

# Roles of gene transcription and PKA subtype activation in maturation of murine oocytes

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The aims of this study were to examine the role of transcription and the coincident involvement of type I and type II protein kinase A (PKA) in the resumption of meiosis in murine cumulus–oocyte complexes (COCs) using the transcriptional inhibitors 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) and  $\alpha$ -amanitin. The first series of experiments was designed to: (i) characterize the role of transcription in gonadotrophin-mediated and spontaneous maturation of murine oocytes; (ii) examine the roles of specific gonadotrophins (FSH versus hCG) and cumulus cells in transcriptionally mediated oocyte maturation; and (iii) determine the reversibility of the transcriptional arrest of meiosis. In the presence of FSH, transcriptional inhibitors arrested germinal vesicle breakdown (GVBD) (DRB:  $2 \pm 2\%$  and control:  $76 \pm 2\%$ ;  $\alpha$ -amanitin:  $4 \pm 4\%$  and control:  $70 \pm 4\%$ ). Furthermore, cumulus cells were required for transcriptional inhibitors to arrest GVBD (DRB with cumulus cells:  $0 \pm 15\%$ ; DRB without cumulus cells:  $94 \pm 13\%$ ;  $\alpha$ -amanitin with cumulus cells:  $15 \pm 2\%$ ;

$\alpha$ -amanitin without cumulus cells:  $99 \pm 2\%$ ). Thus, in mice, FSH-mediated GVBD uses a transcriptional mechanism, which probably occurs within the cumulus cell compartment. In a second series of experiments, the role of transcription in mediating the resumption of meiosis after activation of either type I or type II PKA was examined. Activation of type I PKA in murine COCs resulted in an arrest of GVBD that was independent of a transcriptional event (with DRB:  $7 \pm 9\%$  GVBD; without DRB:  $11 \pm 9\%$  GVBD). In contrast, activation of type II PKA resulted in a resumption of meiosis, which required the occurrence of gene transcription (with DRB:  $12 \pm 9\%$  GVBD; without DRB:  $80 \pm 9\%$  GVBD). As FSH binding to cumulus cells activates the PKA second messenger system, our results indicate that, in cultured murine COCs, FSH binding to cumulus cells results in the activation of type II PKA, which, in turn, mediates a downstream transcriptional event required for the initiation of GVBD.

## Introduction

The resumption of meiosis in oocytes is characterized by chromosome condensation, germinal vesicle breakdown (GVBD) and progression to meiosis I. *In vivo*, this occurs in response to the preovulatory surge of gonadotrophins (Tsafiri *et al.*, 1976). *In vitro*, maturation occurs spontaneously in the absence of gonadotrophins when the oocyte is removed from the intrafollicular environment (Pincus and Enzmann, 1935). As maturation can be prevented by increased cAMP in isolated cumulus–oocyte complexes (COCs) and denuded oocytes (Dekel *et al.*, 1981; Schultz *et al.*, 1983a; Downs and Eppig, 1984; Eppig, 1989), spontaneous maturation is thought to result from a decrease in intra-oocyte cAMP content.

Specific inhibitors of RNA polymerase II, such as  $\alpha$ -amanitin or 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB), have been used to demonstrate that gonadotrophin-mediated resumption of meiosis in pigs (Meinecke and

Meinecke-Tillmann, 1993), sheep (Osborn and Moor, 1983) and cows (Kastrop *et al.*, 1991; Farin and Yang, 1994) requires an initial transcriptional event that occurs in the cumulus cell compartment of COCs. In bovine COCs, transcriptional events initiated by FSH did not occur when COCs resumed meiosis spontaneously or in the presence of hCG (Wolf and Farin, 1996). In mice, it has been suggested that GVBD may not be mediated by a transcriptional event (Crozet and Szollosi, 1980). However, in hypoxanthine-arrested murine COCs,  $\alpha$ -amanitin blocked the induction of meiosis by FSH, indicating a possible role for transcription in this model system (Downs, 1995; Downs *et al.*, 1996).

Signal transduction by FSH occurs after binding to its receptor, which results in stimulation of adenylyl cyclase and activation of cAMP-dependent protein kinase A (PKA) (Hunzicker-Dunn and Jungmann, 1978). PKA is a tetrameric molecule consisting of a regulatory subunit dimer. Each regulatory subunit is bound to one inactive catalytic subunit. There are two isozymes of PKA, designated as type I and type II on the basis of order of elution of their regulatory subunits (RI and RII, respectively) from anion exchange resins (Corbin *et al.*, 1975). There are two cAMP binding sites on each of these regulatory subunits. Within each regulatory subunit,

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the two cAMP binding sites differ on the basis of their rate of exchange and affinity for [ $^3\text{H}$ ]cAMP (Potter and Taylor, 1980; Doskeland and Ogreid, 1984). By using combinations of cAMP analogues that bind selectively to each site on the regulatory subunits, it is possible to activate either type I or type II PKA differentially (Ogreid *et al.*, 1989). Differential activation of PKA isozymes has opposing effects on maturation of murine oocytes. Oocytes were arrested at the germinal vesicle stage after activation of type I PKA, whereas activation of type II PKA stimulated GVBD in oocytes arrested by isobutyl methylxanthine (IBMX) (Downs and Hunzicker-Dunn, 1995).

