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in Meiotic Induction in Mouse Oocytes

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Cyclic adenosine monophosphate (cAMP) has been implicated as an important regulator of meiotic maturation in mammalian oocytes. A decrease in cAMP, brought about by the action of cAMP phosphodiesterase (PDE), is thought to initiate germinal vesicle breakdown (GVB) by the inactivation of cAMP-dependent protein kinase. However, the product of PDE activity, 5′-AMP, is a potent activator of an important regulatory enzyme, AMP-activated protein kinase (AMPK). The aim of this study was to evaluate a possible role for AMPK in meiotic induction, using oocytes obtained from eCG-primed, immature mice. Alpha-1 and -2 isoforms of the catalytic subunit of AMPK were detected in both oocytes and cumulus cells. When 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICA riboside), an activator of AMPK, was tested on denuded oocytes (DO) and cumulus cell-enclosed oocytes (CEO) maintained in meiotic arrest by dbcAMP or hypoxanthine, GVB was dose-dependently induced. Meiotic induction by AICA riboside in dbcAMP-supplemented medium was initiated within 3 h in DO and 4 h in CEO and was accompanied by increased AMPK activity in the oocyte. AICA riboside also triggered GVB when meiotic arrest was maintained with hypoxanthine, 8-AHA-cAMP, guanosine, or milrinone, but was ineffective in olomoucine- or roscovitine-arrested oocytes, indicating that it acts upstream of maturation-promoting factor. Adenosine monophosphate dose-dependently stimulated GVB in DO when meiotic arrest was maintained with dbcAMP or hypoxanthine. This effect was not mimicked by other monophosphate or adenosine nucleotides and was not affected by inhibitors of ectophosphatases. Combined treatment with adenosine and deoxycoformycin, an adenosine deaminase inhibitor, stimulated GVB in dbcAMP-arrested CEO, suggesting AMPK activation due to AMP accumulation. It is concluded that phosphodiesterase-generated AMP may serve as a transducer of the meiotic induction process through activation of AMPK.

Key Words: **AICA riboside; AMP-activated protein kinase; meiotic maturation.**

INTRODUCTION

Mammalian oocyte maturation within healthy, non-atretic ovarian follicles is triggered by gonadotropin at the time of the preovulatory hormonal surge. However, when meiotically competent germinal vesicle-stage oocytes are removed from mature follicles and cultured *in vitro* in a suitable medium, they spontaneously resume meiotic maturation without hormone mediation. This observation, originally reported by Pincus and Enzmann (1935) and since confirmed by numerous laboratories (see Tsafriri and Dekel, 1994; Downs, 1995a for reviews), suggested that the follicle provides an inhibitory influence that holds the competent oocyte in meiotic arrest until the proper meiosis-inducing signal is delivered. One compound thought to play a critical role in this regard is cyclic adenosine monophosphate (cAMP). Evidence indicates that cAMP levels within the oocyte dictate meiotic status such that high levels maintain meiotic arrest while lower levels are permissive for reinitiation of meiotic maturation as manifested by germinal vesicle breakdown (GVB).

The action of cAMP in oocytes is mediated by cAMP-dependent protein kinase (PKA). cAMP binds to the regulatory subunits of PKA, thereby releasing the active catalytic subunits to phosphorylate serine or threonine residues on protein substrates. It has been shown by the use of site-selective cAMP analogs that different PKA isoforms are involved in opposing functions of meiotic regulation in the germ and somatic cell compartments of the follicle. Type I

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PKA mediates the inhibitory action of cAMP in the oocyte, while type II mediates the meiosis-inducing pulse of cAMP that occurs within the cumulus granulosa cells following hormonal stimulation (Downs and Hunzicker-Dunn, 1995). Moreover, oocytes maintained in meiotic arrest by elevated cAMP can be induced to resume maturation by microinjection of PKA inhibitors (Bornslaeger *et al.,* 1986; Eppig, 1989).

An important enzyme controlling PKA activity is cAMP phosphodiesterase (PDE). This enzyme cleaves the phosphodiester bond of cAMP to form 5′-AMP that can no longer bind to the regulatory subunit of PKA to activate it. PDE activity is present within oocytes (Bornslaeger *et al.,* 1984; Downs *et al.,* 1989), and its action is responsible for lowering cAMP levels and permitting spontaneous maturation, presumably by the loss of PKA activity. This is readily demonstrated by the use of established PDE inhibitors that maintain elevated cAMP levels and coincidentally block spontaneous maturation (Cho *et al.,* 1974; Magnusson and Hillensjo, 1977; Dekel *et al.,* 1981; Vivarelli *et al.,* 1983). Hormone-induced maturation *in situ* also apparently requires a drop in oocyte cAMP. Using inhibitors of the oocyte-specific isoform of PDE, Wiersma *et al.* (1998) reported the ovulation of immature, germinal vesicle-stage oocytes in a superovulated rat model. This exciting result not only showed that ovulation and meiotic maturation are separable phenomena but also that PDE is required in the events preceding meiotic resumption.

The model that has emerged for meiotic regulation in mammalian ovarian follicles has cAMP, PDE, and PKA as pivotal components. According to this scheme, cAMP, generated either locally or transferred to the oocyte from the somatic compartment via gap junctions, binds to and activates PKA, thereby maintaining meiotic arrest. A PDE-mediated drop in cAMP inactivates PKA and eliminates the meiosis-arresting influence, allowing meiotic resumption. Thus, the activity of PKA within the oocyte is directly linked to meiotic arrest, with maturation occurring by a "disinhibition" mechanism. However, the transduction system by which the somatic compartment signals the oocyte to trigger meiotic resumption following the preovulatory gonadotropin surge is not well understood.

Although considerable evidence supports cAMP-dependent meiotic arrest, conditions exist whereby mammalian oocyte maturation can apparently occur without a preceding drop in oocyte cAMP. For example, cumulus cell-enclosed oocytes maintained in meiotic arrest with cAMP analogs or phosphodiesterase inhibitors can be stimulated to resume meiotic maturation with gonadotropin, despite the continued presence of the arresting agent (Dekel and Beers, 1978; Downs *et al.,* 1988). Thus, it is unlikely that, prior to GVB, oocyte cAMP falls below the basal level present at the time of isolation; rather, a positive meiotic trigger appears to bypass meiosis-arresting levels of cAMP. Indeed, studies from several vertebrate species, including sheep (Moor and Heslop, 1981), hamster (Racowsky, 1985a; Hubbard, 1986), rabbit (Yoshimura *et al.,* 1992a,b), pig (Racowsky, 1985b; Mattioli *et al.,* 1994), mouse (Salustri *et al.,* 1985; Hashimoto *et al.,* 1985), and frog (Gelerstein *et al.,* 1989), have reported that meiotic resumption can occur without a measurable decline in oocyte cAMP below basal levels. In fact, meiotic maturation in some invertebrate species actually requires an increase in oocyte cAMP (Yamashita, 1988; Freeman and Ridgway, 1988; Stricker and Smythe, 2001). Hence, these studies suggest that alternative, or additional, mechanisms exist other than reduced cAMP levels to bring about meiotic resumption.

