okadaic acid, MAPK activation was induced, followed by GVBD, whereas histone H1 kinase was not activated (Verlhac *et al*., 1994). This indicates that under certain conditions MAPK can substitute for histone H1 kinase in the induction of meiosis in mouse oocytes. Moreover, when okadaic-acid-treated mouse incompetent oocytes were exposed to PD98059, a transient inhibition of MAPK activation and GVBD was observed (de Vantéry *et al*., 2000). These data support our results suggesting that events leading to MEK/MAPK activation are somehow involved in the events leading to GVBD in mouse oocytes.

When GV-stage DOs were continuously cultured in PD98059-supplemented medium they passed through GVBD reaching MII arrest. Moreover, when control MII-stage DOs were exposed to PD98059, we did not observe a decrease in MAPK and histone H1 kinase activity and MII arrest was not abolished as well. It was reported that in maturing mos$^{-/-}$ mouse oocytes MAPK activity and MII arrest were restored only when mos mRNA was microinjected, as the microinjection of constitutively active Raf mRNA was ineffective in this case (Verlhac *et al*., 1996). To explain this we take into account the fact that the MEK inhibitor PD98059 binds to the inactive forms of MEK and prevents its phosphorylation and activation by upstream MEK kinase and Raf1 (Alessi *et al*., 1995; Dudley *et al*., 1995). These facts suggest that in our experiments PD98059 was ineffective in MII-stage oocytes as neither Raf1 nor MEK kinase is involved in the persistence of MAPK activity during MII arrest of mouse oocytes. In contrast, in *Xenopus* and *Urechis caupo* oocytes, the cytostatic-factor-induced MII arrest was abolished in the presence of PD98059 (Bitangcol *et al*., 1998; Gould & Stephano, 1999). These data indicate that in oocytes of other species MII arrest might be MEK-kinase or Raf1-dependent.

As only a transient delay in meiosis resumption and MAPK activation in PD98059-treated DOs was detected we performed a further analysis of the effect of PD98059 on MAPK inhibition in EGF-stimulated OCCs. Western blot analysis revealed that in EGF-stimulated OCCs MAPK phosphorylation was induced. But after EGF-treated OCCs were deprived of cumulus cells MAPK phosphorylation was not detected in denuded oocytes. This indicates that in OCCs MAPK was stimulated in cumulus cells only. When EGF-treated OCCs were cultured subsequently in EGF-free medium a slow decrease in MAPK phosphorylation was observed. These results are in accordance with the data reporting the effect of EGF treatment on MAP kinase activity in porcine granulosa (Keel *et al*., 1995; Cameron *et al*., 1996; Keel & Davis, 1999) and other somatic cells (Lamy *et al*., 1993; Chao *et al*., 1994; Traverse *et al*., 1994). It has been shown that in EGF-stimulated cells dephosphorylation gradually prevails after the peak of MAPK activation has been reached (Griswold-Prenner *et al*., 1993). When EGF-stimulated OCCs were further cultured in PD98059-supplemented medium the MAPK dephosphorylation was accelerated. We conclude that while the new phosphorylation of MAPK in cumulus cells was prevented by PD98059, dephosphorylation of MAPK by specific cellular phosphatases prevailed rapidly. Similarly, in EGF-treated Swiss 3T3 cells PD98059 effectively suppressed MAPK activity (Alessi *et al*., 1995). In mouse fibroblasts transfected with EGF receptor PD98059 attenuated EGF-induced MAPK activation also (Kim *et al*., 2000). And in EGF-stimulated human granulosa cells MAPK phosphorylation and activity was inhibited by PD98059 (Oliver *et al*., 1999). These data indicate that PD98059 efficiently inhibits MAPK phosphorylation and activity in mammalian cells.

We demonstrated that PD98059 was highly effective in our culture conditions as accelerated MAPK dephosphorylation occurred in EGF-stimulated mouse OCCs exposed to PD98059. As PD98059 affected MAPK activation and meiosis resumption only transiently in mouse DOs, we suppose that the MAPK pathway involved in meiosis resumption of mouse oocytes is not fully PD98059-sensitive. For further studies of the role of MAPK in meiosis regulation it will be more effective to use inhibitor(s) acting downstream of PD98059, as this inhibitor of MEK does not completely block MAPK activation in mouse oocytes.

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