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Suppression of Chemically Induced and Spontaneous Mouse Oocyte Activation by AMP-Activated Protein Kinase[1]

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Abstract: Oocyte activation is an important process triggered by fertilization that initiates embryonic development. However, parthenogenetic activation can occur either spontaneously or with chemical treatments. The LT/Sv mouse strain is genetically predisposed to spontaneous activation. LT oocytes have a cell cycle defect and are ovulated at the metaphase I stage instead of metaphase II. A thorough understanding of the female meiosis defects in this strain remains elusive. We have reported that AMP-activated protein kinase (PRKA) has an important role in stimulating meiotic resumption and promoting completion of meiosis I while suppressing premature parthenogenetic activation. Here we show that early activation of PRKA during the oocyte maturation period blocked chemically induced activation in B6SJL oocytes and spontaneous activation in LT/SvEiJ oocytes. This inhibitory effect

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was associated with high levels of MAPK1/3 activity. Furthermore, stimulation of PRKA partially rescued the meiotic defects of LT/Sv mouse oocytes in concert with correction of abnormal spindle pole localization of PRKA and loss of prolonged spindle assembly checkpoint activity. Altogether, these results confirm a role for PRKA in helping sustain the MII arrest in mature oocytes and suggest that dysfunctional PRKA contributes to meiotic defects in LT/SvEiJ oocytes.

Keywords: AMP-activated protein kinase, chemically induced activation, LT/SvEiJ strain, mouse, oocyte, spontaneous activation

Introduction

Mammalian oocytes initiate meiosis during embryonic development and become arrested at the dictyate stage of prophase I around the time of birth. Following a midcycle gonadotropin stimulus, oocytes within preovulatory follicles resume meiosis, complete maturation, and are ovulated at the metaphase II (MII) stage, where they remain until fertilization. Meiosis is driven by M-phase promoting factor (MPF), a dimer containing catalytic CDK1 and regulatory cyclin B1 subunits; MPF activity drops during polar body extrusion, then rises and stays elevated during MII arrest [1]. Binding of sperm triggers activation of the mature ovum, a series of events that includes calcium oscillations, completion of meiosis, release of cortical granules, and initiation of embryonic development [2, 3]. Calcium signaling is a universal component of fertilization in the animal kingdom [4]; in vertebrates, calcium oscillations are an essential feature of egg activation and trigger release from MII arrest [5, 6]. This arrest is controlled by cytostatic factor (CSF), which involves stabilization of maturation promoting factor by the c-Mos/MAPK1/3 pathway and suppression of the anaphase promoting complex (APC) by early mitotic inhibitor 2 (Emi2) [7]. Indeed, interrupting the c-Mos/MAPK1/3 pathway leads to loss of MPF activity and premature egg activation [8–10]; in addition, knockdown of Emi2 allows degradation of cyclin B of MPF and drives eggs into anaphase II [11, 12], mimicking the effect of sperm-induced calcium oscillations [13, 14].

Oocyte aging, chemical treatment, and the intrinsic quality of the oocyte are factors that can lead to mouse egg activation in the absence of sperm [15]. It is well known that freshly ovulated oocytes are resistant to activation but become prone to activation with

increased age and tenure in the oviducts [16–18]. Chemical treatments such as calcium ionophore [19, 20], phorbol ester [20–22], protein synthesis inhibitors [23, 24], ethanol [25–27], and heavy metals [28, 29] are well-characterized inducers of parthenogenesis in oocytes matured both in vivo and in vitro. There are strain-dependent differences in oocyte susceptibility to spontaneous maturation [30], while altering metabolism or length of hormonal priming can induce parthenogenesis in strains that normally exhibit very low rates of activation [31, 32].

The LT/Sv strain is an inbred recombinant mouse line derived from C58 and BALB strains whose oocytes fail to complete meiosis, leading to arrest at metaphase I [33–36], due to persistent MPF activity and a poor anaphase I trigger [36–39]. This MI arrest is associated with premature activation of oocytes within the ovary and a high incidence of ovarian teratomas [40–42]. The parthenotes advance to the egg cylinder stage, but further development is disrupted, with formation of a disorganized mass of differentiated cell types [40]. A significant number of LT/Sv oocytes are ovulated at the metaphase I stage, and this arrest appears necessary but not sufficient to induce parthenogenetic activation [43, 44]. Apparently, the protracted MI arrest brings about changes within the oocyte that predispose it to activation. The exact mutation(s) of the LT/Sv mouse remains unknown, but evidence suggests that the phenotype of LT/Sv oocytes is controlled by multiple alleles [42, 43].

We have previously demonstrated a role for AMP-dependent protein kinase (PRKA) in mouse oocyte maturation. This kinase is a critical regulator of metabolism and cell cycle that responds to changes in stress and energy deficit [45]. The activation of PRKA in mouse oocytes not only induces meiotic resumption [46–48] but also promotes first polar body (PB) formation [32]. Active PRKA localizes to chromosomes and the meiotic spindle poles during maturation [32], and this association pattern is dependent on spindle/microtubule integrity [49]. Moreover, blocking PRKA activity with the specific inhibitor compound C induces premature egg activation, whereas treatment with the PRKA activator AICAR suppresses the activation that occurs during spontaneous maturation of oocytes from unprimed and 1-day-primed mice [32]. This latter finding implicated PRKA in the regulation of activation in mouse oocytes.

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In this study, we further examined the relationship between PRKA and oocyte activation. We report that PRKA activators suppress both chemically induced activation in oocytes from C57BL/6J×SJL F_1 (B6SJL) mice and spontaneous activation in oocytes from LT/SvEiJ mice during maturation in vitro but are less effective in ovulated B6SJL oocytes. This protection by PRKA was manifest during the oocyte maturation period and was associated with high levels of MAPK1/3 activity and the accelerated loss of the spindle assembly checkpoint protein MAD2. Stimulation of PRKA in LT/SvEiJ oocytes rescued the "LT phenotype" by reducing the rate of parthenogenetic activation and promoting anaphase onset and PB formation. These data support a multifunctional role of PRKA in mouse oocytes and suggest that PRKA dysfunction may contribute to meiotic defects in LT/SvEiJ oocytes.

