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MAPK/ERK kinase (MEK) signalling is required for resumption of meiosis in cultured cumulus-enclosed pig oocytes

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Summary

Resumption of meiosis of mammalian oocytes is facilitated by the maturation promoting factor (MPF) and accompanied by activation of mitogen activated protein kinases (MAPK) which are phosphorylated by the MAPK kinase (MEK). In this study we examined the effects of PD 98059, which inhibits the activity of MEK, on *in vitro* maturation of pig oocytes. Cumulus–oocyte complexes (COCs) were cultured in the presence or absence of the drug (50 μM) for various time periods. To elucidate the influence of cumulus cells, COCs were first cultured in inhibitor-free medium, subsequently denuded, and incubated further in PD 98059 supplemented medium. Reversibility of drug action as tested following PD 98059 treatment of COCs by transferring them to drug-free medium. Culture of COCs in medium supplemented with PD 98059 prevents resumption of nuclear maturation in the majority of COCs. This inhibition was reversible and accompanied by a non-activation of both MAP and MPF. Addition of the MEK inhibitor to extracts of *in vitro* matured oocytes revealed that the kinase activities were not directly influenced by the inhibitor, suggesting a link between MAP and MPF kinases. Preincubation of COCs in inhibitor-free medium for 6 h followed by further culture of COCs or denuded oocytes in the presence of PD 98059 for various periods resulted in elevated MAP and MPF kinase activities, indicating an early and transient MEK signalling in the oocyte itself. These results support the idea that MAP and MPF are involved in the induction of germinal vesicle breakdown in porcine oocytes.

Keywords: MAP kinase, Maturation, MEK, PD 98059, Pig oocyte

Introduction

In most vertebrates, fully grown oocytes which are arrested at meiotic prophase I resume meiosis in response to hormonal stimuli and progress to metaphase II when they are again arrested until they finally complete meiosis upon fertilisation. After removal of fully grown oocytes with surrounding cumulus cells from their follicles and culture in suitable media, oocytes will resume meiosis spontaneously (Pincus & Enzmann, 1935; Edwards, 1965). Nuclear maturation of porcine oocytes is characterised by early chromatin condensation and nucleolus disappearance followed by dissolution of the nuclear membrane (GVBD; Hunter & Polge, 1966; Motlik & Fulka, 1976). These morphological signs are accompanied by specific changes in protein phosphorylation controlled by enzymes that are intracellularly modified within specific signal transduction pathways. Activation of maturation-promoting factor (MPF) is the key process leading to resumption of meiosis (Masui & Markert, 1971). This serine/threonine kinase belongs to the family of cyclin-dependent protein kinases and consists of the catalytic subunit $p34^{cdc}$, a homologue of the yeast cdc2 protein kinase, and the regulatory subunit cyclin B (Gauthier *et al.*, 1988). In porcine oocytes MPF activity, determined by its histone H1 kinase activity (Arion *et al.*, 1988), increases around the time of GVBD and oscillates during the progression of meiosis with two maxima at metaphase I and II (Naito & Toyoda, 1991). Activation of MPF triggers the phosphorylation of a number of substrates, including nuclear lamins (Peter *et al.*, 1992) and histone H1 (Langan *et al.*, 1989), which suggests that this kinase is involved in the regulation

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of chromatin condensation and GVBD (Moreno & Nurse, 1990).

In addition to MPF, mitogen-activated protein kinase (MAPK), i.e. the 42 kDa and 44 kDa variants, has been reported to be involved in oocyte maturation in *Xenopus* (Gotoh *et al.*, 1991), mouse (Sobajima *et al.*, 1993), pig (Inoue *et al.*, 1995), goat (Dedieu *et al.*, 1996), cow (Fissore *et al.*, 1996), rat (Zernicka-Goetz *et al.*, 1997), horse (Goudet *et al.*, 1998) and human (Sun *et al.*, 1999). The MAPKs are dual specific protein kinases, which can phosphorylate substrates on both tyrosine and serine/threonine residues (for review see Campbell *et al.*, 1995). They are activated by specific extracellular signals (growth factors, neurotransmitter) that belong to different signal transduction pathways. For this reason, the MAPKs are also called extracellular signal-regulated kinases (ERKs, p42-ERK2, p44-ERK1). The intracellular signal transduction pathway consists of specific enzymes (i.e. c-ras, c-raf, c-mos) that are activating the MAPK kinases or MEKs, which subsequently phosphorylate and activate the MAPK (Pelech & Sanghera, 1992). In pig oocytes MAPK activity, determined using the substrate myelin basic protein (MBP), exhibited significantly elevated levels when the majority of the oocytes analysed had already undergone GVBD (Inoue *et al.*, 1995, 1996; Shimada & Terada, 2001; Shimada *et al.*, 2001). Furthermore, phosphorylated MAPK molecules associate with the spindle during the post-GVBD maturation period (Inoue *et al.*, 1998; Lee *et al.*, 2000; Sugiura *et al.*, 2001); thus one can suggest that the MAPK cascade is required not to initiate resumption of maturation but for microtubule dynamics in the meiotic spindle. On the other hand, prior to GVBD activated MAPK molecules translocate from the ooplasm into the intact germinal vesicle (GV), supporting the idea that these kinases are involved in the transduction pathway of the still unknown maturation-inducing agent and that MAPKs themselves transmit the signal from the cytoplasm into the GV of pig oocytes (Inoue *et al.*, 1998). This view is sustained by three lines of evidence. First, when activated MAPK, purified from matured starfish oocytes, was injected into the GV of denuded porcine oocytes, GVBD occurred in about 20% of oocytes within 5–10 h of subsequent culture (Inoue *et al.*, 1998). Second, when MAPK activities were determined in porcine oocytes removed at hourly intervals during a 48 h cultivation period, MAPK activities increased before GVBD took place (Wehrend & Meinecke, 2001). Third, when MEK was inhibited by UO126, only about 40% of pig cumulus-enclosed oocytes underwent GVBD whereas the majority of the oocytes remained in the GV stage showing no activation of MAP and MPF kinases (Kagii *et al.*, 2000). Concerning the effect of UO126 on meiotic maturation as well as on MAP and MPF kinase activities of denuded pig oocytes (DOs), inconsistent results have been reported. Kagii *et al.* (2000), using 100 µM UO126, observed that GVBD occurred in about 92% of DOs and was accompanied by phosphorylation of ERK 1/ERK 2, suggesting the necessity of cumulus cells for the effects of UO126 on the suppression of MAPK activation. In contrast, Shimada *et al.* (2001) demonstrated that 10 and 20 µM UO126, respectively, significantly decreased MAP and MPF kinase activities and led to chromatin hypercondensation in DOs following a preincubation in inhibitor-free medium for 22 h.