One possible mechanism incorporates a more proactive involvement of PDE. As mentioned above, PDE converts cAMP to 5′-AMP, a compound that has been largely ignored in oocyte physiology, presumed to be an inactive byproduct of the enzyme in oocytes. However, it is important to note that AMP is a potent regulator of a serine/threonine kinase termed AMP-activated protein kinase, or AMPK (for reviews, see Hardie and MacKintosh, 1992; Hardie and Carling, 1997; Winder and Hardie, 1999). AMPK is a member of the AMPK/SNF1 protein kinase family, whose members can be found in a diverse array of organisms (e.g., mammals, plants, and yeast) and are typically activated in response to nutritional and environmental stress. AMPK controls the activity of important rate-limiting enzymes of carbohydrate and fat metabolism (Hardie, 1992; Hardie and Carling, 1997; Hardie *et al.,* 1998; Merrill *et al.,* 1997, 1998), acting as a type of "fuel gauge" in response to changes in cellular energy charge (Hardie and Carling, 1997). Thus, as a result of stresses such as heat shock or arsenite (Corton *et al.,* 1994), high fructose (Moore *et al.,* 1991), ischemia (Kudo *et al.,* 1995) or exercise (Winder and Hardie, 1996), which compromise cellular energy levels, AMPK becomes activated, and one function is to shut down fatty acid and cholesterol synthesis. In general, activation of AMPK switches off anabolic pathways of lipid and carbohydrate metabolism and switches on the corresponding catabolic pathways, in order both to conserve ATP and generate more. It achieves this both via direct phosphorylation of metabolic enzymes and via indirect effects on gene expression (Winder and Hardie, 1999; Hardie and Hawley, 2001).

AMPK is a heterotrimeric protein with a catalytic α subunit and β and γ regulatory subunits, and its activity is sensitively controlled within cells by the AMP/ATP ratio (Hardie *et al.,* 1999). Since the level of ATP in cells is in the millimolar range, while that of AMP is much lower, small increases in AMP or larger decreases in ATP significantly increase the AMP/ATP ratio and bring about activation of the enzyme (Hardie and MacKintosh, 1992). AMP activates AMPK both allosterically and by promoting its phosphorylation by an upstream kinase. An intriguing possibility is that PDE activity within oocytes generates enough AMP from cAMP degradation to activate AMPK that, in turn, provides a positive stimulus for oocyte maturation. Indeed, using differential display analysis of cDNA libraries prepared from mouse eggs, Heyer *et al.* (1997, 1999) have identified a novel kinase, *Melk,* that is a member of the

AMPK/SNF1 family and have proposed an important role for the kinase in signal transduction events during development.

We have tested the hypothesis that stimulation of AMPK within the oocyte participates in the mechanism controlling meiotic resumption. Using Western analysis, enzyme assays, and activators of the enzyme, we demonstrate the presence of AMPK in mouse oocytes and show that its activity is associated with meiotic induction.

MATERIALS AND METHODS

Oocyte Isolation and Culture Conditions

Immature C57BL/6J × SJL/J F_1 mice, 20–23 days old, were used for all experiments. Mice were primed with 5 IU equine chorionic gonadotropin and killed 48 h later by cervical dislocation. Ovaries were removed and placed in culture medium, and large antral follicles were punctured with sterile needles. Cumulus cell-enclosed oocytes (CEO) were collected, washed through two to three additional changes of medium, and transferred in a small volume to plastic culture tubes (Falcon 2058) or stoppered borosilicate glass tubes containing 1 ml of the appropriate culture medium. Denuded oocytes (DO) were prepared by repeated pipetting with a Pasteur pipet. Tubes were gassed with a humidified mixture of 5% CO_2, 5% O_2, and 90% N_2 and placed in a water bath at 37°C for the duration of culture. The medium used in this study was Eagle's minimum essential medium supplemented with 0.23 mM pyruvate, penicillin, streptomycin sulphate, and 3 mg/ml crystallized lyophilized bovine serum albumin (ICN ImmunoBiologicals, Lisle, IL)

AMP Uptake

DO were cultured in medium containing 10 μCi [2-^{3}H] adenosine monophosphate (21.0 Ci/mmol; Amersham Life Sciences, Inc., Arlington Heights, IL). At the end of culture, oocytes from each treatment group were washed through three changes of PBS/BSA to remove external radiolabel and 25 were transferred to a scintillation vial. Radioactivity was determined after addition of 5 ml scintillation fluid. An equal volume of buffer from the last wash dish for each group served as a blank.

AMPK Assay

DO were cultured 4 h in medium containing dbcAMP alone or dbcAMP plus 250 or 500 μm AICA riboside. Oocytes were then washed several times in immunoprecipitation buffer [50 mM Tris/HCl, pH 7.4 at 4°C, 150 mM NaCl, 5 mM Na pyrophosphate, 50 mM NaF, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol (DTT)] containing 3 mg/ml polyvinylpyrrollidone and stored frozen in this buffer at −80°C until assayed. AMPK activity was measured by using a method adapted from Hardie *et al.* (2000). Briefly, frozen oocytes were diluted twofold into immunoprecipitation buffer containing 2% (v/v) Triton X-100, 2 mM benzamidine, 2 mM PMSF, 10 μg/ml soybean trypsin inhibitor and vortexed until completely thawed. AMPK was immunoprecipitated by using protein G-Sepharose coupled to a mixture of affinity-purified sheep antibodies raised against the α1 and α2 isoforms of the catalytic subunits of AMPK (Woods *et al.*, 1996). The final pellet was diluted threefold into Hepes–Brij buffer (50 mM Na Hepes, pH 7.4, 1 mM DTT, 0.02% Brij-35) prior to assay in reaction mixture [0.2 mM [γ-^{32}P]ATP (specific activity 250–500 cpm/pmol), 5 mM $MgCl_2$, 0.2 mM AMP in Hepes–Brij buffer, 0.2 mM AMARA peptide (AMARAASAAASARRR) in Hepes–Brij buffer] at 30°C for 30 min. Aliquots corresponding to 3/5 of the reaction mixture were then spotted onto P81 paper (Whatman) and the reactions stopped with 1% (v/v) phosphoric acid. Radioactivity was measured by liquid scintillation counting. Blank values were determined by omitting the peptide substrate from the assay. AMPK activity is expressed in units of nmole phosphate incorporated into substrate peptide per minute.

Western Analysis of α Subunits of AMPK

DO and oocyte-cumulus cell complexes (OCC) were obtained from primed mice, washed in immunoprecipitation buffer (see above), and stored frozen in this buffer at −80°C until used for Western blotting. Prior to gel electrophoresis, the oocytes were homogenized by using a motorized pellet pestle (Sigma) and concentrated in 1.5 ml vivaspins (Vivascience Ltd.; 30,000 mw cut-off). The proteins were electrophoresed on 10% (w/v) polyacrylamide/SDS gels and transferred to nitrocellulose membranes. Blots were probed first with an affinity purified sheep antibody raised against either the α1 or α2 isoform of the catalytic subunit of AMPK (0.3 μg/ml) and then with protein G coupled to horseradish peroxidase (0.2 μg/ml; Sigma). Protein G binding was detected by using enhanced chemiluminescence reagents (ECL; Amersham Pharmacia).

Chemicals

All culture medium components, AICA riboside, dbcAMP, hypoxanthine, 8-aminohexylamino-cAMP, 8-bromo-cAMP, guanosine, milrinone, 5′-aminodeoxyadenosine, forskolin, olomoucine, roscovitine, α,β-methylene ADP, β-glycerophosphate, and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO). Deoxycoformycin was the kind gift of SuperGen, Inc. (San Ramon, CA).

