Specific Inhibition of Mouse Oocyte Nuclear Protein Phosphatase-1 Stimulates Germinal Vesicle Breakdown

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ABSTRACT
Okadaic acid (OA)-induced germinal vesicle breakdown (GVBD) and localization of protein phosphatase-1 (PP1) in oocyte nuclei are suggestive of PP1’s role in regulating oocyte GVBD. To explore this possibility, we microinjected protein phosphatase (PP) inhibitors OA, anti-PP1 antibody (anti-PP1), PP1 inhibitor I2, and anti-PP2A antibody (anti-PP2A) into nuclei of roscovitine (ROSC)-arrested mouse oocytes. Oocytes were also injected with recombinant PP1 in the absence of ROSC. Oocytes were assessed for GVBD and metaphase II (MII) development at 2 and 18 hr post-injection. Data were analyzed using Cochran-Mantel-Haenszel Statistics adjusted for time. Microinjection of OA significantly enhanced GVBD in comparison to controls at 2 and 18 hr (P < 0.01), yet had no effect on MII development. Similarly, microinjection of anti-PP1 resulted in significantly higher levels of GVBD compared to controls at 2 and 18 hr (P < 0.01). Interestingly, anti-PP1 microinjection also tended to enhance MII development at 18 hr in comparison to controls (P < 0.09). Microinjection of I2, anti-PP2A, and PP1 had no effect on GVBD or MII development. If reduction of PP1 activity was important for GVBD, one would anticipate an endogenous means of regulating PP1 activity at this developmental stage. In somatic cells, phosphorylation of PP1 at Thr320 causes PP1 inactivation. Germinal vesicle-intact oocytes did not contain phosphorylated PP1, as determined using a specific Thr320-Phospho-PP1 antibody, Western blot analysis, and confocal immunocytochemistry. At or around the time of GVBD, oocyte PP1 became phosphorylated at Thr320, which remained phosphorylated through MII development. These data indicate that inhibition of intra-nuclear PP1, through specific antibody neutralization, mimics OA-stimulated GVBD, providing the first direct evidence that nuclear PP1 is involved in regulation of oocyte nuclear membrane integrity. In addition, phosphorylation of PP1 occurs at/or around GVBD indicating that inactivation of PP1 is an important intracellular event in regulation of nuclear envelope dissolution at GVBD. Mol. Reprod. Dev. 65: 96–103, 2003. © 2003 Wiley-Liss, Inc.

Key Words: nuclear lamins; roscovitine; okadaic acid; MPF

INTRODUCTION
Elucidating regulatory mechanisms involved in oocyte meiosis is imperative to fully understand this complex process and allow for optimization of in vitro maturation systems. Mouse oocytes spontaneously resume meiosis once removed from their follicular environment (Pincus and Enzmann, 1935). This process involves the oocyte leaving a state of quiescence at prophase of meiosis I, undergoing dissolution of the nuclear envelope (NE) in a process known as germinal vesicle breakdown (GVBD), and finally re-arresting at metaphase of meiosis II until fertilization. These oocytes are referred to as meiotically competent. Release of the oocyte from its quiescent state, as well as other processes in cell cycle progression, is regulated by intracellular phosphorylation/dephosphorylation events. Numerous kinases have been investigated and implicated in oocyte meiotic regulation, while the role of specific protein phosphatases (PP) has largely been overlooked. Two specific kinases, mitogen-activated protein kinase (MAPK) and maturation promoting factor (MPF) have been studied extensively (reviewed by Abrieu et al., 2001). Maturation promoting factor consists of two subunits, cyclin B and p34cdc2 kinase (Labbe et al., 1989). Through a series of phosphorylation/dephosphorylation events, Numerous kinases have been investigated and implicated in oocyte meiotic regulation, while the role of specific protein phosphatases (PP) has largely been overlooked. Two specific kinases, mitogen-activated protein kinase (MAPK) and maturation promoting factor (MPF) have been studied extensively (reviewed by Abrieu et al., 2001). Maturation promoting factor consists of two subunits, cyclin B and p34cdc2 kinase (Labbe et al., 1989). Through a series of phosphorylation/dephosphorylation events, MPF activation leads to GVBD and completion of meiosis I (Maller, 1993). In meiotically competent oocytes, MPF’s p34cdc2 kinase and cyclin B are predominantly localized within nuclei (Mitra and Schultz, 1996). Inhibition of MPF with the pharmacological inhibitor roscovitine

