Essential Role of Protein Phosphatase 2A in Metaphase II Arrest and Activation of Mouse Eggs Shown by Okadaic Acid, Dominant Negative Protein Phosphatase 2A, and FTY720*

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Vertebrate eggs arrest at second meiotic metaphase. The fertilizing sperm causes meiotic exit through Ca2+-mediated activation of the anaphase-promoting complex/cyclosome (APC/C). Although the loss in activity of the M-phase kinase CDK1 is known to be an essential downstream event of this process, the contribution of phosphatases to arrest and meiotic resumption is less apparent, especially in mammals. Therefore, we explored the role of protein phosphatase 2A (PP2A) in mouse eggs using pharmacological inhibition and activation as well as a functionally dominant-negative catalytic PP2A subunit (dn-PP2Ac-L199P) coupled with live cell imaging. We observed that PP2A inhibition using okadaic acid induced events normally observed at fertilization: degradation of the APC/C substrates cyclin B1 and securin resulting from loss of the APC/C inhibitor Emi2. Although sister chromatids separated, chromatin remained condensed, and polar body extrusion was blocked as a result of a rapid spindle disruption, which could be ameliorated by non-degradable cyclin B1, suggesting that spindle integrity was affected by CDK1 loss. Similar cell cycle effects to okadaic acid were also observed using dominant-negative PP2Ac. Preincubation of eggs with the PP2A activator FTY720 could block many of the actions of okadaic acid, including Emi2, cyclin B1, and securin degradation and sister chromatid separation. Therefore, in conclusion, we used okadaic acid, dn-PP2Ac-L199P, and FTY720 on mouse eggs to demonstrate that PP2A is needed to for both continued metaphase arrest and successful exit from meiosis. Mouse eggs, as with most vertebrates, arrest at metaphase of the second meiotic division (metII)3 until fertilized (1–3). Crucial to this arrest is inhibition of the anaphase-promoting complex/cyclosome (APC/C), thereby preventing cyclin B1 and securin degradation and so maintaining high CDK1 kinase (also known as Maturation Promoting Factor, MPF; CDK1/cyclin B1) and low separase (ESPL1) protease activities, respectively (1, 4–6). At fertilization, a sperm-derived Ca2+ signal causes loss of the APC/C inhibitor Emi2, resulting in a drop in CDK1 activity by cyclin B1 degradation and associated anaphase, driven by the protease separase (7–10).

For successful exit from metaphase in either mitosis or meiosis, it is now becoming clear that phosphatase activity is needed to dephosphorylate CDK1 substrates. In Xenopus eggs, calcineurin activity is essential in the process of exit from metII arrest, but this function is not conserved in mouse eggs (11–13). Mammalian eggs may deviate or differ from Xenopus, so it is not always possible to extrapolate across species with accuracy, and indeed this lack of conservation is observed in more than one aspect of cell cycle control (13–15). Instead, the exit of the mouse egg from metII arrest may more closely match mitotic exit in somatic cells and so rely on phosphatase activity from a member of the protein phosphatase 2A (PP2A) family (16–20).

The heterotrimeric nature of the PP2A holoenzyme, coupled with at least three regulatory B subunit families, makes a pharmacological approach to inhibition attractive. The phosphatase inhibitor okadaic acid (OA), which has a 100-fold greater potency for PP2A over protein phosphatase 1 (PP1) (21), has been used previously to explore the role of phosphatases at various stage of mouse oocyte maturation, metII eggs, and mouse one-cell embryos. OA is most well known for its ability to induce M-phase entry, with nuclear envelope breakdown evident following its addition to prophase I-stage immature oocytes (22) as well as pronuclear one-cell embryos (23, 24). It also interferes with chromosome condensation in maturing oocytes (25). In metII mouse eggs, OA induces spindle lengthening (26) and has been reported to block meiotic exit (27). However, it is clear that mouse oocytes contain PP1 in addition to PP2A, and some of the effects of OA on prophase I mouse oocytes may be due to PP1 inhibition (28, 29).