Statistical Analysis

Oocyte maturation experiments were repeated at least 3 times with at least 30 oocytes per group per experiment. In all cases, viability was maintained at greater than 93%, which is considered normal (Downs and Mastropolo, 1997). Oocyte maturation frequencies were subjected to arcsin transformation, and groups of three or more were analyzed statistically by ANOVA followed by Duncan's multiple range test, while paired comparisons were made with Student's *t* test. Nontransformed data from AMPK assays were analyzed by Student's *t* test. For all statistical analyses, a *P* value less than 0.05 was considered significant.

RESULTS

Western Analysis of Oocytes and OCC

Western analysis was carried out by using affinity purified antibodies to identify the AMPK catalytic subunit isoforms in extracts of oocytes and OCC. The equivalent of 200 OCC and 1000 oocytes was loaded in each lane. As shown in Fig. 1, oocytes contain both α-1 and α-2 subunit

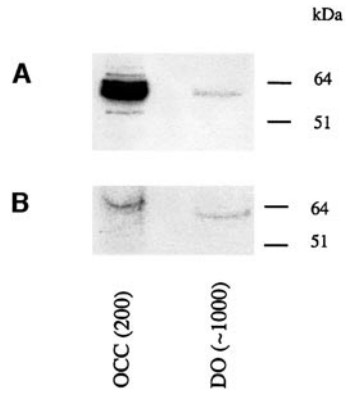

FIG. 1. Western blot analysis of AMPK α subunit isoforms in extracts of denuded oocytes (DO) and oocyte-cumulus cell complexes (OCC). The number of DO and OCC used to load each lane is shown in parentheses. The blots were probed with either an antibody raised against the α1 isoform (a) or with an antibody raised against the α2 isoform (b). The mobilities of molecular weight markers are indicated on the right. The apparent higher molecular weight of the AMPK α2 subunit in the OCC is thought to be due to the high protein concentration in these samples. This does not appear to be a problem for the α1 subunit since this protein represents a much higher proportion of the total protein in these samples.

isoforms, and since the labeled bands are more intense in the lanes loaded with complex extracts, it can be concluded that cumulus cells contain both isoforms as well. It is also apparent that the ratio of the amount of subunit in cumulus cells to that in the oocyte is much higher for the α-1 isoform than for the α-2 isoform. The α-1 subunit from the OCC appeared to migrate as multiple bands, possibly a doublet. This is frequently observed for AMPK α subunits and appears to be due to autophosphorylation at multiple sites (Hawley *et al.*, 1996) and/or multiple phosphorylation by the upstream kinase (Stein *et al.*, 2000).

AICA Riboside and Oocyte Maturation

5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICA riboside) is an adenosine analog that enters cells and is phosphorylated by adenosine kinase to form the AMP analog, AICA ribotide (ZMP), an intermediate in the purine *de novo* pathway (see Fig. 2), but also a potent stimulator of AMPK. AICA riboside is the principle pharmacological agent used to increase AMPK activity in a wide variety of cells and provides a means of activating AMPK without affecting AMP or ATP levels (Sullivan *et al.*, 1994; Corton *et al.*, 1995; Henin *et al.*, 1995). It has been utilized herein to test for a meiosis-inducing action in arrested oocytes.

DO and CEO were cultured 17–18 h in medium containing 300 μM dbcAMP or 4 mM hypoxanthine plus increasing concentrations of AICA riboside before meiotic assessment. In the presence of either inhibitor, AICA riboside increased the frequency of meiotic maturation in a dose-dependent fashion, regardless of the presence or absence of cumulus cells, although it was more potent in DO. In control cultures, DO resumed maturation at a frequency of 5–12%, and the addition of 250 μM AICA riboside increased this to 91–94% (Fig. 3A). In CEO, lower concentrations (50–250 μM) were either slightly inhibitory or had no effect, but higher concentrations proved to be stimulatory, with complete meiotic induction at 1000 μM (Fig. 3B).

To test the time course of meiotic induction, DO and CEO were cultured for varying periods in medium containing 300 μM dbcAMP $\pm$ 250 (DO) or 1000 μM (CEO) AICA riboside. Although AICA riboside had no effect in either DO or CEO after 2 h, significant stimulation of GVB was observed in DO after only 3 h, with a 56.2% increase in GVB after 4 h (Fig. 4). Meiotic induction was slower in CEO, with a comparable increase in GVB requiring more than 6 h of incubation.

To determine whether these rapid kinetics of meiotic induction would occur under other inhibitory conditions, DO were cultured 4 h in medium containing dbcAMP, 8-AHA-cAMP, guanosine, hypoxanthine, or the oocyte-specific cAMP phosphodiesterase inhibitor, milrinone (Tsafriri *et al.*, 1996), in the presence or absence of 250 μM AICA riboside. These data are shown in Fig. 5. Under all five inhibitory conditions, AICA riboside induced meiotic maturation, with increases in GVB of 23–71%.

Since activation of AMP-activated kinase by AICA riboside first requires phosphorylation of the adenosine analog by adenosine kinase (Sabina *et al.*, 1991), it was important to test whether AICA riboside stimulation in mouse oo-

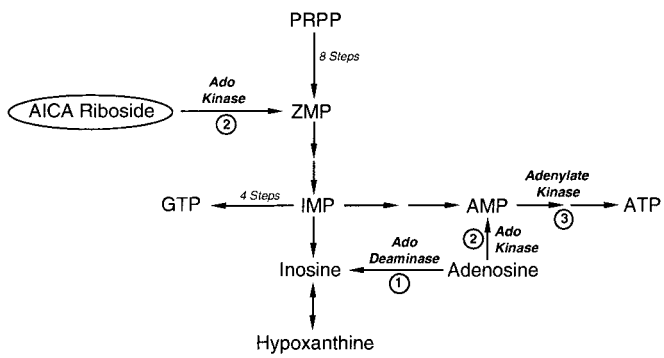

FIG. 2. Relationship between AICA riboside and adenosine with the purine metabolic pathways. ZMP is an intermediate that forms eight steps into the *de novo* purine synthetic pathway. AICA riboside is metabolized by adenosine kinase (2) to ZMP that can then feed directly into *de novo* purine synthesis. ZMP is also an AMP analog and potent stimulator of AMPK. Adenosine is readily phosphorylated to AMP by adenosine kinase (2) and further metabolized by adenylate kinase (3) to other adenyl nucleotides. Alternatively, adenosine is deaminated to inosine by adenosine deaminase (1). Adenylate kinase is usually quite active and limits the amount of AMP that can accumulate in cells.