Therefore the aim of the present study was to examine the role of the MAPK signal transduction pathway(s) on the onset of meiotic resumption in porcine cumulus–oocyte complexes (COCs) and DOs by inhibiting the MEK signal pathway with PD 98059. This flavone derivate is a highly specific inhibitor of the MEK, preventing the activation of the kinase by an allosteric mechanism (Dudley *et al.*, 1995), and has been shown previously to inhibit MAP and MPF kinase activities in pig DOs and cumulus cells (Schilling *et al.*, 1998).

Materials and methods

Oocytes and culture conditions Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% saline at 30–33 °C within 1.5 h of death of the animals. Follicles with a diameter of 3–6 mm were flushed with 0.9% saline containing 1% (v/v) heat-inactivated fetal calf serum (FCS; Sigma Deisenhofen, Germany) using a 20 gauge needle connected to a 20 ml syringe. Oocytes with an evenly granulated cytoplasm and surrounded by a homogeneous cumulus cell layer were washed three times in Dulbecco's phosphate-buffered saline (PBS; Sigma) containing 10% (v/v) FCS. Twenty COCs were placed into 0.5 ml *in vitro* maturation (IVM) medium (TCM 199 (Sigma), 20 µg/ml insulin (Sigma), 50 µg/ml gentamicin (Serva, Heidelberg, Germany), 20% (v/v) FCS) supplemented with 20 IU/ml eCG (Intergonan, Intervet, Toenisvorst, Germany) in a 4-well multidish (Nunc, Roskilde, Denmark). COCs were matured at 39 °C in 5% CO_2 in a humidified atmosphere for the appropriate time depending on the experimental conditions.

Determination of nuclear maturation At the end of the IVM period, COCs were treated with 0.25% (w/v) hyaluronidase (bovine testes, Sigma), and cumulus cells were removed with a fine-bore Pasteur pipette. Oocytes were fixed in ethanol/acetic acid (3:1) for at least 24 h and stained in 2% (w/v) aceto-orcein solution. Nuclear maturation was determined under a phase-contrast microscope (BX 60, Olympus, Hamburg, Germany) according to the method of Motlik & Fulka (1976).

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Table 1 Effect of PD 98059 on MAP and MPF kinase activities of *in vitro* matured porcine oocytes

	Cultivation period		
	0 h	30 h DMSO	30 h PD 98059
MAP kinase	51.0 ± 7.4[a]	166.0 ± 30.5[b]	47.6 ± 6.5[a]
MPF kinase	44.3 ± 13.9[a]	82.6 ± 21.3[b]	37.8 ± 10.0[a]

Kinase activities were determined in oocytes immediately after recovery from follicles (0 h) or following culture of cumulus–oocyte complexes (COCs) in 0.5% (v/v) DMSO (30 h DMSO) and 50 µM PD 98059 (30 h PD 98059) supplemented medium for 30 h. The table presents the enzyme activities in cpm/10 oocytes (mean ± SD) for four replicates.
[a,b]Means with different superscripts differ significantly ($p < 0.05$).

Determination of histone H1 and MAP kinase activity At the end of cultivation COCs were denuded as described above. Ten oocytes were placed in 4 µl PBS and stored at –80 °C until activity determination. Activities of MAP and MPF kinase were determined concomitantly using histone H1 (Sigma) to assess MPF (Arion *et al*., 1988) and MBP (Sigma) to evaluate MAPK activity (Sanghera *et al*., 1990), with modification according to Fissore *et al*. (1996) as described in detail previously (Krischek & Meinecke, 2001). Briefly, oocyte lysates were incubated in the kinase assay buffer containing MBP, histone H1 and 0.1 mCi/ml [γ^{32}P]ATP (Hartmann-Analytic, Braunschweig, Germany) for 30 min at 30 °C. Following termination of the reaction, histone H1 and MBP were separated on a 15% SDS polyacrylamide gel (Laemmli, 1970), stained and dried. Phosphorylation of the substrates was visualized by autoradiography (Biomax MS, Sigma). Radioactivity in the individual histone H1 and MBP bands was measured by a liquid scintillation analyser (model 1600 TR, Canberra-Packard, Dreieich, Germany). Mean values of the blank samples, which contained all the components required for the reaction but without the addition of oocytes, were subtracted from each value to obtain histone H1 and MAP kinase activities.

Experimental design

Experiment 1 COCs were cultured for 30 h in IVM medium supplemented with 50 µM PD 98059 (Calbiochem-Novabiochem, Schwalbach, Germany). Control COCs were either denuded, fixed and stained immediately after recovery from the follicles (0 h) and stored at –80 °C until activity determination, or incubated for 30 h in IVM medium containing 0.5% (v/v) dimethylsulfoxide (DMSO; solvent of PD 98059, Merck, Germany). After termination of culture, nuclear status and MPF and MAPK activities were determined.

Experiment 2 To evaluate possible inhibitory effects of PD 98059 on MAPK and MPF, the activities of the kinases were determined in oocytes either immediately following recovery from the follicle or after culture in IVM medium for 48 h. PD 98059 (50 µM), the solvent of PD 98059 (0.3% v/v DMSO) and an equivalent volume (3 µl) of Aqua bidest, respectively, were added to the kinase assay buffer.

