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Endoplasmic reticulum reorganizations and Ca^{2+} signaling in maturing and fertilized oocytes of marine protostome worms: the roles of MAPKs and MPF

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SUMMARY

Before a proper Ca^{2+} response is produced at fertilization, oocytes typically undergo a maturation process during which their endoplasmic reticulum (ER) is restructured. In marine protostome worms belonging to the phylum Nemertea, the ER of maturing oocytes forms numerous distinct clusters that are about 5 μm in diameter. After fertilization, mature oocytes with such aggregates generate a normal series of Ca^{2+} oscillations and eventually disassemble their ER clusters at around the time that the oscillations cease. Immature oocytes, however, lack prominent ER clusters and fail to exhibit repetitive Ca^{2+} oscillations upon insemination, collectively suggesting that cell cycle-related changes in ER structure may play a role in Ca^{2+} signaling. To assess the effects of meiotic regulators on the morphology of the ER and the type of Ca^{2+} response that is produced at fertilization, nemertean oocytes were treated with pharmacological modulators of mitogen-activated protein kinases (MAPKs) or maturation-promoting factor (MPF) prior to confocal microscopic analyses. Based on such imaging studies and correlative assays of kinase activities, MAPKs of the ERK1/2 type (extracellular signal regulated kinases 1/2) do not seem to be essential for either structural reorganizations of the ER or repetitive Ca^{2+} signaling at fertilization. Conversely, MPF levels appear to modulate both ER structure and the capacity to produce normal Ca^{2+} oscillations. The significance of these findings is discussed with respect to other reports on ER structure, MPF cycling and Ca^{2+} signaling in oocytes of deuterostome animals.

Key words: Fertilization, GVBD, Maturation, Eggs, U0126, ERK, JNK, p38, DiI, Calcium Green, Roscovitine, Colchicine, Ca^{2+} oscillations, *Cerebratulus*, *Micrura*, Nemerteans

INTRODUCTION

In most animals, oocytes must undergo a pre-fertilization maturation process that is ultimately regulated by signaling cascades involving mitogen-activated protein kinases (MAPKs) and maturation-promoting factor (MPF) (Maller, 1990; Maller, 1998; Kishimoto, 1998; Kishimoto, 1999; Sun et al., 1999; Palmer and Nebreda, 2000; Abrieu et al., 2001). At the onset of maturation, the nucleus of each prophase-I-arrested oocyte rapidly disassembles during the germinal vesicle breakdown (GVBD) phase of meiosis. In addition to changes that occur in the nucleus during GVBD, oocyte maturation can also trigger marked alterations in cytoplasmic organelles. For example, the endoplasmic reticulum (ER), which most probably represents the major internal store of Ca^{2+} to be mobilized at fertilization (Eisen and Reynolds, 1985; Han and Nuccitelli, 1990; Terasaki and Sardet, 1991), develops discrete aggregates (i.e. 'clusters' or 'microdomains') in the maturing oocytes of several animal groups that have been investigated (Kline, 2000; Sardet et al., 2002). Accordingly, mature oocytes that have completed such ER reorganizations tend to produce a proper fertilization-induced Ca^{2+} response, whereas immature oocytes typically lack ER clusters and fail to generate normal Ca^{2+} signals (Chiba et al., 1990; Fujiwara et al., 1993; Mehlmann et al., 1995; Shiraishi et al., 1995; Stricker et al., 1994; Stricker et al., 1998; Stricker, 1996a; Machaty et al., 1997).

