The mitogen-activated protein kinase-dependent and the cAMP–protein kinase A-dependent signal transduction pathways were studied in cultured mouse oocytes during induced and spontaneous meiotic maturation. The role of the mitogen-activated protein kinase pathway was assessed using PD98059, which specifically inhibits mitogen-activated protein kinase 1 and 2 (that is, MEK1 and MEK2), which activates mitogen-activated protein kinase. The cAMP-dependent protein kinase was studied by treating oocytes with the protein kinase A inhibitor rp-cAMP. Inhibition of the mitogen-activated protein kinase pathway by PD98059 (25 μmol l⁻¹) selectively inhibited the stimulatory effect on meiotic maturation by FSH and meiosis-activating sterol (that is, 4,4-dimethyl-5α-cholest-8,14,24-triene-3β-ol) in the presence of 4 mmol hypoxanthine 1⁻¹, whereas spontaneous maturation in the absence of hypoxanthine was unaffected. This finding indicates that different signal transduction mechanisms are involved in induced and spontaneous maturation. The protein kinase A inhibitor rp-cAMP induced meiotic maturation in the presence of 4 mmol hypoxanthine 1⁻¹, an effect that was additive to the maturation-promoting effect of FSH and meiosis-activating sterol, indicating that induced maturation also uses the cAMP–protein kinase A-dependent signal transduction pathway. In conclusion, induced and spontaneous maturation of mouse oocytes appear to use different signal transduction pathways.

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maintained by 3-isobutyl-1-methylxanthine (IBMX) (Choi et al., 1996a). A similar effect was observed after microinjection of a constitutively activated MEK. In both cases, the MAPK pathway was activated before GVBD, indicating that the MOS–MAPK pathway has an important role in initiating mammalian oocyte maturation. In contrast, during spontaneous maturation of mouse oocytes, the MAPK pathway seems to be activated only after the occurrence of GVBD (Sobajima et al., 1993; Verlhac et al., 1993). In addition, oocytes from mos kinase knockout mice undergo GVBD normally when cultured in the absence of PDE inhibitors, but display abnormalities during first polar body formation and during the MI–MII transition. Verlhac et al. (1996) also showed that activation of MAPK requires MOS kinase, but synthesis of MOS kinase and activation of MAPK occurs subsequent to GVBD during spontaneous maturation. This finding indicates that MOS kinase functions at a later stage of oocyte maturation, when resumption of meiosis has already been initiated (Hashimoto et al., 1994; Verlhac et al., 1996; Choi et al., 1996b). Spontaneous and induced meiotic maturation are likely to be initiated by different molecular pathways: induced meiosis is dependent and spontaneous maturation is independent of the MAPK pathway.

The mid-cycle surge of gonadotrophins in vivo induces oocyte maturation, overcoming the inhibitory effect of hypoxanthine. A similar effect can be obtained in vitro by stimulating cumulus-enclosed oocytes (CEOs) with FSH in the presence of hypoxanthine, or by adding either one of two naturally occurring meiosis-activating sterols (MAS), FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol) or T-MAS (4,4-dimethyl-5α-cholesta-8,24-diene-3β-ol), both of which are known to induce resumption of meiosis in mouse oocytes (Byskov et al., 1995; Grondahl, 1998; Ruon et al., 1998). FF-MAS was originally isolated and identified from human preovulatory follicular fluid and T-MAS from bull testis, indicating that these sterols play a role in the control of oocyte maturation (Byskov et al., 1995).

The present study examined the role of the MAPK pathway during resumption of meiosis in oocytes in vitro. The effect of the MEKI and MEK2 inhibitor PD98059 (Cross and Smythe, 1998; Pircher et al., 1997), was evaluated on spontaneous and induced maturation. In addition, the effect of the PKA inhibitor rp-cAMP (Rothermel and Botelho, 1988) on FF-MAS-induced maturation was studied.

Materials and Methods

Animals and basic culture media

Immature female C57Bl/2J or B6D2-F1 mice (11–15 g) approximately 3 weeks old (Bombholgtård, Denmark or Charles River, Sulzfeld) were kept in a temperature-controlled room (20 ± 2°C) with a 14 h light:10 h dark cycle (lights on from 06:00 to 20:00 h). Food and water were available ad libitum.