The role of *de novo* gene transcription after specific gonadotrophin stimulation and PKA activation has not been characterized for maturation of murine oocytes. The aims of the present study were to examine the role of transcription and the coincident involvement of type I and type II PKA in the resumption of meiosis in murine COCs.

## Materials and Methods

### Reagents and media

5,6-Dichloro-1- $\beta$ -D-ribofuranosyl-benzimidazole (DRB, Lot 027H4086) and the cAMP analogues,  $N^6$ -monobutyryl-cyclic adenosine monophosphate ( $N^6$ ), aminohexyl-aminocyclic adenosine monophosphate (AHA) and 8-bromo-cyclic adenosine monophosphate (8-Br) were purchased from Sigma Chemical Co. (St Louis, MO).  $\alpha$ -Amanitin was purchased from Boehringer Mannheim (Indianapolis, IN). hCG (75 iu  $\text{l}^{-1}$ ; Sigma Chemical Co.) and FSH (0.5  $\mu\text{g ml}^{-1}$ ; USDA oFSH-18) were used as media supplements. Unless otherwise noted, chemicals for preparation of media were from Sigma Chemical Co. and were of tissue culture grade. The eCG used for superovulation was also obtained from Sigma Chemical Co.

Waymouth medium supplemented with 5% fetal bovine serum, 0.23 mmol pyruvate  $\text{l}^{-1}$ , 7 mmol taurine  $\text{l}^{-1}$ , 50 mg streptomycin sulphate  $\text{l}^{-1}$  and 75 mg penicillin G  $\text{l}^{-1}$  was used for oocyte cultures.  $\alpha$ -Amanitin was dissolved in culture medium (10  $\mu\text{g ml}^{-1}$ ) immediately before use. A stock solution of DRB (20 mg  $\text{ml}^{-1}$ ) was prepared in dimethyl sulphoxide (DMSO) and added to the culture medium at 120  $\mu\text{mol l}^{-1}$ . This dose was chosen after evaluation of doses ranging from 30 to 150  $\mu\text{mol DRB l}^{-1}$  to maintain murine COCs at the germinal vesicle stage for a 3 h culture period (data not shown).

Differential activation of PKA subunits was performed by supplementing media with different combinations of cAMP analogues:  $N^6$  and AHA were used for activation of type I PKA, and 8-Br and  $N^6$  were used for activation of type II PKA (Downs and Hunzicker-Dunn, 1995). The analogues were dissolved in DMSO and used immediately. An equimolar concentration of each analogue was used to achieve the final concentration indicated for each specific experimental group.

### Role of transcription in gonadotrophin-mediated resumption of meiosis

*Oocyte recovery and culture conditions.* Prepubertal

Swiss Albino mice (aged 20–23 days; Taconic, Germantown, NY) were maintained on a 12 h light:12 h dark cycle with access to food and water available *ad libitum*. A minimum of six mice was used in each replicate for all experiments. Mice were given an i.p. injection of 5 iu eCG and were killed by cervical dislocation 48 h later. Ovaries were dissected into culture medium, extracted from the bursa, cleaned of adhering fat, bisected and distributed to dishes containing appropriate treatment media. Each demi-ovary was bisected and the halves were distributed such that each mouse contributed ovarian tissue to all four treatment groups in each experiment. Antral follicles on each quarter-ovary were punctured with a sterile needle and COCs were collected. The COCs were washed once in fresh treatment medium and cultured in multi-well dishes containing 0.5 ml treatment medium. All cultures were conducted at 37°C in an atmosphere of 5%  $\text{CO}_2$  in air with 100% humidity. At the termination of culture, oocytes were denuded of their cumulus cells by manual pipetting and assessed visually for the resumption of meiosis at a magnification of  $\times 60$  using a stereomicroscope. Oocytes without an intact germinal vesicle were classified as having undergone GVBD. Oocytes with a dark or fragmented ooplasm or dark cumulus cells were classified as degenerated. All procedures involving animals were carried out in accordance with NIH guidelines for the care and use of laboratory animals under the approval of the North Carolina State University Institutional Animal Care and Use Committee.