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Serine threonine PPs, which remove phosphate from phosphoproteins, thus antagonizing protein kinases, are also implicated in regulating meiosis (Rime et al., 1990; Gavin et al., 1991; Schwartz and Schultz, 1991; Smith et al., 1998a,b). Of the four types of serine/threonine PPs that exist (PP1, PP2A, PP2B, and PP2C; Ingebritsen and Cohen, 1983; Cohen, 1989), two specific PP's, protein phosphatase-1 (PP1) and PP2A, have been observed in *Xenopus* (Rime et al., 1990), starfish (Picard et al., 1989), and mouse (Smith et al., 1998a) oocytes. Immunocytochemical studies have shown PP1 to be localized to the nucleus of mouse and monkey oocytes following acquisition of meiotic competence, while PP2A is predominantly cytoplasmic (Smith et al., 1998a,b). An important discovery in phosphatase research was the identification of the cell-permeable inhibitor of PP1 and PP2A, okadaic acid (OA; Bialojan et al., 1988). Oocyte microinjection or culture with OA causes premature GVBD in oocytes of several species including starfish (Picard et al., 1989), *Xenopus* (Rime et al., 1990; Goris et al., 1989), monkey (Smith et al., 1998b), and mouse (Alexander et al., 1991). Constant exposure of oocytes to OA results in severe cytoplasmic and spindle aberrations and blocks progression to MII (Rime et al., 1990; Gavin et al., 1991; Lu et al., 2002), while transient exposure to induce GVBD is compatible with normal oocyte development and fertilization (Smith et al., 1998b). Collectively, this would indicate that inhibition of a PP can stimulate GVBD, but some PP activity is necessary for meiotic progression to MII. However, the specific roles of PP1 and PP2A in oocytes remain unknown.

Meiotic resumption involves oocyte NE dissolution at GVBD. The NE consists of a network of filament-type proteins known as nuclear lamins (Stuurman et al., 1998). The three major constituents of the nuclear lamina are A, B, and C type lamins (Eggart et al., 1993). Nuclear lamina have been characterized in amphibian and vertebrate oocytes (Krohne et al., 1981; Schatten et al., 1985; Maul et al., 1987) and mouse oocytes contain all three lamins (Schatten et al., 1985; Maul et al., 1987; Houliston et al., 1988). Nuclear envelope integrity is controlled through phosphorylation and dephosphorylation of nuclear lamins. Hypo-phosphorylation of nuclear lamins maintains NE integrity, while hyper-phosphorylation of lamins results in NE disassembly (Stuurman et al., 1998). In somatic cells, PP1 is involved in lamin-B dephosphorylation (Thompson et al., 1997), while protein kinase C (PKC; Goss et al., 1994; Thompson and Fields, 1996) and possibly p34cdc2 kinase are responsible for phosphorylation of lamin-B (Nikolakaki et al., 1997). A complex containing p34cdc2 kinase and cyclin B, similar to MPF, is implicated in disassembly of clam oocyte NE (Dessev et al., 1991). Thus, in mitotic cells, it appears that NE integrity is determined through protein kinase and PP interactions and balance of these enzymes in regulating the phosphorylation of NE phosphoproteins. However, components regulating NE integrity during meiosis remain to be identified.

Intracellular mechanisms regulating PP1 and PP2A are numerous and diverse. In cell-free extracts PP1, but not PP2A, is sensitive to nanomolar concentrations of cytoplasmic inhibitors I1 and I2 (Ingebritsen and Cohen, 1983). Inactivation of PP1 by I1 involves I1 phosphorylation by protein kinase-A (Endo et al., 1996), whereas I2 does not require phosphorylation. In addition, it has been demonstrated that cdc2 kinase phosphorylation of PP1 on Thr320 causes PP1 inactivation (Dohadwala et al., 1994; Kwon et al., 1997). To date, the regulation of PP1 in the oocyte has not been determined.

The objectives of this study were to determine which OA sensitive PP, PP1, and/or PP2A, is involved in regulating NE dissolution during oocyte GVBD, and to elucidate mechanisms involved in regulating oocyte PP activity at/or around the time of GVBD.