Ovarian stimulation was performed by i.p. administration of Suigonan (Intervent International, Boxmeer) containing equine chorionic gonadotrophin (eCG, 3.7 iu per mouse) and hCG (1.3 iu per mouse) or Humegon (Organon, Oss) containing 20 iu FSH and 20 iu LH-like activity. The animals were killed 46–48 h later by cervical dislocation and the ovaries were transferred to dishes with culture medium supplemented with 4 mmol hypoxanthine l−1 (hypoxanthine medium; Gibco BRL. Life Technologies, Roskilde). The medium consisted of α-minimal essential medium without nucleosides and deoxyribonucleosides (α-MEM, Gibco, BRL), 3 mg BSA ml−1 (Sigma Chemical Co., St Louis, MO), 5 mg HSA ml−1 (Statens Seruminstitut, Copenhagen), 2 mmol l-glutamine l−1, 100 iu penicillin ml−1 and 100 mg streptomycin ml−1 (all from Gibco, BRL). Ovaries were dissected free of surrounding tissues using 27-gauge needles and transferred to a small Petri dish with fresh hypoxanthine medium. The experiments were performed according to the rules of the Danish Authorities for Animal Care, Ministry of Justice and rules approved by the Animal Care Committees at Organon.

Culture of oocytes

Antral follicles were punctured under a dissecting microscope using 27-gauge needles. Cumulus-enclosed oocytes (CEOs) and naked oocytes (NOs) were collected. The oocytes were collected in fresh hypoxanthine medium, washed twice, and then transfer red to the test medium. The oocytes were cultured at 37°C, 100% humidity in 5% CO₂ in air for 20 h in four-well dishes (Nunclon, Roskilde, Denmark) in 400 µl hypoxanthine or test medium. At the end of culture, the oocytes were examined for their meiotic status using an inverted microscope with Hoffman modulation contrast equipment. Oocytes showing a clear nuclear membrane (GV) were classified as meiotically arrested, whereas those showing no nuclear structures were classified as having undergone GVBD. The percentage of GVBD oocytes (including those in which the polar body has been extruded) per total oocytes (% GVBD) and the percentage of polar body oocytes per total GVBD oocytes (% PB) were calculated. Assays were repeated three times.

Test media

Oocytes were washed once in culture medium (similar to the hypoxanthine medium but without hypoxanthine and with 5 mg HSA ml−1 (Statens Seruminstitut, Copenhagen)) and cultured in this medium for 20 h to achieve spontaneous maturation.

FF-MAS was synthesized at the Department of Medical Chemistry of NV Organon, The Netherlands (Org 38408) and characterized as described by Baltsen and Byskov (1999). The appropriate amount of FF-MAS in n-heptane was transferred to a glass test tube and the solvent was evaporated under N₂. The FF-MAS was dissolved in ethanol and transferred to a hypoxanthine medium or dissolved in hypoxanthine medium by sonicating for 3 × 1 min at constant low output (Branson sonifier 250) and cooled with ice-water between sonications.

Test media containing rp-cAMP were prepared by diluting a stock solution of rp-cAMP (Biolog, La Jolla, CA) directly in

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culture media supplemented with 3 mg BSA ml⁻¹. The stock solution contained 50 mmol l⁻¹ dissolved in physiological saline.

FSH (Gonal F, Serono Nordic, Copenhagen) was dissolved in hypoxanthine medium to a concentration of 25 iu l⁻¹. Test media containing PD98059 were prepared by diluting a stock solution of PD98059 (Calbiochem, La Jolla, CA) directly in hypoxanthine medium. The stock solution contained 20 mmol PD98059 l⁻¹ in dimethyl sulphoxide and was stored at −20°C. Oocytes used to evaluate the effect of rp-cAMP on hypoxanthine-inhibited and FF-MAS-induced maturation originated in C57B1/6J mice, whereas oocytes for all other experiments derived from the B6D2F1 strain.

Statistical analysis

Results are expressed as mean ± SEM, and STATGRAPHICS™ software (Manugistics, Inc., Rockville, MD, U.S.A.) was applied for statistical analysis. In the case of two independent samples, the oocyte data were compared after appropriate transformation using Student’s t test. Multiple samples were compared using ANOVA, and Fisher’s least squares difference procedure was used to determine which samples were significantly different (P < 0.05 was considered significant).