Materials and Methods

Chemicals

Unless otherwise noted, chemicals were supplied by Sigma-Aldrich Co. (St. Louis, MO). AICAR was from Toronto Research Chemicals, Inc. (North York, Ontario, Canada) and amphiregulin from R&D Systems (Minneapolis, MN). Highly purified ovine FSH was obtained from the National Hormone and Peptide Program and Dr. A.F. Parlow. Cy3-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmmoResearch (West Grove, PA), Anti-phospho PRKA (PT172) antibody from Cell Signaling Technology (Beverly, MA), and rabbit anti-ERK1/2 and pERK1/2 (MAPK1/3) antibodies from Sigma. Anti-MAD2 antibody was a kind gift from Dr. E.D. Salmon (University of North Carolina).

Oocyte Isolation and Culture Condition

All procedures were approved by the Marquette University Institutional Animal Care and Use Committee. Immature, 20- to 23-day-old C57BL/6J×SJL/J F_1 (B6SJL) and LT/SvEiJ female mice were used for all experiments. Mice were primed with 5 IU equine chorionic gonadotropin (eCG), and oocytes were isolated 2 days later. For superovulation, 5 IU human chorionic gonadotropin (hCG) were administered IP 44–48 h post-eCG, and oocytes were isolated at

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varying times thereafter. Mice were killed by cervical dislocation, and either ovaries or oviducts were removed and placed in a Petri dish containing culture medium. Cumulus cell-enclosed oocytes (CEO) were isolated by puncturing the follicles with sterile needles. Denuded oocytes (DO) were obtained by stripping cumulus cells with a mouth-operated small-bore pipette. Oviducts were punctured with sterile needles to release the expanded cumulus masses, which were then treated briefly with hyaluronidase to remove cumulus cells.

Both CEO and DO were washed twice and transferred to polystyrene round-bottom culture tubes (BD Falcon, Durham, NC) containing 1 ml of the appropriate test medium. The culture medium used was Eagle minimum essential medium (MEM) supplemented with penicillin, streptomycin sulfate, 0.23 mM sodium pyruvate, and 3 mg/ml lyophilized crystallized bovine serum albumin (MP Biomedicals, Solon, OH). Calcium was omitted from the medium when activation was stimulated with strontium or calcium ionophore A23187.

Chemical Activation

Figure 1 depicts the three activation schemes used in this study. CEO or DO were cultured 17 h in control MEM prior to treatment with chemical activators. Cumulus cells were removed from CEO at the end of this initial culture period so that only DO received the activation stimulus. The three activation regimens are as follows:

Activation Protocols

	First Culture		Second Culture		Third Culture
A. Strontium	17 h MEM	---->	2.5 h Strontium	---->	3.5 h MEM
B. Ethanol	17 h MEM	---->	7 min EtOH	---->	6 h MEM
C. Ion/Pur	17 h MEM	---->	5 min Ion	---->	6 h Pur

FIG. 1. Schematic diagram of activation protocols. For all treatment regimens, CEO or DO were first cultured 17 h in control medium to permit completion of maturation. When appropriate, cumulus cells were then removed from CEO, and DO were exposed to chemical activating conditions. A) Oocytes were treated with strontium in calcium-free MEM for 2.5 h, washed, and then transferred to control MEM for 3.5 h. B) Oocytes were exposed to 7% ethanol for 7 min, washed, and then transferred to control MEM for 6 h. C) Oocytes were treated with A23187 for 5 min, washed, and then transferred to puromycin (Pur) for 6 h.

1. *Strontium*. 2.5 h in calcium-free MEM containing 1 mM strontium, wash, and 3.5 h culture in control MEM.

2. *A23187/puromycin*. 5 min in control MEM containing 5 μM calcium ionophore A23187, wash, and 6 h culture in control MEM containing 10 μg/ml puromycin.
3. *Ethanol*. 7 min in control MEM containing 7% EtOH, wash, and 6 h culture in control MEM.

CEO or DO were exposed to PRKA activators (0.2 mM AICAR, 2 mM AMP, or 2 μM RSVA405) during meiotic maturation (prematuration) or during/after the activation procedure (postmaturation). CEO were hormonally stimulated with 0.1 μg/ml FSH or 50 μg/ml amphiregulin during meiotic maturation. Oocytes were considered to be activated if they contained a pronucleus or two polar bodies with or without pronucleus or had undergone cleavage.

Immunofluorescent Staining

Cumulus cell-free oocytes were fixed with 4% formaldehyde in PBS at 4°C overnight. They were then permeabilized 30 min with 0.1% Triton-100 in blocking solution, which contained 10% donkey serum and 0.5 mg/ml saponin in PBS, followed by 1 h in blocking solution. Oocytes were incubated with primary antibody (1:100) overnight at 4°C (either rabbit anti-pPRKA^{Thr172} or rabbit anti-MAD2 antibody) and washed four times at room temperature in blocking buffer. This was followed by 1 h incubation with FITC-conjugated mouse anti-tubulin antibody (1:100) and Cy3-conjugated donkey anti-rabbit IgG antibody (1:100) at room temperature. Some oocytes were stained for actin using Alexa 568 Fluor phalloidin (Life Technologies, Grand Island, NY) during the secondary antibody culture period. Oocytes were washed four times, placed on slides, and mounted with medium containing DAPI (Vector Laboratories, Burlingame, CA).

Confocal Microscopy

Oocytes were observed on a laser scanning confocal microscope (Carl Zeiss Co., Thornwood, NY) with a 63× objective. During scanning, all settings were kept constant (i.e., laser power, detector gain, amplifier offset, amplifier gain, and pinhole size). Digitally recorded images were exported by LSM Examiner software (Carl Zeiss).

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Western Blot Analysis

Oocyte samples were washed with PBS/PVP, then twice with protease inhibitor cocktail (Roche, Indianapolis, IN). Samples were lysed by Laemmli buffer with 20% beta-mercaptoethanol at 95°C for 5 min. Electrophoresis was carried out using NuPAGE 4–12% Bis-Tris Gels (InVitrogen, Carlsbad, CA), and proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk, followed by incubation with rabbit anti-pERK1/2 (pMAPK1/3) antibody (1:1000) at 4°C overnight. Blots were rinsed twice with TBS (pH 7.4) and once with TBS-Tween-20 (0.05%), followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody (1:5000 in 5% nonfat milk; Thermo Scientific Pierce, Rockford, IL) at room temperature for 1 h. After washing, protein signals were detected by super signal west pico chemiluminescent substrate (Thermo Scientific Pierce). Blots were stripped with restore Western blot stripping buffer (Thermo Scientific Pierce) at room temperature for 15 min and reprobed with rabbit anti-MAPK1/3 antibody (1:1000). The pMAPK1/3:MAPK1/3 ratios were quantified with ImageJ software based on protein band density.