### MATERIALS AND METHODS

#### Mouse Stimulation and Oocyte Collection

Female CF1 mice (Harlan; Indianapolis, IN) 19–23 days old were injected with 10 IU eCG (Sigma; St. Louis, MO). Fully-grown GVBD-competent oocytes were collected 42–44 hr post-eCG injection. These GVI oocytes were isolated by manual rupturing of antral ovarian follicles in Hepes-buffered Human Tubal Fluid medium (Quinn et al., 1985; Irvine Scientific, Santa Ana, CA) supplemented with 0.3% w/v polyvinylpyrrolidone (Sigma; HTFH+PVP). In some experiments, oocytes were cultured in Human Tubal Fluid medium (HTF; Irvine Scientific) supplemented with PVP for 2, 7, and 16 hr following GV-intact oocyte collection to isolate GVBD, MI, and MII oocytes, respectively.

#### Roscovitine Dose Titration

Oocytes were denuded through repeated mouth pipeting with hand-pulled pipettes of decreasing diameters (~200 to 90 μm) and placed into organ culture wells in groups of 20–30/900 μl of HTF supplemented with 3 mg/ml fraction V BSA (FisherBiotech; Fair Lawn, NJ) and 0, 6.25, 12.5, 25, or 50 μM ROSC (BioMol, Plymouth Meeting, PA). Oocytes were cultured in 5% CO2 in air at 37°C and assessed for stage of nuclear maturation at 400× on an inverted microscope with Hoffman optics after 2, 4, 6, and 18 hr of culture. This experiment was performed to determine a dose of ROSC that would allow 40–60% GVBD during 16 hr-culture. This tempering of spontaneous GVBD was essential to determine if inhibition of PP1 and/or PP2A stimulates mouse oocyte GVBD.

#### Oocyte Germinal Vesicle Microinjection

Oocytes were collected as described above and placed into HTFH + PVP medium supplemented with 50 μM ROSC for microinjection. Oocytes were microinjected on a Nikon inverted microscope with Hoffman optics equipped with Narishige micromanipulators and a Tritech
microinjector (Mager Scientific, Dexter, MI). Oocyte GV was injected at 400 μl of 1 μM OA (Calbiochem; San Diego, CA), 1 μM I2 (BioMol), 1 mM anti-PP1 antibody (Upstate Biotechnology, Lake Placid) and 1 mM anti-PP2A antibody (Upstate Biotechnology). The microinjected volumes were confirmed as previously described (Corson and Fein, 1983). Controls for each chemical treatment included buffer injection and no injection.

Microinjected and control oocytes were placed into organ culture wells containing 900 μl HTF medium supplemented with 0.3% BSA and 50 mM ROSC and cultured as described above. Oocytes were assessed for stage of nuclear maturation at 2 and 18 hr post-injection at 400× on an inverted microscope. Recombinant PP1 (1 mM; Upstate) was also injected into oocyte nuclei in the absence of ROSC and cultured to assess effects on nuclear maturation at 2 and 18 hr post-injection.

Electrophoresis and Western Blot Analysis

Two hundred frozen cumulus cell-free fully grown GV-intact, GVBD, MI, or MII oocytes were thawed in prewarmed 2 × SDS–PAGE sample loading buffer (80 mM Tris-HCl [pH = 6.8], 20% glycerol, 4% SDS, 4% β-mercaptoethanol, 0.04% bromophenol blue), vortexed and placed on ice for 15 min. Following sonication on ice, samples were denatured at 90°C for 10 min and placed on ice for 5 min. Samples were stored at −20°C until electrophoresis was performed.