Fertilization can also cause ooplasmic ER to undergo dramatic reorganizations, the timing of which depends on the species examined (Kline et al., 1999; Kline, 2000). In animals that produce a single Ca^{2+} wave at fertilization (e.g. sea urchins, starfish and frogs), the ER is rapidly restructured following fertilization (Jaffe and Terasaki, 1993; Jaffe and Terasaki, 1994; Terasaki et al., 2001). Alternatively, ER clusters remain intact for a comparatively longer time in mammals and other species that generate multiple Ca^{2+} waves at fertilization (Stricker et al., 1998; FitzHarris et al., 2003). Collectively, such findings suggest that the particular configuration of the ER may help to modulate the type of Ca^{2+} response that is produced (Kline et al., 1999; Kline, 2000). However, in spite of intensive analyses of fertilization-induced Ca^{2+} signals (reviewed by Sardet et al., 1998; Stricker, 1999; Tarin and Cano, 2000; Carroll, 2001; Runft et al., 2002), the underlying regulatory mechanisms and possible functions of ER reorganizations have yet to be fully defined, particularly in the case of non-mammalian species.

Marine worms in the phylum Nemertea are protostome invertebrates and thus belong to the mollusc/annelid/arthropod lineage, rather than the deuterostome grouping that includes chordates and echinoderms. Fully grown nemertean oocytes

characteristically lack follicle cells and spontaneously mature after being placed in seawater (Stricker, 1987a; Stricker et al., 2001). In the nemertean *Cerebratulus lacteus*, oocytes begin GVBD ~40 minutes after contacting seawater and by 2 hours reach a metaphase I arrest at which they remain until fertilization occurs (Stricker and Smythe, 2000). In previous studies analyzing Ca^{2+} signaling and ER structure during fertilization of *C. lacteus* oocytes, marked differences were described both in the Ca^{2+} responses and the organization of the ER displayed by immature prophase-arrested oocytes versus mature specimens at metaphase I (Stricker et al., 1998).

In order to expand on those studies, the potential roles of MAPKs and MPF in regulating ER structure and Ca^{2+} dynamics in *C. lacteus* are assessed. For such investigations, pharmacological modulators and kinase activity assays are combined with in vivo confocal imaging of ER reorganizations and Ca^{2+} signals. In addition, oocytes of two other nemertean species – *Cerebratulus* sp. and *Micrura alaskensis* – are also analyzed and compared with those of *C. lacteus*.

Collectively, such investigations indicate for the first time that MAPKs belonging to the ERK1/2 type (extracellular signal regulated kinases 1/2) are not required for either ER reorganizations or repetitive Ca^{2+} oscillations at fertilization. Conversely, MPF levels appear to play an essential role in shaping the normal patterns of ER structure and Ca^{2+} signals in nemerteans. The findings presented here are also compared with results obtained from recent investigations of deuterostome oocytes, in which functional interactions between MPF levels, Ca^{2+} signaling, and/or ER organization have been described (Kline et al., 1999; Deng and Shen, 2000; Levasseur and McDougall, 2000; Terasaki et al., 2001; Gordo et al., 2002; FitzHarris et al., 2003).

MATERIALS AND METHODS

Animals

Adult specimens of *Cerebratulus lacteus* were purchased from the Marine Biological Laboratory, Woods Hole, MA, whereas *Micrura alaskensis* and *Cerebratulus* sp. adults were collected at False Bay, San Juan Island, WA. Currently, taxonomic keys do not allow reliable identifications of west coast *Cerebratulus* species (Stricker, 1987b). Hence, the False Bay *Cerebratulus* are simply referred to as *Cerebratulus* sp. Such worms were ~10-50 cm long, 0.5 to 1.5 cm wide, olive-tan to chocolate-brown in color, and their sperm had an unmodified, 5 μm long head (Stricker and Folsom, 1998).

To block spontaneous maturation, oocytes stripped from ripe ovaries were treated with Ca^{2+}-free seawater (CaFSW) (Schroeder and Stricker, 1983) and/or inhibitors of MAPK signaling (Stricker and Smythe, 2000; Stricker and Smythe, 2001). Unless stated otherwise, all assays, confocal runs and blots were repeated at least three times using oocytes from two or more females. Statistical analyses involved Student's *t*-tests and Mann-Whitney-U tests (Stricker and Smythe, 2000).