Experiment 3 COCs were matured in IVM medium for 6, 8, 10, 12, 14, 16 and 22 h, respectively, and subsequently transferred to IVM medium supplemented with 50 µM PD 98059 for an additional 24, 22, 20, 18, 16, 14 and 8 h, respectively. In control experiments, COCs were transferred after 6 h to DMSO (0.5%, v/v) supplemented IVM medium for a further 24 h. At the end of a culture nuclear maturation and, in specific experiments, MPF and MAP kinase activities were determined.

Experiment 4 COCs were initially incubated for 6 or 22 h in IVM medium, subsequently denuded and cultured for a further 24 or 8 h in medium supplemented with PD 98059 (50 µM) and DMSO (0.5%, v/v), respectively. At the end of culture nuclear maturation and, in specific experiments, MAP and MPF kinase activities were determined.

Experiment 5 COCs were cultured for 30 h in IVM medium supplemented with 50 µM PD 98059 and subsequently transferred to inhibitor-free medium for 18, 24, 30 and 48 h, respectively. At the end of cultivation nuclear maturation was determined.

Statistical analysis

All experiments were repeated at least four times. The results were statistically analysed (SAS) using the *t*-test. A p value of 0.05 or less was considered significant.

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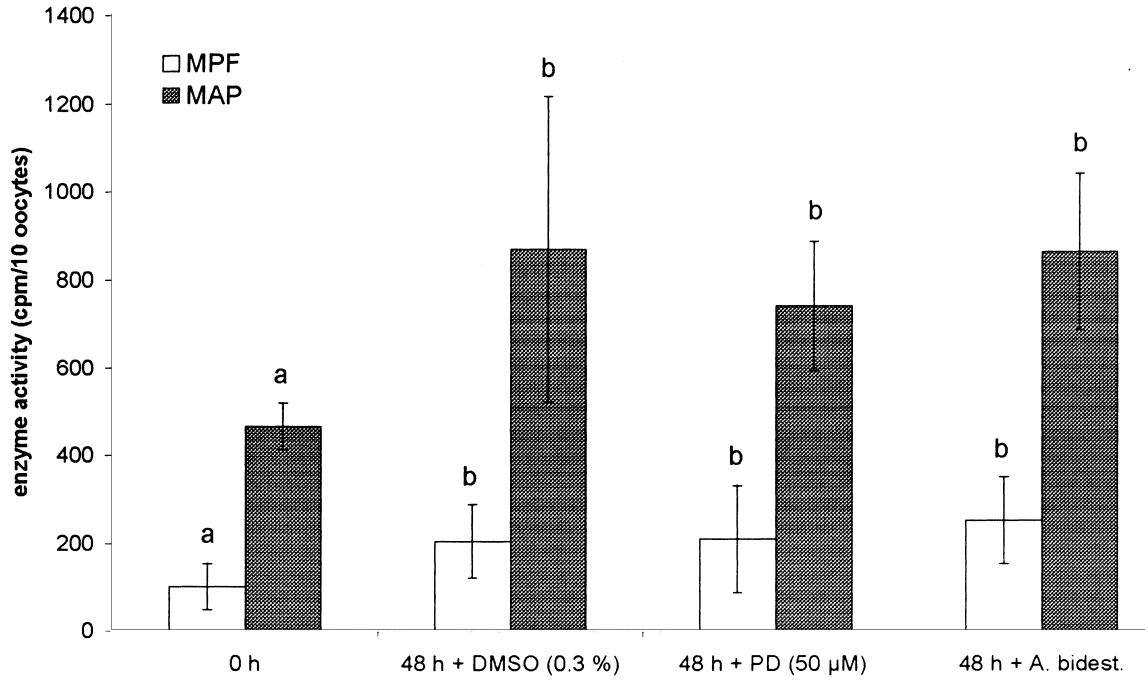

Figure 1 MAP and MPF kinase activities of oocytes analysed immediately after recovery from follicles (0 h) or following culture in inhibitor-free medium for 48 h. Denuded oocytes were subjected to MAP/MPF kinases assay in the presence of 0.3% (v/v) DMSO, 50 µM PD 98059 and Aqua bidest, respectively. Each column represents the mean (± SD) for four replicates of 10 oocytes, respectively. [a,b]Means with different superscripts in a line differ significantly ($p < 0.05$).

Results

Experiment 1

Immediately after recovery from the follicle 83.8 ± 15.6% (n = 163) of the oocytes exhibited an intact GV I stage, whereas 16.1 ± 15.8% of the oocytes showed signs of chromatin condensation (GV II to GV IV stages). Following culture for 30 h in IVM medium supplemented with 50 µM PD 98059, 66.4 ± 11.1% (n = 108) of the COCs exhibited a GV I stage. Conversely, only 2.4 ± 2.9% (n = 223) of the COCs incubated in IVM medium supplemented with 0.5% DMSO for 30 h remained in the GV I stage. This observation demonstrates that PD 98059 prevented chromatin condensation and resumption of meiosis in the majority of oocytes.

After maturation of COCs in IVM medium supplemented with 50 µM PD 98059 for 30 h, MAP and MPF activities remained as low as in oocytes analysed immediately after recovery from follicles, whereas control oocytes cultured in DMSO supplemented medium exhibited significantly elevated kinase values (Table 1). It is emphasised that both MAP and MPF kinase activities were influenced by the MEK inhibitor.

Experiment 2

Supplementation of the kinase buffer with 50 µM PD 98059, 0.3% (v/v) DMSO and Aqua bidest, respectively, resulted in non-significant differences in MAP and MPF kinase activities of oocytes cultured for 48 h in IVM medium (Fig. 1). The MAP and MPF kinase activities of oocytes cultured for 48 h in IVM medium were significantly higher compared with oocytes analysed immediately following recovery from follicles (Fig. 1). This observation indicates that PD 98059 did not directly inhibit MAP or MPF kinase activities or oocytes.

