Introduction

Mammalian oocytes are arrested in the prophase of the first meiotic division, a stage that is characterized by the presence of the nuclear membrane known as the germinal vesicle. After the preovulatory surge of gonadotrophins, the fully grown oocyte resumes meiosis and the nuclear membrane breaks down (germinal vesicle breakdown; GVBD), a process referred to as 'oocyte maturation'. Completion of the first meiotic division results in the extrusion of the first polar body (PB). When fully grown oocytes are released from the follicle in vitro, they spontaneously resume meiosis, presumably due to release from the maturation inhibiting effect of hypoxanthine and other purines present in follicular fluid (mouse: Downs et al., 1985; Eppig et al., 1985; Downs and Eppig, 1986; Eppig and Downs, 1987; bovine: Sirard and First, 1988; monkey: Warikoo and Bavister, 1989). It has been suggested that hypoxanthine is the main inhibitory substance of mouse oocyte maturation in vivo (Downs, 1993).

In mice, most isolated oocytes remain arrested at the germinal vesicle stage when cultured in the presence of physiological concentrations of hypoxanthine, and resume meiosis if hypoxanthine is removed (Downs, 1997).

Two sterols of the cholesterol biosynthetic pathway induce resumption of meiosis in mouse oocytes in vitro. The sterols, termed meiosis-activating sterols (MAS), have been isolated from human follicular fluid (FF-MAS, 4,4-dimethyl-5α-cholest-8,14,24-triene-3β-ol) and from bull testicular tissue (T-MAS, 4,4-dimethyl-5α-cholest-8,24-diene-3β-ol). FF-MAS is the first intermediate in the cholesterol biosynthesis from lanosterol and is converted to T-MAS by sterol Δ14-reductase. An inhibitor of Δ7-reductase and Δ14 reductase, AY9944-A-7, causes cells with a constitutive cholesterol biosynthesis to accumulate FF-MAS and possibly other intermediates between lanosterol and cholesterol. The aim of the present study was to evaluate whether AY9944-A-7 added to cultures of cumulus–oocyte complexes (COC) from mice resulted in accumulation of MAS and meiotic maturation. AY9944-A-7 stimulated dose dependently (5–25 µmol l⁻¹) COC to resume meiosis when cultured for 22 h in α minimal essential medium (α-MEM) containing 4 mmol hypoxanthine l⁻¹, a natural inhibitor of meiotic maturation. In contrast, naked oocytes were not induced to resume meiosis by AY9944-A-7. When cumulus cells were separated from their oocytes and co-cultured, AY9944-A-7 did not affect resumption of meiosis, indicating that intact oocyte–cumulus cell connections are important for AY9944-A-7 to exert its effect on meiosis. Cultures of COC with 10 µmol AY9944-A-7 l⁻¹ in the presence of [³H]mevalonic acid, a natural precursor for steroid synthesis, resulted in accumulation of labelled FF-MAS, which had an 11-fold greater amount of radioactivity incorporated per COC compared with the control culture without AY9944-A-7. In contrast, incorporation of radioactivity into the cholesterol fraction was reduced 30-fold in extracts from the same oocytes. The present findings demonstrate for the first time that COC can synthesize cholesterol from mevalonate and accumulate FF-MAS in the presence of AY9944-A-7. Furthermore, AY9944-A-7 stimulated meiotic maturation dose dependently, indicating that FF-MAS, and possibly other sterol intermediates of the cholesterol synthesis pathway, play a central role in stimulating mouse oocytes to resume meiosis. The results also indicate that oocytes may not synthesize steroids from mevalonate.
The inhibitory effect of hypoxanthine on the resumption of meiosis in cultured mouse oocytes, cumulus-oocyte complexes (COC), as well as cumulus-deprived naked oocytes, can be overcome by adding either one of the two meiosis-activating sterols, FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol) or T-MAS (4,4-dimethyl-5α-cholest-8,24-diene-3β-ol). FF-MAS was isolated and identified from human follicular fluid and T-MAS from bull testis (Byskov et al., 1995). The sterols are intermediates in the cholesterol biosynthetic pathway and are immediate products of lanosterol (Schroepfer et al., 1972) (Fig. 1). The synthesis of FF-MAS from lanosterol is catalysed by cytochrome P450 lanosterol 14α-demethylase (P45014DM) encoded by the CYP51 gene (Rozman et al., 1996; Strömstedt et al., 1996). The next intermediate is T-MAS, which is produced through the activity of sterol Δ14-reductase (Kim et al., 1995). Like most other drugs designed for treatment of hypercholesterolaemia by inhibiting enzymes involved in the cholesterol biosynthesis, AY9944-A-7 has a chemical structure totally unrelated to sterols and the sterols are intermediates in the cholesterol biosynthetic pathway. AY9944-A-7 has a structural similarity to the cholesterol producing cells accumulate FF-MAS when cultured in the presence of AY9944-A-7, for example, in the central nervous system (Ramsey and Fischer, 1977) and in rat hepatocytes (Aoyama and Yoshida, 1986; Kim et al., 1995). AY9944-A-7 (0.1 µmol l⁻¹) acts as a competitive inhibitor of the Δ14-reductase in cultured rat hepatocytes (Kim et al., 1995).