Total protein from two hundred mouse oocytes was loaded in each lane and separated by one-dimensional SDS–PAGE. Resolving gels were cast using 12% acrylamide; stacking gels contained 5% acrylamide. Approximately 5 μg of cdc2 phosphorylated PP1 HeLa cell lysate (Cell Signaling Technology Inc., Beverly, MA) was used as a positive control for recognizing PP1 and Phospho-PP1. Gels were equilibrated and transferred to Hybond-P PVDF transfer membrane (Amersham Life Science, Little Chalfont Buckinghamshire, England) by Semi-Dry Electrophoretic Transfer (Bio-Rad Laboratories, Hercules, CA). Blots were blocked in 5% nonfat milk in TBST for 1 hr and incubated with anti-PP1 antibody at a 1:200 dilution or a polyclonal anti-phospho-PP1 antibody at a 1:50 dilution for 1 hr at 37°C. After washing with 0.3% BSA + 0.1% (v/v) Tween-20 in PBS, oocytes were incubated in the same wash buffer for 90 min at 37°C. Samples were then reacted with anti-rabbit Alexa 488-conjugated secondary antibody (Molecular Probes, Eugene, OR) at a 1:1,000 dilution for 1 hr at 37°C. Following washing, slides were incubated with 5 μg/ml propidium iodide (PI) in PBS containing 0.1% (w/v) BSA for 20 min at 37°C. Coverslips were then mounted on glass slides with 90% glycerol in PBS for fluorescence microscopic visualization with a Bio-Rad MRC-600 confocal scanning laser microscope.

Immunocytochemistry and Confocal Microscope Analysis

In order to identify the localization on PP1 and phospho-PP1 during meiotic resumption, GV-intact and GVBD oocytes were collected, attached to polylysine coated coverslips, and fixed in 2% (wt/vol) paraformaldehyde with 0.04% (v/v) Triton X-100. Oocytes were then blocked overnight with 0.3% (w/v) BSA in PBS at 4°C, and incubated with anti-PP1 antibody at a 1:200 dilution or a polyclonal anti-phospho-PP1 antibody at a 1:50 dilution for 1 hr at 37°C. After washing with 0.3% BSA + 0.1% (v/v) Tween-20 in PBS, oocytes were incubated in the same wash buffer for 90 min at 37°C. Samples were then reacted with anti-rabbit Alexa 488-conjugated secondary antibody (Molecular Probes, Eugene, OR) at a 1:1,000 dilution for 1 hr at 37°C. Following washing, slides were incubated with 5 μg/ml propidium iodide (PI) in PBS containing 0.1% (w/v) BSA for 20 min at 37°C. Coverslips were then mounted on glass slides with 90% glycerol in PBS for fluorescence microscopic visualization with a Bio-Rad MRC-600 confocal scanning laser microscope.

Statistical Analysis

In ROSC dose–response experiments, data were collected over six replicates and analyzed using Fisher’s Exact test. Microinjection data were collected over three to four replicates for each treatment and GVBD data were analyzed using Cochran-Mantel-Haenszel Statistics adjusted for time. Metaphase II development data were analyzed using Mantel-Haenszel Chi-Square. Differences were considered significant at $P < 0.05$.

RESULTS

In initial experimentation, we determined a dose of ROSC that would partially inhibit GVBD in mouse oocytes. This was important for subsequent microinjection experiments examining effects of various PP inhibitors on GVBD. Under normal circumstances, a very high percentage of mouse oocytes spontaneously resume meiosis and undergo GVBD. To determine if PP inhibition stimulated GVBD, a tempering of spontaneous GVBD was necessary. The goal was to establish a minimal dose of ROSC that would partially inhibit the population’s ability to undergo GVBD without complete inhibition. Culturing oocytes in 6.25 μM ROSC did not significantly alter GVBD compared to controls at any time point (Fig. 1). Culture of mouse oocytes in presence of 12.5, 25, and 50 μM roscovitine significantly inhibited GVBD compared to controls at all time points examined ($P < 0.05$; Fig. 1). The presence of 12.5 μM ROSC did not alter GVBD compared to oocytes cultured in the presence of 6.25 μM ROSC at any time point examined (Fig. 1). Culture in the presence of 25 μM significantly prevented GVBD more than culture in 6.25 and 12.5 μM ROSC at all time points ($P < 0.05$; Fig. 1). Addition of 50 μM ROSC to oocyte culture media significantly decreased GVBD compared to all other treatments at all time points examined ($P < 0.05$; Fig. 1). The 50-μM
dose of ROSC permitted between 30 and 50% of oocytes to spontaneously resume meiosis within 2 to 16 hr of culture, respectively. Based on these data, we chose a dose of 50 μM for subsequent microinjection experiments.