*Effect of gonadotrophins on inhibition of meiotic maturation by DRB or  $\alpha$ -amanitin.* Two experiments were conducted. For both Expts 1a and 1b, COCs were distributed to one of four treatments in a  $2 \times 2$  factorial arrangement. In Expt 1a, the main factors were transcriptional inhibitor (DRB versus vehicle control) and gonadotrophin treatment (FSH plus hCG versus medium control). In Expt 1b, the main factors were transcriptional inhibitor ( $\alpha$ -amanitin versus medium control) and gonadotrophin treatment (FSH plus hCG versus medium control). Oocytes were assessed for GVBD after 3 h culture. Each experiment was replicated four times and included an average of  $145 \pm 28$  COCs (Expt 1a) and  $143 \pm 27$  COCs (Expt 1b) per replicate.

*Effect of supplementation with either hCG or FSH on inhibition of meiotic maturation by DRB or  $\alpha$ -amanitin.* Two experiments were conducted. Each experiment included treatments in a  $2 \times 2$  factorial arrangement with the major factors being presence or absence of specific gonadotrophins (FSH, hCG). In Expt 2a, all media were supplemented with 120  $\mu\text{mol DRB l}^{-1}$ . In Expt 2b, all media were supplemented with 10  $\mu\text{g } \alpha\text{-amanitin ml}^{-1}$ . Oocytes were denuded and assessed for GVBD after 3 h culture. Each experiment was replicated four times and included an average of  $129 \pm 18$  COCs (Expt 2a) and  $136 \pm 30$  COCs (Expt 2b) per replicate.

*Effect of removal of cumulus cells on the inhibition of oocyte maturation by DRB or  $\alpha$ -amanitin.* For these two

experiments, all media were supplemented with 0.5  $\mu\text{g}$  FSH  $\text{ml}^{-1}$ . When appropriate, COCs were denuded by manual pipetting in treatment medium before initiation of culture. In Expt 3a, treatment groups were in a  $2 \times 2$  factorial arrangement with the major factors being presence or absence of transcriptional inhibitor (DRB) and presence or absence of cumulus cells surrounding the oocyte. In Expt 3b, the major treatment factors were transcriptional inhibitor ( $\alpha$ -amanitin) and the presence or absence of cumulus cells. For both experiments the cultures continued for 3 h. After culture, all COCs were denuded by manual pipetting and assessed for GVBD. The experiments were replicated four times and included an average of  $127 \pm 25$  COCs (Expt 3a) and  $135 \pm 24$  COCs (Expt 3b) per replicate, respectively.

**Reversibility of DRB- and  $\alpha$ -amanitin-mediated arrest of GVBD.** All media for Expts 4a and 4b were supplemented with 0.5  $\mu\text{g}$  FSH  $\text{ml}^{-1}$ . In Expt 4a, COCs were distributed to one of five treatment groups: without DRB, 3 h (culture for 3 h in control medium); with DRB, 3 h (culture for 3 h in medium supplemented with DRB); with DRB reverse (culture for 3 h in medium supplemented with DRB followed by transfer to inhibitor-free medium and culture for an additional 3 h); without DRB reverse (culture for 3 h in control medium, transfer to fresh control medium, culture for an additional 3 h); with DRB, 6 h (culture for 3 h in DRB-supplemented medium, transfer to DRB-supplemented medium, culture for an additional 3 h). This final group was considered as an experimental control and was included to demonstrate that if the oocytes were cultured continuously for 6 h in the presence of the inhibitor, the arrest of maturation would be maintained. At the termination of treatment, COCs from each group were denuded and assessed for the occurrence of GVBD.

In Expt 4b, the treatment groups were the same as in Expt 4a with the exception that  $\alpha$ -amanitin was used as the transcriptional inhibitor in place of DRB. The experiments were each replicated four times and included an average of  $164 \pm 50$  COCs (Expt 4a) and  $82 \pm 13$  COCs (Expt 4b) per replicate, respectively.

#### *Role of PKA isozymes in facilitating resumption of meiosis*

**Oocyte recovery and culture conditions.** Prepubertal Swiss albino mice (age 20–23 days) were housed and super-ovulated as described previously. For all experiments, a minimum of 12 mice was used in each replicate. Ovaries were collected as described previously and then bisected. Each demi-ovary was distributed randomly to dishes containing treatment media such that no mouse would contribute more than one demi-ovary to the same treatment. Cultures were performed in the absence of gonadotrophin stimulation unless specifically noted. COCs were cultured for 3 or 4 h, after which the COCs were stripped by manual pipetting and assessed for GVBD.

**Effect of differential activation of type I or type II PKA on resumption of meiosis.** The analogues used for activation of type I PKA were  $\text{N}^6$  and AHA, whereas the analogues used for the activation of type II PKA were  $\text{N}^6$  and 8-Br (Downs and Hunzicker-Dunn, 1995). In the first two replicates of this experiment, the analogues used for activation of either type I or type II PKA were used at concentrations of 0, 50 or 100  $\mu\text{mol l}^{-1}$ . In the second two replicates, two additional concentrations (5 and 10  $\mu\text{mol l}^{-1}$ ) were also included. Cultures were continued for 3 h, after which time the oocytes were stripped from their cumulus cells and assessed for GVBD. The experiment was replicated four times and included an average of  $212 \pm 25$  COCs per replicate.