Statistical Analysis

All experiments were repeated at least three times and data presented as mean ± SEM. Percentages of activation, polar body, or MAD2 staining underwent arcsin transformation, and data were analyzed statistically by analysis of variance followed by the Duncan multiple range test. Student *t*-test was used to compare paired treatments in some experiments. A P value < 0.05 was considered significant.

Results

Effects of PRKA Activators on Chemically Induced Activation in B6SJL Oocytes

We have previously shown that the PRKA activator AICAR prevents premature activation in spontaneously activating oocytes from B6SJL mice when present during meiotic maturation [32]. To test

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if PRKA stimulation would also block activation induced by chemical treatments, we adopted three different protocols depending on the agent(s) used to stimulate activation: strontium, A23187/puromycin, or ethanol. For all three treatments, CEO or DO were cultured 17 h in control MEM to allow for meiotic maturation. Remaining cumulus cells were then removed from CEO, and these cumulus cell-free oocytes as well as the mature DO were exposed to chemical activating conditions (Fig. 1).

Strontium-induced activation.

The first series of experiments utilized the activating agent strontium, and cultures were carried out in calcium-free medium since the absence of calcium has previously been demonstrated to augment activation by this agent [50]. After removing cumulus cells from matured CEO, oocytes were exposed to 1 mM strontium in calcium-free MEM for 2.5 h, followed by 3.5 h incubation in control, calcium-containing medium. In control oocytes not exposed to strontium, only 4% were activated compared to 70% activation in strontium-treated oocytes (Fig. 2A). When 200 μM AICAR were included in both the activation and the postactivation media, a modest reduction in the activation percentage was observed (57%); however, when supplementation of AICAR was restricted to the initial 17 h oocyte maturation period, prior to strontium treatment, activation was reduced to 15% (Fig. 2A). In the next experiment, the direct effects of AICAR were tested on strontium activation of matured DO, utilizing the same protocol that was used with CEO. In the absence of cumulus cells, no activation was observed in the control group, but strontium stimulated 34–35% of the oocytes to activate. AICAR blocked strontium-induced activation, and the effect of exposure during meiotic maturation was comparable to that when exposure followed maturation (Fig. 2A).

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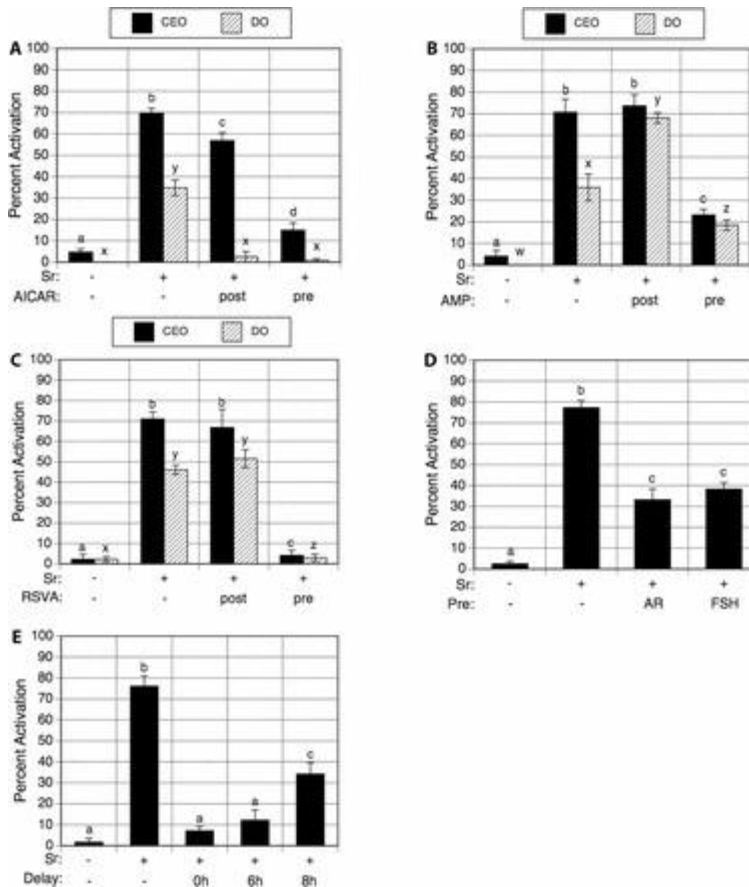

FIG. 2. Effect of PRKA stimulators and hormones on strontium-induced activation. Treatment with AICAR (A), AMP (B), or RSVA405 (C) during oocyte maturation (prematuration) prevented strontium-induced activation in both CEO and DO, but supplementation after the 17-h maturation period (postmaturation) was relatively ineffective. D) Amphiregulin (AR) or FSH suppressed strontium-induced activation in CEO when present during the initial maturation period. E) Effect of delaying AICAR treatment on strontium-induced activation. The inhibitory effect of AICAR on activation in CEO was decreased when AICAR treatment was initiated 8 h but not 6 h after the start of culture. All data are presented as mean percent activated ± SEM. Groups with no common letter are significantly different (CEO, a–d; DO, w–z).

AICAR is an analog of AMP, and when the experiment was repeated with native AMP, similar results were obtained. Exposure to AMP during oocyte maturation reduced the activation rate in CEO from 71% to 23% (compared to 4% in controls), whereas exposure to AMP postactivation had no effect (Fig. 2B). When the experiment was repeated in DO, a similar suppression of activation was obtained if AMP treatment occurred during the maturation period; however, surprisingly, treatment postmaturation with AMP increased the level of activation (Fig. 2B).

A small-molecule PRKA activator has recently been described that is an analog of resveratrol, RSVA405 [51]. Supplementary experiments demonstrated that, like AICAR, RSVA405 promoted GVB and polar body formation, and its action was suppressed by the PRKA inhibitor compound C (Supplemental Fig. S1, available online at www.biolreprod.org). We therefore repeated the strontium activation experiments, using RSVA405 as the PRKA activator. Consistent with the AICAR and AMP results, RSVA405 suppressed strontium-induced activation in both CEO and DO at concentrations that stimulate meiotic resumption and polar body formation but was effective only when present during the initial maturation period (Fig. 2C).