Morphology and Ca^{2+} imaging

For assessments of ER morphology, dejellied oocytes were attached to protamine-sulfate-coated specimen dishes and microinjected with the ER-specific probe DiI (Terasaki and Jaffe, 1993) before examination with a BioRad MRC-600 confocal microscope (Stricker et al.,1998). For these studies, serial *z*-sections taken at 10 μm intervals with a 20×, 0.7 NA objective were routinely stacked together by means of a maximum projection algorithm to produce a compressed image of the entire oocyte (Stricker et al., 1998).

Confocal imaging was also used to monitor fertilization-induced Ca^{2+} signaling in specimens injected with Calcium-Green- and Rhodamine-B dextrans (Stricker and Whitaker, 1999; Stricker, 2000). Graphs were expressed as uncalibrated changes in fluorescence intensity relative to either the pre-stimulus CG/RB ratio (R_o) or the initial non-ratioed CG fluorescence (F_o). In nearly all cases (see exception in Fig. 7), Ca^{2+} imaging was carried out on separate batches of oocytes from those used to observe ER dynamics, as double injections of DiI-saturated oil droplets and aqueous solutions of Ca^{2+}-sensitive probes led to reduced viability.

Protein analysis

To analyze MAPK activities using western blots, one to several hundred oocytes that had been dejellied via a Nitex mesh were lightly pelleted at each timepoint of the run before the overlying seawater solution was removed and replaced with 30 μl of lysis buffer (Carroll et al., 2000). The samples were then frozen in liquid nitrogen (LN) and maintained at –80°C. Thawed preparations were centrifuged for 15 minutes at 14,000 ***g*** at 4°C, and 2 μl of the resulting supernatant was used in Bradford protein assays before boiling the remainder in sample buffer. Based on such determinations, each lane of the 12% SDS-PAGE mini-gels received an equal amount of total protein ranging from 10 to 50 μg, with 15 μg being the typical load. After electrophoresis, the separated proteins were transferred to Gelman PVDF filters, probed with primary and secondary antibodies, and subsequently detected by enhanced chemiluminescence. X-ray films of the blots were subjected to background-subtracted densitometry using MetaMorph image processing software (Molecular Devices, Sunnyvale, CA).

In addition, MPF activity was monitored by a phosphorylation assay that used the retinoblastoma (Rb) protein as a substrate specifically targeted by active MPF (Lees et al., 1991), as attempts to use the more conventional substrate histone H1 produced only a weak signal. For such assays, LN-frozen samples were thawed and assayed for protein content. Based on such determinations, 10 μg of total protein in lysis buffer from each timepoint was incubated in kinase buffer (Cell Signaling) to comprise a total of 18 μl. To each tube with 18 μl of sample in kinase buffer, 2 μl was added of the following constituents: 0.5 μl of a 10 mM ATP solution (Cell Signaling), 0.25 μl of a C-terminal retinoblastoma fusion protein (Cell Signaling) and 1.25 μl kinase buffer. After incubation at 30°C for 30 minutes, the samples were boiled in sample buffer and processed for western blotting using an antibody against phospho-Rb (Cell Signaling). In addition, because ELK-1 phosphorylation can be used to monitor ERK1/2 activity in marine invertebrate eggs (Carroll et al., 2000), some Rb samples were co-incubated with 0.25 μl of ELK-1 fusion protein (Cell Signaling) plus 0.75 μl kinase buffer for subsequent detection with a phospho-ELK-1 antibody (Cell Signaling). Further descriptions of both the Rb and ELK-1 phosphorylation assays are currently being prepared (T.L.S. and S.A.S., unpublished)

Materials

All inhibitors of MAPK signaling, except for curcumin, were purchased from Tocris (Ballwin, MO), whereas curcumin, roscovitine and 5-HT were from Sigma-Aldrich (St Louis, MO). Primary and secondary antibodies were from Cell Signaling Technology (Beverly, MA) and Accurate Chemical and Scientific (Westbury, NY), respectively. Buffers for electrophoresis and blotting were purchased from BioRad (Hercules, CA), whereas DiI and the fluorescent dextrans were from Molecular Probes (Eugene, OR).