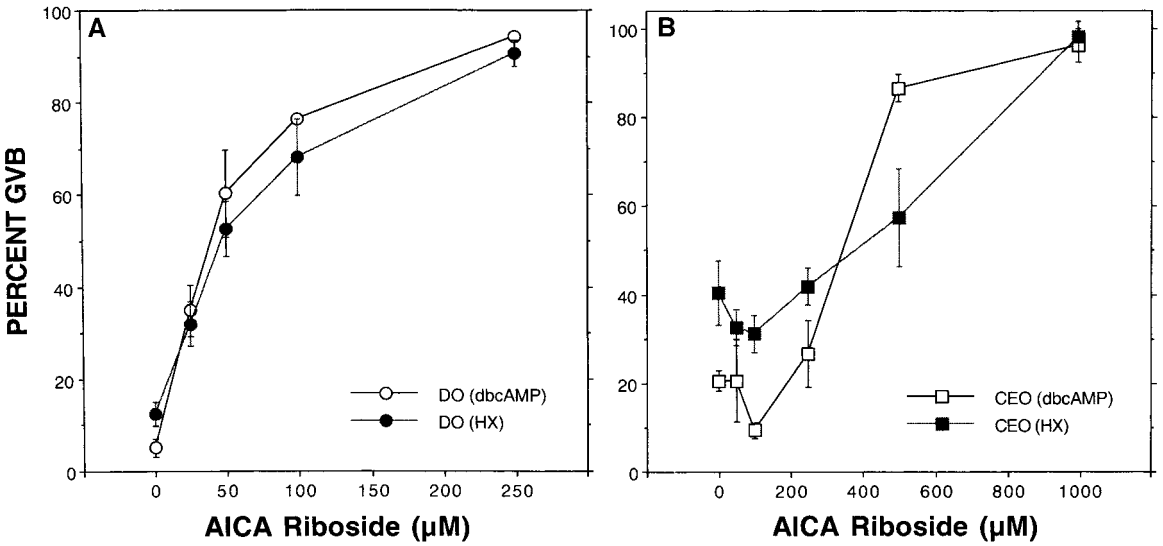

FIG. 3. Dose response effect of AICA riboside on GVB in dbcAMP- and hypoxanthine-arrested oocytes. DO (A) and CEO (B) were cultured 17–18 h in medium containing 300 μm dbcAMP or 4 mM hypoxanthine (HX) plus increasing concentrations of AICA riboside. Oocytes were then assessed for GVB. Each data point represents the mean ± SEM of three determinations.

cytes is also dependent on its phosphorylation by this enzyme. To this end, we used the adenosine kinase inhibitor, 5′-amino-deoxyadenosine. DO were cultured 4 h in medium containing 300 μM dbcAMP ± 250 μM AICA riboside, and 5′-amino-deoxyadenosine was added at 10 μM, a concentration previously shown to block adenosine phosphorylation in mouse OCC (Downs, 1999). AICA riboside again induced a significant number of oocytes to

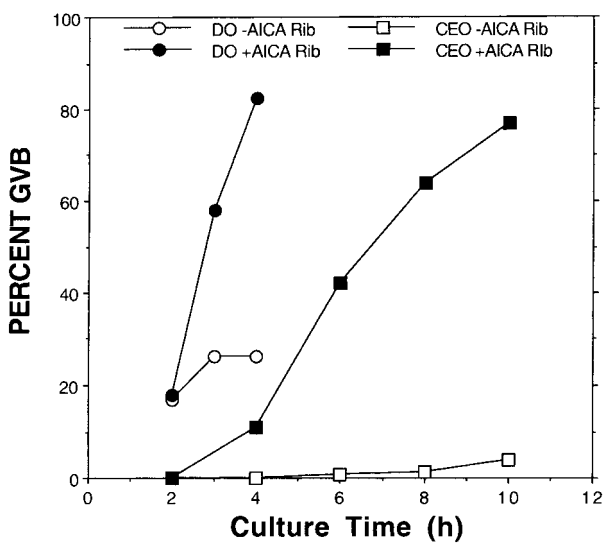

FIG. 4. Kinetics of AICA riboside-induced oocyte maturation. DO or CEO were cultured for varying periods of time in medium containing 300 μm dbcAMP plus 250 (DO) or 1000 μM (CEO) AICA riboside. Oocytes were then assessed for GVB. Each data point represents the mean of three determinations.

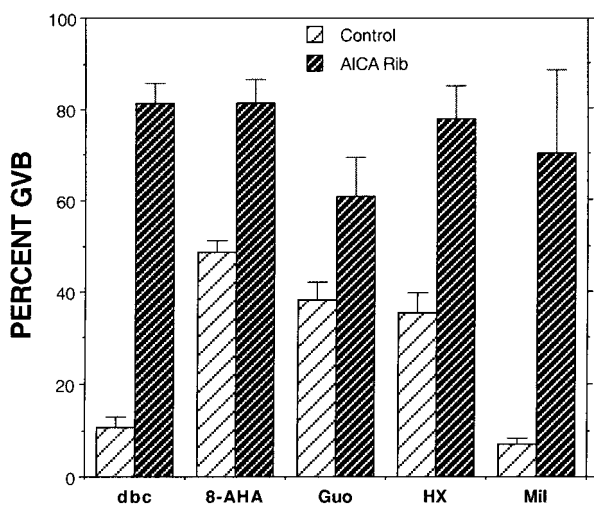

FIG. 5. Effect of AICA riboside on the maturation of DO in different meiotic inhibitors. DO were cultured 4 h in medium containing one of five different inhibitors: 300 μM dbcAMP (dbc), 300 μM 8-aminohexylamino-cAMP (8-AHA), 1 mM guanosine (Guo), 4 mM hypoxanthine (HX), or 2 μM milrinone ± 250 μm AICA riboside. Oocytes were then assessed for GVB. Each data point represents the mean ± SEM of three determinations. In all treatment groups, AICA riboside stimulated a significant portion of the oocytes to resume maturation, by Student's *t* test.

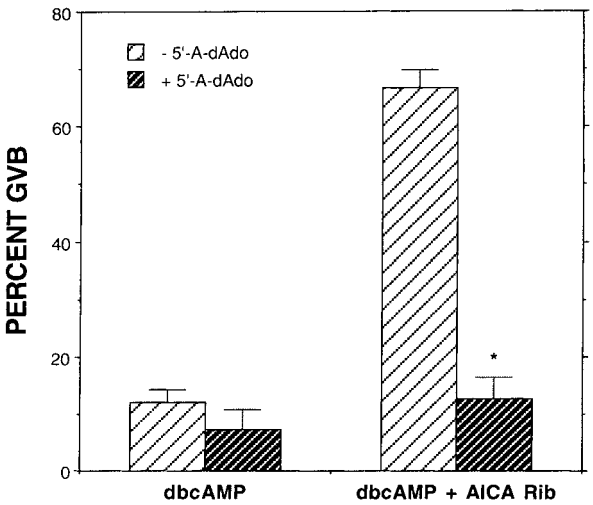

FIG. 6. Effect of inhibition of adenosine kinase on AICA riboside-induced maturation. DO were cultured 4 h in medium containing 300 μM dbcAMP ± 10 μM 5′-amino-deoxyadenosine (5′-A-dAdo), with AICA riboside added at a concentration of 250 μM. Oocytes were then assessed for GVB. Each data point represents the mean ± SEM of three determinations.

resume maturation (from 12 to 67% GVB; Fig. 6). While 5′-amino-deoxyadenosine had no effect in the absence of AICA riboside, the analog completely blocked AICA riboside-induced maturation, consistent with the idea that phosphorylation by adenosine kinase is required for its meiosis-inducing activity.