Both FSH and the EGF-like peptide amphiregulin (AR) are potent stimulators of meiotic maturation of mouse CEO in vitro, with downstream activation of oocyte PRKA preceding GVB [48]. We predicted that, because of their PRKA-stimulating capability, treatment of CEO with either of these peptides would block strontium-induced activation. For this experiment, FSH and AR were present only during the initial maturation period. These results are shown in Figure 2D. As predicted, FSH and AR reduced the activation rate, from 77% to 38% and 33%, respectively.

Since the data indicate that early activation of PRKA within the oocyte is important for its protective effect on activation, an experiment was conducted in which exposure of CEO to AICAR was delayed during the maturation period to determine the required temporal exposure to achieve maximum protection. There was no loss of protection when exposure to AICAR was delayed by 6 h; however, delaying AICAR treatment by 8 h reduced its protective effect by 27% (Fig. 2E). This suggests that maximum protection requires exposure within 6 h of the initiation of culture, a time point corresponding to the early stages of spindle formation.

Since the cMos-MAPK1/3 pathway is important for maintaining MII arrest [8–10], we tested the effect of PRKA activation in CEO on MAPK1/3 activity following strontium activation. Oocytes were collected for Western blot at 17 h (after maturation), 19.5 h (poststrontium treatment), and 23 h (after final recovery). As shown in Figure 3, control oocytes that were treated with calcium-free medium alone maintained a high level of MAPK1/3 activity at all three

time points. In contrast, oocytes that were treated with strontium exhibited decreased MAPK1/3 activity after the second and third culture periods. Consistent with their preventive effect on activation, both AMP- and AICAR-treated oocytes maintained high MAPK1/3 activity at all time points tested.

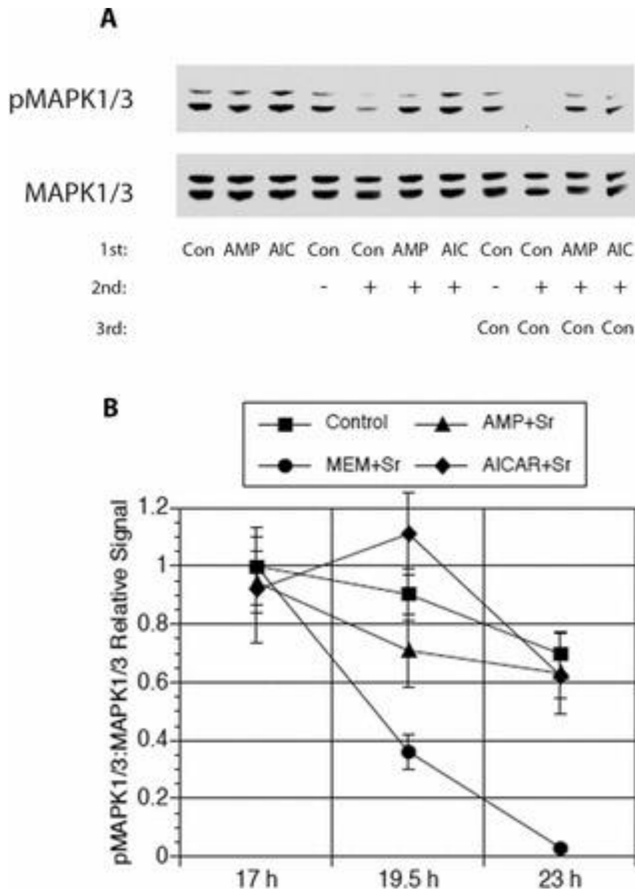

FIG. 3. Maintenance of MAPK1/3 activity in oocytes treated with AICAR (AIC) or AMP during maturation. A) Representative Western blot analysis of p-MAPK1/3 in oocytes treated as described in Figure 1A. Denuded oocyte extracts were subjected to Western blot analysis at the end of each of the three treatment phases. +/− in the second culture indicates the presence or absence of strontium. Con, control. B) Quantitative analysis of pMAPK1/3 signals from Western blot, presented as a ratio of the band intensity of pMAPK1/3 to that of nonphosphorylated MAPK1/3. Mean ± SEM of three blots. Treatments with either AICAR or AMP maintained high pMAPK1/3 levels that were comparable to the control group. Data are presented as mean ratio ± SEM.

A23187/puromycin-induced activation.

CEO were used to test the ability of AICAR to reverse the activation resulting from sequential treatment of calcium ionophore A23187 followed by the protein synthesis inhibitor puromycin. Mature

oocytes were cultured 5 min in 5 μM calcium ionophore A23187 followed by 6 h in 10 μg/ml puromycin (Fig. 1). In the control group treated with ionophore alone, only 12% of the oocytes underwent activation (Fig. 4A). When ionophore-treated oocytes were subsequently exposed to puromycin, the activation rate was increased to 66%. If oocytes were exposed to AICAR during puromycin exposure, there was no significant reduction in activation (43%); however, a significant decrease occurred when AICAR was present during the initial maturation period (31% activation; Fig. 4A).

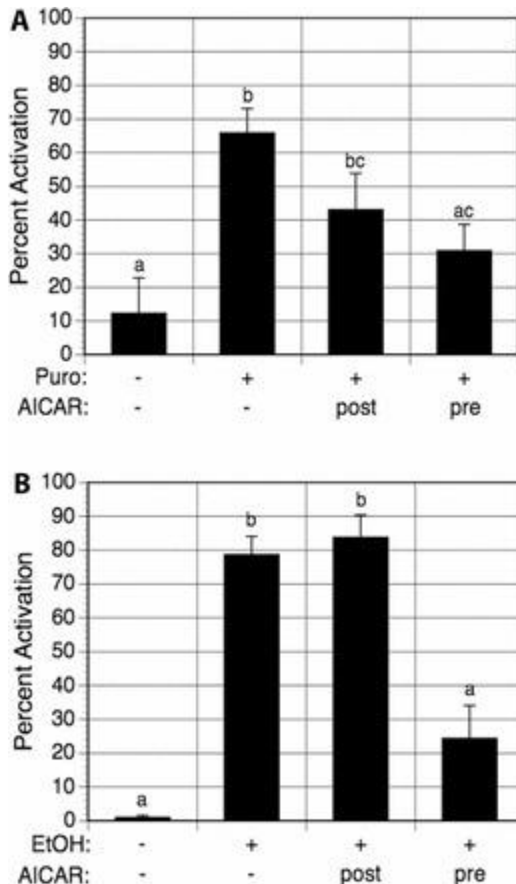

FIG. 4. Treatment with AICAR during but not following oocyte maturation blocked A23187/puromycin-induced (A) and ethanol-induced (B) activation in CEO. Data are presented as mean percent activated ± SEM. Groups with no common letter are significantly different.