In this study, we decided to further examine the cell cycle effects of OA on mouse metII eggs. We wanted to determine the outcome of OA addition on cyclin B1 and securin degrada-

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3 The abbreviations used are: metII, second meiotic division; APC/C, anaphase-promoting complex/cyclosome; PP2A, protein phosphatase 2A; OA, okadaic acid; PP1, protein phosphatase 1; PB2, second polar body; PN, pronucleus; dn, dominant-negative; Δ90 cyclin B1, non-degradable cyclin B1.
on metII eggs before, and it would be insightful to determine whether PP2A activation could block any actions of OA.

**EXPERIMENTAL PROCEDURES**

**Animals**—Animals were used in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the University of Newcastle Animal Care and Ethics Committee. F1 hybrid mice from crosses of C57Bl6 females x CBA males were used. They were bred and housed at the University of Newcastle Animal Services Unit and kept under a 12-h day, 12-h night cycle. Three- to 4-week-old female mice were superovulated by intraperitoneal injection of human chorionic gonadotrophin in M2 medium, which mimics the action of sperm by inducing an oscillatory Ca2+-containing medium, with second polar body (PB2) extrusion and pronucleus (PN) formation evident by 8 h (Fig. 1A). However, when eggs were co-incubated in Sr2+-containing medium, with or without OA, the vast majority of eggs (80%, n = 42) were parthenogenetically activated in Sr2+-containing media, with second polar body (PB2) extrusion and pronucleus (PN) formation evident by 8 h (Fig. 1A). However, when eggs were co-incubated in Sr2+ and OA, both morphological events of egg activation were blocked. To explore the nature of the block further and so determine the status of the chromatin, eggs were Hoechst-stained 8 h after Sr2+-induced egg activation. For chromatin staining, eggs were incubated with 10 μg/ml Hoechst 33258 for 15 min and then mounted on glass slides with Citifluor (Citifluor Ltd., UK).

Images were acquired using an Olympus FV1000 CLSM equipped with a 60 × 1.2 NA UPLSAPO oil-immersion objective lens. Z-stacks were performed with an interval of 2 μm. Images were analyzed using Fiji (Universal Imaging Corp., PA) or MetaMorph v6.1 (Universal Imaging Corp., PA) were used for data analysis.

**Immunoblotting**—Immunoblots on eggs were performed as described previously (45). Eggs were probed with anti-cyclin B1 (1:500, catalog no. ab72, Abcam, UK) antibody, anti-securin (1:200, catalog no. ab3305, Abcam), and anti-GAPDH (1:5000, G9545, Sigma). All images were taken using a Fuji LAS4000.

**RESULTS**

Okadaic Acid Blocks Sr2+-induced Egg Activation—OA is a phosphatase inhibitor that has selectivity for PP2A over PP1. To examine the effects of phosphatase inhibition on meiotic exit from metII arrest, eggs were cultured in Sr2+-containing medium, which mimics the action of sperm by inducing an oscillatory Ca2+ signal (38, 46, 47). OA was added at the beginning of the experiment with a dose of 400 nM that has been reported to preferentially inhibit PP2A over PP1 and can achieve >80% PP2A inhibition (16, 17).

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Okadaic Acid Induces Spindle Elongation and Sister Chromatid Disjunction—Disruption to the spindle was the likely reason why PB2 extrusion was inhibited in OA-treated eggs. To observe this process in more detail and to determine whether this happens in the absence of Sr²⁺/H₁₁₀₀₁, metII eggs were exposed to OA for different times before fixation and immunofluorescence for chromatin and tubulin. In these experiments, no parthenogenetic stimulus was added. At 1 h, OA treatment led to a very elongated spindle, although chromatin still appeared aligned at the spindle equator in the majority of eggs (Fig. 2A). However, by 3 h, the chromatin was observed to have dispersed, with long microtubules evident in the cytoplasm that started to lack any spindle structure (Fig. 2A). By 5 h, tubulin staining was very weak, and the condensed chromatin was now widely dispersed, a feature that was maintained at later time points (Fig. 2A).