**Role of transcription in regulating meiosis after differential activation of type I or type II PKA.** On the basis of the previous analysis, two effective concentrations for activation of type I or type II PKA were chosen for further study. The COCs were distributed randomly to one of 12 treatment groups and cultured for 4 h. The 12 treatment groups were arranged in three subgroups: control groups:  $n = 4$  groups that included all combinations of treatments with and without FSH and with and without DRB; type I activation groups:  $n = 4$  groups that included 10 or 50  $\mu\text{mol l}^{-1}$  type I cAMP analogues in the presence or absence of DRB; and type II activation groups:  $n = 4$  groups that included 5 or 10  $\mu\text{mol l}^{-1}$  type II cAMP analogues in the presence or absence of DRB. After termination of culture, COCs were stripped of their cumulus cells by manual pipetting and assessed for GVBD. The experiment was replicated four times with an average of  $201 \pm 94$  COCs per replicate.

#### *Statistical analysis*

Data on the percentage of oocytes undergoing GVBD were analysed both as raw data and as arcsine-transformed data by ANOVA using general linear model procedures. When a significant  $F$  statistic was found, the means were separated using Duncan's new multiple-range test (SAS, 1988). All data are reported as least squares means  $\pm$  SE and the significant differences were based on the analysis of the transformed data. The number of oocytes per replicate is expressed as mean  $\pm$  SD.  $P < 0.05$  was considered significant.

## **Results**

### *Role of transcription in gonadotrophin-mediated resumption of meiosis*

**Effect of gonadotrophins on inhibition of meiotic maturation by DRB or  $\alpha$ -amanitin.** Meiotic maturation occurring in the presence of gonadotrophins was sensitive to transcriptional inhibition by either DRB or  $\alpha$ -amanitin (Fig. 1a,b). In contrast, neither inhibitor was effective at arresting GVBD when COCs were cultured in the absence of gonadotrophins. On the basis of these observations, nascent gene transcription was required for gonadotrophin-mediated resump-

tion of meiosis. In contrast, resumption of meiosis in the absence of gonadotrophins (spontaneous maturation) did not require a transcriptional event. A subsequent experiment was conducted to determine whether transcriptional activation occurred in response to a specific gonadotrophin (hCG or FSH).

*Effect of hCG or FSH on inhibition of meiotic maturation by DRB or  $\alpha$ -amanitin.* GVBD initiated in the presence of FSH and hCG or FSH alone was effectively blocked by treatment with either DRB or  $\alpha$ -amanitin (Fig. 1c,d). GVBD initiated in the presence of hCG alone or in the complete absence of gonadotrophins was not sensitive to transcriptional inhibition. Thus, FSH-mediated resumption of meiosis occurred through a transcriptional pathway that was not used during either spontaneous maturation or hCG-mediated resumption of meiosis.

*Effect of removal of cumulus cells on the inhibition of oocyte maturation by DRB or  $\alpha$ -amanitin.* In the presence of cumulus cells, both DRB and  $\alpha$ -amanitin effectively inhibited FSH-mediated GVBD (Fig. 1e,f). However, when cumulus cells were removed, neither transcriptional inhibitor was effective at preventing GVBD. These data indicate that the transcriptional event initiated during FSH-mediated GVBD probably occurred in the cumulus cell compartment.

*Reversibility of DRB- and  $\alpha$ -amanitin-mediated arrest of GVBD.* As  $\alpha$ -amanitin has been associated with oocyte and embryo degeneration (Kidder *et al.*, 1985; Hunter and Moor, 1987), the capacity of murine COCs to continue maturation after culture in the presence of either transcriptional inhibitor was examined. Transcriptional inhibition of FSH-mediated GVBD was reversible and was not associated with oocyte degeneration (Fig. 1g,h). Ninety-eight per cent of COCs cultured for 3 h in the presence of DRB and 81% of COCs cultured in the presence of  $\alpha$ -amanitin remained at the germinal vesicle stage (with DRB, 3 h and with  $\alpha$ -amanitin, 3 h, respectively). After 6 h of culture in the presence of DRB, 89% of COCs remained at the germinal vesicle stage and after 6 h of culture in the presence of  $\alpha$ -amanitin 85% remained at the germinal vesicle stage (with DRB, 6 h and with  $\alpha$ -amanitin, 6 h, respectively). When COCs were exposed to DRB or  $\alpha$ -amanitin and then transferred to inhibitor-free medium and cultured for an additional 3 h, approximately 92% of COCs (with DRB reverse group) and 66% (with  $\alpha$ -amanitin reverse group) underwent GVBD. Compared with treatments with DRB, differences in reversibility associated with  $\alpha$ -amanitin were not due to an increased incidence of oocyte degeneration, as < 1.5% (28/2009) of oocytes were degenerate after treatment with  $\alpha$ -amanitin.