Experiment 3

When COCs were transferred following a first culture period in inhibitor-free medium for 6, 8 and 10 h, respectively, into PD 90859 supplemented medium for further 24, 22 and 20 h, respectively, about 60% of the oocytes remained at the GV I stage (Table 2). Prolongation of the initial inhibitor-free cultivation period to 12, 14, 16 and 22 h, respectively, resulted in a significant increase in the proportion of oocytes resuming meiotic maturation, indicating that COCs require an inhibitor-free culture period of at least 12 h to resume meiosis even in the presence of 50 µM PD 98059. However, a 22 h inhibitor-free incubation period is necessary for COCs to reach equivalent proportions of post-GVBD stages to those observed in control oocytes which were cultured initially in inhibitor free medium for 6 h and subsequently for 24 h in DMSO supplemented medium (Table 2). This

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Table 2 Effect of oocyte preincubation on PD 98059 inhibition of nuclear maturation of pig oocytes

First culture inhibitor-free (h)	Second culture in PD 98059 (P) or DMSO (D) (h)	*n*	Oocytes in GV I stage (%)	Oocytes in D, MI, AI, TI stage (%)
6	24 (P)	167	63.1 ± 7.3^{a}	10.6 ± 8.6^{a,b}
8	22 (P)	114	66.5 ± 12.6^{a}	15.1 ± 7.9^{b}
10	20 (P)	194	62.3 ± 15.5^{a}	11.6 ± 8.4^{a,b}
12	18 (P)	249	44.4 ± 12.1^{b}	38.2 ± 18.5^{c}
14	16 (P)	164	49.0 ± 14.7^{b}	30.9 ± 19.5^{b,c}
16	14 (P)	144	34.1 ± 4.0^{c}	57.3 ± 9.4^{d}
22	8 (P)	110	20.8 ± 9.5^{d}	67.1 ± 10.3^{d,e}
6	24 (D)	128	16.6 ± 8.8	76.2 ± 12.8^{e}

COCs were first cultured in IVM medium (6, 8, 10, 12, 14, 16 and 22 h, respectively) and subsequently transferred to medium supplemented with 50 µM PD 98059. Maturation was terminated after 30 h. Control COCs were matured for 6 h in IVM medium and subsequently transferred to DMSO (0.5%, v/v) supplemented medium for a further 24 h. The table presents the percentages (mean ± SD) of oocytes in germinal vesicle I (GVI),* diakinesis (D), metaphase I (MI), anaphase I (AI) and telophase I (TI) stages from at least four replicates.

*For clarity proportions of oocytes in GV II–IV are not shown.

^{a,b,c,d,e}Means with different superscripts in a column differ significantly ($p < 0.05$).

observation demonstrates that PD 98059 may also influence later nuclear maturation stages.

Determination of MAP and MPF kinase activities in oocytes cultured in inhibitor-free medium for 6, 12 and 16 h, respectively, and subsequently transferred to PD 98059 supplemented medium, revealed a significant increase in enzyme activities in comparison with oocytes analysed immediately after recovery from the follicles (Fig. 2). These elevated enzyme values were as high as enzyme activities of control oocytes cultured in DMSO supplemented medium for 30 h (Fig. 2). These results suggest that MEK activation occurs during the first 6 h of inhibitor-free cultivation of COCs, leading to an increase in MAP and MPF kinase activities even in the presence of the MEK inhibitor.

Experiment 4

An initial culture of COCs in inhibitor-free medium for 6 and 22 h, respectively, and a subsequent incubation period of the denuded oocytes in medium supplemented with PD 98059 or DMSO for 24 and 6 h, respectively, resulted in GVBD > 90% of oocytes (Table 3) and a significant increase in MAP (DMSO: 142.3 ± 30.9 cpm/10 oocytes, $n = 4$; PD 98059: 235.6 ± 57.1 cpm/10 oocytes, $n = 4$) and MPF (DMSO: 66.3 ± 29.8 cpm/10 oocytes, $n = 4$; PD 98059: 128.4 ± 68.9 cpm/10 oocytes, $n = 4$) kinase activities in comparison with oocytes analysed immediately following recovery from