During activation, sister chromatids have to undergo disjunction for the egg to become haploid. We wanted to determine whether the spindle elongation and eventual dispersal of chromatin in the egg observed with OA was also associated with sister disjunction. This seemed plausible on the basis of casual inspection of sister chromatids in eggs at 6 h following OA addition (e.g. Fig. 2A, where >20 chromatids can be counted). To examine this directly, we labeled sister kinetochores in eggs following 6 h of OA treatment using CREST antibodies. Nearly 50% of eggs were observed to contain separated sister chromatids (Fig. 2B), with the majority of these eggs having more than half of their sisters separated (Fig. 2C). Therefore, we conclude that OA treatment is associated with sister chromatid disjunction, although this process is not as efficient as that observed following parthenogenetic activation with agents such as Sr²⁺, in which nearly 100% disjunction occurs (data not shown).

Okadaic Acid Induces Cyclin B₁ and Securin Degradation—Securin degradation is a normal feature of egg activation caused by an oscillatory Ca²⁺ signal such as that triggered by sperm, which allows the protease separase to break the cohesive forces holding sister chromatids together. It was therefore important to determine whether the observed sister chromatid disjunction following OA addition (Fig. 2B and C) was also associated with a loss in securin. Because APC/C activation during egg activation causes a loss in both securin and the CDK1 regulatory subunit cyclin B₁, we decided to examine for OA-induced changes in levels of both these substrates.

Immunoblotting on eggs for cyclin B₁ and securin was performed at up to 7 h following 400 nM OA addition. OA-treated eggs were compared with those that were non-treated, with
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FIGURE 3. Okadaic acid induces cyclin B1 and securin degradation. A, immunoblot for cyclin B1 and securin in which eggs were treated with 7% ethanol for 7 min to parthenogenetically activate them, or incubated with 400 μM OA for the times indicated. GAPDH was used as loading control. The blot is representative of two other independent blots with 50 eggs per lane, and times are relative to OA or ethanol addition. B, fluorescence in eggs microinjected with fluorescent protein labeled cyclin B1 or securin cRNA as indicated. At 0 h, eggs were incubated with OA or with no OA additions. At hourly intervals, the loss in fluorescence measured in OA-treated eggs is measured as a percentage of the non-treated eggs. To allow comparison among eggs, all fluorescence reading were normalized (F/Fmax × 100). Plots are mean ± S.D. Error bars, n = 50 per time point. **, p < 0.05; ***, p < 0.005.

Non-otic-exposed eggs at either metII or parthenogenetically activated with 7% ethanol. Ethanol rather than Sr2+ was used here because it gives a more synchronous activation profile, which is beneficial on timed immunoblotting. Interestingly, immunoblotting demonstrated that OA caused decreases in levels of both securin and cyclin B1 over a 5-h period, which were comparable with that achieved by parthenogenesis (Fig. 3A). Degradation of these two APC/C substrates was not evident at 1 h after OA addition but was at 3 h and appeared complete by 5 h. No such decrease was evident in the absence of OA (data not shown), in keeping with the observation that eggs have a capacity to maintain CDK1 activity over at least a 30-h window post-ovulation (48).

Such observations on endogenous proteins could also be observed in real-time measurements of fluorescent protein-tagged cyclin B1 and securin (Fig. 3B). In these experiments, eggs were microinjected with cRNA to either cyclin B1- or securin-fluorescent protein (YFP or GFP), and fluorescence was measured following OA addition. Loss of both fluorescent protein-tagged cyclin B1 and securin was first apparent at 3 h after OA addition, in agreement with endogenous proteins. However, endogenous protein was far more rapidly degraded than these expressed proteins (compare Fig. 3, A and B), and this was presumed to be due to the increased amount of APC/C substrate when these exogenous proteins are expressed as well as continued cRNA translation during the period of degradation.