A meiosis promoting substance is produced by the cumulus cells of intact cumulus-enclosed oocyte complexes when stimulated with FSH (Dekel and Beers, 1978; Downs et al., 1988). In cumulus-enclosed oocytes of mice, the intact connections between cumulus cells and the oocyte are essential for FSH (Byskov et al., 1997) and forskolin (Guoliang et al., 1994) to initiate the production of a meiosis-activating substance by the cumulus cells. These connections seem to be crucial for a signal transduction from the oocyte to the cumulus cells that triggers the production and release of the meiosis-activating substance from the cumulus cells of the COC. The FSH-induced meiosis-activating substance is heat stable and thus probably not a protein, but more likely a peptide or a lipid (Byskov et al., 1997). It has been proposed that the active substance is FF-MAS, but its chemical nature has not yet been elucidated.

An indirect way of clarifying whether the active substance is FF-MAS would be to inhibit the activity of Δ14-reductase with AY9944-A-7 in cultured COC, thus lowering the conversion of FF-MAS to T-MAS. An accumulation of FF-MAS in COC cultured with AY9944-A-7 would demonstrate that COC are capable of synthesizing these sterols, and would support the proposal that the meiosis-activating substance from COC is FF-MAS.

The aim of the present study was to evaluate the capacity of AY9944-A-7 to induce accumulation of FF-MAS in cultured COC to investigate whether promotion of endogenous accumulation of FF-MAS in COC and naked oocytes affects resumption of meiosis in vitro. In addition, the effect of AY9944-A-7 on resumption and completion of the first meiotic division in the presence of hypoxanthine was investigated. Finally, the effect of AY9944-A-7 on co-cultures of cumulus-deprived oocytes and their cumulus cells (split COC) was investigated to determine the importance of cumulus–oocyte connections in sterol

**Fig. 1.** Biosynthesis of cholesterol from mevalonate in mammals. The enzymes that mediate the conversion of lanosterol to zymosterol are indicated. The inhibitor of sterol Δ14-reductase and Δ7-reductase used in the present study, AY9944-A-7, is shown. FF-MAS: meiosis-activating sterol isolated from human follicular fluid (4,4-dimethyl-5α-cholest-8,14,24-triene-3β-ol); T-MAS: meiosis-activating sterol isolated from bull testicular tissue (4,4-dimethyl-5α-cholest-8,24-diene-3β-ol).
production. A combined effect of AY9944-A-7 and FF-MAS was also tested.

Materials and Methods

Animals and basic culture media

Immature female C57Bl/2J B6D2 mice (11–15 g) were injected intraperitoneally with Conadoplex (Leo, Ballerup) containing equine chorionic gonadotrophin (eCG; 5 iu per mouse) and hCG (2.5 iu per mouse). The animals were killed 46–48 h later by cervical dislocation and the ovaries were transferred to hypoxanthine medium, which consisted of α-MEM (Gibco, BRL, Tåstrup) with 4 mmol hypoxanthine l–1 (Sigma, Copenhagen), 3 mg BSA ml–1 (Cohns Fraction V, Sigma), 2 mmol L-glutamine l–1, 100 iu penicillin ml–1, 100 µg streptomycin ml–1 (all from Gibco, BRL) and 5 mg human serum albumin ml–1 (Cohns Fraction V, The Serum Institute, Copenhagen). Ovaries were isolated from attached tissues and transferred to fresh hypoxanthine medium.