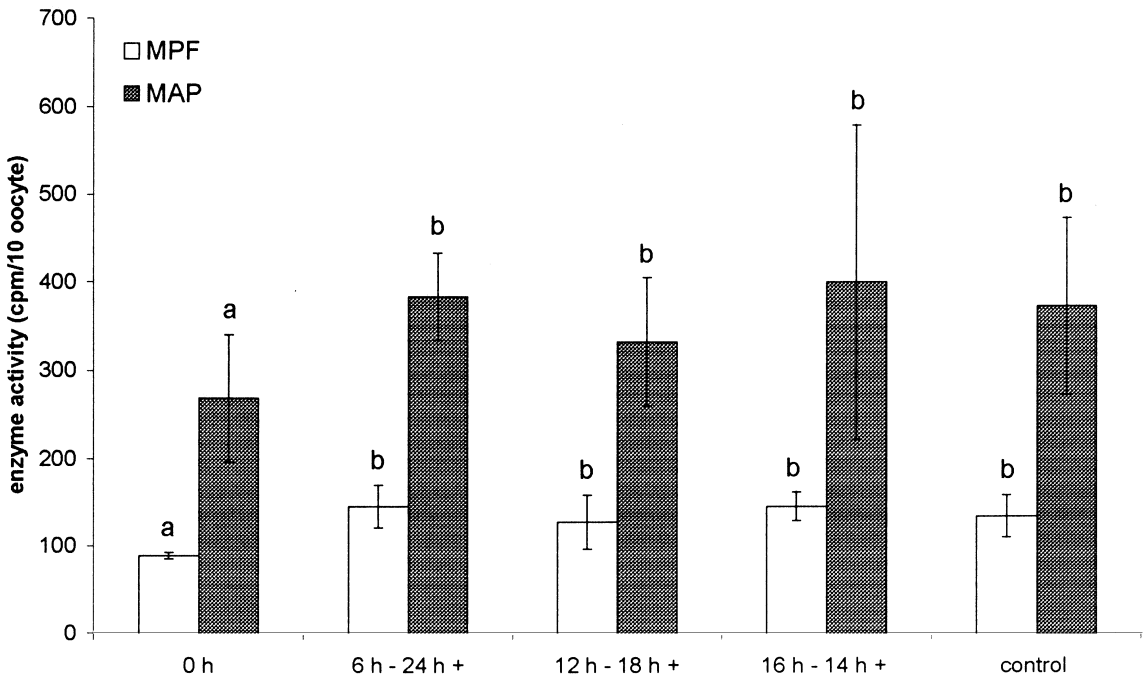

Figure 2 MAP and MPF kinase activities of oocytes cultured first in inhibitor-free medium (0 h, 6h–, 12 h–, 16 h–) and subsequently in PD 98059 (50 µM) supplemented medium (24 h+, 18 h+, 14 h+). Control oocytes were incubated in inhibitor-free medium supplemented with 0.5% (v/v) DMSO for 30 h. Each column represents the mean (± SD) for four replicates of 10 oocytes, respectively. ^{a,b}Means with different superscripts in a line differ significantly ($p < 0.05$).

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Table 3 Effect of denudation of cumulus–oocyte complexes on nuclear maturation of pig oocytes

First culture inhibitor-free (h)	Denudation and second culture in PD 98059 (P) or DMSO (D) (h)	*n*	Oocytes in GV I stage (%)	Oocytes in D, MI, AI, TI stage (%)	Oocytes in MII stage (%)
6	24 (P)	97	4.7 ± 4.0[a]	30.5 ± 9.7[a]	66.4 ± 9.8[a]
6	24 (D)	122	1.4 ± 3.9[a]	18.3 ± 13.8[a]	75.9 ± 21.8[a]
22	8 (P)	120	8.9 ± 6.5[a]	81.1 ± 11.3[b]	0[b]
22	8 (D)	125	4.6 ± 2.2[a]	90.3 ± 4.8[b]	0.7 ± 1.3[b]

COCs were first cultured in IVM medium for 6 or 22 h, subsequently denuded and transferred to medium supplemented with 50 µM PD 98059 or 0.5% (v/v) DMSO. Maturation was determined after 30 h. The table presents the percentages (mean ± SD) of oocytes in germinal vesicle I (GVI),* diakinensis (D), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII) stages from at least four replicates.
*For clarity proportions of oocytes in GV II–IV are not shown.
[a,b]Means with different superscripts in a column differ significantly ($p < 0.05$).

follicles (MAP kinase: 51.1 ± 7.5 cpm/10 oocytes, $n = 4$; MPF: 44.4 ± 13.9 cpm/10 oocytes, $n = 4$). When COCs were denuded after 6 h of incubation in inhibitor-free medium, about 66% and 76%, respectively, of the oocytes reached the metaphase II stage in the presence of PD 98059 and DMSO, respectively, in the course of the subsequent 24 h culture period (Table 3). In contrast, when COCs were denuded after a 22 h incubation period in inhibitor-free medium, hardly any oocytes reached the metaphase II stage during the following 8 h culture period in PD 98059 or DMSO supplemented medium (Table 3). These results demonstrate that denudation of COCs after an initial 6 h culture period led to an acceleration of meiosis progression to metaphase II in comparison with removal of cumulus cells after an initial cultivation period for 22 h. Above all, the activation of MAP and MPF kinase activities was not influenced by the presence of PD 98059 in the culture medium.

Experiment 5

When COCs were cultured in PD 98059 supplemented medium for 30 h and subsequently transferred into inhibitor-free medium for a further 24, 30 and 48 h, respectively, oocytes underwent GVBD and progressed to metaphase II (Table 4). However, in comparison with oocytes which were incubated solely in inhibitor-free medium for 24 and 30 h, respectively, significantly more oocytes reached metaphase II following an initial culture period in PD 98059 supplemented medium and a subsequent incubation in inhibitor-free medium for 24 and 30 h (Table 4). These data demonstrate that PD 98059 treatment of COCs is reversible but that the temporal course of nuclear maturation following previous inhibition by PD 98059 is accelerated.

Discussion

This study investigated the involvement of MAPK in the initiation of *in vitro* maturation of porcine oocytes. To elucidate possible functions of the MAPK, a specific inhibitor of the upstream MEK was used. The results show that PD 98059 (50 µM) prevents MAP and MPF kinase activation in COCs and that this non-activation is accompanied by a failure to resume meiosis in the majority of oocytes. These observations correspond to the results of Kagii *et al.* (2000) who used UO126 as an MEK inhibitor. Furthermore, in the present study additional experiments showed that PD 98059 does not exert a direct influence on the MAP and MPF kinase activities. This indicates that the reduced activity levels detected in the oocytes following exposure to the inhibitor resulted from a non-activation of the upstream MEK. However, since both MAP and MPF kinases are affected by MEK inhibition, a definite function during induction of maturation of either kinase could not be demonstrated. It is likely that there is simultaneous reaction(s) of the kinases to MEK inhibition, since the MAPK and MPF pathway seems to be mutually regulated during the G2/M phase transition of the mitotic (Wright *et al.*, 1999) and meiotic (Palmer & Nebreda, 2000) cell cycle. In *Xenopus* oocytes, for example, it has been reported that Rsk proteins (mammalian homologue: $p90^{rsk}$) associate with the c-terminus of Myt 1 and that Rsk can phosphorylate and inactivate Myt 1 (Palmer *et al.*, 1998). Since Myt 1 is one of the two $p34^{cdc2}$-inactivating kinases identified in animal cells and Rsk proteins are well-characterised substrates for MAP kinases, Rsk is a possible candidate for one link between the MAPK cascade and the activation of the catalytic subunit of MPF. Moreover, the ability of activated forms of Mos, MEK and MAPK to cause MPF activation (GVBD) in *Xenopus* oocytes implies that

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Table 4 Nuclear maturation of COCs following PD 98059 treatment for 30 h

