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AMPK Regulation of Mouse Oocyte Meiotic Resumption in Vitro

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Keywords
Mouse oocytes, AMP-activated protein kinase, Meiotic resumption, Compound C

Abstract

We have previously shown that the adenosine analog 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), an activator of AMP-activated protein kinase (AMPK), stimulates an increase in AMPK activity and induces meiotic resumption in mouse oocytes [Downs, S.M., Hudson, E.R., Hardie, D.G., 2002. A potential role for AMP-activated protein kinase in meiotic induction in mouse oocytes. Dev. Biol, 245, 200–212]. The present study was carried out to better define a causative role for AMPK in oocyte meiotic maturation. When microinjected with a constitutively active AMPK, about 20% of mouse oocytes maintained in meiotic arrest with dibutyryl cAMP (dbcAMP) were stimulated to undergo germinal vesicle breakdown (GVB), while there was no effect of catalytically dead kinase. Western blot analysis revealed that germinal vesicle (GV)-stage oocytes cultured in dbcAMP-containing medium plus AICAR possessed elevated levels of active AMPK, and this was confirmed by AMPK assays using a peptide substrate of AMPK to directly measure AMPK activity. AICAR-induced meiotic resumption and AMPK activation were blocked by compound C or adenine 9-beta-d-arabinofuranoside (araA, a precursor of araATP), both inhibitors of AMPK. Compound C failed to suppress adenosine uptake and phosphorylation, indicating that it did not block AICAR action by preventing its metabolism to the AMP analog, ZMP. 2′-Deoxycoformycin (DCF), a potent adenosine deaminase inhibitor, reversed the inhibitory effect of adenosine on oocyte maturation by modulating intracellular AMP levels and activating AMPK. Rosiglitazone, an anti-diabetic agent, stimulated AMPK activation in oocytes and triggered meiotic resumption. In spontaneously maturing oocytes, GVB was preceded by AMPK activation and blocked by compound C. Collectively, these results support the proposition that active AMPK within mouse oocytes provides a potent meiosis-inducing signal in vitro.

Introduction

Mammalian oocytes are arrested in the diplotene stage of the first meiotic prophase soon after initiating meiosis. The meiotically arrested oocyte is characterized by a prominent nucleus, termed the germinal vesicle (GV), which is maintained throughout the period of oocyte growth. Fully grown oocytes within healthy, non-atretic secondary follicles are triggered to resume meiotic maturation in response to a preovulatory gonadotropin surge. This process is manifested by germinal vesicle breakdown (GVB), a morphological change commonly used to monitor meiosis initiation.

The mechanism by which oocytes overcome the prophase I meiotic arrest is not well understood. Oocytes cultured within their follicles in vitro are maintained in meiotic arrest unless stimulated by gonadotropin. When the oocyte is removed from the intrafollicular environment and cultured under permissible conditions, maturation occurs spontaneously in the absence of hormone stimulation, indicating a dependence on the follicular somatic compartment for maintenance of prophase I arrest. If cumulus cell-enclosed oocytes are isolated from follicles and maintained at the GV stage in vitro with exogenous inhibitor, meiotic resumption can be elicited by gonadotropin. This phenomenon is mediated by the cumulus cells that produce a signal driving the oocyte into meiotic maturation.

Cyclic adenosine monophosphate (cAMP) plays an important role in the regulation of meiotic resumption in oocytes. Agents that elevate cAMP levels, cAMP analogs or factors that prevent degradation of intracellular cAMP reversibly suppress oocyte maturation. cAMP prevents GVB through the activation of cAMP-dependent protein kinase (PKA). Indeed, mouse 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). In *Xenopus* oocytes, PKA phosphorylates and inactivates cdc25 phosphatase, thereby blocking activation of maturation promoting factor (cdc2 kinase) and contributing to meiotic arrest (Duckworth et al., 2002). Recently, protein tyrosine phosphatase nonreceptor type 13 (PTPN13), a substrate of PKA in both mouse and *Xenopus* oocytes, has been implicated in the regulation of oocyte meiotic resumption (Nedachi and Conti, 2004).

Oocyte cAMP synthesis and hydrolysis are regulated via adenylyl cyclase and cyclic nucleotide phosphodiesterase (PDE), respectively. PDE3A is the predominant PDE isoform expressed in rodent oocytes (Shitsukawa et al., 2001, Conti et al., 2002), and its activity is increased prior to oocyte maturation (Richard et al., 2001). PDE3A-null female mice are infertile as oocytes are ovulated in the immature GV stage (Masciarelli et al., 2004). PDE3A-null oocytes lack cAMP-specific PDE activity, contain increased cAMP levels, are persistently maintained in the GV stage in vivo and failed to undergo spontaneous maturation in vitro. These data indicate that PDE activity is required for oocyte maturation.

The product of PDE activity and cAMP degradation, 5′-AMP, is a potent stimulator of the stress response kinase, AMP-activated protein kinase (AMPK), and has been suggested to have an important meiosis induction function via activating AMPK (Downs et al., 2002). AMPK, a serine/threonine kinase, composed of an α catalytic subunit and β and γ regulatory subunits, is a pivotal enzyme in the regulation of cellular energy charge (Hardie and Hawley, 2001, Hardie, 2004, Kahn et al., 2005). AMPK is activated by an upstream kinase, recently identified as the tumor repressor, LKB1 (Hawley et al., 2003, Woods et al., 2003), via phosphorylation of Thr-172 on the α subunit (Hawley et al., 1996). Binding of AMP or ATP to CBS domains on the γ subunit (Scott et al., 2004) regulates the activation state of the kinase (reviewed by Hardie and Hawley, 2001, Hardie, 2004). AMP promotes phosphorylation, and thereby activation, of AMPK by directly binding to the γ subunit of AMPK and changing the conformation of the kinase, making it a better substrate for the upstream kinase (Hawley et al., 2002) and a worse substrate for the phosphatase (Davies et al., 1990). By controlling the activity and expression of important rate-limiting enzymes of carbohydrate, fat and protein metabolism, AMPK regulates the cell energy status through conservation of ATP levels.

5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), an analog of adenosine, is widely used to activate AMPK in vitro. After entering cells, it is phosphorylated to form AICA ribotide (ZMP), an analog of AMP. ZMP, though less effective than AMP, mimics all the activating effects of AMP in the AMPK cascade (Corton et al., 1995). Thus, AICAR provides a means of activating AMPK without affecting AMP or ATP levels.

In a previous study, AICAR was shown to rapidly stimulate GVB in mouse oocytes maintained in meiotic arrest with a variety of inhibitors (Downs et al., 2002). AICAR was more potent in denuded oocytes (DO) than cumulus cell-enclosed oocytes (CEO), identifying the oocyte as the target of AICAR action.

Meiotic resumption was also induced by AMP in dbcAMP-arrested oocytes. Therefore, these data were consistent with the possibility that activation of AMPK provides a potent meiosis-inducing signal.

In the present study, we determined if AMPK has a causative role in GVB in mouse oocytes by modifying AMPK activity and relating it to meiotic status. Using four different strategies to expose oocytes to active AMPK and using two putative inhibitors to block AMPK activity, we show that stimulation of AMPK in prophase-I-arrested oocytes leads to meiotic resumption and that preventing AMPK activation abolishes the meiotic response.

Materials and methods

Oocyte isolation and culture conditions

C57BL/6JxSJL/J F1 mice, 19–23 days old, were used for all experiments. Mice were primed with 5 IU equine choronic gonadotropin and killed 48 h later by cervical dislocation. Ovaries were removed and placed in the culture medium, and cumulus cell-enclosed oocytes (CEO) were obtained by puncturing large antral follicles with sterile needles. Denuded oocytes were prepared by repeated pipetting with a Pasteur pipette. Oocytes were cultured in glass tubes for long-term culture (17–18 h) or in plastic tubes for short-term culture (≤4 h). 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 culture medium used for most experiments was Eagle's minimum essential medium with Earle's salts (GIBCO), supplemented with 0.23 mM pyruvate, penicillin, streptomycin sulfate and 3 mg/ml crystallized lyophilized bovine serum albumin (ICN ImmunoBiologicals, Lisle, IL) and buffered with 26 mM bicarbonate. For oocyte microinjection, medium was buffered with 25 mM HEPES, pH 7.2, with bicarbonate reduced to 6 mM.