Microinjection of OA, an inhibitor of both PP1 and PP2A, into GV of ROSC-arrested oocytes significantly enhanced GVBD in comparison to controls at 2 and 18 hr post injection. (2 hr: OA-73%, buffer-41%, no injection-40%; 18 hr: OA-85%, buffer-49%, no injection-61%; P < 0.01; Fig. 2a). Even though OA GV-microinjection of ROSC-arrested oocytes stimulated GVBD, it had no significant effect on MII development at 18 hr (OA-n = 39, 38%; buffer-n = 39, 38%; no injection-n = 75, 40%).

Similar to OA injection, anti-PP1 antibody microinjection into GV of ROSC arrested oocytes significantly increased GVBD at 2 and 18 hr post-injection (2 hr: anti-PP1-59%, buffer-40%, no injection-35%; 18 hr: anti-PP1-71%, buffer-56%, no injection-50%; P < 0.01; Fig. 2b). Interestingly, microinjection of anti-PP1 antibody into oocytes tended to enhance MII development at 18 hr (anti-PP1-51%, buffer-32%, no injection-32%; P < 0.09).

Microinjection of the PP1 cytoplasmic inhibitor, I2, had no effect on GVBD compared to controls (2 hr: I2-n = 77, 54%; buffer-n = 42, 50%; no injection-n = 59, 55%; 18 hr: I2-79%, buffer-69%, no injection-66%; Fig. 3). Similarly, I2 microinjection into ROSC arrested oocytes had no effect on MII development (I2-33%, buffer-36%, no injection-36%).

To verify PP1 was indeed the PP responsible for maintaining NE integrity, anti-PP2A was also microinjected into ROSC-arrested oocytes. Germinal vesicle-microinjection of anti-PP2A into ROSC arrested oocytes had no effect on GVBD at 2 or 18 hr (anti-PP2A-58%, buffer-57%, no injection-59%; 18 hr: anti-PP2A-76%, buffer-74%, no injection-73%; Fig. 3) or MII development at 18 hr post-injection (anti-PP2A-36%, buffer-42%, no injection-42%).

Finally, we wished to determine if microinjection of purified recombinant PP1 into nonROSC-arrested oocyte GV could prevent GVBD. Microinjection of PP1 had no effect on GVBD at 2 hr (PP1-n = 32, 76%; buffer-n = 35, 80%; no injection-n = 30, 77%) or MII development at 18 hr post-injection (PP1-59%, buffer-61%, no injection-60%).

Due to the lack of effect on GVBD of microinjection of recombinant PP1, we decided to perform Western blot analysis, followed by immunocytochemistry and confocal image analysis to determine whether the phosphorylated state, and therefore, the activity of PP1, varies during oocyte maturation. Western blot analysis showed the presence of PP1 in GV-intact, GVBD, MI, and MII stage oocytes. However, phosphorylated PP1 was only present following GVBD (Fig. 4). Confocal image analysis verified the presence of PP1 in the GV-intact mouse oocyte, while phosphorylated PP1 was
The presence of phosphorylated PP1 in GVBD oocytes was confirmed through immunocytochemistry and confocal image analysis (Fig. 5).

**DISCUSSION**

A large majority of mouse oocytes spontaneously resume meiosis when removed from their follicular environment, making it difficult to detect treatment-enhanced rates of GVBD. Therefore, when assessing factors that stimulate GVBD, it is helpful to control the event by chemical inhibition. Through ROSC treatment, it was possible to modulate the number of oocytes undergoing GVBD, without completely or irreversibly inhibiting the system. By using ROSC at a dose of 50 μM, mouse oocyte GVBD was held at intermediate levels, which allowed for manipulation of the system, as well as detection of increased oocyte GVBD due to chemical manipulation. It is important to realize that mechanisms of ROSC inhibition, and possible interaction with PP’s, were not the focus of these experiments. This ROSC treatment was designed as a model system, or a means to an end, in attempting to elucidate which PP, PP1, or PP2A is involved in regulating the event of GVBD during meiosis.