#### *Role of PKA isoenzymes in facilitating resumption of meiosis*

*Effect of activation of type I or type II PKA on meiotic resumption: dose-response analysis.* Increasing concentrations of type I PKA activators resulted in a dose-dependent

decrease in the incidence of GVBD in cultured murine COCs (Fig. 2). As activation of type II PKA was reported to initiate resumption of meiosis in dbcAMP-arrested COCs (Downs and Hunzicker-Dunn, 1995), the most effective concentrations for activation of type II PKA were defined as the lowest concentrations used that did not interfere with oocyte maturation. These concentrations were 5 and 10  $\mu\text{mol l}^{-1}$  ( $84.1 \pm 14.3$  and  $88.5 \pm 14.3\%$  GVBD, respectively; Fig. 2).

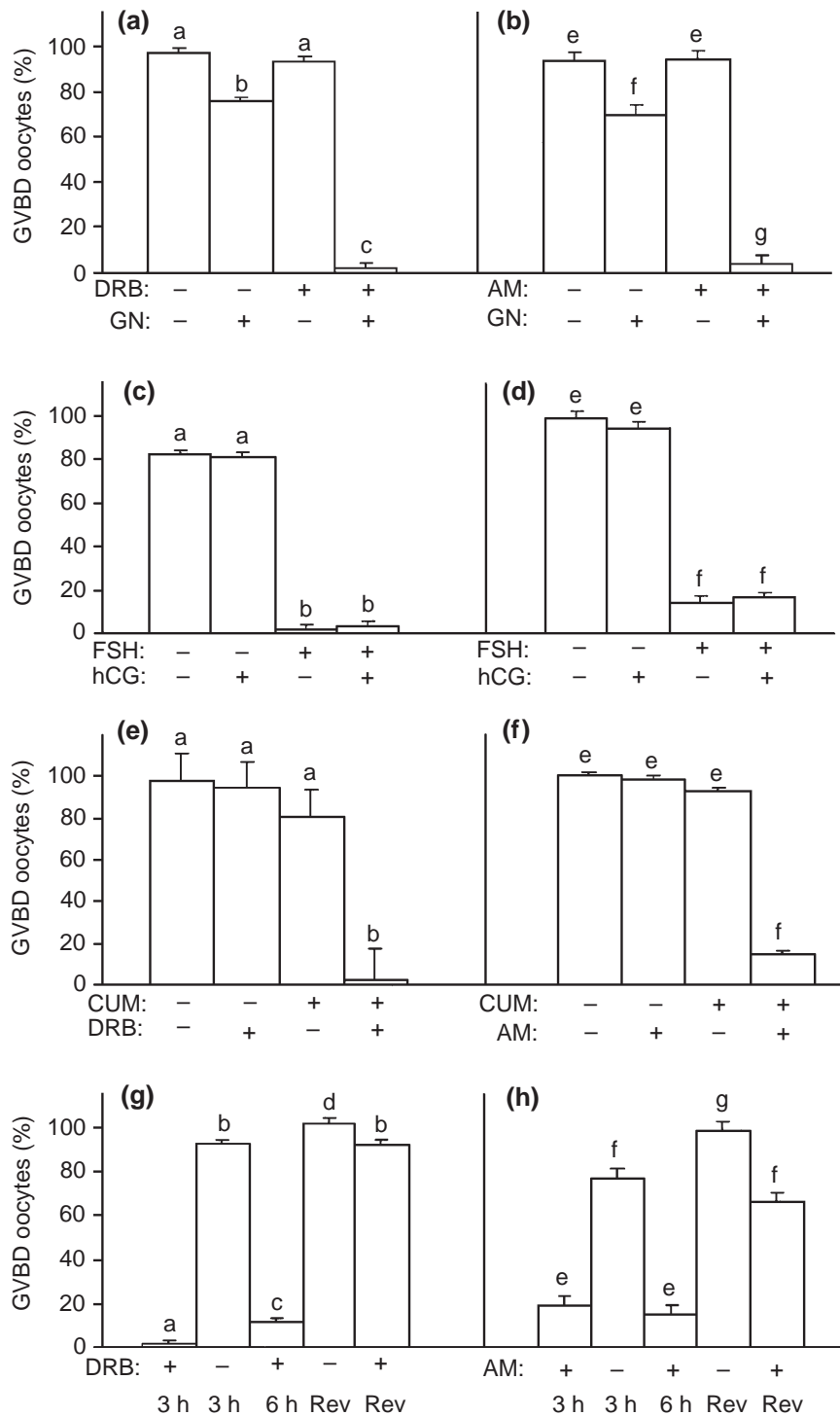
*Role of transcription in regulating meiosis after differential activation of type I or type II PKA.* In the absence of FSH, treatment of murine COCs with type I PKA activators at 10 or 50  $\mu\text{mol l}^{-1}$  significantly inhibited the occurrence of GVBD ( $P < 0.001$ ). There was no effect of treatment with DRB on the percentage of oocytes remaining in germinal vesicle arrest after activation of type I PKA. These observations indicate that inhibition of GVBD by activation of type I PKA may not require a transcriptional event. In contrast, activation of type II PKA resulted in resumption of meiosis requiring a transcriptional event. GVBD was inhibited when DRB was added to media in the presence of activators of type II PKA (Fig. 3). In addition, this degree of inhibition was not different from that found when DRB was used in the presence of FSH ( $20.7 \pm 8.4$  and  $12.5 \pm 8.4\%$  GVBD, respectively).