Meiotic resumption in mammalian oocytes is normally preceded by an increase in the activity of maturation-promoting factor (MPF), comprised of regulatory cyclin and catalytic $p34^{cdc2}$ kinase subunits, which drives germ cells through the G_2/M transition (Nurse, 1990). To determine whether AICA riboside acts upstream or downstream of MPF, its action was tested on meiotic inhibition imposed on DO by two established inhibitors of the cyclin-dependent kinase, olomoucine (Vesely *et al.*, 1994; Abraham *et al.*, 1995) and roscovitine (Meijer *et al.*, 1997). To this end, DO were cultured 4 h in medium containing 400 μM olomoucine or 50 μM roscovitine in the presence or absence of 250 μM AICA riboside, while dbcAMP-arrested DO served as a positive control. Both olomoucine and roscovitine suppressed oocyte maturation when compared to control oocytes, but AICA riboside was unable to reverse the arrest by either inhibitor (Fig. 7), although this agent stimulated GVB in dbcAMP-arrested DO. These results suggest that AICA riboside (and presumably AMPK) acts upstream in the cascade leading to MPF activation.

To establish that AICA riboside is able to stimulate AMPK activity in mouse oocytes, DO were cultured 4 h in medium containing 300 μM dbcAMP ± AICA riboside and then assayed for activity as described in Materials and Methods, using a peptide substrate specific for the enzyme. In the absence of AICA riboside, AMPK activity in dbcAMP-treated oocytes was detected at a level of 0.043–0.071 mU/100 oocytes (Fig. 8). While there was a trend toward increased activity in response to 250 μM AICA riboside, this difference was not significant; however, significant stimulation was observed with 500 μM AICA riboside (a 2.4-fold increase). These results demonstrate the presence of AMPK activity in mouse oocytes and show it to be a target for AICA riboside.

AMP and Oocyte Maturation

Since AICA riboside is thought to act on AMPK by its conversion to an AMP analog, it was important to test the action of native AMP on oocyte maturation. DO were cultured 17–18 h in medium containing 300 μM dbcAMP or 4 mM hypoxanthine, plus increasing concentrations of AMP from 0.1 to 2 mM. As shown in Fig. 9, AMP dose-dependently stimulated the resumption of maturation in oocytes arrested by either inhibitor, with increases in GVB of 21.4–24.6% at 2 mM.

To test the nucleotide specificity of this effect, DO were maintained in meiotic arrest for 17–18 h by 300 μM dbcAMP and exposed to a series of nucleotides at 2 mM. AMP again produced a significant stimulation of GVB (from 23.7 to 49.1%), but none of the other nucleotides was able to mimic this effect (Fig. 10).

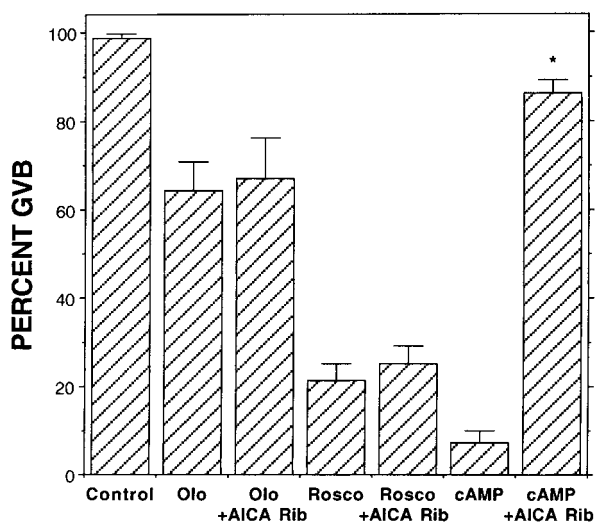

FIG. 7. Effect of AICA riboside in the presence of MPF inhibitors. DO were cultured 4 h in control medium or medium containing 300 μM dbcAMP (cAMP), 400 μM olomoucine (Olo), or 50 μM roscovitine (Rosco), with AICA riboside added at a concentration of 250 μM. Oocytes were then assessed for GVB. Each data point represents the mean ± SEM of three determinations. An asterisk denotes a significant difference from the corresponding group minus AICA riboside.

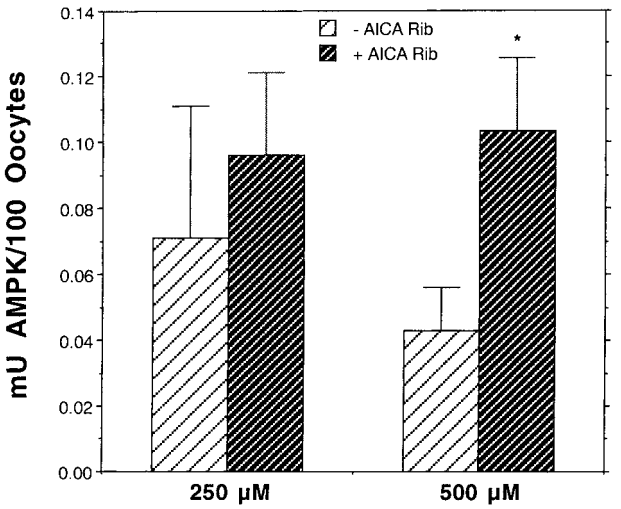

FIG. 8. Effect of AICA riboside on AMPK activity in DO. DO were cultured 4 h in medium containing 300 μm dbcAMP plus either 250 or 500 μm AICA riboside. Oocyte extracts were then analyzed for AMPK activity. Each data point represents the mean ± SEM of four determinations. An asterisk denotes a significant difference by Student's *t* test.

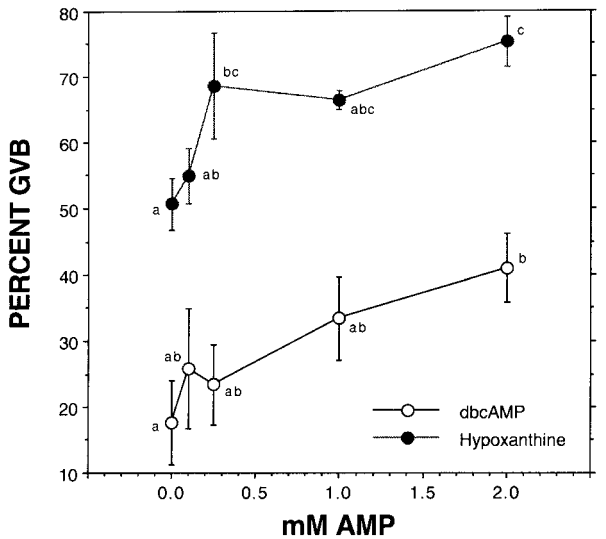

FIG. 9. Effect of AMP on dbcAMP- and hypoxanthine-maintained meiotic arrest in DO. DO were cultured 17–18 h in medium containing 300 μm dbcAMP or 4 mM hypoxanthine (HX) plus increasing concentrations of AMP. Oocytes were then assessed for GVB. Each data point represents the mean ± SEM of three (HX-treated) or five (dbcAMP-treated) determinations. Groups from each inhibitor treatment were analyzed separately by ANOVA followed by Duncan's multiple range test. Groups with no common letters are significantly different.