Non-degradable Cyclin B1 Can Block OA-induced Spindle Changes—Non-degradable cyclin B1 (Δ90 cyclin B1) expressed in mouse eggs can inhibit not only the loss in CDK1 activity normally associated with cyclin B1 degradation but can also prevent sister chromatin disjunction (5, 38). This latter effect is due to the ability of high CDK1/cyclin B1 activity to bind to and inhibit separase (49, 50). Given that OA addition was able to induce cyclin B1 and securin degradation, we wanted to establish if any of the changes in spindle structure associated with OA addition could be blocked if CDK1 activity levels were sustained by Δ90 cyclin B1 expression, which, as we have previously shown, will also compensate for securin loss by inhibiting separase (5, 38). It seemed possible that OA was exerting its effects on the spindle indirectly through the associated loss of cyclin B1 and so CDK1 activity, a mechanism made plausible by the observations that CDK1 phosphorylates a number of microtubule regulatory proteins such as MAP4, OP18/stathmin, MCAK, and MAP215/TOG (51–56).

Δ90 cyclin B1 cRNA was microinjected into metII eggs at a concentration that we used previously to maintain CDK1 activity and block sister chromatid disjunction (5, 38). Eggs were cultured for 3 h to allow Δ90 cyclin B1 expression, and then OA was added for a further 4 h. In control eggs, the metII spindle was present and had not been noticeably affected by culture, but in all eggs treated with OA alone there were no discernible spindle structures remaining (Fig. 4, A and B). However, when OA treatment was performed in eggs expressing Δ90 cyclin B1, then 44% of eggs were observed to retain an intact metII spindle (Fig. 4, A and B). The shape of the spindle, measured by the pole-to-pole length, was not affected in untreated eggs by the period in culture (Fig. 4C). However, in Δ90 cyclin B1-expressing eggs treated with OA, the metII spindle pole-to-pole length was approximately three times longer than all other control, non-OA treated eggs (Fig. 4C). Therefore, these observations demonstrate that Δ90 cyclin B1 has the capacity to ameliorate OA-induced spindle changes, they also show that it is likely that phosphatase inhibition is also directly affecting spindle dynamics.

OA Induces Emi2 Degradation—In Xenopus eggs, CDK1 phosphorylates the APC/C inhibitor Emi2, and this is antagonized by PP2A (30–32). CDK1-phosphorylated Emi2 is less able to inhibit the APC/C and is also more unstable, leading to an autoregulatory loop through APC/C activation and cyclin B1 degradation, which would cap CDK1 activity in metII eggs (30). Based on this regulatory cycle for Emi2, it would be predicted that OA-induced PP2A inhibition would lead to increased phosphorylated Emi2, which has less APC/C affinity. In fact, if this were so, then this would explain the degradation of cyclin B1 and securin observed following OA addition.

Eggs were microinjected with Emi2-Venus cRNA to measure real-time changes in its stability following expression. We have previously used this construct and demonstrated that it behaves like endogenous Emi2 (7). In the presence of OA, Emi2-Venus levels gradually decreased over a 10-h period (Fig. 5A). The loss of this fluorescent protein construct had similar dynamics to that of exogenous cyclin B1 and securin (Fig. 3).
PP2A inhibition were indeed due to inhibition of this phosphatase. This was achieved by preincubating eggs with 2.5 μM FTY720, a PP2A activator, for 3 h before OA addition (34). Importantly, FTY720 has no ability to activate PP1 (see “Discussion”). FTY720 preincubation greatly reduced the Emi2-Venus degradation otherwise observed following OA treatment (Fig. 5B). Indeed, there was little to no Emi2-Venus degradation in the presence of FTY720 and OA, suggesting that the destabilizing actions of OA on Emi2 had largely been neutralized by FTY720 at this dose. This suggested that in its presence there could be insufficient APC/C activity to cause substantial cyclin B1 or securin degradation. This was tested directly by immuno- blotting eggs preincubated with FTY720 before OA for these APC/C substrates. Confirming our hypothesis, very little loss in either securin or cyclin B1 was evident over a 5-h period (Fig. 5C). This contrasts with their very evident loss seen in eggs incubated with OA alone (compare Fig. 3A with 5C).