First culture in PD 98059 (h)	Second culture inhibitor-free (h)	*n*	Oocytes in GV I stage (%)	Oocytes in D, MI, AI, TI stage (%)	Oocytes in MII stage (%)
30	24	97	17.6 ± 8.6^{b}	38.3 ± 8.7^{b}	42.2 ± 12.3^{b}
30	30	72	5.3 ± 4.1^{c,e}	33.7 ± 12.8^{b,c}	50.3 ± 10.4^{b}
30	48	73	1.8 ± 2.7^{c}	23.7 ± 12.4^{c}	70.6 ± 15.3^{c}
0	24	202	45.1 ± 8.6^{d}	42.4 ± 16.6^{b}	0^{a}
0	30	107	8.1 ± 2.0^{e}	86.0 ± 5.1^{d}	4.3 ± 4.5^{a}
0	48	122	2.9 ± 2.4^{c}	8.7 ± 3.8^{a}	86.3 ± 2.4^{d}

COCs were first cultured for 30 h in IVM supplemented with 50 µM PD 98059, then transferred to inhibitor-free medium for 24, 30 and 48 h, respectively. Control COCs were cultivated for 24, 30 and 48 h, respectively, in inhibitor-free medium. The table presents the percentages (mean ± SD) of oocytes in germinal vesicle I (GVI),* diakinensis (D), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII) stages from at least four replicates.
*For clarity proportions of oocytes in GV II–IV are not shown.
^{a,b,c,d,e}Means with different superscripts in a column differ significantly ($p < 0.05$).

there is a downstream pathway from MAPK leading to MPF activation (for review see Yamashita *et al*., 2000). Whether or not a similar kinase network acts in oocytes of domestic animal species is not proved. However, the detection of Mos protein in cow and pig oocytes (Newman & Dai, 1996; Wu *et al*., 1997) and its phosphorylation around the time of GVBD in cow oocytes (Tatemoto & Terada, 1995), as well as the activation of MAPK by Mos mRNA injection into bovine oocytes (Fissore *et al*., 1996), argue in favour of the presence of the Mos/MAPK signal transduction pathway.

In the present study MAP and MPF kinase activities already exhibited elevated values when COCs were cultivated in inhibitor-free medium for 6 h and subsequently cultured in PD 98059 supplemented medium for 24 h. A further extension of the inhibitor-free incubation period did not change the activities of the kinases. This indicates that during the first 6 h of culture, when MEK signalling was allowed, all the biochemical steps required for MAP and MPF kinases activation occurred. Above all, the almost constant activity levels of both kinases even in the presence of PD 98059, suggest that an early and transient MEK signal(s) led to downstream activation of MAP and MPF kinases. However, these biochemical events were not cytologically evident until an inhibitor-free culture period of at least 12 h, when a significant proportion of oocytes left the GV I stage. This lag period is probably accounted for by the time needed by the oocyte to prepare for and execute the complex molecular steps leading to chromatin condensation and GVBD. Fulka *et al.* (1986) reported that pig oocytes require about 12 h of undisturbed protein neosynthesis for GVBD.

Despite the continuous presence of 50 µM PD 98059, about one-third of the oocytes resumed meiosis, an observation also described by Kagii *et al.* (2000) using 100 µM UO126. Additionally, the authors demonstrated that following UO126 treatment ERK 1 and ERK 2 in GV-arrested oocytes were not phosphorylated whereas both MAPK isoforms became phosphorylated in oocytes which matured in the presence of the MEK inhibitor. These corresponding data lead to the question of why UO126 treatment prevents resumption of meiosis in some oocytes, whereas others escape MEK inhibition. Kagii *et al.* (2000) concluded that UO126 suppressed the activity of MAPK in porcine oocytes but incompletely, thus permitting nature maturation of about 40% of the COCs. The present results allow an alternative explanation. About 20–30% of COCs analysed immediately following recovery from the follicles already exhibited signs of chromatin condensation within the GV still intact (GV II to GV IV), which is in accordance with previous studies (Grupen *et al*., 1997; Gutherie & Garrett, 2000). This indicates that in such oocytes activation of the MEK/MAPK pathway may already have occurred when the COCs were exposed to the MEK inhibitor. This assumption is supported by the present observation that PD 98059 was unable to suppress MAP and MPF kinase activities in oocytes which were exposed to the inhibitor following a 6 h inhibitor-free cultivation period.

In porcine DOs UO126 treatment (100 µM) remained without any effect on GVBD and ERK1/ERK2 phosphorylation, indicating the requirement of cumulus cells for its action within the oocyte (Kagii *et al*., 2000). In contrast, cultivation of DOs in UO126 (10 and 20 µM) supplemented medium revealed significant suppression of MAP and MPF kinase activities and chromatin clustering in post-GVBD oocytes, suggesting an inhibiting influence of UO126 even in the absence of cumulus cells (Shimada *et al*., 2001). Concerning the effects of PD 98059 on

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porcine Dos, an inhibitory influence on meiosis resumption has been detected. Furthermore, MAP and MPF kinase activities of GV-arrested DOs following a 24 h PD 98059 (50 μM) treatment remained as low as those in oocytes analysed immediately after recovery from follicles, suggesting the requirement of MEK activity prior to GVBD in the oocyte itself (Schilling, 1999). In addition, the present finding that COCs cultured in inhibitor-free medium for 6 h, subsequently denuded and further cultivated for 24 h in the presence of PD 98059 exhibited high MAP and MPF kinase activities and progressed to the metaphase II stage suggests that the signal(s) inducing resumption of meiosis was generated in the cumulus cells and transduced within 6 h into the oocyte itself where it allows MEK signalling and subsequently progression of meiosis even in the presence of PD 98059. Thus, PD 98059 requires the presence of cumulus cells for at least the initial 6 h of culture to exert its MEK inhibition effect in the oocyte itself.

When COCs were incubated in inhibitor-free medium for 22 h, subsequently denuded and transferred into PD 98059 supplemented medium, significantly fewer oocytes progressed to metaphase II in comparison with COCs cultured for 6 h in inhibitor-free medium then denuded and transferred into medium containing the MEK inhibitor. This indicates that cumulus cells may exert an inhibitory effect on the kinetics of nuclear maturation, a phenomenon already described in pig (Isobe *et al*., 1996) and cow oocytes (Tatemoto *et al*., 1994). The influence of cumulus cells on the progression of meiosis I may even affect post-GVBD stages in pig oocytes as suggested by Shimada & Terada (2001) and Shimada *et al.* (2001), who presented evidence that cumulus cells are involved in the regulation of MAP kinase activity in oocytes via the PI3-kinase signalling pathway during meiotic maturation beyond metaphase I.