Microinjection of active/dead AMPK

To test the effects of microinjected AMPK on oocyte maturation, denuded oocytes were maintained in meiotic arrest with 300 µM dbcAMP and microinjected with a preparation of either constitutively active AMPK (T172D) or dead (inactive) kinase (D139A), each at a concentration of 7.5 mg/ml. Oocytes were placed into a micro-culture dish at room temperature, and approximately 3–10 pl of solution containing active or inactivate kinase was injected into the cytoplasm of germinal vesicle-stage oocytes using femtotip II needles with an Eppendorf Transjector 5246 system (Eppendorf, Madsion, WI) under a Nikon Diaphot 200 inverted microscope equipped with Nomarski optics. Following microinjection, oocytes were transferred to 500 µl of dbcAMP-containing medium under mineral oil and placed in a 37°C incubator for 22 h. A positive control was included in which non-injected oocytes were cultured in medium containing dbcAMP plus 250 µM AICAR. At the conclusion of the incubation period, oocytes were assessed for GVB.

Construction and expression of GST-AMPK α1 catalytic domain proteins (T172D, D139A)

Plasmid DNA encoding GST-tagged AMPK α1 catalytic domain has been previously described (Scott et al., 2002). This DNA was used in site-directed mutagenesis (QuickChange, Stratagene) to generate constitutively active (T172D) and kinase dead (D139A) GST-tagged AMPK α1 catalytic domain proteins.

The following synthetic oligonucleotides were used: T172D mutation, GGT GAA TTT TTA AGA GAC AGC TGT GGC TCG CCC (sense) and GGG CGA GCC ACA GCT GTC TCT TAA AAA TTC ACC (antisense), D139A mutation ATG GTG GTC CAC AGA GCT TTG AAA CCT GAA AAC (sense) and GTT TTC AGG TTT CAA AGC TCT TGT GAC CAC CAT (antisense). The underlined bases indicate the sites on the codon where the mismatch was placed. All proteins were expressed in *E. coli* (BL21) and purified on glutathione sepharose as previously described (Scott et al., 2002).

Western blots

DO and oocyte–cumulus cell complexes (OCC) were washed in phosphate-buffered saline (PBS, pH 7.4)/PVP (3 mg/ml) plus protease inhibitors (Protease Inhibitors Cocktail Tablets, 1 mM Na orthovanadate, 2 μg/ml pepstatin, 50 mM β-Glycerophospate) and then added to an equal volume of 2× Laemmli's buffer containing 20% β-mercaptoethanol. After heating at 95°C for 5 min, samples were stored frozen at −80°C until used for Western blotting. For Western analysis, proteins were electrophoresed on a 3–8% Tris–Acetate mini Gel (Invitrogen) for 1 h at 150 V and then transferred to nitrocellulose at 100 V for 1 h. To obtain the sharpest bands for active AMPK (PT172) in Fig. 2B, samples were electrophoresed on a 4–12% Bis–Tris SDS mini Gel (Invitrogen) for 50 min at 100 V and then in a semi-dry system transferred to nitrocellulose at 400 mA for 2.5 h at 4°C. Blots were blocked with 5% nonfat milk for 2 h at room temperature and then incubated with primary antibodies (1:250) overnight at 4°C, washed in Tris-buffered saline (TBS pH 7.4) 3× 15 min, and incubated with HRP-conjugated IgG (1:2000) for 1 h at room temperature. Detection was performed with Supersignal Western Pico Chemiluminescent Substrate (Pierce; Rockford, IL) and exposed on auto radiographic films. Blots were stripped (7 μl/ml BME, 2% SDS, room temperature 30 min) and reprobed with regular ACC antiserum (1:2000) as a loading control. Bands were quantified by UVP BioImaging Systems (UVP, Inc.; Upland, CA), and ratios of pACC/ACC were presented as the mean of two determinations.

Anti-pACC was purchased from Upstate Biotechnology Inc. (Lake Placid, NY); anti-ACC antibodies were generated in Dr. Hardie's laboratory; anti-PT172 antibody was obtained from Cell Signaling Technology (Beverly, MA).

AMPK assay

AMPK activity was measured as previously described (Downs et al., 2002, Hardie et al., 2000a, Hardie et al., 2000b). Four hundred GV-stage oocytes from each treatment group were collected and washed several times in immunoprecipitation (IP) buffer and frozen at −80°C. IP buffer for oocytes contained: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na orthovanodate, protease inhibitor cocktail tablet (Roche, Germany), Na pyrophosphate (0.5834 g/250 ml) and 1 mM dithiothreitol.

DNA staining

After 17–18 h of culture, DO were fixed in 3% formaldehyde in PBS for 0.5–1 h. Oocytes were then transferred to a microscope slide and dried on a warming tray. 10 μl glycerol:PBS (1:1) containing 1

μg/ml Hoechst 3342 (Polysciences, Warrington, PA) was added, and a cover slide was immediately placed on the oocytes and sealed with nail polish. Meiotic status was determined using a Leitz Ortholux II fluorescent microscope.

AMP/ATP level measurement

Denuded oocytes were cultured for varying periods in organ culture dishes containing 1 ml medium. After the designated culture times, individual oocytes were assayed for AMP or ATP.

AMP was assayed as described in Chang et al. (2004). ATP was measured as specified in Chi et al. (2002). Briefly, after different treatments, individual oocytes were extracted by 0.5 μl 0.1 N NaOH at room temperature for 20 min, 0.5 μl of the extract was heated to 80°C for 20 min, and a 0.2 μl mixture of 0.2 N HCl and 0.1 M Tris–HCl (pH 6.8) was added. The $NADP^+$ cycling reagent was used for the ATP assay, containing 100 mM imidazole HCl (pH 7.0), 7.5 mM α-ketoglutarate, 5 mM glucose 6-phosphate, 25 mM NH_4Ac, 0.02% BSA, 100 μM ADP, 100 μg/ml beef liver glutamate dehydrogenase and 10 μg/ml *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase. The indicator reaction for the $NADP^+$ cycling step involves adding 10 μl of the reaction to 1 ml of the indicator, 6-phosphogluconate reagent, containing 50 mM imidazole HAc (pH 7.0), 1 mM EDTA, 30 mM NH_4Ac, 5 mM $MgCl_2$, 100 μM $NADP^+$ and 2 μg/ml yeast 6-phosphogluconate dehydrogenase.

Statistical analysis

Oocyte maturation experiments were repeated at least 3 times with at least 25 oocytes per experiment. Data are reported as mean percentage GVB ± SEM. Maturation frequencies were subjected to arcsin transformation and analyzed statistically by ANOVA followed by Duncan's multiple range test. For all statistical analyses, a P value less than 0.05 was considered significant.

Results

Microinjection of active AMPK induces oocyte maturation

To directly determine if active AMPK could stimulate meiotic resumption, bacterially expressed constitutively active (T172D) AMPK was injected into meiotically arrested oocytes. Denuded oocytes (DO) maintained in meiotic arrest with dbcAMP were microinjected with either a constitutively active AMPK or a catalytically inactive (dead) AMPK preparation. DO cultured with AICAR served as a positive control. The results of two experiments are shown in Fig. 1. After 21–22 h of culture, AICAR stimulated most of the arrested oocytes to resume maturation (94–100% GVB compared to 0–2% of control oocytes), indicating the system is responsive to an appropriate meiosis-inducing stimulus. About 20% of the oocytes receiving active AMPK were stimulated to undergo maturation. Although this was a smaller effect than that of AICAR, the catalytically inactive kinase had no effect.

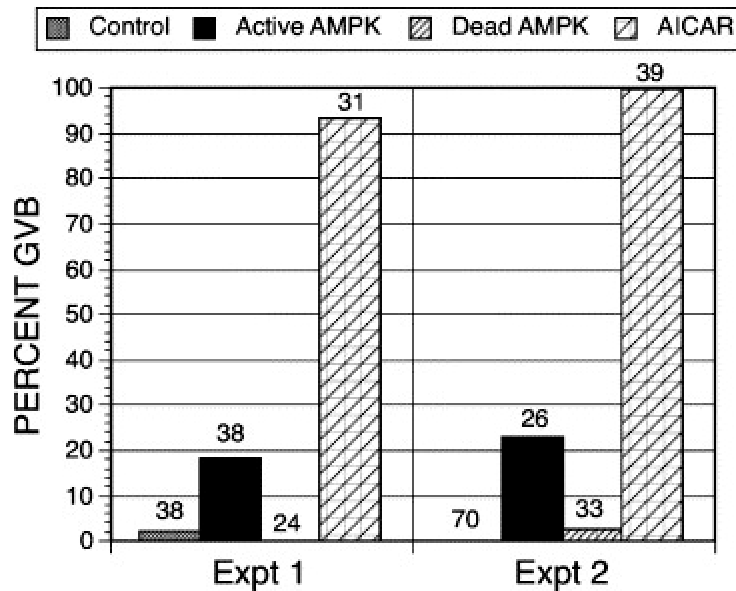

Fig. 1. Microinjection of constitutively active AMPK induces meiotic resumption in dbcAMP-arrested oocytes. Oocytes were maintained in meiotic arrest with 300 μM dbcAMP and microinjected with either a constitutively active AMPK or a catalytically inactive (dead) AMPK preparation (injection volume approximately 3–9 pl). Culture was continued for 20–21 h before assessing GVB. The number of oocytes in each group is shown at the top of the bar.