The ability of FTY720 to block the dispersal of chromatin caused by OA was examined. Consistent with the previous experiments already described, in eggs treated with OA alone the chromatin that was initially observed on the metaphase plate (Fig. 5D, arrow) began to disperse between 1 and 1.5 h following OA addition (Fig. 5D, asterisks in right upper panels). In contrast, in eggs pretreated with FTY720, OA had little impact on the metaphase plate (Fig. 5D, lower panels) over the 2-h observation period. In addition, we did not observe any morphological changes in eggs that were treated with FTY720 alone over a 3-h period. Similarly, FTY720 had the ability to block the actions of OA on spindle lengthening (Fig. 5E). We therefore conclude that all the actions attributed to OA on mouse eggs can be blocked by preincubation with the PP2A activator FTY720.

**Effects of Dominant-Negative PP2Aα on Mouse Eggs**—As an additional method of confirming that PP2A inhibition leads to the changes observed following OA addition, we used PP2A-L199P, a dominant-negative construct of PP2AC (dn-PP2A) (57, 58). dn-PP2A cRNA microinjected into metII eggs induced similar changes to that of OA following a 6-h culture. Thus, chromatin became misaligned or dispersed within the cytoplasm (Fig. 6, A and B, n = 16), and sister chromatids were observed to have separated in the vast majority of eggs (Fig. 6C, 81%). The same as OA, degradation of cyclin B1 and securin was apparent following dn-PP2A expression (Fig. 6D). None of these changes were observed when microinjections of GFP/YFP cRNA were made.

Together we conclude that PP2A activity plays an essential role in maintenance of metII arrest and prevention of premature egg activation in mouse eggs by keeping spindle integrity.

**DISCUSSION**

This study has used the PP2A inhibitor OA and activator FTY720 to show a regulatory role for this phosphatase in mouse eggs during metII arrest and meiotic resumption. PP2A inhibition led to both cyclin B1 and securin degradation caused by increased APC/C activity associated with loss of its meiotic inhibitor Emi2. All events associated with OA addition could be prevented by pretreatment with FTY720, a PP2A activator and could be mimicked by expression of dn-PP2A.

**PP2A and metII Arrest**—In *Xenopus* egg extract, the association of Emi2 with the APC/C is uncoupled by high CKD1 activity, forming an essential feedback loop to control excessive buildup of this kinase during the protracted and indeterminate period of metII arrest (10, 30–32). Essential for the reestablishment of APC/C inhibition following activation of this loop in *Xenopus* eggs is PP2A, whose association with Emi2 is enhanced by the c-mos-MAPK-p60^{Rsk} pathway (31). In *Xenopus*, at least, it appears that the main ability of the c-mos pathway to cause metII arrest is due to this stabilizing and activating effect on Emi2 (59, 60), and this would account for the long-held view that c-mos is central to metII arrest in

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**FIGURE 4. Δ90 cyclin B1 prevents OA-induced spindle disassembly.** A and B, metII eggs, some expressing Δ90 cyclin B1, were incubated with or without 400 μM OA for 4 h as indicated. Spindles were examined and classified as being intact or as dispersed. A, representative images of intact and dispersed spindles. B, percentage of total analyzed having either an intact or dispersed spindle. All of the eggs to which no additions were made possessed an intact spindle, but none of those did that were treated with OA. Δ90 cyclin B1 expression in OA-treated eggs preserved spindle integrity. All eggs had a total culture time of 7 h. Above each bar is the number of oocytes in this condition/total oocytes analyzed. C, spindle length was measured in those eggs in A and B as well as those metII eggs that had not been incubated for 4 h with/without OA, as indicated. Spindle length in Δ90 cyclin B1 and OA-treated eggs was 3-fold greater than controls. Spindle length was not possible to calculate in eggs treated with OA alone, as the spindle had been completely dispersed. Total number of oocytes analyzed are in parentheses.***, *p < 0.005.
both frog and mammalian eggs (61–63). The OA-induced loss in Emi2, cyclin B1, and securin observed here did lead to events that resemble exit from meiII arrest. This is not unexpected, given that the loss of these proteins is required for completion of meiosis. Most notable was the disjunction of sister chromatids, a process that is physiologically blocked during meiII arrest by securin (5) but which can also be prevented by CDK1 activity (38).