Results

PD98059 and spontaneous maturation

When oocytes were cultured in the presence of the inhibitor of MEK, PD98059, for 20 h in the absence of hypoxanthine, 84 ± 12% of the NOs and 85 ± 9% of the CEOs resumed meiosis (Fig. 1a). The frequency of GVBD was similar for the CEO and NO groups cultured in the absence of PD98059 and in the presence of 50–200 μmol PD98059 l⁻¹, indicating that spontaneous meiosis was independent of signal transduction through MEK and MAPK. In addition, PD98059 did not affect the frequency of the first polar body formation (Fig. 1b).

PD98059 and FF-MAS-induced maturation of cumulus-enclosed oocytes and naked oocytes

PD98059 inhibited in a dose-dependent manner the resumption of meiosis induced by 3 μg ml⁻¹ (7 μmol l⁻¹) FF-MAS in both CEOs and NOs, although significance was reached only in the NO group (Fig. 2a,b). The proportion of oocytes that underwent GVBD in the presence of FF-MAS (62 ± 3% and 54 ± 5% of the CEO and NO groups, respectively) was significantly higher than that of the control group (30 ± 4% and 13 ± 3% of the CEO and NO groups, respectively, P < 0.001). When oocytes were cultured in the presence of 12.5, 25 and 50 μmol PD98059 l⁻¹, the occurrence of GVBD was reduced to 36 ± 4%, 29 ± 3% and 27 ± 2% in the CEO group, respectively, and 34 ± 6%, 25 ± 7% and 10 ± 2% in the NO group, respectively.

The percentage of PD98059-treated oocytes that underwent GVBD and extruded a first polar body was similar to that of oocytes cultured in hypoxanthine medium only or hypoxanthine medium supplemented with FF-MAS (Fig. 2, insert). The frequency of GVBD after treatment with 50 μmol PD98059 l⁻¹ in the absence of FF-MAS was similar to that in control CEO and NO groups (Fig. 2).

PD98059 and FSH-induced maturation of cumulus-enclosed oocytes

When CEOs were cultured for 20 h in hypoxanthine medium in the presence of 25 iu FSH l⁻¹, a significant increase in GVBD was observed (67 ± 5% GVBD) compared with CEOs cultured in hypoxanthine medium (28 ± 4% GVBD) (Fig. 3a). This increase was efficiently abolished by PD98059. In the presence of 12.5, 25 and 50 μmol PD98059 l⁻¹ and 25 iu FSH l⁻¹, 39 ± 5%, 41 ± 8% and 14 ± 3% of the CEOs, respectively, underwent GVBD during a 20 h culture period.
None of these percentages was significantly higher than for control oocytes (28 ± 6% GVBD) or for oocytes cultured in hypoxanthine medium supplemented with PD98059 alone (Fig. 3a).

The percentage of GVBD oocytes that had extruded a first polar body was not significantly different in PD98059-treated oocytes compared with oocytes cultured in hypoxanthine medium only or hypoxanthine medium supplemented with FSH (Fig. 3a, insert).

Neither PD98059 nor FSH affected the frequency of maturation and extrusion of the first polar body of the NOs (Fig. 3b).

**FF-MAS-induced meiosis and PKA inhibitor rp-cAMP**

Increasing concentrations of PKA inhibitor (0, 1 and 5 mmol l⁻¹) increased the incidence of GVBD in a dose-dependent manner in oocytes cultured both in the absence and presence of 5 μmol FF-MAS l⁻¹ (14 ± 6%, 42 ± 12% and

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Fig. 2. Effect of PD98059 (0–50 μmol l⁻¹) on meiotic maturation induced by FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol) in mice. Cumulus-enclosed oocytes (a) or naked oocytes (b) were cultured with PD98059 in a hypoxanthine medium in the absence (–) or presence (+) of 3 μg FF-MAS ml⁻¹. After 20 h of culture, the percentage of oocytes undergoing germinal vesicle breakdown (GVBD) and the percentage of GVBD oocytes that had extruded a first polar body (PB/GVBD, insert) were determined. Bars represent mean ± SEM of three to five determinations in three experiments. Asterisks indicate a significant difference between the treatment and control groups (cumulus-enclosed and naked oocytes, respectively, cultured without PD98059): **P < 0.001; *P < 0.01 (Student’s t test). The numbers on the x-axis of the insert panel refer to the same treatment groups as that of the main panel, in the same order. The absence of a bar indicates that the value is zero.