## Discussion

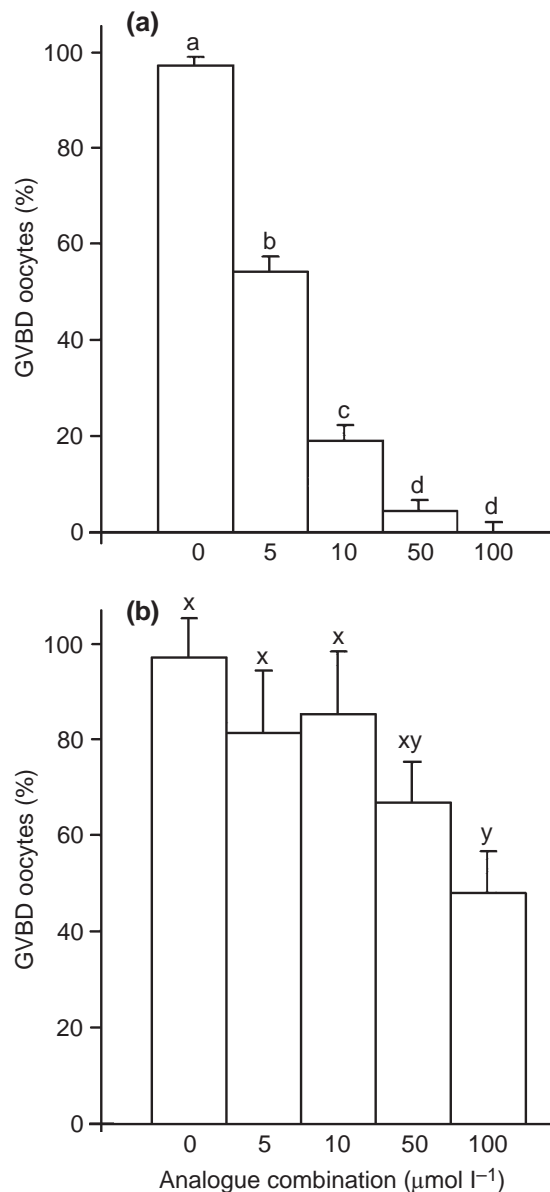
The data from the present study demonstrate that, in cultured murine COCs, FSH-mediated GVBD requires gene transcription. The use of two inhibitors, each of which prevents transcription by different mechanisms, led to similar conclusions. DRB, an analogue of the nucleoside adenosine, acts by preventing the formation of a stable transcription initiation complex (Zandomeni *et al.*, 1983). In contrast,  $\alpha$ -amanitin acts by inhibiting nucleoplasmic RNA polymerase II specifically (Zubay, 1983). Treatment with either inhibitor did not induce oocyte degeneration during the short-term cultures used throughout these experiments, as only 85 of 4282 oocytes (2%) were classified as degenerate after culture. In addition, COCs were not required to be maintained in a hypoxanthine-induced meiotic arrest (Downs, 1995; Downs *et al.*, 1996) for either transcriptional inhibitor to prevent gonadotrophin-mediated GVBD.

When gonadotrophins were included in the culture medium, GVBD was initially inhibited in murine (Eppig *et al.*, 1983; Schultz *et al.*, 1983b) and bovine (Farin and Yang, 1994) COCs. This transient inhibition was followed by an acceleration in the rate of GVBD compared with that observed during spontaneous maturation (Downs, 1990b; Farin and Yang, 1994). It has been suggested that the initial inhibitory phase results from an increase in cAMP occurring in response to hormone binding (Eppig and Downs, 1988; Downs, 1990a).