AMPK is activated prior to GVB in AICAR-treated oocytes

AICAR, an AMPK activator, is a very potent inducer of oocyte maturation and stimulates AMPK activity in mouse oocytes (Downs et al., 2002). To assess the activation state of AMPK on Western blots, two different antibodies were used, anti-PT172 and anti-phospho-acetyl CoA carboxylase (Ser-79-pACC). Anti-PT172 antibody recognizes phosphorylated Thr-172 on the catalytic subunit and correlates with AMPK activity. ACC, an enzyme involved in fatty acid metabolism, is a major substrate of AMPK (Davies et al., 1992), and its phosphorylation state on Ser-79 is commonly used as an indirect assay for AMPK activity. Extracts were prepared from 230 oocyte–cumulus cell complexes (OCC) cultured for 4 h in 300 μM dbcAMP plus or minus 1 mM AICAR. Phosphorylation of Thr-172 was evident in freshly isolated complexes, but this decreased in intensity after culture, while addition of AICAR to the culture medium restored the high level of AMPK activity (Fig. 2A). A similar pattern was seen in the pACC blots.

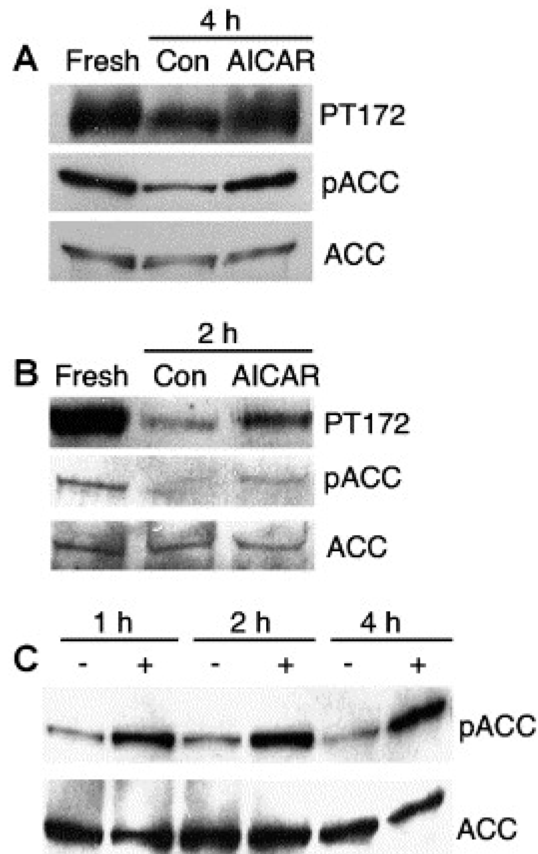

Fig. 2. Western analysis of phosphorylation of AMPK (at pT172) and phosphorylation of ACC (pACC) in OCC and DO. (A) Extracts were prepared from freshly isolated OCC or OCC cultured 4 h in 300 µM dbcAMP with (AICAR) or without (Con) 1 mM AICAR (230 OCC per lane). Blots were stripped and reprobed with anti-ACC antibody as a loading control. (B) AMPK and pACC levels in freshly isolated DO or DO cultured 2 h in 300 µM dbcAMP with (AICAR) or without (Con) 500 µM AICAR. Only germinal vesicle (GV)-stage oocytes were collected and assayed (500 oocytes per lane). (C) DO were freshly isolated or cultured in 300 µM dbcAMP with (+) or without (−) AICAR (250 µM) for 1, 2, or 4 h (235 oocytes per lane). All oocytes were at the GV stage, except for the 4 h AICAR-treated group, which contained only germinal vesicle breakdown (GVB)-stage oocytes.

The experiments were repeated in DO. DO were cultured in 300 µM dbcAMP plus or minus AICAR for 2 h, and only GV-stage oocytes were collected for analysis. For the above blot, extract from OCC was electrophoresed on a 3–8% Tris–Acetate gel, which produces distinct bands for pACC but more blurred bands for PT172. Initial attempts at using this gel for detection of PT172 in oocyte extracts proved unsuccessful; consequently, we carried out electrophoresis using a 4–12% Bis–Tris gel. Under these conditions, phosphorylation of Thr-172 on AMPK was detected in freshly isolated oocytes that decreased after 2 h of culture in dbcAMP alone but was increased by AICAR treatment (Fig. 2B). Ser-79 phosphorylation on ACC showed the same pattern, but the bands were less distinct. That the phosphorylation of Ser-79 on ACC parallels that of Thr-172 on ACC confirms the usefulness of the anti-pACC antibody as a marker of AMPK activity in mouse oocytes. The elevated phosphorylation of AMPK

in the AICAR-treated GV-stage DO suggests that AMPK is activated prior to oocyte meiotic resumption, which supports the idea that AMPK triggers oocyte maturation.

Using the anti-pACC antibody, a more detailed time course of AMPK activation by AICAR in DO was determined. DO were cultured in 300 µM dbcAMP with or without AICAR (250 µM) for 1, 2 or 4 h before probing extracts for AMPK activity with anti-pACC antibody. All oocytes used for analysis were at the germinal vesicle (GV) stage, except for the 4 h AICAR-treated group, which contained only germinal vesicle breakdown (GVB)-stage oocytes. For this and all subsequent pACC Western blotting, we utilized 3–8% Tris–Acetate gels. Results of the time course experiment show that AMPK is activated within 1 h of culture (Fig. 2C), well before the initiation of meiotic induction, which is not manifested until after 2 h of culture under these conditions (Downs et al., 2002).

To directly measure AMPK activity in AICAR-treated GV-stage oocytes, SAMS peptide phosphorylation assays were performed. SAMS peptide is a peptide sequence based on the unique phosphorylation site (Ser-79) of ACC for AMPK and is a specific substrate for the kinase (Davies et al., 1989). Extracts were assayed from GV-stage fresh DO or from GV-stage DO cultured for 2 h in dbcAMP-containing medium in the presence or absence of AICAR. Fresh oocytes contained a high level of AMPK activity that was reduced by 86% after 2 h of culture under meiosis-arresting conditions (Fig. 3). In agreement with the results of Western blotting, AICAR increased the AMPK activity 3.4-fold (Fig. 3), thereby confirming that an increase in AMPK precedes GVB.

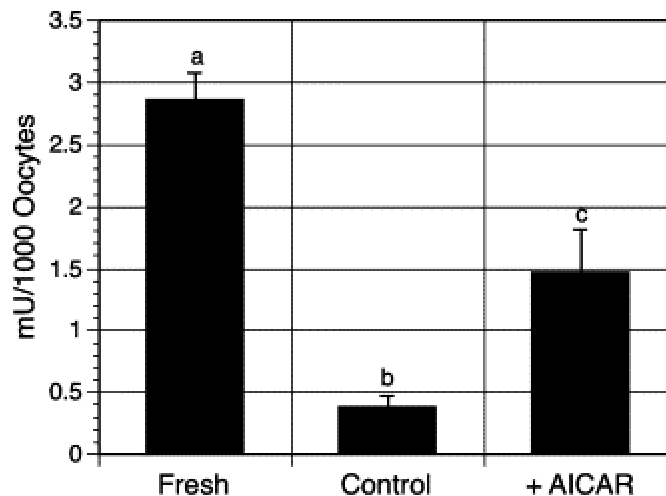

Fig. 3. Effect of AICAR on AMPK activity in GV-stage oocytes. AMPK activity was analyzed in extracts from freshly isolated oocytes or from GV-stage oocytes after 2 h culture in 300 µM dbcAMP alone (Control) or dbcAMP plus 500 µM AICAR (+AICAR). A different letter denotes a significant difference.

AMPK inhibitors block AMPK activation and AICAR-induced oocyte maturation

Compound C, a potent small-molecule AMPK inhibitor (Zhou et al., 2001), was tested on AICAR-induced oocyte maturation. DO were preincubated with increasing concentrations of compound C in dbcAMP-containing medium for 0.5 h before addition of 250 µM AICAR, and cultures were continued

for 4 h before GVB assessment. A parallel group of DO was cultured 4.5 h in dbcAMP-supplemented medium containing increasing concentrations of compound C but without added AICAR. In the absence of AICAR, 34% of the control oocytes resumed maturation, and compound C exerted a modest inhibitory effect (Fig. 4A). AICAR treatment increased the maturation percentage to 83%, and this stimulation was dose-dependently eliminated by compound C. Inhibition was reversible as it was eliminated by washing out compound C and further culture in dbcAMP-free medium (data not shown).