Fig. 3. Effect of PD98059 (0–50 μmol l⁻¹) on FSH-induced oocyte maturation in mice. Cumulus-enclosed oocytes (a) or naked oocytes (b) were cultured with PD98059 in the absence (–) or presence (+) of 25 iu FSH ml⁻¹ in a hypoxanthine medium. After 20 h of culture, the percentage of oocytes undergoing germinal vesicle breakdown (GVBD) and the percentage of GVBD oocytes that had extruded a first polar body (PB/GVBD, insert) were determined. Bars represent mean ± SEM of three to five determinations in three experiments. Asterisks indicate a significant difference between treatment and control group (cumulus-enclosed and naked oocytes, respectively, cultured without PD98059): *P < 0.05 (Students t test). The numbers on the x-axis of the insert panel refer to the same treatment groups as that of the main panel, in the same order. The absence of a bar indicates that the value is zero.
89 ± 6%; and 57 ± 5%, 96 ± 6% and 100 ± 0%, respectively) (Fig. 4).

Discussion

This study shows that spontaneous and induced resumption of meiosis in mature mouse oocytes most likely follow different intracellular regulatory pathways. The FF-MAS and FSH-induced oocyte maturation is sensitive to inhibition of the two MAPK activating kinases MEK1 and MEK2 by PD98059, whereas spontaneous oocyte maturation is unaffected by the presence of this inhibitor. This finding indicates that spontaneous maturation is independent of the MAPK pathway, whereas induced maturation is dependent on its activation. The notion of two parallel pathways leading to GVBD is supported by the observations that FF-MAS and the PKA inhibitor rp-cAMP have an additive effect on GVBD.

Hegele-Hartung et al. (1999) showed that GVBD occurs much faster in oocytes undergoing spontaneous maturation compared with those undergoing FF-MAS-induced meiotic maturation, implying different mechanisms of regulation of spontaneous and induced meiotic maturation. Other studies have indicated different regulatory pathways for the resumption of meiosis. In mouse oocytes arrested in the GV stage by IBMX, the phosphatase 1 and 2A inhibitor, okadaic acid, induced an increase in MAPK activity within 1 h and GVBD occurred 1 h later (Gavin et al., 1994), in contrast to spontaneously matured oocytes, in which a similar increase in MAPK was not observed until 1 h after GVBD (Sobajima et al., 1993; Verlhac et al., 1993; Gavin et al., 1994).

The results of the present study support and extend those of previous studies indicating that induced and spontaneous resumption of meiosis are regulated differently. Spontaneous maturation of oocytes can be considered as the default route, which becomes activated when the mature oocyte is relieved from the inhibitory effect of various purines and other components within the follicular compartment.

A detailed knowledge of the role of MAPK during induced resumption of meiosis is not yet available. Some studies show that MAPK remains non-activated until GVBD has been achieved (Verlhac et al., 1994, 1996; Choi et al., 1996b; Hashimoto et al., 1996), indicating that MAPK functions later in the maturation process and is not required for GVBD. In contrast, other studies show that MAPK is activated before GVBD (Zhao et al., 1990; Sobajima et al., 1993; Choi et al., 1996a). Although these differences may be related to species differences, studies in mice show that activation of the MAPK cascade by microinjection of mos mRNA or by direct microinjection of MAPK into GV oocytes induces meiotic maturation. In addition, the present study is in agreement with reports that inhibition of the MAPK cascade inhibits GVBD, strongly indicating that MAPK is directly involved in initiating meiotic maturation, at least in mice (Choi et al., 1996a; Inoue et al., 1998).

A number of studies have now used PD98059 as a specific inhibitor of the activation of MAPK in vitro and in vivo (Alessi et al., 1995; Lazar et al., 1995; Pircher et al., 1997; Cross and Smythe, 1998). Given the lipophylic nature of PD 98059
(Lazar et al., 1995) it is not surprising that all these studies have reported a quick (that is, within minutes) and consistent uptake of PD98059, followed by a specific inhibition of the MAPK activity, in oocytes and granulosa cells. In the present study, culture conditions were similar for all groups and a differentiated effect of PD98058 was apparent. Therefore, it is reasonable to assume that the uptake of PD98059 was similar among the groups of the present study, and that the observed differences actually relate to the importance of the inhibition of MEK1 and MEK2 during resumption of meiosis.