Assay for resumption of meiosis in mouse oocytes

Antral follicles were punctured using 27-gauge needles to release the oocytes. The oocytes were collected and washed three times in hypoxanthine medium, using a mouth-controlled fine glass pipette and divided into COC and spherical naked oocytes in the germinal vesicle stage.

Some intact COC as well as COC in which the cumulus cells only covered parts of the oocyte were denuded mechanically by repeated flushing through a fine-bore mouth-controlled pipette. As naked oocytes and denuded oocytes do not differ significantly in their response to MAS (L. Leonardsen, A. G. Byskov, C. Yding Andersen, unpublished), these oocytes were pooled and collectively termed naked oocytes.

In one experiment, cumulus cells from COC were separated from the oocytes as described above and the denuded oocytes with their cumulus cells were co-cultured in test and control media (split COC).

The oocytes were cultured at 37°C in 100% humidity and 5% CO₂ in air for 20–22 h. After culture, oocytes in the germinal vesicle stage, in GVBD stage and with polar bodies were counted. The percentage of oocytes in GVBD (including those oocytes with polar bodies) per total number of oocytes (referred to as the percentage of GVBD) and the percentage of oocytes with polar bodies per total number of GVBD (referred to as the percentage with polar bodies) were calculated.

Oocytes from 10–15 mice were pooled and randomly divided into the different test groups. The tests were performed using four-well dishes (Nunclon, Roskilde), each well containing 20–50 oocytes in 400 µl culture medium. Each dish had one well serving as a control and the three other wells as test wells.

Test media

All test media were prepared using hypoxanthine medium. AY9944-A-7 was kindly provided by Wyeth-Ayerst (Princeton, NJ). A stock solution of AY9944-A-7 in water was stored at −20°C, and was added directly to the hypoxanthine medium immediately before oocyte culture. The control media were supplemented with similar concentrations of water. FF-MAS was purified from human follicular fluid by HPLC and detected using a photo-diode array system (Waters, Copenhagen) essentially as described by Baltsen and Byskov (1999) (see also below). The FF-MAS media were prepared by transferring the appropriate amount of FF-MAS, which is stored in n-heptane or ethanol, to a glass tube and evaporating the solvent under helium. The FF-MAS was dissolved in hypoxanthine medium by sonication for 3 × 1 min at approximately 20 W (Branson sonifier 250) and cooled with ice-water between sonications.

Incorporation of [³H] mevalonic acid into 4,4-dimethyl-sterols in COC

Culture of oocytes. In both control and test cultures, 250 COC per well were cultured overnight in 400 µl hypoxanthine medium supplemented with 90 µCi [³H] mevalonate (38 Ci mmol⁻¹; NEN Life Science Products, Boston, MA). In test cultures, the medium contained 10 µmol AY9944-A-7 l⁻¹, and the control medium was supplemented with the same amount of AY9944-A-7 vehicle (water).

Extraction and HPLC purification of MAS, lanosterol and cholesterol. Culture medium and COC were transferred to 4 ml teflon-capped glass vials and acidified by 50 µl of 0.3 mol NaH₂PO₄ l⁻¹, pH 1.0. Two millilitres of 75% n-heptane (hiPerSolv™; BDH Laboratories, Poole): 25% isopropanol (Baker, Deventer, analytical grade) was added and the solution was shaken vigorously for 3 h. The organic phase, containing extracted sterols and other non-polar compounds, was isolated by centrifugation (15 min at 2000 g), transferred to new glass tubes and stored at −20°C until further analysis.

Lanosterol, FF-MAS and T-MAS and cholesterol were identified and purified by HPLC as described by Baltsen and Byskov (1999) with minor modifications. Briefly, organic phases were dried in a vacuum centrifuge (1 mbar, 40°C, 25 min), reconstituted in 100 µl n-heptane and separated by straight-phase HPLC (column: ChromSpher™ Si, 5 µm, 250 mm × 4.6 mm; mobile phase: 99.5% n-heptane, 0.5% isopropanol (v/v); flow rate: 1.00 ml min⁻¹; 28°C). Two fractions were collected from the eluate, one containing lanosterol, FF-MAS and T-MAS, and the other containing cholesterol.