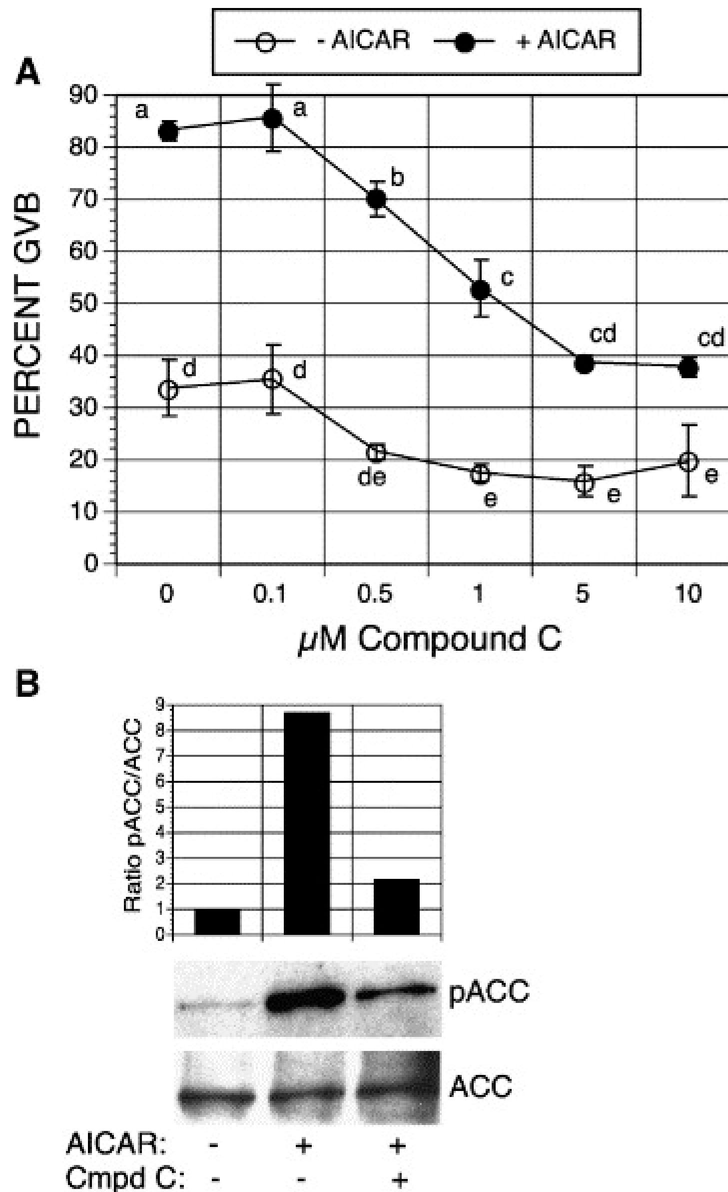

Fig. 4. Effects of compound C on AICAR-induced meiotic maturation and ACC phosphorylation. (A) DO were preincubated 0.5 h in medium containing 300 μm dbcAMP with or without increasing concentrations of compound C. AICAR was then added to some of the tubes, and cultures were continued for 4 h before assessment of GVB. (B) Western analysis of pACC in extracts of GV-stage DO (250 oocytes per lane). Extracts were prepared from GV-stage DO cultured 2 h in medium containing

300 µM dbcAMP, dbcAMP plus AICAR (500 µM) or dbcAMP plus both AICAR and compound C (5 µM). The mean pACC/ACC ratio of two blots is shown in the corresponding graph, normalized to the dbcAMP alone control group.

To assess the effect of compound C on AMPK activity in these oocytes, pACC levels were determined in GV-stage oocytes by Western blot analysis. After 2 h of culture, very little phosphorylation of ACC was detected in oocytes cultured in dbcAMP alone, but this was increased by AICAR treatment. Exposure of AICAR-treated oocytes to compound C significantly reduced phosphorylation of pACC, indicating suppression of AMPK activity within oocytes (Fig. 4B).

To rule out the possibility of a general toxic effect, compound C was tested on okadaic acid (OA)-induced oocyte maturation. DO were pre-treated with dbcAMP plus increasing concentrations of compound C for 0.5 h before adding OA, and GVB was assessed after 4 additional hours of culture. Compound C had no inhibitory effect on OA-induced oocyte maturation even at a concentration 10-fold higher than that which effectively blocked AICAR-induced maturation (Fig. 5A). The kinetics of OA-stimulated GVB was only slightly delayed by 5 µM compound C (data not shown). Extracts from GV-stage oocytes after 1.5 h treatment with 1 µM OA revealed only a modest activation of AMPK when compared to AICAR-treated oocytes (Fig. 5B). These results show that compound C is not a non-specific inhibitor of maturation and indicate that AMPK activation is not the principal means by which OA stimulates meiotic resumption.

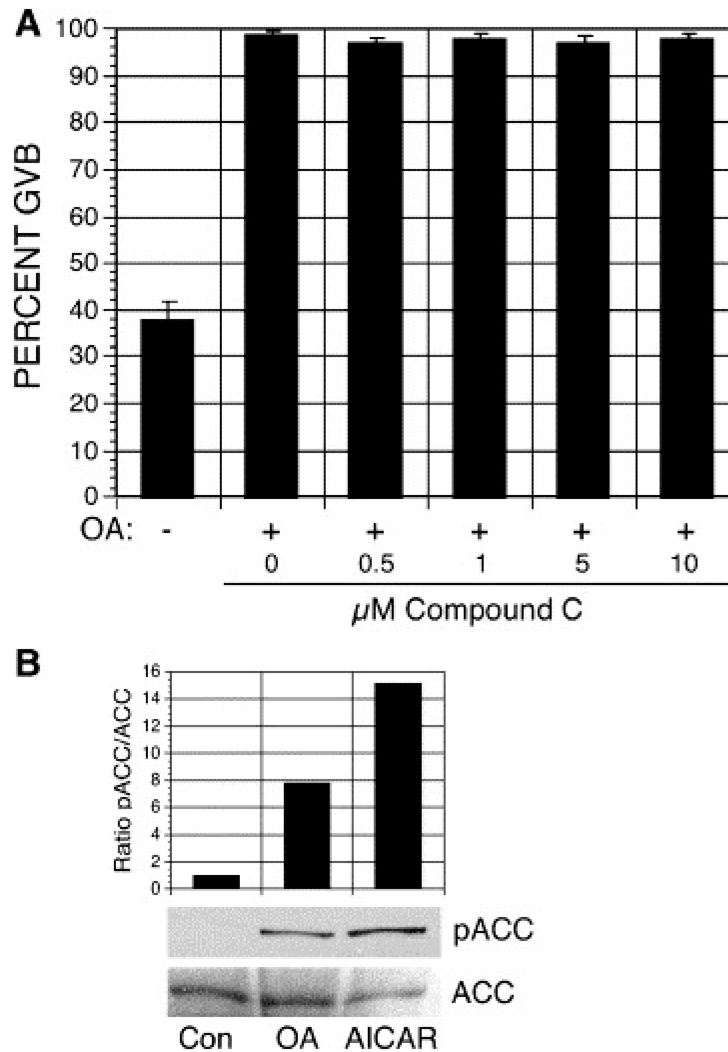

Fig. 5. Effects of compound C on okadaic-acid (OA)-induced meiotic resumption and ACC phosphorylation. (A) DO were preincubated with increasing concentrations of compound C for 0.5 h before adding 1 µM OA in 300 µM dbcAMP-containing medium. GVB percentage was assessed after 4 h of culture. (B) Western analysis of pACC levels in OA-treated GV-stage oocytes (250 oocytes per lane). DO were treated in 300 µM dbcAMP containing medium with 1 µM OA or 500 µM AICAR for 1.5 h. The mean pACC/ACC ratio of two blots is shown in the corresponding graph, normalized to the dbcAMP alone control group.

AICAR is transported into cells via the adenosine transport system and is then phosphorylated by adenosine kinase to form the AMP analog, ZMP (Corton et al., 1995, Gadalla et al., 2004). To discount the possibility that compound C blocks AMPK activation by inhibition of AICAR transport into the oocytes or its conversion to ZMP, we tested the effect of compound C on: (1) the uptake of radiolabeled adenosine by mouse oocytes and (2) adenosine-stimulated ATP production in oocytes. To measure possible effects on AICAR uptake, DO were cultured 30 min in control medium or medium containing 2 mM uridine or increasing concentrations of compound C. Tritiated adenosine was then added, and cultures were continued for 3 h. Uridine served as a positive control for adenosine

transport inhibition (Downs, 1999). Fig. 6 shows the mean of two experiments. Compound C did not affect the uptake of adenosine at concentrations that completely suppressed AICAR-induced maturation, while uridine totally eliminated uptake (Fig. 6A). To test compound C for an effect on adenosine kinase, DO were cultured for 3 h in control medium or medium containing 250 µM adenosine, in the presence or absence of compound C, and individual oocytes were then analyzed for ATP. As shown in Fig. 6B, adenosine stimulated an increase in ATP levels that was not affected by the presence of compound C. These data demonstrate that the ability of the oocyte to take up and convert AICAR to ZMP was not compromised by compound C.