Reversibility of PD 98059 treatment is illustrated by the high proportion of oocytes which progressed to metaphase II in inhibitor-free medium subsequent to MEK inhibition for 30 h. The data from this experiment also suggest that nuclear maturation is accelerated after removal of the inhibitor, since significantly more oocytes released from PD 98059 treatment progressed to metaphase II during a 24 and 30 h incubation period, respectively, as compared with controls. This observation could mean that in the course of MEK inhibition molecules may accumulate in the COCs which, following removal of the inhibitor, lead to an accelerated nuclear maturation. Whether this acceleration is accompanied by a simultaneously rise in MAP and MPF activities remains to be established.

In conclusion, it can be stated that the treatment of pig COCs with PD 98059, a specific MEK inhibitor, led to a reversible arrest of meiotic resumption *in vitro*. This inhibition was accompanied by a non-activation of MAP and MPF kinase activities in the oocytes. Furthermore, evidence is presented suggesting early and transient MEK signalling in the oocyte itself which causes increasing MAP and MPF kinase activities prior to GVBD. The results thus support the idea of MAP as well as MPF kinases being involved in the induction of GVBD.

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References

Arion, D., Meijer, L., Brizuela, L. & Breach, D. (1988). Cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. *Cell* **55**, 371–8.

Campbell, J.S., Seger, R., Graves, J.D., Graves, L.M., Jensen, A.M. & Krebs, E.G. (1995). The MAP kinase cascade. *Rec. Prog. Horm. Res.* **50**, 131–59.

Dedieu, T., Gall, L., Crozet, N., Sevellec, C. & Ruffini, S. (1996). Mitogen-activated protein kinase activity during goat oocyte maturation and the acquisition of meiotic competence. *Mol. Reprod. Dev.* **45**, 351–8.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A. & Saltiel, R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* **92**, 7686–9.

Edwards, R.G. (1965). Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature* **208**, 349–51.

Fissore, R.A., He, C.L. & Vande Woude, G.F. (1996). Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. *Biol. Reprod.* **55**, 1261–70.

Fulka, J. Jr., Motlík, J., Fulka, J. & Jílek, F. (1986). Effect of cycloheximide on nuclear maturation of pig and mouse oocytes. *J. Reprod. Fertil.* **77**, 281–5.

Gautier, J., Norbury, C., Lohka, M., Nurse, P. & Maller, J. (1988). Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle. *Cell* **54**, 433–9.

Gotoh, Y., Nishida, E., Matsuda, S., Shina, X., Kosaka, H., Shiokawa, K., Akiyama, T., Ohta, K. & Sakai, H. (1991). *In vitro* effects on microtubule dynamics of purified *Xenopus* M phase-activated MAP kinase. *Nature* **349**, 251–4.

Goudet, G., Belin, F., Bézard, J. & Gérard, N. (1998). Maturation-promoting factor (MPF) and mitogen activated protein kinase (MAPK) expression in relation to oocyte competence for *in vitro* maturation in the mare. *Mol. Hum. Reprod.* **4**, 563–70.

Grupen, C.G., Nagashima, H. & Nottle, M.B. (1977). Asynchronous meiotic progression in porcine oocytes