The fractions were dried and dissolved in 100 µl acetonitrile (HPLC reagent, Baker) for further purification by reverse-phase HPLC (column: LiChrospher™ RP-8, 5 µm, 250 mm × 4.6 mm; mobile phase: 92.5% acetonitrile, 7.5% water (v/v); flow rate: 1.00 ml min⁻¹; 40°C). Standards of lanosterol, FF-MAS and T-MAS were added to the fraction containing lanosterol and MAS before loading on the column to avoid loss of radioactive sterols due to unspecific binding.
Quantification of radioactivity in each of the purified fractions was carried out in a liquid scintillation counter (Beckman LS 1801) after addition of 4 ml Ultima Gold scintillation fluid (Packard, Groningen).

The biosynthesis of lanosterol, FF-MAS, T-MAS and cholesterol were quantitated by measuring the incorporated radioactivity. The effect of AY9944-A-7 was evaluated by calculating the ratio of c.p.m. per COC cultured with and without AY9944-A-7.

Statistical analysis

All experiments were performed at least three times. Results are expressed as mean ± SEM, and for statistical analysis STATGRAPHICS™ software (Manugistics, Inc., Rockville, MD) was applied. In the case of two independent samples, the oocyte data were compared using Student’s t test. The correlation of dose of AY9944-A-7 with resulting percentage of GVBD was examined by least square regression analysis. Multiple samples were compared using Fisher’s least squares difference procedure. P values < 0.05 were considered to indicate significant differences.

Results

Dose-dependent effect of AY9944-A-7 on COC, naked oocytes and split COC pooled from several mice

AY9944-A-7 stimulated oocyte maturation of COC in a dose-dependent manner (Fig. 2a). Concentrations of 5, 10 and 25 µmol AY9944-A-7 l−1 increased GVBD significantly compared with the controls (P < 0.05, < 0.001 and < 0.001, respectively). At 25 µmol l−1, AY9944-A-7 decreased the percentage with polar bodies in COC to 8 ± 5% compared with the mean percentage (55 ± 11%) at the lower concentrations of AY9944-A-7, but otherwise the oocytes appeared normal (data not shown). Concentrations of AY9944-A-7 between 0.2 and 10 µmol l−1 did not affect GVBD in naked oocytes, but a small increase in GVBD was observed at 25 µmol l−1. In naked oocytes, the percentage with polar bodies decreased to 7 ± 4% in cultures with 25 µmol AY9944-A-7 l−1 compared with the mean at the lower AY9944-A-7 concentrations (36 ± 8%), and the oocyte cytoplasm contained numerous granules and was shrunken, leaving an enlarged space between the oocyte and zona pellucida. The decrease in the percentage with polar bodies and the altered morphology indicated toxic effects of AY9944-A-7 at 25 µmol l−1, thus 10 µmol AY9944-A-7 l−1 was used in the subsequent experiments.

The percentage of GVBD in split COC cultured with 10 µmol AY9944-A-7 l−1 did not differ from that in the control split COC (53 ± 9% and 44 ± 6%, respectively), indicating that AY9944-A-7 has no effect on split COC. However, the percentage of GVBD in split COC cultured in AY9944-A-7 was significantly higher (P < 0.05) than that in control COC and control naked oocytes (23 ± 5% and 18 ± 3%, respectively) (P < 0.001) (Fig. 3). These experiments showed a significant effect of AY9944-A-7 on intact COC (66 ± 5% and 23 ± 5%, with and without AY9944-A-7, respectively) (P < 0.001).

Effect of FF-MAS on GVBD with and without AY9944-A-7

Five micrograms FF-MAS ml−1 had no effect on COC, whereas 10 µg ml−1 stimulated the percentage of GVBD significantly (P < 0.005) (Fig. 4a). At 5 and 10 µg ml−1, FF-MAS induced a dose-related increase in the percentage of GVBD in naked oocytes (P < 0.01 and P < 0.0005, respectively) (Fig. 4b). Addition of 10 µg FF-MAS ml−1 to COC did not increase the effect of AY9944-A-7 significantly (Fig. 5a). The percentage of GVBD was 74 ± 6% and 84 ± 5% after AY9944-A-7 and AY9944-A-7 + FF-MAS treatment, respectively. The presence of 10 µmol AY9944-A-7 l−1 did not affect the stimulatory effect of 10 µg FF-MAS ml−1 on the percentage of GVBD in naked oocytes (69 ± 4% and 67 ± 6% with and without 10 µmol AY9944-A-7 l−1, respectively) (Figs 4b and 5b).