Ethanol-induced activation.

CEO were used to test the effect of AICAR on ethanol-induced activation. When oocytes were exposed for 7 min to 7% ethanol

followed by 6 h culture in control medium (Fig. 1), 78% of the oocytes underwent activation compared to 1% of controls (Fig. 4B). If CEO were treated with AICAR during the initial maturation period, ethanol-induced activation was reduced by 54%, while restricting AICAR treatment to the last 6 h had no effect.

Effect of PRKA Activation on In Vivo-Aged B6SJL Oocytes

It has been well documented that increased aging of ovulated oocytes within the oviduct increases their susceptibility to spontaneous activation [16–18]. This was confirmed when eggs were retrieved from the oviducts of superovulated B6SJL mice 16, 20, and 24 h post-hCG and cultured 6 h in control medium. The percentage of activation increased in direct relationship to the time post-hCG the eggs were retrieved (2%, 12%, and 32% activation, respectively); there was a modest trend for suppression of activation at all time points when AICAR was present during 6 h culture (0%, 8%, and 22% activation, respectively; Fig. 5).

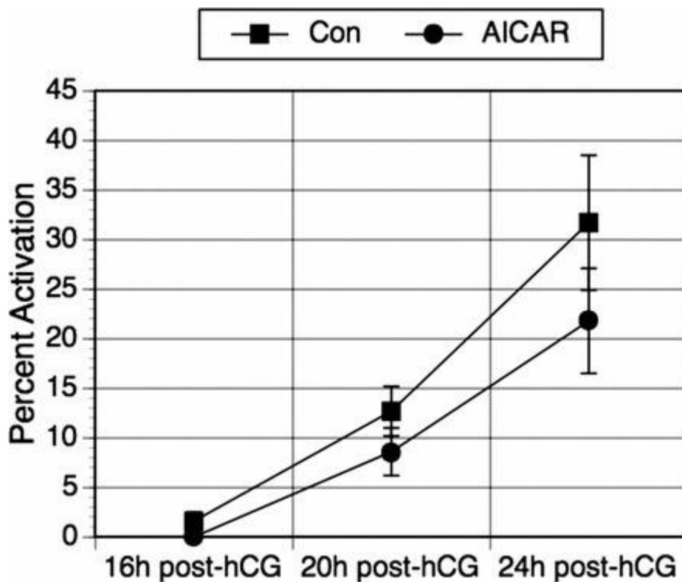

FIG. 5. Effect of PRKA on activation of in vivo-matured oocytes. Oocytes were retrieved from oviducts 16, 20, and 24 h post-hCG and cultured 6 h with or without AICAR. Although there was a trend toward less activation in AICAR-treated oocytes, differences were not significant. Data are presented as mean percent activated ± SEM.

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Effects of PRKA Activators on Oocyte Activation in the LT/SvEiJ Strain

Oocytes from the LT/Sv mouse strain exhibit a high rate of spontaneous activation [43]. It was therefore important to determine if PRKA activators would also suppress this type of oocyte activation. We initially compared spontaneous activation rates in oocytes from B6SJL and LT/SvEiJ (hereafter designated LT) mice following 30 h culture in the presence or absence of AICAR. In the absence of AICAR, activation rates were 2% and 21%, respectively, while AICAR reduced these rates to 0% and 4% (Fig. 6A). When LT CEO were treated with RSVA405, a similar reduction in activation was observed (Fig. 6B).

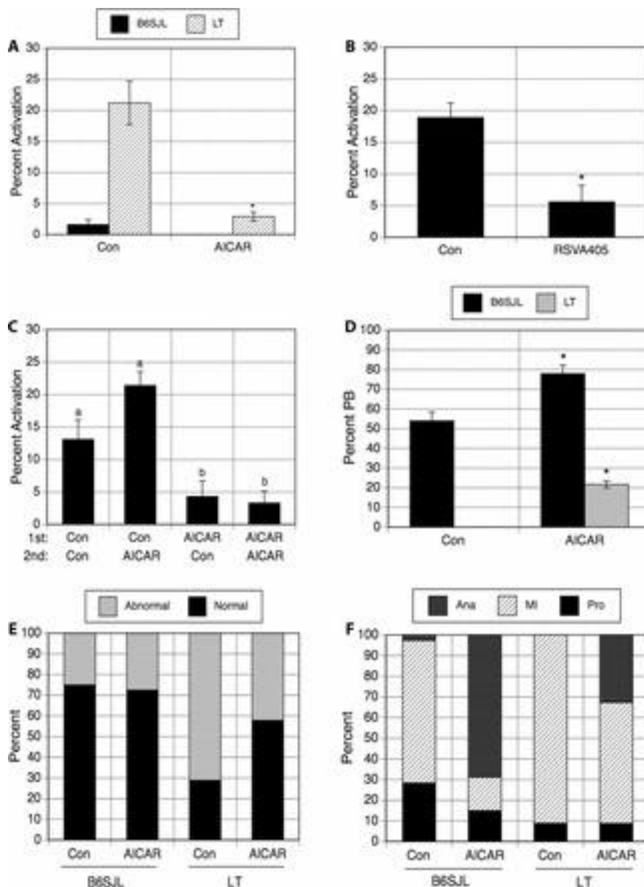

FIG. 6. Effects of PRKA stimulation on LT/SvEiJ oocytes. A) AICAR blocked spontaneous activation in LT oocytes during 30 h cultures. B) RSVA405 had a similar inhibitory action on LT activation. Asterisks in A and B denote a significant difference from controls (Con). C) AICAR blocked activation in LT oocytes when present during but not after oocyte maturation. LT/Sv oocytes were first cultured in control MEM (Con) or AICAR for 17 h and then transferred to control or AICAR-supplemented

medium for another 13 h before activation was assessed. A different letter denotes a significant difference. D) AICAR stimulated PB formation in both B6SJL and LT oocytes during 17 h cultures. An asterisk denotes a significant difference from controls (Con). Data in A–D are presented as percent activation or PB ± SEM. E and F) pPRKA (PT172) localization (E) and stage of meiosis (F) in MI stage oocytes after 8 h of culture. AICAR increased the percentage of normal PT172 spindle pole localization in LT oocytes and increased the percentage of anaphase I (Ana) in oocytes from both the B6SJL and LT strains. Pro, prometaphase.