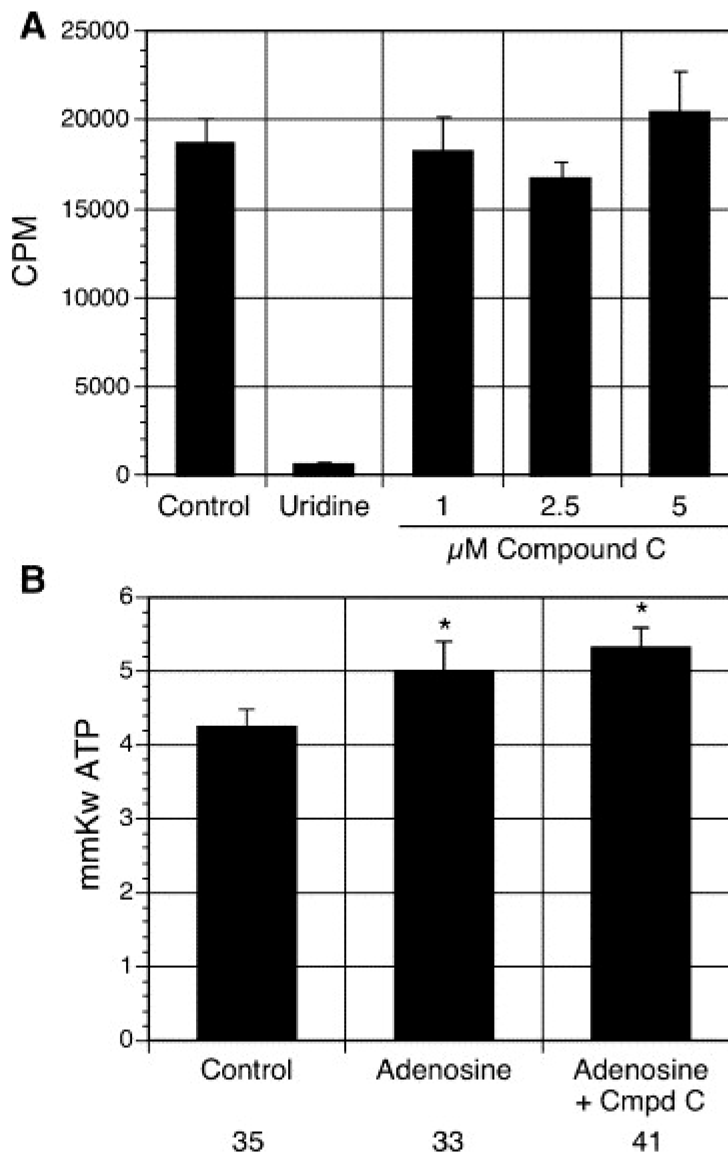

Fig. 6. Effects of compound C on adenosine uptake and adenosine phosphorylation in DO. (A) DO were cultured 30 min in control medium or medium containing 2 mM uridine or increasing concentrations of compound before the addition of tritiated adenosine and the continuation of culture for 3 h. Groups of 25 oocytes were analyzed for radioactivity by scintillation spectroscopy (mean ± SEM of three experiments). (B) DO were cultured 3 h in control medium or medium containing 250 µM adenosine or

adenosine plus 5 µM compound C. Individual oocytes were then analyzed for ATP. The number of oocytes assayed is given below each bar. An asterisk denotes a significant difference from the control group.

Adenine 9-β-d-arabinofuranoside (araA) is the precursor of araATP, a competitive inhibitor of AMPK (Henin et al., 1996). We tested this inhibitor on AICAR-induced maturation to determine if it could duplicate the inhibitory action of compound C. Oocytes were pretreated with dbcAMP plus increasing concentrations of araA for 0.5 h before adding 250 µM AICAR, and GVB percentages were determined after 4 additional hours of culture. AICAR increased the maturation percentage from 20% to 80%, and this was blocked by araA in a dose-dependent fashion (Fig. 7A). Western analysis demonstrated an increased phosphorylation of ACC in response to AICAR that was suppressed by araA (Fig. 7B).

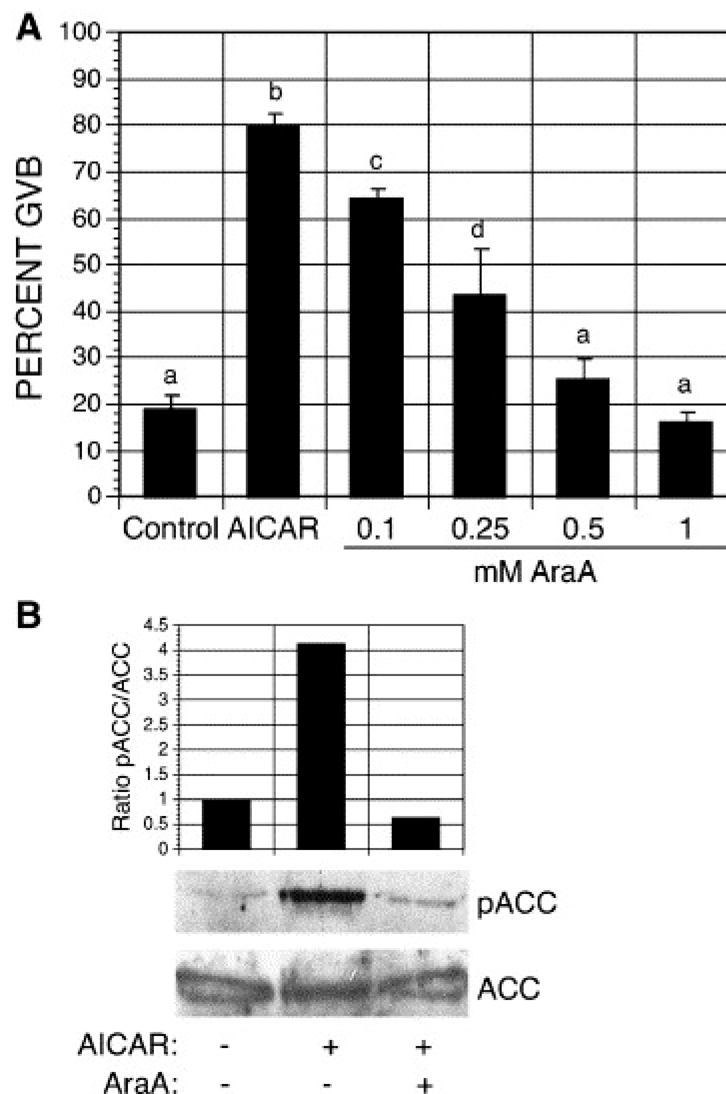

Fig. 7. Effects of araA on AICAR-induced oocyte maturation and ACC phosphorylation. (A) DO were pretreated 0.5 h in 300 µM dbcAMP-supplemented medium with increasing doses of araA; 250 µM AICAR was then added, and cultures were continued for 4 h before assessment of GVB. A different

letter denotes a significant difference. (B) Western analysis of pACC levels in araA and AICAR-treated GV-stage oocytes (250 oocytes per lane). DO were preincubated with 1 mM araA for 0.5 h in 300 µM dbcAMP before adding 500 µM AICAR, and cultures were continued for 2 h. The mean pACC/ACC ratio of two blots is shown in the corresponding graph, normalized to the dbcAMP control group.

AICAR and its metabolites may have effects unrelated to AMPK activation (Kemp et al., 1999), and it was therefore important to consider alternative methods to stimulate the activity of the kinase and relate the activity to meiotic status. To accomplish this end, we utilized two additional strategies, (1) manipulation of adenosine metabolism and (2) treatment of oocytes with the anti-diabetic agent rosiglitazone.

DCF plus adenosine induces oocyte maturation and AMPK activation

Adenosine, a component of follicular fluid, exerts a meiosis-arresting action on oocytes (Eppig et al., 1985, Miller and Behrman, 1986, Salustri et al., 1988, Downs, 1999). This nucleoside has two main metabolic routes within cells—phosphorylation by adenosine kinase to generate AMP and deamination by adenosine deaminase to produce inosine. Deoxycoformycin (DCF), an adenosine deaminase inhibitor, has no effect on oocyte maturation on its own but can reverse the inhibitory effect of adenosine in CEO (Downs, 1999, Downs et al., 2002). This led to the idea that blocking deamination of adenosine might divert increased adenosine through adenosine kinase, causing an accumulation of AMP, activation of AMPK and meiotic induction.