Effect of AY9944-A-7 on accumulation of sterols in COC

Culturing of COC with 10 µmol AY9944-A-7 l−1 increased the amount of lanosterol and FF-MAS and decreased the
In three experiments.

the synthesis of progesterone was

incorporation of radioactivity was decreased by incubation

that is an 11-fold increase. For T-MAS and cholesterol, the

incorporated radioactivity (c.p.m.) between controls without

radioactivity into the purified sterols. The ratio of

used to measure biosynthesis as the incorporation of

Fractiob

Table 1. Effect of AY9944-A-7 on biosynthesis of sterols from [3H]mevalonic acid in mouse cumulus-oocyte complexes (COC)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>c.p.m. per 100 COC</th>
<th>Ratio (AY9944-A-7:control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanoster</td>
<td>7167 ± 1567</td>
<td>1</td>
</tr>
<tr>
<td>FF-MAS</td>
<td>256 ± 26</td>
<td>0.2</td>
</tr>
<tr>
<td>T-MAS</td>
<td>1704 ± 126</td>
<td>0.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>41,929 ± 18,841</td>
<td>0.05</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1281 ± 100</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM from three independent experiments.

The radiolabelled sterol-precursor [3H]mevalonic acid was used to measure biosynthesis as the incorporation of radioactivity into the purified sterols. The ratio of incorporated radioactivity (c.p.m.) between controls without AY9944-A-7 and test with AY9944-A-7 was 1:2.3 for lanosterol, that is a 2.3-fold increase, and 1:11 for FF-MAS, that is an 11-fold increase. For T-MAS and cholesterol, the incorporation of radioactivity was decreased by incubation with AY9944-A-7; the ratio between cultures without and with AY9944-A-7 was 5:2.1 and 30:1, respectively. In the presence of AY9944-A-7, the synthesis of progesterone was increased 2.8-fold (Table 1). The total c.p.m. per 100 COC of the five metabolites of the controls was 52,337, but only half of that amount was found in COC cultured with AY9944-A-7 (24,690 c.p.m.).

Discussion

This study demonstrates that an endogenous production of MAS in COC caused by the sterol synthesis inhibitor AY9944-A-7 results in a dose-dependent stimulation of resumption of meiosis in mouse cumulus-oocyte complexes (COC) at 10 μg ml⁻¹ and in mouse naked oocytes in a dose-dependent manner. COC (a) or naked oocytes (b) were cultured in hypoxanthine medium alone or containing 5 or 10 μg FF-MAS ml⁻¹. After 20–22 h, the percentage of oocytes in GVBD (including those with polar bodies) per total number of oocytes was scored. Values represent the mean ± SEM from three to nine independent experiments. n = total number of oocytes. Different letters indicate significant differences (Fisher’s least significant difference procedure). AY9944-A-7 caused an effective accumu-

Fig. 3. Stimulation by AY9944-A-7 of germinal vesicle breakdown (GVBD) in intact mouse cumulus-oocyte complexes (COC), but not in mechanically split and re-pooled COC or naked oocytes. Oocytes pooled from several mice were divided in COC, split COC (split) and naked oocytes (NO). After culture for 20–22 h with and without 10 μmol AY9944-A-7 (AY) l⁻¹, the percentage of oocytes in GVBD (including those with polar bodies) per total number of oocytes was scored. Values represent the mean ± SE from three to six observations in three experiments. n = total number of oocytes. Bars with at least one identical letter are not significantly different at the 95% confidence level (Student’s t test).
meiosis in vitro induced by AY9944-A-7 was associated with an accumulation of FF-MAS, consistent with the hypothesis that FF-MAS exerts the stimulating effect in this process.