Inhibition of GVBD by ROSC was successfully overcome through GV microinjection of both OA and neutralizing PP1 antibodies, but not anti-PP2A antibodies. This suggests that PP1 may be the antagonistic phosphatase to MPF's cdc2 kinase, responsible for regulating the phosphorylated state of nuclear lamins and maintenance of NE integrity. Microinjection or culture of oocytes in OA can overcome meiotic inhibition caused by chemical treatments such as cycloheximide (Gavin et al., 1991), 6-DMAP and IBMX (Kalos et al., 1993). These meiotic inhibitors are believed to interfere with meiotic resumption upstream of MPF through inhibition of protein synthesis (cyclin B) or alteration of cAMP levels. These upstream interruptions may have effects on other regulators of oocyte meiotic progression, thus making it difficult to draw any conclusions on interactions of MPF and protein phosphatases. Our results indicate GV microinjection of OA can also overcome meiotic inhibition by ROSC. Roscovitine inhibits meiotic resumption directly through MPF's p34cdc2 kinase by occupying the ATP binding site of cdc2 kinase (Mermillod et al., 2000). Through antibody neutralization of oocyte nuclear PP1, under specific inactivation of MPF, our data suggests PP1 is the OA-sensitive PP responsible for maintaining NE integrity. With this said, it is not implied that protein kinases do not also play an important regulatory role in maintenance of NE integrity. We would suggest that both MPF and PP1 are important in regulating the phosphorylated state of nuclear phosphoproteins, like lamins, that ultimately influence NE integrity. This theory is further supported by studies showing MPF subunits, cdc2 kinase and cyclin B, predominately localized in mouse (Mitra and Schultz, 1996) and starfish (Ookata et al., 1992) oocyte nuclei. Maturation promoting factor moves into nuclei of mouse oocytes in conjunction with acquisition of meiotic competence (Mitra and Schultz, 1996). In starfish oocytes, MPF traffics to the nucleus prior to resumption of meiosis (Ookata et al., 1992). PP-1 also translocates from the cytoplasm to the nucleus in conjunction with acquisition of meiotic competence (Smith et al., 1998a). In addition, immunolocalization studies show PP1 translocates to the nucleus of mitotic cells during the G2 phase (Fernandez et al., 1992) prior to the mitotic G2/M phase transition. Thus, both MPF and PP1 are present in...
the nucleus at a time when they can regulate phosphorylation of nuclear phosphoproteins important for maintaining NE integrity or stimulating NE dissolution and GVBD.

In somatic cells, trafficking of PP1 to the NE is required for assembly of nuclear lamins (Steen et al., 2000). PP-1 anchoring protein(s) exist within the assembling mitotic NE and are requisite for NE re-assembly (Steen et al., 2000). Specific inhibition of PP1 in mitotic cells demonstrates that this OA-sensitive PP is the major serine/threonine PP responsible for dephosphorylation of lamin-B, a process necessary for NE re-assembly and maintenance of NE integrity (Thompson et al., 1997). Dephosphorylation of lamin-B by purified PP1 allows re-assembly of lamina polymers (Peter et al., 1991). It has also been shown that p34<sup>cdc2</sup> kinase acts directly on lamin-B receptors to cause hyperphosphorylation in mitotic cells (Nikolakaki et al., 1997) and evidence exists to suggest MPF is involved in NE disassembly in clam oocytes (Dessev et al., 1991). These findings suggest that PP1 and MPF likely regulate phosphorylation of NE-associated proteins and dictate the integrity of the NE during mitosis and meiosis.

Monkey oocytes transiently exposed to low concentrations of OA have increased rates of MII development compared to control treatments (Smith et al., 1998b), primarily due to enhanced meiotic initiation. However, prolonged exposure of oocytes to OA results in severe cytoplasmic aberrations (Smith et al., 1998b) and microtubule abnormalities (Alexander et al., 1991). These observations, coupled with those indicating PP2A is associated with cytoskeletal elements such as microtubules, centromeres, and spindles (Van Dolah and Ramsdell, 1992; Sontag et al., 1995; Tournebize et al., 1997; Lu et al., 2002) suggests PP2A involvement with microtubule polymerization, chromatin segregation, and completion of meiosis. Oocytes injected with anti-PP1 antibodies showed higher rates of MII development compared to OA injected even though they had comparable GVBD rates. This is likely due to inhibitory effects of OA exposure on PP2A and compromised meiotic progression beyond GVBD, further supporting the idea that prolonged inhibition of PP2A may prevent oocytes from completing meiosis. Nuclear microinjection of anti-PP2A had no effect on MII development in our study. From these studies, it is unclear if nuclear anti-PP2A microinjection was capable of neutralizing the cytoplasmic PP2A following GVBD. Currently, experiments are being performed addressing the issue of antibody neutralization of PP2A following GVBD and subsequent microtubule polymerization, chromatin segregation, and normal development to MII.