There is no universal explanation at present to account for the observations in the present and previous studies relating to the intracellular pathway for resumption of meiosis. However, the results of the present study indicate a model with the following elements: FSH induces oocyte maturation indirectly via the cumulus cells, since no receptors are present on the oocyte itself (Eppig and Downs, 1987; Downs et al., 1988; Byskov et al., 1997). The FSH receptor, located in the plasma membrane of the cumulus cells, is coupled to a trimeric G-protein that activates a cAMP-regulated pathway (El-Tayar et al., 1996). However, the molecular mechanisms that take place between FSH-receptor activation in the cumulus cells and resumption of meiosis in the oocyte are presently unknown. Leonardsen et al. (2000) have shown that cumulus cells of CEOs produce and accumulate FF-MAS under the influence of AY9944-A-7, which inhibits the conversion of FF-MAS to T-MAS. This accumulation occurs concomitantly with resumption of meiosis. On the basis of this and other studies, it is suggested that FF-MAS produced by the cumulus cells of CEOs upon FSH stimulation acts as a trigger for mammalian meiotic maturation. The mechanism by which MAS induces oocyte maturation has yet to be identified. However, the results presented here show that oocyte maturation induced by FSH and FF-MAS requires activation of the MAPK cascade.

The indications are that FSH induces the resumption of meiosis via FF-MAS, although this has not been proven. FF-MAS and T-MAS are both intermediates in the biosynthetic pathway of cholesterol after the synthesis of lanosterol (Byskov et al., 1995). Ketokonazole is a drug used to combat infections with fungicides and one of its modes of action is to inhibit the lanosterol 14-demethylase (P45014DM), thereby preventing the formation of FF-MAS (Brajtburg et al., 1990). Ketokonazole inhibits the FSH-induced resumption of meiosis in a dose-dependent manner in cultured mouse oocytes, indicating that the FSH-inducing effect on GVBD acts via FF-MAS (Lu et al., in press).

FF-MAS and the PKA inhibitor rp-cAMP were capable of inducing GVBD separately, but their effects were additive. In the system used in the present study, 5 μmol FF-MAS 1-1 elicited a maximal response, which is in agreement with other studies that report that a concentration of 7 μmol FF-MAS 1-1 results in a maximal response of 86 ± 2 % GVBD (Gröndahl et al., 1998). Therefore, the enhancement of the rate of GVBD resulting from combining 5 μmol FF-MAS 1-1 and rp-cAMP observed in the present study shows that PKA and FF-MAS may be involved in two different pathways. However, it is possible that some cross-talk occurs between the pathways. Yang et al. (1996) have shown that PKA inhibits MOS. Mos is a germ cell-specific protooncogene that activates MEK1 and MEK2, and the mos product is produced at the onset of oocyte maturation, and phosphorylated and activated at the time of GVBD (Tatemoto and Terada, 1995; Gebauer and Richter, 1997). Inhibition of MOS kinase activity by microinjection of anti-MOS antibody blocks spontaneous maturation in mouse oocytes (Zhao et al., 1990). At present, it is unclear how such interplay between the pathways is regulated. Apparently, PKA does not control spontaneous maturation solely by inhibiting MOS, and thereby the MAPK cascade, since inhibition of MAPK activation by PD98059 had no effect on spontaneous maturation. Therefore, it is proposed that, during induced maturation, an interaction between the cAMP–PKA and the MAPK pathway takes place, while spontaneous maturation acts only via the cAMP–PKA pathway.

In the present study, NOs rather than denuded cumulus-enclosed oocytes were used. It is usually assumed that CEOs derive from the more healthy follicles, whereas NOs derive from smaller and less healthy follicles. Therefore, these two groups of oocytes may represent different developmental competencies, making a comparison difficult. However, when the meiotic competence of NOs and denuded oocytes have been studied (L. Leonardsen, M. Balsen, C. Yding Andersen and A. G. Byskov, unpublished), no difference in any of the studied parameters was observed, thereby justifying a comparison of results from CEOs and NOs.

In conclusion, the present study indicates that different molecular pathways regulate induced and spontaneous resumption of meiosis. Spontaneous maturation occurs in response to a release from an inhibitory effect of hypoxanthine acting through the cAMP–PKA pathway. When hypoxanthine is removed, the FDEA in the oocyte degrades cAMP, leading to decreased PKA activity. However, stimulated meiotic maturation, induced by FSH or FF-MAS, is an MAPK dependent pathway, which leads to decreased cAMP–PKA activity despite the presence of hypoxanthine.

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