Monophosphate nucleotides might be susceptible to ecto-5′-nucleotidase or other phosphatases that could cleave the phosphate from extracellular AMP and generate adenosine. This is an important consideration, since adenosine is readily taken up by cumulus cell-free oocytes (Downs *et al.*, 1986; Downs, 1999) and could conceivably mediate the action of AMP on DO. Therefore, the effect of AMP on oocyte maturation was tested in the presence of α,β−methylene ADP (MeADP), an inhibitor of 5′-nucleotidase, and β-glycerophosphate, an inhibitor of nonspecific phosphatases. DO were cultured 17–18 h in medium containing 300 μM dbcAMP ± 2 mM AMP, and MeADP and β-glycerophosphate were added at 100 μM and 10 mM, respectively. The concentration chosen for each inhibitor was dictated by a literature search that determined them to be maximally effective in other systems. AMP produced a modest stimulation of maturation that was not significant. However, since the frequency of maturation in the presence of AMP plus inhibitors was significantly greater than that in their absence, it can be concluded that the inhibitors failed to prevent AMP stimulation of GVB (Fig. 11). The inhibitors also had no influence on maturation in the absence of AMP.

The direct action of AMP on the oocyte suggests it is taken up by germ cells and acts internally. To address whether AMP is taken up by oocytes, DO were cultured for 3 h in the presence of ^{3}H-AMP and were collected at hourly intervals to assess accumulation. A parallel group of DO was cultured in the presence of MeADP and β-glycerophosphate. In a time-dependent fashion, oocytes accumulated a significant amount of radiolabel, but this was suppressed by 93% after the addition of phosphatase inhibitors (Fig. 12). These results suggest that a major portion of the uptake was indirect, resulting from metabolic conversion of AMP to adenosine, although compe-

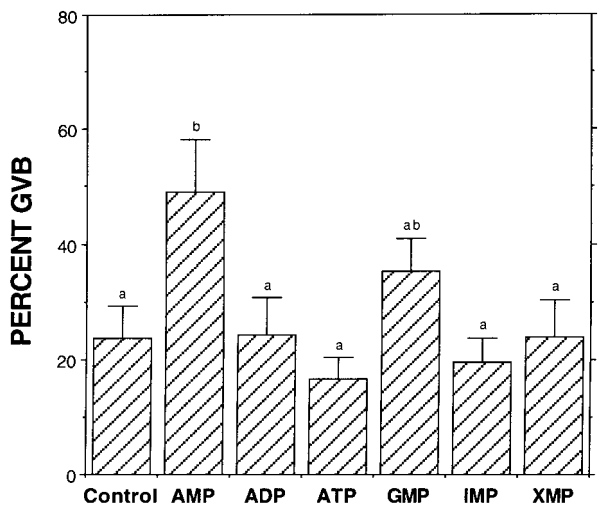

FIG. 10. Specificity of AMP-induced meiotic maturation in DO. DO were cultured 17–18 h in medium containing 300 μM dbcAMP plus one of six different nucleotides at a concentration of 2 mM. Oocytes were then assessed for GVB. Each data point represents the mean ± SEM of three determinations. Groups with no common letters are significantly different.

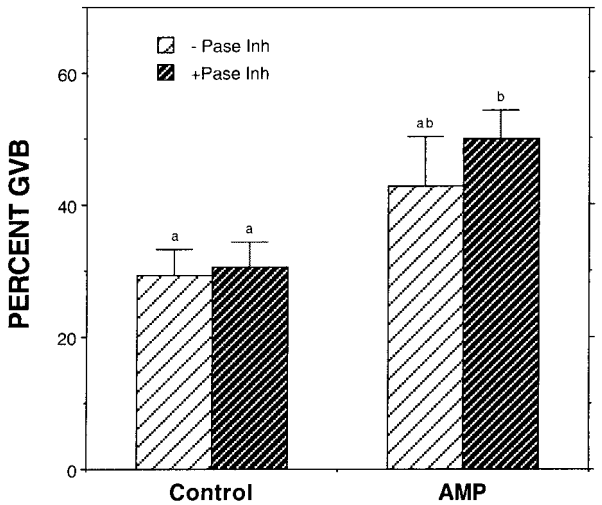

FIG. 11. Effect of phosphatase inhibitors on AMP-induced meiotic maturation. DO were cultured 17–18 h in control medium or medium containing 10 mM β-glycerophosphate and 100 μM α,β-methylene ADP, ± 2 mM AMP. Oocytes were then assessed for GVB. Each data point represents the mean ± SEM of six determinations. Groups with no common letters are significantly different.

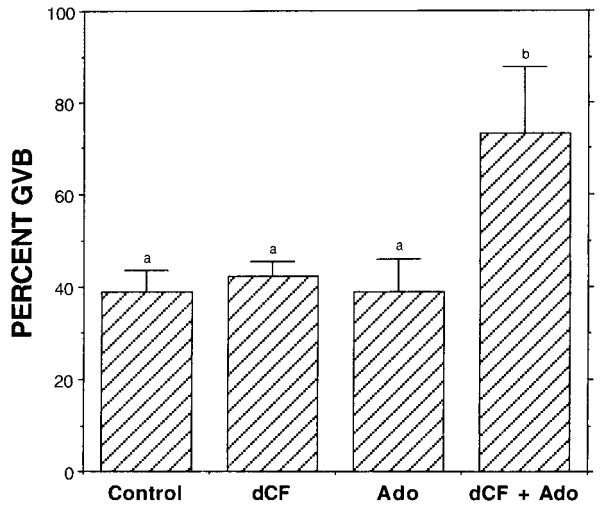

FIG. 13. Effect of adenosine and deoxycoformycin on dbcAMP-maintained meiotic arrest. CEO were cultured 17–18 h in medium containing 150 μm dbcAMP alone, or dbcAMP plus 250 μm adenosine (Ado), 250 μm deoxycoformycin (dCF), or the two agents together. Oocytes were then assessed for GVB. Each data point represents the mean ± SEM of four determinations. Groups with no common letters are significantly different .

tition for the purine transporter cannot be discounted. Nevertheless, uptake was still detected in the presence of inhibitor, consistent with the idea that the oocyte has the capacity to directly take up AMP.

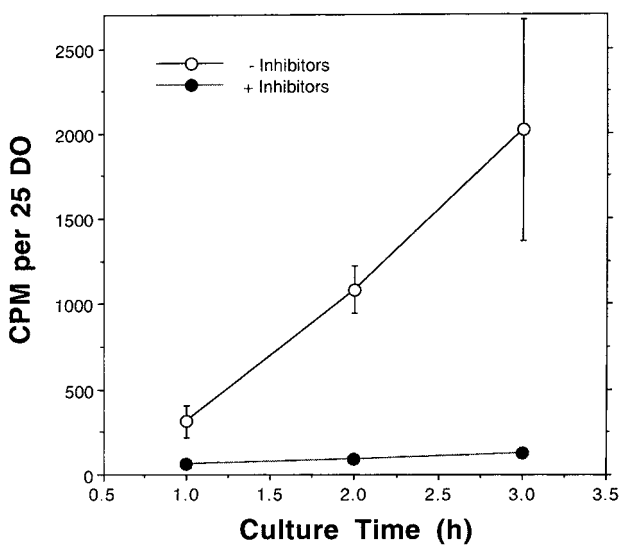

FIG. 12. Effect of phosphatase inhibitors on AMP uptake by DO. DO were cultured for 1, 2, or 3 h in control medium or medium containing 10 mM β-glycerophosphate and 100 μM α,β-methylene ADP, plus 10 μCi ^{3}H-AMP. Uptake of AMP by oocytes was then determined by scintillation spectroscopy. Each data point represents the mean ± SEM of three determinations.