Previous reports have shown that nanomolar concentrations of I2 completely abolish PP1 activity (Huang and Glinsmann, 1976; Thompson et al., 1997; Huang et al., 1999). It has been shown that I2 is present in oocytes of starfish (Pondaven and Cohen, 1987) and Xenopus (Foulkes and Maller, 1982). Research in our lab has also verified the presence of I2 mRNA and protein in mouse oocytes. Microinjection of I2 into nuclei of ROSC-arrested oocytes had no effect on GVBD. I2 is a cytoplasmic regulator of PP1 and, therefore, may not convey activity when microinjected into nuclei. This may be the result of blockage of PP1/I2 interactive sites by PP1 binding to nuclear tethering proteins or substrates. It is known that I2 binds to multiple sites on PP1 catalytic subunit (Yang et al., 2000) and only of these interactions mediate PP1 inhibition (Connor et al., 2000). Alternatively, the dosage of I2 used may not have been sufficient to inhibit PP1 activity. Yet this alternative is unlikely since similar doses of I2 have been demonstrated to inhibit cytoplasmic PP1 activity in numerous somatic cell systems (Thompson et al., 1997; Huang et al., 1999). A nuclear inhibitor of PP1, NIPP1, has been detected in rat liver nuclei (Jagiello et al., 1995) and bovine thymus (Buellens et al., 1992). However, studies in our lab failed to identify this enzyme in fully-grown mouse GV-intact oocytes. Lack of this nuclear regulator of PP1 activity in mouse oocyte nuclei raises the question of how nuclear PP1 is regulated in mouse oocytes.

Based on results of previous experiments, purified recombinant PP1 was also injected into oocyte GV, without inhibition of p34<sup>cdc2</sup> kinase activity, to determine whether NE integrity could be maintained. Microinjection of active PP1 in an anaphase mitotic cell affects subsequent cell cycle activity, resulting in accelerated...
cytokinesis and re flattening of the injected cell (Fernandez et al., 1992). Failure of PP1 GV-microinjection to effect subsequent meiotic progression by preventing GVBD in our study may be due to the subsequent phosphorylated state of endogenous and injected PP1. In mitotic cells, MPF’s cdc2 kinase phosphorylates PP1 on Thr320, resulting in its inactivation (Dohadwala et al., 1994; Kwon et al., 1997). Interestingly, both PP1 and MPF move into the oocyte nucleus around the same time during meiosis (Mitra and Schultz, 1996; Smith et al., 1998a). Using Western blot analysis and confocal imaging, we found the phosphorylated state, and therefore the activity of PP1, changes during oocyte maturation. Due to the regulatory role of MPF on PP1 in mitotic cells, it is reasonable to assume the activity of MPF may have already reached levels sufficient enough to phosphorylate and, therefore, inhibit injected PP1 in the oocyte. Alternatively, amounts of PP1 injected may not have been adequate to elicit an effect. However, this is not likely, as previous studies have shown an effect of PP1 on mitotic cell cycle activity at concentrations far lower than those used in this study (Fernandez et al., 1992). In the future, it will be important to determine the developmental timing of PP1 phosphorylation and whether this phosphorylation occurs during in vivo meiotic maturation.

CONCLUSIONS

In conclusion, we report here that inhibition of oocyte nuclear PP1, through specific antibody neutralization, can overcome ROSC induced block of GVBD and meiotic resumption. This provides the first direct evidence that nuclear PP1 is involved in regulation of oocyte nuclear membrane integrity. Utilizing Western blot analysis and confocal immunocytochemistry, we also demonstrate phosphorylation of PP1 at Thr320, and therefore inactivation of PP1 at or around the time of GVBD. These results reveal a cell-cycle dependent regulatory role of PP1 in nuclear envelope dynamics and oocyte meiotic progression.

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