matured *in vitro*: a cause of polyspermic fertilization? *Reprod. Fertil. Dev.* **9**, 187–91.
Gutherie, H.D. & Garrett, W.M. (2000). Changes in porcine oocyte germinal vesicle development as follicles approach preovulatory maturity. *Theriogenology* **54**, 389–99.
Hunter, R.H.F. & Polge, C. (1966). Maturation of follicular oocytes in the pig after injection of human chorionic gonadotrophin. *J. Reprod. Fertil.* **12**, 525–31.
Inoue, M., Naito, K., Aoki, F., Toyoda, Y. & Sato, E. (1995). Activation of mitogen-activated protein kinase during meiotic maturation in porcine oocytes. *Zygote* **3**, 265–71.
Inoue, M., Naito, K., Nakayama, T. & Sato, E. (1996).Mitogen-activated protein kinase activity and microtubule organisation are altered by protein synthesis inhibition in maturing porcine oocytes. *Zygote* **4**, 191–8.
Inoue, M., Naito, K., Nakayama, T. & Sato, E. (1998). Mitogen-activated protein kinase translocates into the germinal vesicle and induces germinal vesicle breakdown in porcine oocytes. *Biol. Reprod.* **58**, 130–6.
Isobe, N., Fujihara, H. & Terada, T. (1996). Cumulus cells suppress meiotic progression in pig oocytes cultured *in vitro*. *Theriogenology* **45**, 1479–89.
Kagii, H., Naito, K., Sugiura, K., Iwamori, N., Ohashi, S., Goto, S., Yamanouchi, K. & Tojo, H. (2000). Requirement of mitogen-activated protein kinase activation for the meiotic resumption of porcine oocytes. *J. Reprod. Dev.* **46**, 249–56.
Krischek, C. & Meinecke, B. (2001). Roscovitine, a specific inhibitor of cyclin-dependent protein kinases, reversibly inhibits chromatin condensation during *in vitro* maturation of porcine oocytes. *Zygote* **9**, 309–16.
Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–5.
Langan, T.A., Gautier, J., Lohka, M., Hollingsworth, R., Moreno, S., Nurse, P., Maller, J. & Sclafani, R.A. (1989). Mammalian growth-associated H1 histone kinase: a homolog of cdc+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells. *Mol. Cell Biol.* **9**, 3860–8.
Lee, J., Miyano, T. & Moor, R.M. (2000). Spindle formation and dynamics of γ-tubulin and nuclear mitotic apparatus protein distribution during meiosis in pig and mouse oocytes. *Biol. Reprod.* **62**, 1184–92.
Masui, Y. & Markert, C.L. (1971). Cytoplasmic control of nuclear behaviour during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177**, 129–46.
Motlik, J. & Fulka, J. (1976). Breakdown of the germinal vesicle in pig oocytes *in vivo* and *in vitro*. *J. Exp. Zool.* **198**, 155–62.
Moreno, S. & Nurse, P. (1990). Substrate for $p34^{cdc2}$: *in vivo* veritas? *Cell* **61**, 549–51.
Naito, K. & Toyoda, Y. (1991). Fluctuation of histone H1 kinase activity during meiotic maturation in porcine oocyte. *J. Reprod. Fertil.* **93**, 467–73.
Newman, B. & Dai, Y. (1996). Transcription of c-*mos* protooncogene in the pig involves both tissue-specific promoters and alternative polyadenylation sites. *Mol. Reprod. Dev.* **44**, 275–88.
Palmer, A., Gravin, A.C. & Nebreda, A.R. (1998). A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *EMBO J.* **17**, 5037–47.
Palmer, A. & Nebreda, A.R. (2000). The activation of MAP kinase and p34cdc2/cyclin B during the meiotic maturation of *Xenopus* oocytes. *Prog. Cell Cycle Res.* **4**, 131–43.
Pelech, S.L. & Sanghera, J.S. (1992). MAP kinase: charting the regulatory pathways. *Science* **257**, 1355–6.
Peter, M., Sanghera, J.S., Pelech, S.L. & Nigg, E.A. (1992). Mitogen-activated protein kinases phosphorylate nuclear lamins and display sequence specificity overlapping that of mitotic protein kinase $p34^{cdc2}$. *Eur. J. Biochem.* **205**, 287–94.
Pincus, G. & Enzmann, E.V. (1935). The comparative behavior of mammalian eggs *in vivo* and *in vitro*. 1. The activation of ovarian eggs. *J. Exp. Med.* **62**, 665–75.
Sanghera, J.S., Paddon, H.B., Bader, S.A. & Pelech, S.L. (1990). Purification and characterization of a maturation-activated myelin base protein kinase from sea star oocytes. *J. Biol. Chem.* **265**, 52–7.
Schilling, A., Wehrend, A. & Meinecke, B. (1998). Inhibition of the mitogen-activated protein kinase (MAPK) pathway in denuded porcine oocytes prevents nuclear maturation *in vitro*. *J. Reprod. Fertil. Abstr. Ser.* **21**, 35.
Schilling, A. (1999). Experimentelle Untersuchungen zur Proteinsynthese- und MAP-Kinase-Hemmung waehrend der In-vitro-Reifung der Schweineeizelle. Dissertation, Tierärztliche Hochschule, Hannover.
Shimada, M. & Terada, T. (2001). Phosphatidylinositol 3-kinase in cumulus cells and oocytes is responsible for activation of oocyte mitogen-activated protein kinase during meiotic progression beyond the meiosis I stage in pigs. *Biol. Reprod.* **64**, 1106–14.
Shimada, T., Zeng, W.X. & Terada, T. (2001). Inhibition of phosphatidylinositol 3-kinase or mitogen-activated protein kinase kinase leads to suppression of $p34^{cdc2}$ kinase activity and meiotic progression beyond the meiosis I stage in porcine oocytes surrounded with cumulus cells. *Biol. Reprod.* **65**, 442–8.
Sobajima, T., Aoki, F. & Kohmoto, K. (1993). Activation of mitogen-activated protein kinase during meiotic maturation in mouse oocytes. *J. Reprod. Fertil.* **97**, 389–94.
Sugiura, K., Naito, K., Iwamori, N., Kagii, H., Goto, S., Ohashi, S., Yamanouchi, Y. & Tojo, H. (2001). Germinal vesicle materials were not required for the activation of MAP kinase in porcine oocyte maturation. *Mol. Reprod. Dev.* **59**, 215–20.
Sun, Q.Y., Blumenfeld, Z., Rubinstein, S., Goldman, S., Gonen, Y. & Breitbart, H. (1999). Mitogen-activated protein kinase in human eggs. *Zygote* **7**, 181–5.
Tatemoto, H. & Terada, T. (1995). On the c-*mos* proto-oncogene product during meiotic maturation in bovine oocytes cultured *in vitro*. *J. Exp. Zool.* **272**, 159–62.
Tatemoto, H., Horiuchi, T. & Tereda, T. (1994). Effects of cycloheximide on chromatin condensation and germinal vesicle breakdown (GVBD) of cumulus-enclosed and denuded oocytes in cattle. *Theriogenology* **42**, 1141–8.
Wehrend, A. & Meinecke, B. (2001). Kinetics of meiotic progression, M-phase promoting factor (MPF) and mitogen-activated protein kinase (MAP kinase) activities during *in vitro* maturation of porcine and bovine oocytes: species

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specific differences in the length of the meiotic stages. *Anim. Reprod. Sci.* **66**, 175–84.

Wright, J.M., Munar, E., Jameson, D.R., Andreassen, D.R., Margolis, R.L., Seger, R. & Krebs, E. (1999). Mitogen-acvtivated protein kinase kinase activity is required for the G2/M transition of the cell cycle in mammalian fibroblasts. *Proc. Natl. Acad. Sci. USA* **96**, 11335–40.

Wu, B., Ignotz, G., Currie, W.B. & Yang, X. (1997). Dynamics of maturation-promoting factor and its constituent proteins during *in vitro* maturation of bovine oocytes. *Biol. Reprod.* **56**, 253–9.

Yamashita, M., Mita, K., Yoshida, N. & Kondo, T. (2000). Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects. *Prog. Cell Cycle Res.* **4**, 115–29.

Zernicka-Goetz, M., Verlhac, M.-H., Géraud, G. & Kubiak, J.Z. (1997). Protein phosphates control MAP kinase activation and microtubule organization during rat oocyte maturation. *Eur. J. Cell Biol.* **72**, 30–8.

https://doi.org/10.1017/S0967199403001023 Published online by Cambridge University Press