The results of the present study show that COC incorporate radioactivity into FF-MAS after culture with radiolabelled mevalonic acid in the presence of AY9944-A-7. In these experiments, the percentage of oocytes in GVBD (including those with polar bodies) per total number of oocytes was scored. Values represent the mean ± SE of the observations in three to eleven experiments. Bars with different letters are significantly different at the 95% confidence level (Student’s t test).

Fig. 5. Effect of meiosis-activating sterol isolated from human follicular fluid (FF-MAS; 4,4-dimethyl-5α-cholest-8,14,24-triene-3β-ol) in combination with AY9944-A-7 on resumption of meiosis in mouse oocytes. Cumulus–oocyte complexes (COC) (a) or naked oocytes (b) were cultured in hypoxanthine medium with 10 µmol AY9944-A-7 (AY) with or without 10 µg FF-MAS ml−1. After 22 h, the percentage of oocytes in GVBD (including those with polar bodies) per total number of oocytes was scored. Values represent the mean ± SE of the observations in three to eleven experiments. Bars with different letters are significantly different at the 95% confidence level (Student’s t test).

meiosis in vitro induced by AY9944-A-7 was associated with an accumulation of FF-MAS, consistent with the hypothesis that FF-MAS exerts the stimulating effect in this process.

The results of the present study show that COC incorporate radioactivity into FF-MAS after culture with radiolabelled mevalonic acid in the presence of AY9944-A-7, and that COC are capable of cholesterol biosynthesis from mevalonate. Since AY9944-A-7 has no effect on naked oocytes, with or without cumulus cells added, except at a very high concentration, sterol synthesis may be very low or absent in the oocyte. Thus, Δ14-reductase may be localized mainly or exclusively in the cumulus cells of the COC and be at a low concentration or not present in the oocyte. However, the experiments presented here are not sufficiently sensitive to resolve this issue.

In the present study, mevalonic acid was used as a precursor for the sterol synthesis. Mevalonic acid is an intermediate in the sterol synthesis pathway from acetate. In mammals, the conversion of 3β-hydroxy-3β-methylglutaryl-CoA (HMG-CoA) to mevalonic acid by HMG-CoA-reductase is the rate limiting enzyme in the formation of lanosterol (Schroepfer et al., 1972). Therefore, de novo sterol biosynthesis from acetate by COC remains to be demonstrated.

In cultures of yeast (Aoyama and Yoshida, 1986) and in hepatocytes from rats fed with a diet containing AY9944-A-7 (Kim et al., 1995), AY9944-A-7 attenuates metabolism of T-MAS, leading to an accumulation of FF-MAS. The concentration of AY9944-A-7 (5 µmol l−1) required to affect GVBD in COC is higher than the concentration needed to inhibit the activity of Δ14 reductase in rat hepatocytes (0.1 µmol l−1) (Kim et al., 1995). This may be related to a different capacity of cholesterol synthesis in the two types of cell, or to differences in binding to other proteins or uptake of the inhibitor. In addition, in the present study an indirect biological effect of AY9944-A-7 on the oocyte was measured, since the AY9944-A-7-induced active stimulatory substance seems to derive from the cumulus cells of AY9944-A-7-stimulated COC. Thus, in contrast to the hepatocytes, in which the effect is measured directly on the target cell, the signal transduction pathway in COC from the effect of AY9944-A-7 to the measured effect (GVBD) must involve both cumulus cells and oocyte.

The low concentration of T-MAS in the presence of AY9944-A-7 indicates that the resumption of oocyte meiosis may not require high concentrations of T-MAS in vitro. However, T-MAS is able to stimulate meiotic resumption in vitro (Byskov et al., 1995) and T-MAS as well as FF-MAS are present in human follicular fluid (Baltsen and Byskov, 1999). It is possible that natural gonadotrophin-stimulated oocyte resumption of meiosis is the result of a combined effect of a family of meiosis-activating sterols.

The results of this study showed that AY9944-A-7 only induced GVBD in intact COC. When the COC were separated into cumulus cells and oocyte and pooled to be cultured together, AY9944-A-7 did not stimulate resumption of meiosis compared with the split and co-cultured controls. AY9944-A-7 only stimulated GVBD in naked oocytes at a high concentration (25 µmol l−1). However, 25 µmol AY9944-A-7 l−1 had a toxic effect on the COC and inhibited the development of polar bodies in both COC and naked oocytes, indicating that the results at this concentration could be an artefact. At the lower concentrations of AY9944-A-7, there were no indications of toxic effects in either naked oocytes or COC, and the rate of polar body extrusion was not affected.