RESULTS

Reorganization of the ER during oocyte maturation

In a previous study of *C. lacteus* oocytes injected with the ER-

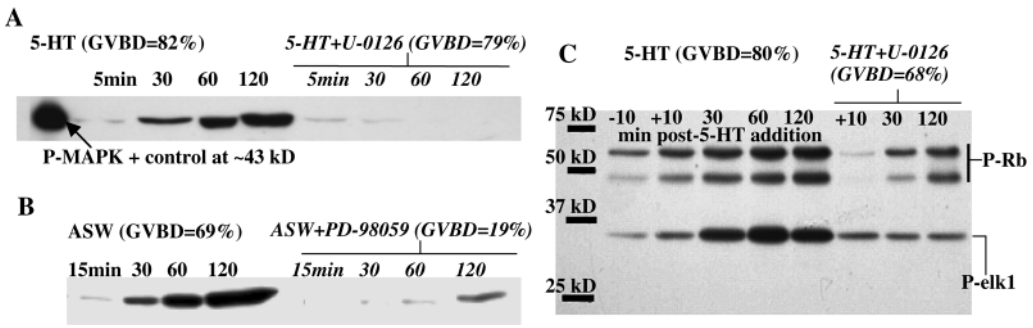

Fig. 1. (A) *C. lacteus* oocytes treated with 1 μM 5-HT in the presence or absence of 20 μM U0126 and probed for MAPK activity with anti-phospho-ERK1/2 Ab. Note normal levels of GVBD in U0126-treated samples even though MAPK signal is greatly reduced. 'Positive control' was supplied by Cell Signaling as material used to generate antibody. (B) Although 5-HT-treated oocytes mature at high levels without MAPK activity, similar incubations in U0126 or 25 μM PD98059 reduce spontaneous GVBD. Each blot is representative of five replicates. (C) Activity assays of MPF and ERK1/2 MAPK in maturing *C. lacteus* oocytes. Upper two bands at ~66 and 48 kDa represent full-length and truncated Rb fusion protein probed with a phospho-Rb-specific antibody to track MPF activity. Phospho-ELK-1 band at ~34 kDa depicts ERK1/2 activity. Note MPF activation continues in the absence of substantial MAPK activity.

specific probe DiI, a few fully grown oocytes failed to mature after removal from the ovary, and such specimens typically possessed a homogeneous ER without obvious clusters (or 'microdomains') when viewed by confocal microscopy (Stricker et al., 1998). Subsequently, methods were devised to block spontaneous GVBD (Stricker and Smythe, 2000; Stricker and Smythe, 2001) by treating prophase-arrested oocytes with either CaFSW or ASW (artificial sea water) containing U0126 or PD98059 to inhibit the activating kinase of ERK1/2 MAPKs (Dudley et al., 1995; Favata et al., 1998). Conversely, it was shown that maturation could be re-initiated through the addition of the neurohormone serotonin (5-HT) to CaFSW or ASW solutions, even in the presence of MAPK inhibitors (Stricker and Smythe, 2000; Stricker and Smythe, 2001).

By controlling the maturation state of nemertean oocytes via such protocols, a broader sampling of oocytes could be checked for ER structure prior to hormone-induced GVBD, and in all 58 *C. lacteus* oocytes examined before GVBD, a conspicuous clustering of the ER was lacking. Similarly, GV-containing oocytes of both *Cerebratulus* sp. and *Micrura alaskensis* failed to display marked ER clusters, although such structures typically formed after GVBD (Stricker et al., 2001) (S.A.S., unpublished).