We first tested the effects of DCF on adenosine-mediated meiotic arrest. DO were cultured for 17–18 h in medium containing 150 µM dbcAMP plus increasing concentrations of adenosine in the absence or presence of 250 µM DCF. This lower concentration of dbcAMP was used to achieve a suboptimal level of inhibition that would then be augmented by the addition of adenosine (cf. Downs, 1999), because adenosine alone has a very limited inhibitory effect. As shown in Fig. 8, adenosine dose-dependently suppressed oocyte maturation, while at each concentration DCF completely reversed this effect. A time course experiment showed that the meiotic induction brought about by DCF treatment was slow: a small increase in maturation was evident after 6 h of culture, with complete reversal achieved by 18 h (Fig. 8B).

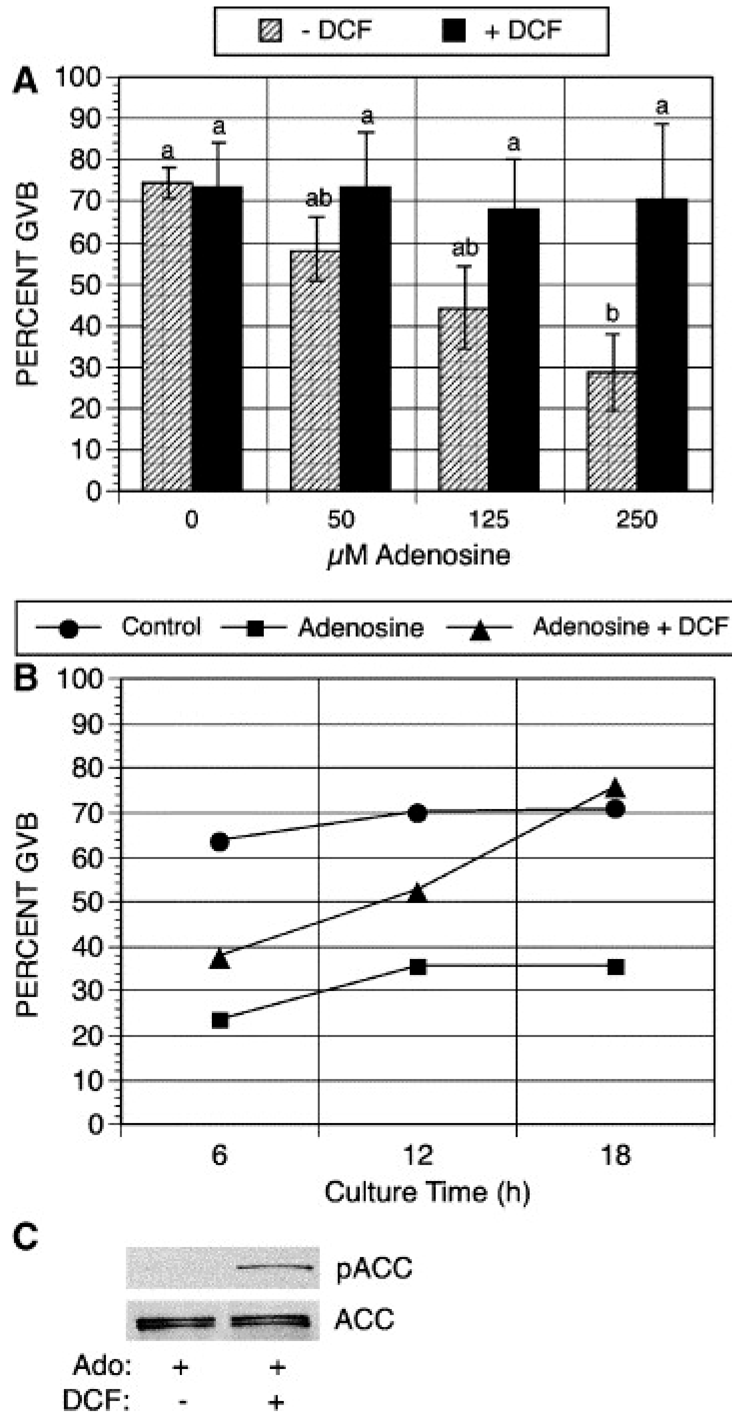

Fig. 8. DCF reversal of the inhibitory effect of adenosine on oocyte meiotic resumption. (A) DO were cultured in 150 µM dbcAMP or adenosine plus 250 µM DCF, with increasing concentrations of adenosine. After 17–18 h culture, oocytes were fixed and stained with Hoechst, and GVB was assessed under a fluorescent microscope. Groups with at least one identical letter are not significantly different. (B) Kinetics of DCF effects on adenosine-treated oocyte maturation. DO were cultured in 150 µM dbcAMP, dbcAMP plus 250 µM adenosine or dbcAMP plus adenosine and 250 µM DCF. Meiotic resumption was determined at the indicated times. (C) Western analysis of pACC in DCF-treated GV-

stage oocytes (250 oocytes per lane). DO were cultured 6 h in 150 µM dbcAMP, dbcAMP plus 250 µM adenosine or dbcAMP plus adenosine and 250 µM DCF.

Western blot analysis was next performed to assess the phosphorylation of ACC in oocytes treated with or without DCF, in the presence of adenosine. GV-stage oocytes were collected after 6 h of treatment with adenosine alone or with adenosine plus DCF. Little phosphorylation of ACC was observed in the adenosine alone group, but significant phosphorylation was stimulated upon addition of DCF (Fig. 8C).

To further clarify the effect of DCF on adenosine-treated oocytes, experiments were performed to measure oocyte AMP and ATP levels. In individual oocytes, ATP was measured in freshly isolated oocytes or oocytes cultured 5 h in control inhibitor-free medium or in medium containing adenosine alone, DCF alone or adenosine plus DCF. Fresh oocytes contained 3.55 mmol ATP per kg wet wt of oocyte, and this decreased to 2.5 mmol per kg after culture in control medium for 5 h (Fig. 9A). Adenosine or DCF alone had a modest stimulatory effect on ATP levels, but the combination of adenosine plus DCF profoundly increased ATP, even well above the levels observed in fresh oocytes. These results demonstrate that DCF treatment elicits a significant shift in adenosine metabolism towards phosphorylation.

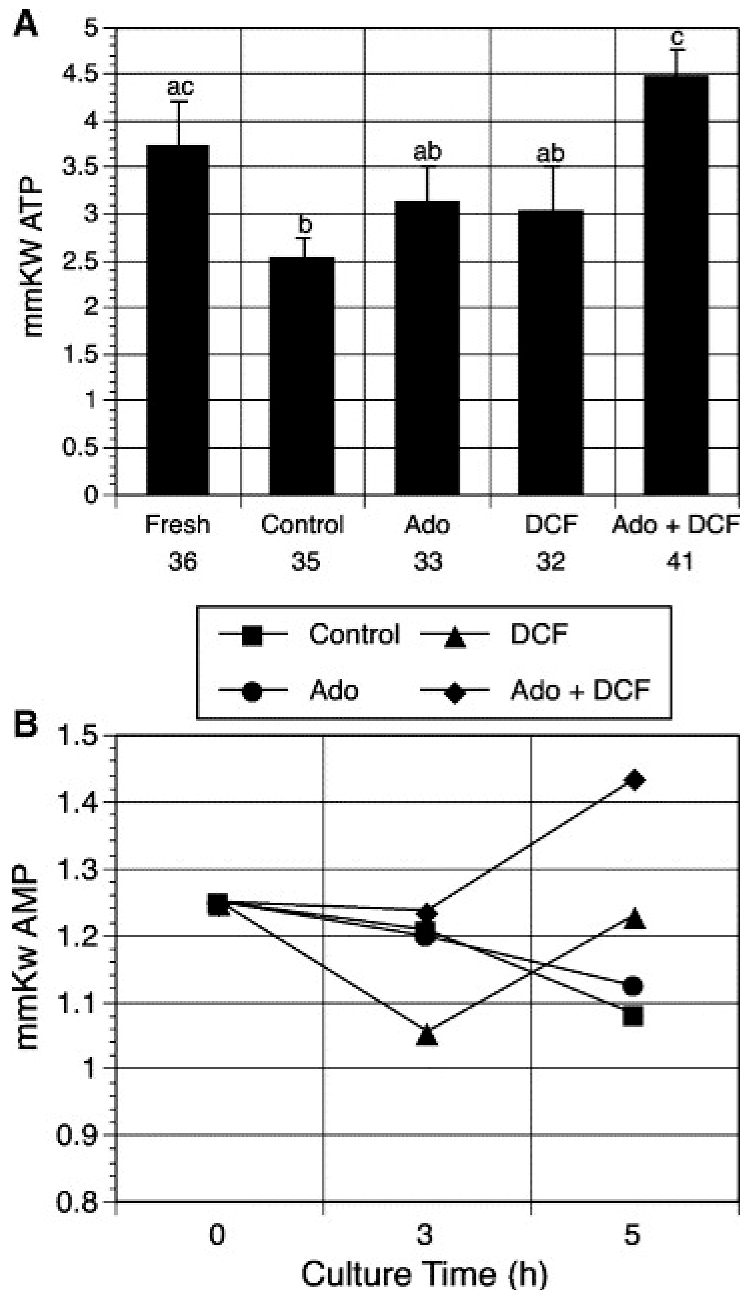

Fig. 9. AMP/ATP levels in DCF- and adenosine-treated oocytes. (A) ATP was measured in fresh DO or DO cultured 3 h in control medium or medium containing 250 µM adenosine, 250 µM DCF or adenosine plus DCF. The number of oocytes assayed in each group is given below each bar. Groups with at least one identical letter are not significantly different. (B) AMP was measured in fresh oocytes or in oocytes cultured 3 or 5 h in control medium or in medium containing 250 µM adenosine, 250 µM DCF or dbcAMP plus DCF. The number of oocytes assayed per treatment group ranged from 31 to 40.