Effect of the Combined Treatment of Adenosine and Deoxycoformycin on Oocyte Maturation

Adenosine has consistently been shown in previous studies to exert a meiosis-arresting action on oocytes (Eppig *et al.*, 1985; Miller and Behrman, 1986; Tornell *et al.*, 1990; Salustri *et al.*, 1988; Shim *et al.*, 1992; Downs, 1999). Nevertheless, it was hypothesized that blocking deamination of adenosine might cause an accumulation of AMP and lead to AMPK activation, thereby stimulating meiotic resumption (see Fig. 2). An experiment was therefore carried out to determine whether the combined treatment of adenosine and the adenosine deaminase inhibitor, deoxycoformycin (dCF), provided a stimulus for meiotic induction in dbcAMP-arrested oocytes. CEO were cultured 17–18 h in medium containing 150 μM dbcAMP, plus 250 μM adenosine, 250 μM dCF, or the two compounds together. As shown in Fig. 13, neither adenosine nor dCF alone had a significant effect on oocyte maturation. However, consistent with the hypothesis, when these compounds were added together, a significant increase in oocyte maturation from 39 to 73.2% GVB was observed. The same experiment was attempted with DO, but adenosine at similar concentrations proved toxic to the oocyte during overnight culture.

DISCUSSION

In this study, we report the presence of AMPK catalytic subunits in mouse oocytes and OCC. In addition, through

pharmacological activation of AMPK, a direct relationship between enzyme activity and meiotic induction in arrested oocytes has been demonstrated. Further evidence suggests that an increase in oocyte AMP stimulates AMPK activity. It is proposed that AMPK participates in meiotic induction by its involvement in a metabolic cascade downstream of PDE but upstream of MPF activation.

AMPK Is Present in OCC

Initial experiments demonstrated the presence of both α1 and α2 isoforms of AMPK in both the oocyte and cumulus cells. While the tissue distribution of α1 appears to be ubiquitous, α2 has been reported to be expressed principally in liver and skeletal and cardiac muscle (Stapleton *et al.*, 1996). Thus, OCC can be added to the limited number of tissues that express the α2 isoform. AMPK complexes containing the α2 subunit have been shown to be partially localized in the nucleus, while complexes containing the α1 subunit are located principally in the cytoplasm; in addition, α2-containing complexes are more responsive to AMP than those containing α1 (Salt *et al.*, 1998). Although both isoforms are apparently present in oocytes, they may not have equivalent potential for meiotic induction.

Evidence That AMPK Stimulates Meiotic Resumption in Mouse Oocytes

As a potent activator of AMPK in intact cells (Sullivan *et al.*, 1994; Corton *et al.*, 1995; Henin *et al.*, 1995), AICA riboside is an important pharmacological tool used for assessing AMPK involvement in various physiological processes. AICA riboside stimulation of both GVB and AMPK activity in meiotically arrested mouse oocytes provides compelling evidence that AMPK participates in meiotic induction. Metabolic conversion of AICA riboside to the AMP analog, AICA ribotide (ZMP), likely mediates its meiosis-inducing action, since the adenosine kinase inhibitor, 5′-aminodeoxyadenosine, completely blocked AICA riboside-induced GVB. A physiological role for AMPK was supported by the rapid kinetics of GVB: AICA riboside-induced maturation in dbcAMP-arrested DO was initiated within 3 h, a time frame similar to that observed *in situ* in eCG-primed mice treated with hCG (Eppig and Downs, 1988). Moreover, AICA riboside action was not restricted by the type of arresting agent, since this compound triggered GVB in DO maintained in meiotic arrest by a wide variety of inhibitory compounds. These included cAMP analogs, hypoxanthine and milrinone, the latter an inhibitor of oocyte-specific type III PDE (Tsafriri *et al.*, 1996; Shitsukawa *et al.*, 2001), which indicates that AMPK can stimulate nuclear maturation under conditions that restrict cAMP degradation and therefore suggests an action downstream of PDE.

Interestingly, higher concentrations of AICA riboside were required to stimulate GVB in CEO. In fact, there was an inhibitory trend at lower concentrations, which may be due to metabolic conversion to purine nucleotides, since AICA ribotide (ZMP) is a natural intermediate in the *de novo* purine synthetic pathway (see Fig. 2). Indeed, AICA riboside competes with ^{3}H-glycine for purine nucleotide production by OCC (Downs, 1997). No inhibition was detected in DO at any concentration of AICA riboside, but the nucleotide-producing capacity of the oocyte has yet to be determined. The cellular target for the stimulatory action of AICA riboside in the OCC cannot be discerned from these data, but its high potency in DO makes the oocyte a likely candidate. It may be that at high concentrations a stimulatory action in the oocyte overrides a suppressive action mediated by the cumulus cells.

In an earlier study (Downs, 1997), it was shown that AICA riboside could overcome a block to follicle-stimulating hormone- (FSH) induced meiotic maturation brought about by inhibitors of purine *de novo* synthesis. It was concluded that entry of AICA riboside into the *de novo* purine synthetic pathway downstream of the block was responsible for the restoration of meiotic resumption. However, in control groups lacking FSH, AICA riboside exhibited a modest meiosis-inducing ability on its own that was hard to reconcile in terms of *de novo* purine synthesis (Downs, 1997). Based on the data obtained in the present study, this AICA riboside effect on oocyte maturation in the absence of FSH may have been due to stimulation of AMPK.

Since AMP is a potent activator of AMPK (Moore *et al.*, 1991), dbcAMP- and hypoxanthine-arrested DO were exposed to increasing concentrations of AMP to test for a meiosis-inducing action. Under either inhibitory condition, 2 mM AMP stimulated more than 20% of the oocytes to resume maturation. This result was not duplicated by other nucleotides, nor was it due to ecto-5′-nucleotidase or other phosphatase activity. Such stimulation was somewhat surprising, considering the polar nature of AMP and its presumed poor permeability through cell membranes. Nevertheless, uptake studies using ^{3}H-AMP showed significant accumulation by DO. Although the majority of uptake may be attributable to initial extracellular conversion to adenosine, significant uptake above background was consistently detected in the presence of phosphatase inhibitors. Hence, it is concluded that external AMP may enter the oocyte to stimulate AMPK. It is doubtful that adenosine produced from AMP enters the cells to mediate a positive action on maturation, since oocytes exposed to this nucleoside exhibit increased levels of ATP (Salustri *et al.*, 1988) and cAMP (Downs *et al.*, 1989), conditions usually associated with meiotic arrest. In addition, although we cannot eliminate the possibility of an external effect of AMP or adenosine via purinoceptor binding, such a mechanism seems unlikely, because there is no precedence for a meiosis-inducing action of these compounds at the oocyte surface. On the contrary, it has been proposed that an oocyte receptor-mediated mechanism contributes to a meiosis-arresting action of adenosine (Salustri *et al.*, 1988).