OA Block of Egg Activation

Despite cyclin B1 and securin loss, there was no morphological manifestation of egg activation or PB2 or PN formation following OA or dn-PP2A addition. The chromatin in the egg remained condensed. Further-
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more, OA had the ability to block both chromosome decondensation and nuclear envelope formation even in the presence of Sr²⁺, an otherwise effective parthenogenetic agent. This is most likely because mouse eggs have the same essential requirement for PP2A as that recently described in human mitotic cells (20), where loss in PP2A was observed to block both chromosome decondensation and nuclear envelope formation. Additionally, in mouse eggs, OA has been demonstrated to elevate MAPK activity (23, 27), a process that also occurs in small meiotically incompetent oocytes (64) and fully grown mature oocytes (65). Raised MAPK is known to inhibit pronucleus formation in eggs (23), and this effect on chromatin is possibly mediated by changes in histone phosphorylation (66).

The lack of PB2 following Sr²⁺ addition to OA-treated eggs is explained by the inability of the egg to be able to set up a cleavage furrow when the chromatin becomes misaligned or dispersed. This seems the most likely cause, given that a similar block to polar body extrusion has been observed in other instances when the integrity of the meiotic spindle has been disrupted (67, 68).

**PP2A and Microtubules**—The dramatic action of OA on spindle-associated microtubules, which led to spindle elongation and eventual complete dissolution, has previously been observed in oocytes (26, 69). We found that the longer-term action of OA in inducing spindle disassembly, normally observed by 4 h, could be prevented by expression of Δ90 cyclin B1, suggesting that some of the effects of OA on spindle morphology were due to changes in CDK1 activity. A view consistent with the known phosphorylation of a number of microtubule associated proteins by CDK1 (51–56). However, even with Δ90 cyclin B1, OA-induced spindle lengthening was still evident, with spindle length being three times that of controls. In fact, the action of OA on increasing spindle pole-to-pole length could only be prevented by preincubation with the PP2A activator FTY720 (Fig. 5). PP2A has been reported to have direct actions on microtubules via dephosphorylation of oncoprotein 18/stathmin, which are consistent with the observations here. Inhibition of PP2A would enhance phosphorylation of oncoprotein 18/stathmin, thus stabilizing microtubules and causing spindle lengthening (70, 71). Thus, based on the co-culture experiments with Δ90 cyclin B1 or FTY720, we conclude that the immediate actions of OA on spindle length are attributable to PP2A inhibition but that the longer-term effects over several hours are more likely to be due to the loss in CDK1 activity brought about by the actions of OA on APC/C activity (i.e. Emi2 loss).

FTY720 as a Tool for PP2A Activation—OA is reported to have much greater potency for PP2A than PP1, and, as such, in some studies its actions have been attributed to PP2A inhibition solely on this basis. Here, however, we have used FTY720 to confirm the involvement of PP2A as the OA-sensitive phosphatase. FTY720 is a synthetic sphingosine-1-phosphate analog that has recently been approved as an immunosuppressive drug for multiple sclerosis. The activity of FTY720 has been useful in autoimmune conditions as well as in solid organ transplantation because of its immunosuppressive function in clinical trials (72). It has become clear that FTY720 has the capacity to activate PP2A independently of its immunosuppressive functions both in cultured cells (33) and in vitro (34) and that its anticancer action may be mediated by PP2A activation (35, 36). In contrast, FTY720 has no ability to activate PP1 (34), and thus aids us in concluding that the actions of OA on metII eggs are attributable to PP2A rather than PP1 inhibition.

Future studies are needed to examine which members of the PP2A family are required for metII arrest. The heterotrimeric holoenzyme is made up of one of a number of regulatory B subunits that are expressed by up to 15 genes interacting with the catalytic subunit (PP2AC) and structural core subunit (PP2A-A/PR65) (73). One attractive B subunit would be B55α, given that recent evidence suggests PP2A-B55α is the relevant phosphatase for mitotic exit in adult somatic cells (20).

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