To determine the temporal window for AICAR exposure that protects the LT oocyte from activation, oocytes were first cultured in control medium or medium containing AICAR for 17 h, then transferred to control MEM or AICAR-supplemented medium for an additional 13 h before activation was assessed. As shown in Figure 6C, high levels of activation were observed in the control/control or control/AICAR oocyte groups, while significantly lower levels of activation were observed in the AICAR/AICAR and AICAR/control groups. These results are consistent with earlier experiments in B6SJL oocytes showing a protective effect of AICAR during the initial period of oocyte maturation.

In addition to high rates of spontaneous activation, LT oocytes also fail to progress past metaphase I both in vivo and during spontaneous maturation in vitro [33, 38, 52]. Thus, we tested whether PRKA stimulation could overcome this meiotic defect. CEO from B6SJL and LT mice were cultured in either control or AICAR-containing medium for 17 h and assessed for polar body formation. In B6SJL oocytes, 53% formed polar bodies in control medium, while AICAR increased this number to 78% (Fig. 6D). No polar bodies were observed in LT oocytes cultured in control medium, but 22% of AICAR-treated oocytes extruded a polar body.

When LT oocytes were stained for active PRKA after 17 h culture in control medium, we observed a high incidence of abnormal localization. Instead of staining at both spindle poles, which is the typical staining pattern in B6SJL oocytes, in most LT oocytes the localization was either shifted away from the poles or absent at one or both of the poles (data not presented). Since LT oocytes are arrested at MI for a prolonged period of time, to eliminate the possibility that this phenotype was due to an aging effect, we stained oocytes for active PRKA after 8 h in both B6SJL and LT strains, when MI has been freshly established, and compared MI localization patterns between the

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two strains. Seventy-five percent of B6SJL MI-stage oocytes cultured in control medium exhibited active PRKA at both spindle poles (Figs. 6E, E,7A).7A). However, only 28% of LT MI-stage oocytes had similar bipolar staining, (Figs. 6E, E,7B,7B, and 7C). Treatment with AICAR had no effect in B6SJL oocytes but doubled the percentage of LT oocytes with normal PRKA staining (57%).

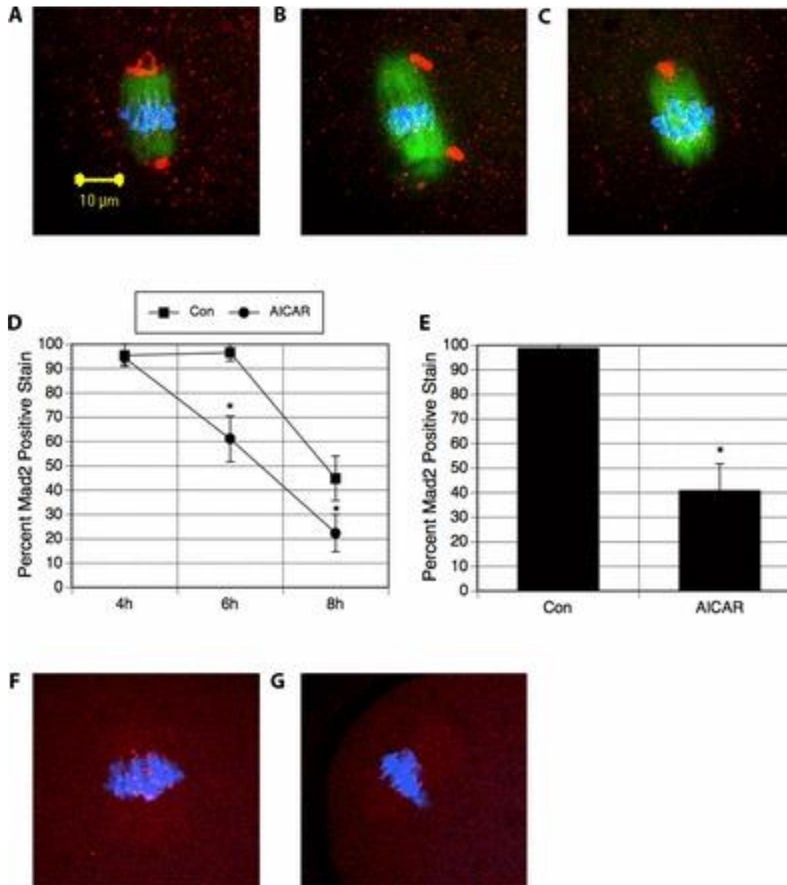

FIG. 7. **A–C**) Immunofluorescent staining of active PRKA in LT oocytes. **A**) Normal spindle pole localization of active PRKA. **B**) Shifted spindle pole localization. **C**) Missing spindle pole localization. Green, α-tubulin; red, pPRKAThr172; blue, DAPI. **D–G**) AICAR treatment accelerated loss of MAD2 in kinetochores. **D**) Kinetics of centromeric MAD2 loss in B6SJL oocytes in the presence or absence of AICAR. **E**) AICAR treatment eliminated prolonged MAD2 kinetochore localization in LT oocytes. The data in **D** and **E** are presented as percent positive MAD2 stain ± SEM; the asterisk denotes a significant difference from −AICAR controls. **F** and **G**) MAD2 stain in LT oocytes that were cultured 8 h in control (**F**) or AICAR-supplemented (**G**) medium. Red, MAD2; blue, DAPI.

A comparison of meiotic status in these same oocytes showed that 97% of B6SJL oocytes cultured 8 h in control medium reached prometaphase or metaphase I, with 3% in anaphase (Fig. 6F).

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Similarly, 100% of LT oocytes were at metaphase I or prometaphase. The addition of AICAR accelerated meiotic progression such that 71% of B6SJL oocytes were at anaphase or beyond after 8 h of culture. In addition, 33% of LT oocytes were at anaphase after exposure to AICAR, which, along with the polar body data above, demonstrates AICAR-induced rescue from metaphase arrest in a significant percentage of the oocytes.