In cultured FSH-stimulated COC, a meiosis-activating, heat stable substance is released to the culture medium. These culture media were able to induce GVBD in naked oocytes. However, FSH-stimulated split cumulus–oocyte cultures did not release a meiosis-activating substance (Byskov et al., 1997). Similar results were found when forskolin was used as a stimulator (Guoliang et al., 1994). It appears that the cumulus cells must be in intimate contact with the oocyte to enable the production and secretion of the active substance. In these two studies, as well as in the present study, the GVBD rates were high in oocytes of the split COC cultures, in both tests and controls. The cumulus cells of the intact COC may protect against GVBD or it is possible that the disruption of the cumulus–oocyte junctions leads to changes in the phenotype of both the cumulus cells and oocyte. The slightly higher percentage of GVBD of the split COC cultured with AY9944-A-7 compared with the controls may be the result of accumulation and secretion of MAS by the isolated cumulus cells.
Inhibition of sterol Δ14-reductase and meiotic resumption

The controlling activity from the oocyte has not yet been identified, but the crucial role of the oocyte in follicular granulosa and cumulus cell luteinization has been established since Westman showed that the oocyte prevents granulosa cell luteinization (Westman, 1934). Many studies have documented this role and it is well established that the oocyte interacts with the cumulus cells through gap junctions (Anderson and Albertini, 1976; Moor et al., 1980; Fagbohun and Downs, 1991), as well as in a paracrine manner.

The oocyte secretes a multitude of factors that act locally, for example a cumulus expanding-enabling factor (Buccione et al., 1990; Vanderhyden et al., 1990) and factors that promote granulosa cell proliferation (Vanderhyden et al., 1992, Lanuza et al., 1998). The oocyte inhibits progesterone and oestradiol synthesis (Coskun et al., 1995; Vanderhyden and Tonary, 1995), inhibits plasminogen activator production by the cumulus and granulosa cells (Canipari et al., 1995) and suppresses expression of LH receptor mRNA in granulosa cells (Eppig et al., 1995) (Vanderhyden and Macdonald, 1998) found that fully grown oocytes and even ovulated oocytes secrete a factor that inhibits accumulation of progesterone by co-cultured cumulus cells. In spite of the action of this factor, maturing cumulus–oocyte complexes accumulate progesterone during luteinization, possibly as a result of desensitization of the cumulus to the factor after the gonadotrophin surge (Vanderhyden and Macdonald, 1998). The results of the present study indicate that the action of AY9944-A-7 may share some similarities with the action of gonadotrophins, resulting in oocyte maturation and accumulation rather than inhibition of progesterone.

In the present study, 5 µg FF-MAS ml−1 induced GVBD in naked oocytes, whereas 10 µg FF-MAS ml−1 was required to stimulate GVBD in COC. Synthetic FF-MAS induces GVBD in mouse COC in the presence of hypoxanthine at concentrations of 0.7 and 7.0 µmol l−1 (Grøndahl et al., 1998). At 7 µmol l−1, FF-MAS could also overcome GVBD inhibition by isobutylmethylxanthine and dibutyryl CAMP. It is unclear why at concentrations as high as 10 µg ml−1 FF-MAS only exerts a rather modest induction of GVBD in COC. The most likely explanation is that Δ14-reductase metabolizes FF-MAS to T-MAS and that other enzymes in the cumulus cells immediately convert T-MAS into metabolically inactive steroids or sterols, leaving only a fraction of FF-MAS to reach the oocyte. Natural gonadotrophin-stimulated steroidogenesis by the preovulatory follicle affects enzyme activity involved in steroid and steroid synthesis (Gore-Langton and Armstrong, 1994); progesterone upregulation is the most well characterized effect. Cholesterol biosynthesis is directly affected by gonadotrophins (Douglas et al., 1978; Baranao and Hammond, 1986). Natural resumption of oocyte meiosis in the preovulatory follicle may thus be the combined effect of an altered sterol biosynthesis of the granulosa and cumulus cells, resulting in accumulation of MAS, and a direct transfer of MAS from the cumulus cells to the oocyte. If this altered enzyme activity is not induced in the cumulus cells, exogenous MAS is most likely converted immediately to cholesterol and possibly to steroids. It is proposed that during the natural oocyte maturation, factors in the follicular environment may regulate the enzyme activities that metabolize MAS in a similar manner to that observed when AY9944-A-7 stimulates oocyte maturation in vitro, for example by inhibiting Δ14-reductase. AY9944-A-7 also reduced the total incorporation of radioactivity in the five metabolites to half of that in the controls. Although not all metabolites of cholesterol biosynthesis were measured or quantified, the results indicate that AY9944-A-7 may have reduced the total metabolic flux. More detailed studies of the enzymes and metabolites involved are required to understand better the role of sterols in natural and artificial induction of meiosis.