To confirm that the lack of ER substructuring was not simply a sign of cell death caused by the treatments used to block GVBD, prophase-arrested oocytes of *C. lacteus* were injected with DiI in low-Ca^{2+} (75 nM) ASW and subsequently incubated in a 1 μM solution of 5-HT in ASW. Based on time-lapse confocal microscopy, controls that were not treated with 5-HT typically did not mature or form ER clusters (data not shown). Conversely, hormone addition routinely triggered GVBD, and 86% of these maturing oocytes developed distinct ER clusters (n=28) (Table 1). Such clusters were typically 3-6 μm in size (Stricker et al., 1998) and associated with normal development, as oocytes with these ER aggregates cleaved properly when fertilized (Stricker et al., 1998).

The role of MAP kinases in ER cluster formation

To examine the possible roles of ERK1/2 MAP kinases in ER cluster assembly, *C. lacteus* oocytes were matured in 5-HT-containing ASW solutions supplemented with 20 μM U0126 or 25 μM PD98059. Such treatments dramatically reduced MAPK activity in western blots using a phospho-specific antibody to activated ERK1/2 (Fig. 1A,B). Nevertheless, GVBD continued in the absence of substantial MAPK activity at a rate that was only marginally (<15%) reduced compared with controls (Fig. 1A). This lack of inhibition was not simply due to a complete ineffectiveness of U0126 or PD98059, because similar incubations in the absence of 5-HT

Table 1. Effects of MAPK inhibitors on ER cluster formation in mature oocytes

Species	Inhibitor	Putative MAPK signal affected by inhibitor	% of mature specimens with ER clusters (no. of oocytes; no. of females)
Cerebratulus lacteus	None	None	85.7% (n=28; 4)
Cerebratulus lacteus	25 μM PD98059	ERK1/2	89.2% (n=65; 4)
Cerebratulus sp.	15 μM U0126	ERK1/2	90.2% (n=51; 3)
Micrura alaskensis	15 μM U0126	ERK1/2	80.0% (n=15; 3)
Cerebratulus lacteus	20 μM curcumin	JNK	90.1% (n=11; 2)
Cerebratulus sp.	20 μM curcumin	JNK	92.6% (n=27; 3)
Cerebratulus sp.	20 μM SP600125	JNK	2.9% (n=68; 6)*
Cerebratulus sp.	20 μM SB202190	p38	90.5% (n=21; 2)

Oocytes obtained from ripe females were continuously incubated in ASW solutions containing a MAPK inhibitor before and after DiI injection followed by 5-HT-stimulated GVBD and confocal microscopy to assess the presence or absence of ER clusters.

*Significantly less than other MAPK inhibitors or controls; P<0.05.

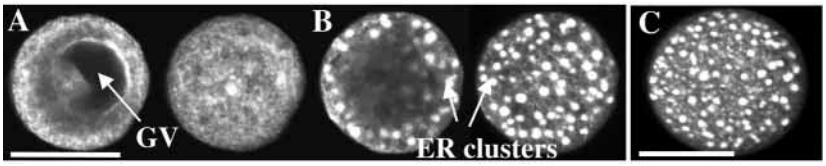

Fig. 2. (A,B) Single oocyte of *Micrura alaskensis* continuously bathed in 20 μM U0126. (A) single confocal plane (left) and compressed *z*-series (right) before GVBD. (B) Same oocyte in mature state 2 hours after treatment with 5-HT plus U0126, showing ER clusters: single optical plane view (left) and compressed z-series (right). (C) Compressed *z*-series of a mature *Cerebratulus* sp. oocyte with ER clusters 2 hours after treatment with 5-HT+U0126. Scale bars: 50 μm. GV, germinal vesicle.

significantly reduced both MAPK activity and spontaneous GVBD (Fig. 1B). The validity of using the phosphorylated MAPK (P-MAPK) signal to track MAPK activity was also verified by means of ELK-1 phosphorylations, which revealed similar patterns to those obtained with the phospho-MAPK-specific antibody (Fig. 1C). In addition, MPF assays using Rb phosphorylations demonstrated that in oocytes treated with 5-HT and U0126, MPF rose dramatically without marked MAPK activation (Fig. 1C).