In bovine, pig and ovine COCs, the transcriptional event required for gonadotrophin-induced GVBD occurs within



**Fig. 1.** Characterization of gonadotrophin-mediated germinal vesicle breakdown (GVBD) in murine cumulus–oocyte complexes. (a,b) Effect of gonadotrophins on inhibition of meiotic maturation by 5,6-dichloro-1- $\beta$ -D-ribofuranosyl-benzimidazole (DRB) or  $\alpha$ -amanitin (AM) (GN: FSH plus hCG). (c,d) Effect of hCG or FSH on inhibition of meiotic maturation by DRB or  $\alpha$ -amanitin. All treatments depicted in (c) included 120  $\mu$ mol DRB  $l^{-1}$ . All treatments depicted in (d) included 10  $\mu$ g  $\alpha$ -amanitin  $ml^{-1}$ . (e,f) Effect of removal of cumulus cells on inhibition of oocyte maturation by DRB or  $\alpha$ -amanitin (CUM: cumulus cells; all treatment groups included 0.5  $\mu$ g FSH  $ml^{-1}$ ). (g,h) Reversibility of DRB- and  $\alpha$ -amanitin-mediated arrest of GVBD (all treatments included 0.5  $\mu$ g FSH  $ml^{-1}$ ). Rev: reverse. <sup>a-d</sup> $P < 0.001$  and <sup>e-g</sup> $P < 0.001$  within each panel.



**Fig. 2.** Effect of activation of (a) type I or (b) type II protein kinase A (PKA) with various concentrations of cAMP analogues on the incidence of germinal vesicle breakdown (GVBD) in murine cumulus-oocyte complexes. Cultures were continued for 3 h in the presence of either type I activators (*N*<sup>6</sup>-monobutyl-*N*-butyryl-cAMP and 8-aminohexylamino-cAMP) or type II PKA activators (*N*<sup>6</sup>-monobutyl-*N*-butyryl-cAMP and 8-bromo-cAMP) in the absence of gonadotrophin supplementation. <sup>a-d</sup>*P* < 0.001 and <sup>xy</sup>*P* < 0.001 within each panel.

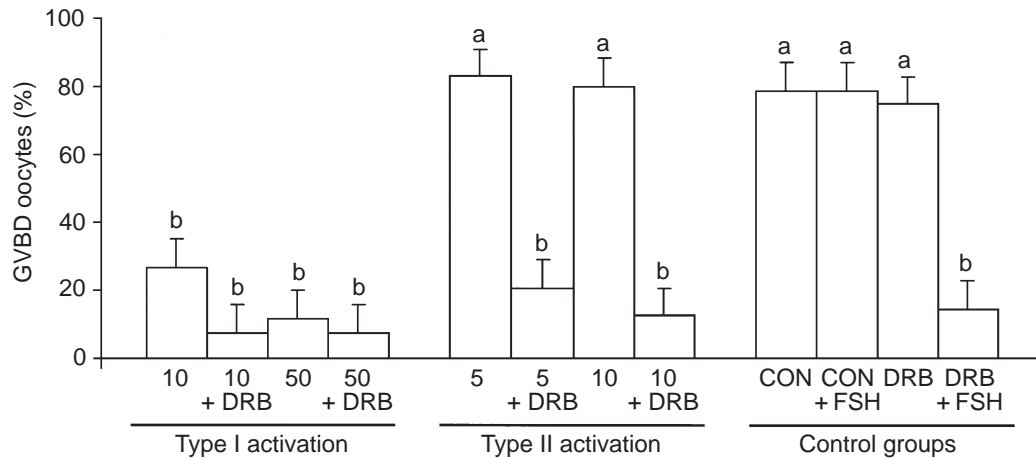
1 h of the initiation of culture (Osborn and Moor, 1983; Kastrop *et al.*, 1991; Meinecke and Meinecke-Tillmann, 1993). As the kinetics of maturation in murine oocytes are rapid (Schultz *et al.*, 1983a; Downs *et al.*, 1988), it is likely that this transcriptional event occurs within minutes of the initiation of culture. In cattle and sheep, gonadotrophin-mediated maturation required the presence of cumulus cells (Osborn and Moor, 1983; Hunter and Moor, 1987;

Fagbohun and Downs, 1991). In the present study, removal of cumulus cells rendered both DRB and  $\alpha$ -amanitin ineffective in blocking murine GVBD. These data are consistent with a model in which FSH induces GVBD by a transcriptional event that occurs in the cumulus cells.

It is unlikely that the lack of effectiveness of the transcriptional inhibitors in blocking GVBD in denuded COC resulted from decreased access of the inhibitor to the oocyte. Treatment of bovine COCs with DRB resulted in > 94% reduction in [<sup>3</sup>H]uridine incorporation into COC mRNA, regardless of the presence or absence of gonadotrophins in the culture medium (Farin and Yang, 1994). In addition, injection of  $\alpha$ -amanitin directly into denuded pig oocytes was ineffective in blocking GVBD (Meinecke and Meinecke-Tillmann, 1993).