AMP levels were next examined under the same culture conditions, with measurements taken at 0, 3 and 5 h. These results are shown in Fig. 9B. Fresh oocytes contained 1.25 mmol AMP per kg wet wt of oocyte. In control medium or in medium containing adenosine or DCF alone, AMP remained

unchanged or decreased after 3 and 5 h of culture. However, in the adenosine plus DCF group, AMP was significantly increased by 5 h. These results are consistent with AMP-induced activation of AMPK as the mechanism for the DCF-mediated reversal of adenosine-maintained meiotic arrest.

Rosiglitazone-induced meiotic resumption and AMPK activation

Rosiglitazone is an anti-diabetic agent that has recently been shown to activate AMPK by increasing the cellular AMP/ATP ratio (Fryer et al., 2002a). We therefore tested the effect of rosiglitazone on meiotic maturation and AMPK activation. Because preliminary attempts to treat dbcAMP-arrested oocytes with rosiglitazone produced inconsistent results on oocyte maturation, we report the actions of the drug on oocytes maintained in meiotic arrest with hypoxanthine. DO or CEO were cultured for 17–18 h in 4 mM hypoxanthine plus increasing concentrations of rosiglitazone before assessment of GVB. Although it did not affect oocyte maturation in CEO, rosiglitazone at 100 µM increased the frequency of meiotic resumption in DO from 61% to 79% (Fig. 10A). Higher concentrations of rosiglitazone were toxic to oocytes, increasing the level of cell death (data not shown) and inhibiting oocyte maturation. Western blot analysis revealed increased phosphorylation of ACC in GV-stage oocytes cultured for 2 h in 300 µM dbcAMP plus rosiglitazone compared to oocytes cultured in dbcAMP alone, with phosphorylation of ACC being greatest in the AICAR-treated oocytes (Fig. 10A).

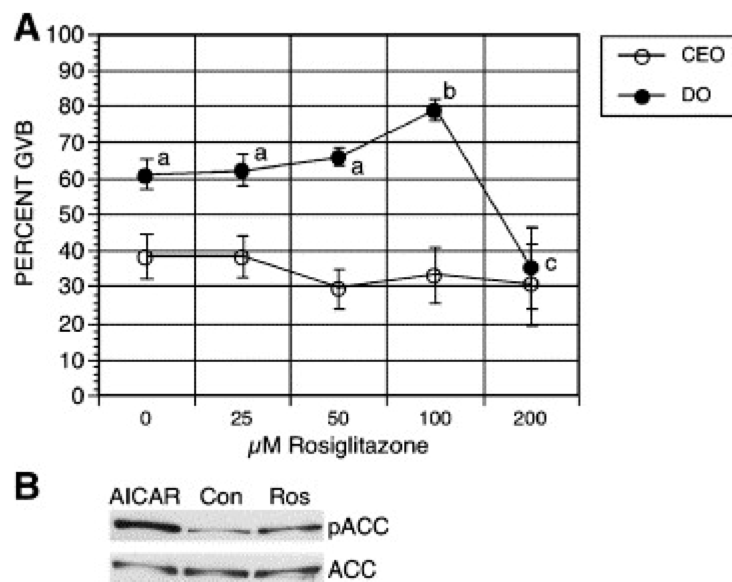

Fig. 10. Effects of rosiglitazone on meiotic resumption and ACC phosphorylation. (A) DO or CEO were cultured 17–18 h in 4 mM HX with increasing concentrations of rosiglitazone and then assessed for GVB. There was no significant difference between any of the CEO groups. Within the DO groups, a different letter denotes a significant difference. (B) Western analysis of GV-stage DO after 2 h of treatment with (Ros) or without (Con) 100 µM rosiglitazone (500 oocytes per lane). 500 µM AICAR-treated oocytes were shown as a positive control.

AMPK and spontaneous maturation

Two types of experiments were carried out to test for possible AMPK involvement in spontaneous maturation. In the first experiment, DO were held in meiotic arrest for 0.5 h with 300 µM dbcAMP in the presence or absence of 5 µM compound C. Oocytes were then washed free of dbcAMP, and cultures were continued for varying periods of time. As shown in Fig. 11A, compound C produced a significant delay in oocyte maturation. To determine if AMPK is activated in spontaneously maturing oocytes prior to GVB, DO were maintained in meiotic arrest with dbcAMP for 2 h, after which they were split into two groups, one remaining in dbcAMP for 0.5 h and the other washed free of dbcAMP and cultured for 0.5 h in control inhibitor-free medium. The 2-h preincubation assured a drop in phosphorylated ACC from fresh levels before the release from dbcAMP arrest. Extracts from GV-stage oocytes were then processed for pACC Western analysis. When oocytes were maintained in meiotic arrest for 2.5 h, very little phosphorylation of ACC was detected, whereas oocytes cultured in the absence of dbcAMP for the last 0.5 h exhibited a prominent pACC band, indicating active AMPK (Fig. 11B).

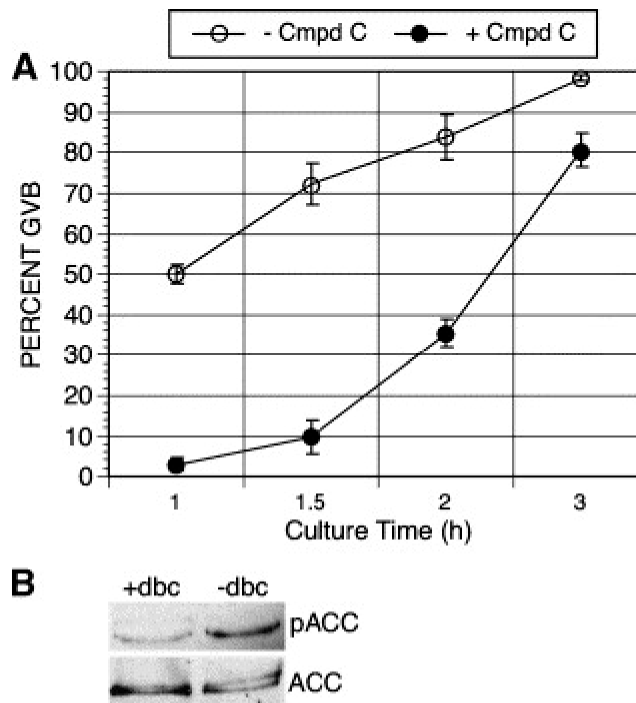

Fig. 11. AMPK involvement in spontaneous maturation. (A) DO were cultured 0.5 h in medium containing 300 µM dbcAMP with or without 5 µM compound C. They were then washed free of dbcAMP and cultured for varying periods up to 3 h in the presence or absence of 5 µM compound C before assessment of GVB. (B) Western analysis of GV-stage DO after 2 h preincubation in 300 µm dbcAMP followed by either 0.5 h in dbcAMP (+dbc) or 0.5 h in inhibitor-free control medium (−dbc) (250 oocytes per lane).

Discussion

In the present study, we present results that strongly support a causal role for AMPK in meiotic maturation. The new evidence may be summarized as follows:

(1) Microinjection of constitutively active, but not catalytically inactive, kinase into dbcAMP-arrested oocytes stimulated GVB.

(2) Both Western blot analysis and kinase assays indicated that AMPK activity was increased in AICAR-treated oocytes prior to meiotic resumption.

(3) Compound C and araA, two pharmacological inhibitors of AMPK, blocked AICAR-induced oocyte meiotic resumption and decreased the activity of AMPK in AICAR-treated, GV-stage oocytes, as judged by phosphorylation of ACC.

(4) Activation of AMPK by two additional means, i.e., rosiglitazone or adenosine plus DCF, also triggered meiotic maturation.

(5) AMPK activity increased prior to GVB in spontaneously maturing oocytes; moreover, compound C transiently blocked spontaneous maturation.