We recently showed that adenosine-suppressed matura-

tion in hypoxanthine-treated CEO could be reversed by the addition of the adenosine deaminase inhibitor, dCF (Downs, 1999). It was concluded from this result that adenosine deamination contributes to the meiosis-arresting action of the nucleoside. However, results of the present study have forced us to reevaluate the significance of these earlier data. An alternative explanation is that blocking adenosine deaminase did not suppress an inhibitory pathway but, rather, turned on a positive pathway. Thus, at a high adenosine concentration (250 μM), phosphorylation of AMP by adenylate kinase may become saturated, and with the alternative deamination pathway blocked by dCF (see Fig. 2), the resulting AMP accumulation brings about the activation of AMPK and subsequent meiotic induction. Such a mechanism has been demonstrated in cultured hepatocytes for inhibition of autophagy (Samari and Seglen, 1998). Support for this mechanism in oocytes comes from the finding that, when added to dbcAMP-containing medium in the presence of dCF, adenosine actually stimulated a significant increase in GVB. It should be stressed that this response is uncharacteristic for adenosine, requiring the presence of dCF and, thus, represents an alteration in normal adenosine metabolism.

Is a Loss of Oocyte PKA Activity Required for Meiotic Resumption?

If one assumes that (1) meiotic arrest requires the continuous activity of PKA and (2) the cAMP content of germinal vesicle-stage oocytes at the time of isolation is sufficient to maintain PKA activity at meiosis-arresting levels, it necessarily follows that resumption of meiosis would require a reduction in oocyte cAMP. However, conditions exist in which meiotic maturation can resume in mammalian oocytes without a decline in oocyte cAMP below basal, presumably inhibitory, levels. For example, Moor and Heslop (1981) reported an increase in sheep oocyte cAMP in response to follicular stimulation by gonadotropin, but no decrease below fresh, nonstimulated levels during the early stages of maturation. Racowsky (1985) found that cAMP levels did not fall during release of hamster CEO from forskolin-mediated meiotic arrest. Treatment of mouse CEO with FSH (Salustri *et al.,* 1985) or a 2-h exposure of mouse follicles to forskolin (Hashimoto *et al.,* 1985) caused a transient rise in oocyte cAMP, but GVB commenced before the cAMP returned to pretreatment levels. This relationship was even more apparent in follicle-enclosed rabbit oocytes exposed to hCG or forskolin (Yoshimura *et al.,* 1992a,b), and these authors proposed that a transient cAMP rise in both the somatic and germ cell compartments is essential for effective meiotic induction. Similar results with follicle-enclosed hamster oocytes led Hubbard (1985, 1986) to conclude that maturation requires an increase followed by a decline in oocyte cAMP. Interestingly, a requirement for increased oocyte cAMP in meiotic resumption has been demonstrated in lower forms such as hydrozoans (Freeman and Ridgway, 1988), brittle stars (Yamashita, 1988), and marine nemertean worms (Stricker and Smythe, 2001), but this effect has been attributed to PKA activity.

Such data in mammalian systems are difficult to reconcile solely in terms of PKA control of meiotic maturation. Unless PKA can be inactivated by means other than lowered cAMP levels, meiotic maturation in the face of elevated cAMP suggests an alternative mechanism for meiotic resumption. The participation of AMPK in meiotic induction provides an answer to these seemingly conflicting data. A common finding in the above reports is that, while the meiosis-inducing stimulus produces an increase in oocyte cAMP, a significant drop in the peak cAMP level precedes meiotic resumption, even though it may not fall below the prestimulatory level. This fall in cAMP concentration, mediated by oocyte PDE, could produce AMP at levels above the stimulatory threshold required for AMPK activation and lead to AMPK-induced GVB. Such activation of AMPK would require PDE-mediated degradation of cAMP, but not necessarily a reduction below that normally associated with maintenance of meiotic arrest. Hence, GVB might be triggered even with a background of active PKA, as suggested by the actions of AICA riboside in oocytes arrested by a variety of meiotic inhibitors, although high levels of PKA activity may restrict AMPK effectiveness. In addition, it should be noted that, even if oocyte cAMP levels fall below the presumed inhibitory threshold, AMPK may still contribute to the meiosis-inducing effect.

CONCLUSION

The results of this study show that the oocyte contains AMPK that is activated coincidentally with GVB during AICA riboside treatment. Further indirect evidence implicates AMP, acting through AMPK, as having an important meiosis-inducing function. A model for the participation of AMPK in meiotic regulation that incorporates these observations is presented in Fig. 14.

Although AMPK is considered to be principally a stress-response enzyme, the participation of this kinase family in meiotic processes is not without precedence. In yeast, fermentable carbon sources such as glucose block meiosis through suppression of the AMPK homolog, SNF1; removal of glucose from the culture medium activates the kinase and drives yeast cells into meiosis (Mitchell, 1994; Kupiec *et al.,* 1997). Moreover, inactive *snf1* mutants are incapable of undergoing meiosis regardless of the presence or absence of glucose (Carlson *et al.,* 1981; Honigberg and Lee, 1998). We have shown previously that when CEO are cultured in medium containing a meiotic inhibitor plus millimolar pyruvate, glucose exerts an inhibitory effect on meiotic resumption that is dependent on glycolysis (Fagbohun and Downs, 1992; Downs and Mastropolo, 1994; Downs, 1995b). It is tempting to speculate that, under these special conditions, glycolytic generation of ATP lowers AMP/ATP below the AMPK activation threshold and thereby contrib-

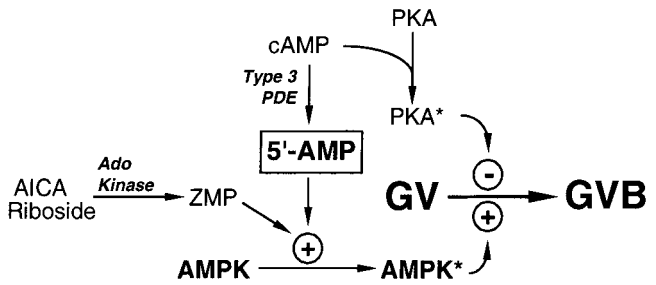

FIG. 14. A model for AMPK involvement in meiotic regulation. Oocyte PDE3 converts cAMP to 5′-AMP, thereby inactivating PKA but also activating AMPK. The loss of PKA activity combined with a gain in AMPK activity leads to meiotic resumption. AICA riboside is metabolized to ZMP via adenosine kinase and can stimulate AMPK and trigger GVB in the absence of PDE activity. Since AICA riboside induction of maturation bypasses the PDE-dependent step, GVB may be possible without a significant loss of PKA activity. The active states of PKA and AMPK are denoted by an asterisk.

utes to meiotic suppression. Such regulation by glucose resembles that present in yeast and may represent a primitive system for meiotic control. However, in complex organisms, a more sophisticated means of meiotic control has evolved that incorporates a hormone-triggered signal cascade. AMPK may have been retained as a participant in this process, but apparently with a different transduction system, since hormone-stimulated maturation in mouse CEO *requires* glucose (Fagbohun and Downs, 1992; Downs and Mastropolo, 1994).

Evaluation of AMPK involvement in meiotic induction promises to yield important new insights into how meiosis is regulated. Confirmation of such involvement will necessitate establishing where AMPK is situated in the cascade of events leading to meiotic induction. It will be important to determine whether increased AMPK activity precedes and is essential for ligand-induced, as well as spontaneous, maturation, not only in the mouse but in other species as well. Even if it proves not to be required for normal meiotic induction, its presence in the oocyte indicates a mechanism for the oocyte to respond to stressful conditions. In addition, it is anticipated that the recent development of AMPK-specific inhibitors (e.g., Zhou *et al.*, 2001) and other molecular and pharmacological tools will facilitate a thorough examination of the physiological role of AMPK in meiosis.

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