AICAR Promotes the Loss of Chromosomal MAD2 Staining

Hupalowska et al. [53] previously reported that prolonged spindle assembly checkpoint (SAC) activity contributes to MI arrest in LT oocytes. To test the hypothesis that stimulation of PRKA promotes anaphase onset by overcoming SAC activity, oocytes were stained for MAD2, one of the major components of SAC [54]. We first determined the kinetics of centromeric MAD2 loss in B6SJL CEO (Fig. 7D). Although MAD2 staining intensity was reduced in control oocytes by 6 h, 95% of them still remained positive. Between 6 and 8 h, the number of MAD2-positive oocytes decreased by 50%. AICAR treatment accelerated the loss of MAD2, with only 61% and 23% positively staining oocytes at 6 and 8 h, respectively. In LT CEO, 98% stained positive for MAD2 after 8 h of culture, whereas in the AICAR-treated group, it was reduced to 41% (Fig. 7, E, F, and G).

Discussion

In this study, we demonstrate the suppressive effects of PRKA on parthenogenetic activation in mouse oocytes. Stimulation with the PRKA activators AICAR, AMP, or RSVA405 during oocyte maturation prevented both chemically induced activation in B6SJL mice and spontaneous activation in LT mice, while exposure of oocytes to these agents after maturation had little effect on either type of activation. Stimulating PRKA in ovulated, in vivo-matured B6SJL eggs was relatively ineffective in preventing activation, likely due to the fact that the eggs were already mature at the time of treatment. Additional effects of PRKA activation on LT oocytes included changes in active PRKA localization, release from metaphase arrest, and a reduction in spindle checkpoint activity.

Treatment of mouse oocytes with strontium produces repetitive calcium oscillations that mimic the effect of sperm binding, making it one of the most effective chemical activating agents [20, 50, 55, 56]. Nevertheless, exposure to AICAR, AMP or RSVA405 was sufficient to block strontium-induced activation in both CEO and DO, indicating a direct action on the oocyte. Twice as many oocytes were activated in CEO compared to DO, confirming that the presence of cumulus cells during the maturation period promotes activation [57, 58]. The protection by AMP and AICAR was associated with maintenance of MAPK1/3 phosphorylation, which suggests that PRKA may prevent activation by blocking dephosphorylation of MAPK1/3 and keeping MPF activity high [7]. Activating the cMos/MAPK1/3 pathway in vertebrate oocytes can induce a CSF arrest [59, 60], while treating oocytes with inhibitors of MAP2K1 (MEK1), the immediate upstream MAPK1/3 kinase, alleviates metaphase arrest and stimulates activation [9, 61]. Because treatment with PRKA activator and strontium were temporally separated, it is possible that this action of PRKA may reflect an indirect upstream effect, preventing an activation stimulus that acts on the MAPK1/3 pathway. Strontium-induced oocyte activation involves the interaction of phospholipase and InsP3 receptor [62], and it is possible that PRKA activation also interferes with these processes [63].

Calcium ionophore A23187 and protein synthesis inhibitors alone each modestly stimulate oocyte activation, but when oocytes are treated sequentially with these two agents, synergistic activation is observed [55, 64–66]. Brief exposure to ethanol is also an effective trigger for parthenogenetic activation [56, 67, 68]. Though both treatment regimens cause only single calcium transients, they still bring about significant levels of activation, and this was confirmed in the present study, with activation frequencies comparable to those achieved with strontium. More important, PRKA activators suppressed both A23187/puromycin- and ethanol-induced activation with a potency resembling that for strontium-treated oocytes. These results are important because they demonstrate a consistent activation-suppressing response to PRKA stimulation regardless of the type of chemical activating stimulus.

Chemically induced parthenogenesis was also suppressed by hormonal stimulation. The action of FSH and AR in CEO may be mediated by PRKA within the oocyte because we have previously

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shown that these ligands activate the oocyte kinase in stimulated CEO [48]. However, it should also be noted that, under such stimulation, the CEO activation rate was only partially reduced to the level of DO. Thus, it is also possible that the proactivation cumulus cell influence is neutralized by hormone-triggered uncoupling of the oocyte from gap junction-mediated cumulus signals [57], thereby rendering it a physiologically denuded oocyte that is less sensitive to strontium stimulation.

PRKA activation during oocyte maturation was essential for optimal protection from chemically induced activation; later treatment with PRKA activators during either the activation or the recovery period was largely ineffective. This relationship was very consistent for CEO. However, for DO, while prematuration exposure to PRKA activators was always inhibitory, the results of postmaturation treatment on activation varied between treatment groups: RSVA405 had no effect, AICAR was inhibitory, and, curiously, AMP was stimulatory. The reason for these differences is not readily apparent but could relate to the comparative potency of the treatments in the presence or absence of the cumulus cells, or perhaps in the case of AMP there are additional direct actions on the oocyte unrelated to PRKA activity. This need for PRKA activity during oocyte maturation to safeguard against activation is consistent with our in vivo oocyte maturation data since AICAR only marginally prevented the spontaneous activation of oocytes when exposure occurred after they completed maturation in vivo. Further, the AICAR timing experiment showed that treatment with kinase activator could be delayed up to 6 h after the initiation of culture and still achieve full protection against activation, a time point that roughly corresponds to metaphase spindle formation. After 6 h, physiological changes begin to manifest in the oocyte that make it more susceptible to activating conditions that PRKA activators cannot overcome. Interestingly, this temporal sensitivity is different than that for PRKA promotion of PB formation, which requires earlier exposure to PRKA activators, around the time of GVB [32]. Thus, PRKA not only induces initiation of meiotic resumption and promotes PB but also suppresses oocyte activation, though the data suggest its action is required at different time points during the maturation period to achieve these effects.

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Most LT oocytes are unable to complete meiosis in a timely fashion, arresting at the MI stage [33–36], and this meiotic attenuation is important in the spontaneous activation phenotype [43]. Exposure to PRKA activators was not only effective against chemically induced activation in B6SJL oocytes but also blocked the spontaneous activation of LT oocytes; again, the PRKA activity was needed during the initial maturation period for optimal protection. Based on results of the Western blot analysis of MAPK1/3 phosphorylation in B6SJL oocytes, it is reasonable to propose that PRKA also suppressed MAPK1/3 dephosphorylation in LT oocytes and sustained elevated MPF activity that protected against activation. Consistent with this idea, MAPK1/3 activity remains high in LT oocytes throughout the period of MI arrest [35]. Activation of PRKA could prevent the loss of this activity and the escape from MI arrest that accompanies activation. Hirao and Eppig [38, 39] suggested a role for CSF in the prolonged MI arrest in LT oocytes; however, in wild-type oocytes, high CSF activity did not initiate the MI arrest but only delayed the metaphase-to-anaphase transition, which suggested that CSF is not the primary cause of metaphase I arrest in this strain, though it may help maintain it.