Eliciting GVB by microinjection of active, but not inactive, AMPK catalytic subunit demonstrates that AMPK can directly stimulate meiotic resumption. In these experiments, we used a constitutively active (T172D) mutant that would not be sensitive to the protein phosphatases that would be present in the oocytes. Microinjection of active kinase stimulated an increase in GVB of about 20%, while the meiotic induction achieved by AICAR was much higher. There could be a number of reasons why stimulation of GVB by active kinase may be limited compared with the effects of AICAR. For example, only the kinase domain of the α1 subunit was injected. The α1 and α2 isoforms appear to have rather similar specificity in solution (Woods et al., 1996), but the kinase domain represents only about 25% of the total sequence of an αβγ hetertrimer, and the α1 kinase domain may lack targeting sequences that direct the kinase to the substrate required for the effect on oocyte maturation. Despite the limited size of the effect, the ability of constitutively active AMPK to stimulate meiotic resumption strongly supports a positive role for the kinase in meiosis. In a previous study, we showed that AICAR, known to be a potent activator of AMPK in somatic cells, stimulated meiotic resumption and AMPK activation in mouse oocytes (Downs et al., 2002). In the present study, we provide evidence that the ability of AICAR to stimulate maturation is due to AMPK activation. Using both anti-PT172 and anti-pACC antibodies as probes, Western blot analysis revealed that AMPK was activated at the GV stage by AICAR, and this was confirmed by AMPK kinase assays using the SAMS peptide as substrate. While GVB is initiated by AICAR after more than 2 h of culture (Downs et al., 2002), AMPK is already activated within 1 h. Thus, AMPK activation precedes GVB, a finding consistent with its function as a meiotic inducer.

Further evidence supporting a causal role for AMPK in meiotic resumption was provided by experiments with inhibitors. Compound C was the first potent and selective inhibitor of AMPK to be described (Zhou et al., 2001), while araA, a precursor of araATP, has also been shown to competitively

suppress AMPK activity (Henin et al., 1996). Both inhibitors blocked AICAR-induced maturation in a dose-dependent fashion and prevented AMPK activation as assessed by phosphorylation of ACC. While the specificity of these inhibitors for AMPK may not be absolute, their ability to block both AICAR-induced maturation and AMPK activation, despite the fact that they inhibit AMPK via different mechanisms, lends credence to the idea that AMPK is a meiotic inducer.

One point of concern for compound C was the possibility that it might act by preventing AICAR uptake and accumulation of ZMP (Fryer et al., 2002a, Fryer et al., 2002b). Since AICAR is an adenosine analog, we tested the effects of compound C on adenosine uptake and its metabolism to ATP. Neither uptake of adenosine nor its stimulation of ATP production was adversely affected by compound C, thereby discounting a negative influence in ZMP generation. It should also be noted that compound C completely blocked meiotic induction by three different stresses, none of which involved metabolic processing of exogenous nucleoside (LaRosa and Downs, in press).

To determine if compound C exerts a non-specific inhibitory influence on meiosis, we tested its effect on okadaic acid (OA)-induced maturation. OA is an inhibitor of protein phosphatase 1 and 2A that has been shown to stimulate oocyte maturation in many species (Alexandre et al., 1991, Gavin et al., 1991, Schwartz and Schultz, 1991). Recent work shows that protein phosphatase 1 is the OA-sensitive protein phosphatase important in the regulation of the acquisition of meiotic competence, nuclear events during meiotic arrest and GVB in mouse oocytes (Wang et al., 2004). OA is involved in regulation of protein kinase B (PKB) activity, which plays a role in CDK1 activation and meiotic resumption in mouse oocytes (Kalous et al., 1999). In mouse oocytes, OA is a potent inducer of meiosis, stimulating GVB within 1.5 h in dbcAMP-arrested oocytes, with induction completed by 3 h (LaRosa and Downs, 2005). In this study, compound C had no inhibitory effect on OA-induced maturation during overnight culture, and only marginally attenuated the kinetics of OA-induced maturation, thereby demonstrating specificity in the inhibitory actions of the drug. Moreover, Western blot analysis revealed that, although GV-stage oocytes after 1.5 h of OA treatment contained relatively higher amounts of phosphorylated ACC compared to control oocytes cultured in dbcAMP alone, these levels were still lower than in AICAR-treated oocytes. These results provide evidence that OA treatment may lead to some degree of AMPK activation but that such activation is not necessary for OA-induced maturation.

Despite AICAR serving as an effective stimulator of both AMPK activation and meiotic maturation, it remained possible that it could influence meiosis by means unrelated to AMPK activation (Kemp et al., 1999). It was therefore important to employ additional strategies for generating active kinase and relating that activity to meiotic status. To achieve this, we utilized two different treatments: (1) adenosine plus DCF and (2) rosiglitazone. DCF, a potent adenosine deaminase inhibitor, reversed the inhibitory effect of adenosine on oocyte maturation in CEO (Downs, 1999, Downs et al., 2002). We hypothesized that, by blocking adenosine deaminase, adenosine metabolism was diverted towards phosphorylation, resulting in AMP accumulation, activation of AMPK and meiotic induction (Downs et al., 2002). In the present study, we confirmed that there was a stimulatory action of DCF on oocyte maturation and showed that AMPK activity was, indeed, high prior to GVB. It was also shown that DCF caused a diversion of adenosine towards the phosphorylation pathway and a subsequent accumulation of adenyl nucleotides. A buildup of oocyte AMP in the presence of DCF was consistent with activation of AMPK and stimulation of GVB. Interestingly, increases in both AMP and ATP were observed in

oocytes treated with adenosine and DCF. However, as the affinity of the CBS domains on the γ subunit for AMP is higher than that for ATP (Scott et al., 2004), a small increase in AMP should displace the ATP and increase the kinase activity. We propose that the elevated AMP in oocytes is sufficient to overcome the inhibitory effect of ATP on AMPK. Whether alterations in oocyte AMP levels normally contribute to the signal triggering meiotic resumption in vivo remains to be determined.

We also tested the effect of the anti-diabetic thiazolidinedione drug rosiglitazone on oocyte maturation and AMPK activation. This drug is an inhibitor of complex I of the respiratory chain (Brunmair et al., 2004) and has been reported to increase the AMP/ATP ratio with concomitant activation of AMPK in a muscle cell line (Fryer et al., 2002a). Rosiglitazone produced a small, but significant, increase in GVB in HX-arrested DO, whereas it had no effect on meiotic induction in CEO. The poor potency of rosiglitazone in meiotic induction when compared to AICAR may be due to a weaker stimulation of AMPK in mouse oocytes since GV-stage oocytes after 2 h of treatment with rosiglitazone exhibited less intense phosphorylation of ACC than AICAR-treated oocytes. It should also be noted that rosiglitazone at high concentrations is toxic to oocytes. We suspect that this may be due to a reduction of ATP concentrations below the threshold required to maintain oocyte viability. Thus, there appears to be a narrow window of rosiglitazone concentrations in which a small drop in ATP leads to a weak activation of AMPK and a small effect on GVB but that at higher concentrations any effect on meiotic resumption is prevented by loss of viability.

Surprisingly, freshly isolated DO or OCC contained a high level of phosphorylation of AMPK on Thr-172 and ACC on Ser-79, indicating a high AMPK activity, and this was confirmed by directly assaying oocyte extracts for AMPK activity. However, this activity was greatly reduced within 1 h of culture. AMPK is a stress-activated enzyme, and even gentle physical manipulation can stimulate the kinase (Hardie et al., 2000a, Hardie et al., 2000b). It therefore seems likely that the process of oocyte isolation, involving ovary manipulation, piercing follicles with needles, oocyte denudation and pipetting could contribute to the high level of active AMPK in fresh oocytes. Yet, we cannot discount the possibility that oocyte AMPK activity is high at the time of isolation and that AICAR and the other meiosis-inducing stimuli used in this study act to maintain an already active kinase. Work is in progress to address this issue.

Two lines of evidence suggest that AMPK is involved also in spontaneous maturation. First, compound C suppressed spontaneous GVB at a concentration that was also effective on meiotic induction; second, AMPK activity increased prior to GVB in spontaneously maturing DO, as indicated by pACC Western analysis. While these results support the participation of AMPK, they do not establish whether AMPK activity is essential for spontaneous maturation, particularly since the arrest by compound C was transient.

The primary purpose of the present study was to establish whether AMPK can stimulate meiotic resumption in mouse oocytes. Although the methods employed to activate AMPK may not have mimicked signals occurring in vivo, the results nevertheless provide compelling evidence that AMPK is a potent inducer of maturation. Current research is aimed at determining what physiological role, if any, AMPK plays in the control of oocyte maturation. Although the data indicate involvement in both spontaneous maturation and meiotic induction, the necessity for AMPK participation in meiotic regulation remains to be demonstrated.

Acknowledgments

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