Neither DRB nor  $\alpha$ -amanitin was effective at preventing spontaneous maturation or hCG-mediated maturation of COCs. In contrast, in the presence of FSH, both transcriptional inhibitors were effective at preventing meiotic resumption. These observations are consistent with those for bovine COCs (Wolf and Farin, 1996) and indicate that GVBD initiated in the presence of FSH involves a transcriptional step that is not required when GVBD occurs spontaneously or under hCG stimulation. It should be noted that COCs from these eCG-stimulated follicles may have few, if any, receptors for LH (Peng *et al.*, 1991; Eppig *et al.*, 1997, 1998). Therefore, these COCs may have been unresponsive to hCG treatment and, as a result, they may have undergone spontaneous GVBD even in the presence of hCG.

On the basis of the data from both the present study and previous studies, a general consensus can be reached regarding the mechanisms of mammalian oocyte maturation for *in vitro*-cultured COCs. Oocytes undergo either spontaneous or gonadotrophin-mediated maturation. Spontaneous maturation occurs as a result of a decrease in intra-oocyte cAMP content after removal of the oocyte or COC from an inhibitory intrafollicular environment. This would be consistent with the essentially linear kinetics observed for spontaneously maturing murine (Downs and Eppig, 1984) and bovine (Farin and Yang, 1994) COCs. In contrast, gonadotrophin-mediated maturation probably occurs through a mechanism involving FSH binding to cumulus cells, initiating an increase in intracellular cAMP and activation of type I and type II PKAs. Activation of type I PKA results in a temporary inhibition of oocyte maturation by an intracellular mechanism not involving transcription. Simultaneously, activation of type II PKA would initiate a new gene transcription event that subsequently leads to the resumption of meiosis. This hypothesized mechanism would be consistent with the observations in the present study and with the observations of Downs and Eppig (1984) and Farin and Yang (1994) that culture of COCs in the presence of gonadotrophins first inhibited and then accelerated the rate of GVBD in mice and cattle, respectively. On the basis of the data in the present study, it becomes apparent why oocytes did not revert to spontaneous maturation



**Fig. 3.** Role of transcription in germinal vesicle breakdown (GVBD) after activation of type I or type II protein kinase A (PKA). Mouse cumulus–oocyte complexes (COCs) were cultured for 3 h in the presence of type I or type II PKA activators at two different concentrations (10 or 50  $\mu\text{mol l}^{-1}$ ) in the presence or absence of 5,6-dichloro-1- $\beta$ -D-ribofuranosyl-benzimidazole (DRB). CON: COCs cultured in the presence or absence of FSH without DRB supplementation; DRB: COCs cultured in the presence of DRB and in the presence or absence of FSH. <sup>ab</sup>Comparisons are made across all treatment groups ( $P < 0.001$ ).

when arrested at the germinal vesicle stage by transcriptional inhibitors in the presence of FSH. In this case, it is likely that FSH-induced increases in cAMP continued to stimulate type I PKA, which then maintained oocytes in germinal vesicle arrest. However, the simultaneous activation of transcription that is mediated by stimulation of type II PKA and is required for GVBD was blocked by the actions of DRB or  $\alpha$ -amanitin.

On the basis of this model, it would follow that neither transcriptional nor translational inhibitors would be effective in the absence of an FSH stimulus in any species. This is consistent with reports for mice (Downs, 1990b), rats (Ekholm and Magnusson, 1979), pigs (Fulka *et al.*, 1986; Meinecke and Meinecke-Tillmann, 1993) and cows (Farin and Yang, 1994). Conversely, in the presence of an FSH stimulus it would be anticipated that both transcriptional and translational inhibitors would interrupt GVBD. This has been clearly established in cows and pigs (Fulka *et al.*, 1986; Hunter and Moor, 1987; Kastrop *et al.*, 1991; Meinecke and Meinecke-Tillmann, 1993; Farin and Yang, 1994). Furthermore, murine COCs that were meiotically arrested with hypoxanthine and then cultured in the presence of FSH were also inhibited from undergoing GVBD by treatment with cyclohexamide (Downs *et al.*, 1996) or  $\alpha$ -amanitin (Downs, 1995; Downs *et al.*, 1996). The data from the present study demonstrate that, in mice, transcriptional inhibition by either DRB or  $\alpha$ -amanitin occurred only in the presence of FSH, could be mimicked by activation of type II PKA and did not require previous hypoxanthine-induced meiotic arrest. Together, these observations strongly support the conclusion that transcriptional and translational regulation of GVBD in both murine and livestock COCs is remarkably similar, particularly when assessed under analogous culture conditions.

In conclusion, the mechanisms for FSH-mediated GVBD in cultured murine COCs are similar to those described for *in vitro*-matured COCs of other species, including cattle. Thus, in cultured murine COCs, FSH binding to cumulus cells results in the activation of type II PKA, which, in turn, mediates a transcriptional event required for the initiation of GVBD.

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