If the MI arrest in LT oocytes is, indeed, linked to parthenogenesis, then it is not surprising that PRKA also stimulated meiotic progression past this arrest point. To further address possible downstream mediators of PRKA, we investigated whether PRKA altered spindle assembly checkpoint (SAC) dynamics. SAC is a surveillance mechanism that monitors the integrity of the meiotic spindle and proper attachment of chromosomes to spindle microtubules and controls activation of the APC [69–71]. MAD2 is a SAC protein in mouse and rat oocytes that localizes to unattached kinetochores, sequestering the main component of APC, Cdc20, thereby inhibiting APC activity and keeping MPF activity high [72, 73]. MAD2 is required in mouse oocytes for maintaining MI and preventing premature anaphase [73–75], and similar involvement in the prolonged MI arrest in LT oocytes was recently reported [53, 76]. Also, as LT oocytes [53] or mice [77] age, the MI arrest weakens, coincident with declining SAC activity. We have shown herein that AICAR activation stimulated the loss of chromatin MAD2 staining, in concert with promoting anaphase onset. It is therefore likely that PRKA activator-induced loss of MAD2 kinetochore binding contributes to both its acceleration of meiosis in

B6SJL oocytes and rescue of LT oocytes from MI arrest and activation. Whether the action of PRKA on MAD2 dynamics is direct or indirect and whether the mechanisms involved in the two strains are identical remain to be determined.

Ciemerych and Kubiak [36] proposed that the lack of an appropriate anaphase I trigger in LT oocytes may be the critical determinant of the extended MI arrest. AICAR and, presumably, PRKA stimulation provided such a trigger in the present study. Our data support an alternative mechanism for PRKA action in which stimulation of meiotic progression past MI—for example, by altering MAD2 binding dynamics—eliminates the attenuated maturation that is essential for activation such that oocytes driven past MI fail to activate. Indeed, on treatment of LT oocytes with AICAR, the reduction in activation (18%; Fig. 6A) was offset by the number of oocytes that progress to MII (21%; Fig. 6D). Hence, it is tempting to speculate that the LT oocytes that would have activated in the absence of AICAR are those same oocytes stimulated to reach MII in its presence and that the nearly complete suppression of activation is due to meiotic escape from MI. Why the remaining MI-arrested oocytes do not activate is not clear, but it appears that only about one-fifth of the LT oocytes are prone to activation in our culture conditions.

Curiously, the percentage of PRKA-treated LT oocytes that entered anaphase (33%) was higher than the number extruding a PB (21%). This suggested that karyokinesis occurred in the absence of cytokinesis in a small population of these oocytes. Indeed, when we stained actin filaments with phalloidin, 4% of AICAR-treated oocytes after 17 h of culture contained separated homologous chromosomes with a MII spindle yet no sign of a PB or contractile ring (data not shown). It is also possible that a small number of oocytes had segregated homologous chromosomes that reaggregated after anaphase onset [78, 79]. Furthermore, myosin regulatory light chain (MRLC), an important protein involved in the regulation of contractile ring formation, has recently been shown to be a downstream target of PRKA [80–82]. It would be interesting to see if MRLC localization and function is affected in LT oocytes and how it is related to the partial rescue of meiosis I arrest by AICAR.

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When LT oocytes were stained for active PRKA after 8 h of culture, abnormal localization was frequently observed. This was not due to an aging effect following prolonged MI arrest because it was observed in oocytes with newly formed meiotic spindles. Although some B6SJL oocytes also showed abnormal spindle pole localization, it was much less frequent. Importantly, supplementation of AICAR significantly lowered the incidence of PRKA mislocalization in LT oocytes, which suggests that LT oocytes may have insufficient PRKA function. It has been reported that metaphase I-arrested LT oocytes have more organized centrosomal material on one spindle pole than the other [83]. This could help explain the missing or shifted spindle pole localization of active PRKA in these oocytes since active PRKA colocalizes with microtubule organizing centers [49].

Our results support the possibility that defective control of PRKA activity contributes to the LT phenotype. Genes for the two PRKA catalytic subunits, *Prkaa1* and *Prkaa2*, are located on mouse chromosomes 15 and 4, respectively. However, a whole genome scan determined that regions in chromosome 1 and 9 were likely responsible for LT oocyte defects [44]. It may be that dysregulation of PRKA is a downstream consequence of abnormal genetic control in these regions. Regardless, it is apparent that PRKA has a profound influence on meiotic progression and regulation of parthenogenetic activation in both B6SJL and LT oocytes. Up to this point, our experiments have depended largely on pharmacological manipulation of PRKA, and genetic ablation experiments are needed for confirmation. Both isoforms of the catalytic subunit have been knocked out in mice, and each is viable and able to reproduce; however, the double knockout is an embryonic lethal [84]. It will be important to eliminate both isoforms in the oocyte to determine if PRKA is a requisite player or merely serves to fine-tune these physiological processes. We are currently in the process of generating PRKA-null oocytes to test this.

Supplementary Material

Supplemental Data:

Supplemental Figure S1. Effects of RSVA405 on oocyte maturation. **A**) CEO or DO were cultured 17-18 h in medium containing 300 μM dibutyryl cyclic adenosine monophosphate plus increasing concentrations of RSVA405 before

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assessing germinal vesicle breakdown (GVB). Meiotic resumption was induced in both groups of oocytes (by 25% in DO and 73% in CEO). **B**) CEO were cultured 17-18 h in dbcAMP alone or with additional supplements of 2 μM RSVA405 ± 2.5 μM compound C (PRKA inhibitor). The inhibitor completely blocked RSVA40s-induced maturation. **C**) CEO were cultured 15-16 h in control medium containing increasing concentrations of RSVA405 and assessed for polar body formation. The drug stimulated polar body formation in dose-dependent fashion. Data are presented as mean percent GVB or polar body (PB) ± SEM. For **B** and **C**, groups with no common letter are significantly different.

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