The role of MAS on maturation in vivo was investigated using another cholesterol biosynthesis inhibitor, ketoconazole. This drug inhibits cytochrome P450 14α-demethylase, which catalyses the conversion of lanosterol to FF-MAS in vitro (Yoshida et al., 1996), and thus blocks the natural synthesis of FF-MAS. Ketoconazole suppressed follicular progesterone accumulation in vitro, but did not inhibit spontaneous maturation of rat oocytes in vitro or LH-induced maturation in vivo. The toxic effect by ketoconazole on isolated rat oocytes (Tsafiri et al., 1998) is in accordance with the finding that isolated mouse oocytes degenerate or mature without forming polar bodies when cultured in the presence of 10 µmol ketoconazole l−1 (M. Strömstedt, L. Leonardsen, A. G. Byskov, M. Baltsen, unpublished). Spontaneous meiotic maturation and FSH-induced maturation are mediated by different intracellular pathways (Coticchio and Fleming, 1998). Similarly, it has been suggested that induced maturation is mediated by the generation of a stimulatory signal, which overrides an inhibitory input from the follicle, whereas this stimulatory signal is unnecessary for spontaneous maturation to occur (Downs 1993). The present study indicates that there is a similar difference between MAS-induced and spontaneous maturation, which may explain how rat oocytes mature spontaneously despite an inhibitory action of ketoconazole on MAS biosynthesis. However, the contradictory findings between the action of ketoconazole and the proposed action of FF-MAS in resumption of meiosis cannot be explained, except by possible species differences.

Ketoconazole inhibits other cytochrome P450 enzymes, including P450 CYP3A (Zhang et al., 1998; Gibbs et al., 1999), CYP11A1 (P450 cholesterol side-chain cleavage (scC) enzyme) (Wacht et al., 1996; Nnane et al., 1998; Gal et al., 1994), CYP11B1 (Johansson et al., 1998), CYP17 (17α-hydroxylase/C17,20-lyase), CYP19 (P450-aromatase) (Ayub and Levell, 1990), oestrogen 2-hydroxylase (Purba et al., 1994), and 17-ketosteroid-reductase (Malozowski et al., 1986). P450 CYP3A is a major drug metabolizing enzyme in liver. CYP11B1 is involved in adrenal corticosterone synthesis, and CYP17 (17α-hydroxylase/C17,20-lyase) regulates an early step in the synthesis of testosterone and other androgens in both the gonads and the adrenal glands (Weber et al., 1993). Similarly, oestrogen 2-hydroxylase, 17-ketosteroid-reductase, P450scC and P450 aromatase are key steroidogenic cytochromes (Thomson, 1998). In addition, ketoconazole interferes with covalent modifications of hormone receptor family proteins by steroids and PGE2 (Takahashi and Breitman, 1992), and binds to the benzodiazepine receptor (Fahey et al., 1998; Thomson, 1998).
Therefore, some of the effects of ketoconazole on rat oocytes observed by Tsafiriri et al. (1998) may be due to interference with physiologically important mechanisms that are unrelated to FF-MAS synthesis.

In conclusion, inhibition of steroid Δ4-reductase by AY9944-A-7 in vitro results in the accumulation of endogenously produced FF-MAS in the cumulus cells and the resumption of meiosis in oocytes of COC, but not in naked oocytes. Therefore, it is proposed that MAS biosynthesis takes place in the cumulus cells of COC, and not in the oocyte itself, and that this may be responsible for resumption of meiosis in oocytes in vitro.

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