In correlative studies of ER morphology, 58/65 (89%) of the mature specimens that were continually incubated in PD98059 developed ER clusters after GVBD (Table 1). Similarly, post-GVBD oocytes of either *Cerebratulus* sp. (46/51; 90%) or *Micrura alaskensis* (12/15; 80%) formed ER clusters when treated with 5-HT in the continuous presence of U0126 (Table 1; Fig. 2).

To test whether other kinds of MAP kinases might also be dispensable for ER cluster development, prophase-arrested specimens of *Cerebratulus* sp. and *C. lacteus* were incubated in 5-HT solutions containing a 20 μM dose of either curcumin or SB-202190, in order to block the JNK (jun-N-terminal kinase) or p38 forms of MAPKs (Chen et al., 2001). Neither curcumin, which is an inhibitor of JNK (Chen and Tan, 1998), nor the p38 blocker SB-202190 (Frantz et al., 1998; Nemoto et al., 1998) stopped ER clusters from forming in mature oocytes (Table 1; Fig. 3A). However, both drugs reduced spontaneous maturation in Ca^{2+}-containing ASW solutions lacking 5-HT (data not shown). Thus, the blockage of spontaneous GVBD by these inhibitors indicates that their inability to alter ER cluster organization was not solely due to oolemmmal impermeability.

Conversely, 5-HT-containing ASW solutions supplemented with an alternative JNK inhibitor, SP600125 (Bennett et al., 2001), blocked ER cluster formation in *Cerebratulus* sp., as only 2/68 (3%) of the oocytes from six females tested showed conspicuous ER aggregates after undergoing GVBD in the presence of SP600125 (Table1; Fig. 3B). The effects of SP600125 on the ER of other nemertean oocytes have not been investigated. However, based on the ability of SP600125-treated *Cerebratulus* sp. oocytes to generate fertilization-induced Ca^{2+} signals (see section 'Fertilization-induced Ca^{2+} signaling'), the lack of ER clustering was not simply due to morbidity.

Reorganization of the ER after fertilizations or roscovitine treatments

In the absence of fertilization, mature *C. lacteus* oocytes retained their ER clusters for at least a day (Stricker et al., 1998), but fertilization caused the clusters to disassemble as the oocytes resumed meiosis and formed polar body 1 and 2 at ~45 and 90 minutes post-insemination, respectively (Stricker, 1996b; Stricker et al., 1998). In *C. lacteus*, such reorganizations of the ER typically began ~40 minutes after sperm addition and were completed by about an hour post-fertilization in 80-90% of the oocytes examined (Stricker et al., 1998) (S.A.S., unpublished).

Fertilization also caused the ER clusters of metaphase-I-arrested oocytes to disassemble both in the case of *Micrura alaskensis* (Stricker et al., 2001) (S.A.S., unpublished) and in *Cerebratulus* sp., where 88% of the oocytes possessing ER clusters prior to insemination underwent cluster disassembly after fertilization (*n*=17). In *Cerebratulus* sp. where the precise timing of ER disassembly was tracked, the ER clusters began to be disassembled as early as 15-20 minutes post-fertilization, which in turn was similar to the ~20 minutes post-fertilization timepoint of first polar body formation in this species. Unlike the ~60 minute timeframe observed for *C. lacteu*s, the clusters of *Cerebratulus* sp. usually disappeared by 30 minutes after sperm addition (Fig. 4A; Table 2). This in turn corresponded to between first polar formation and the production of the second polar body, which was usually achieved by ~45 minutes post-fertilization.

Similarly, in metaphase-I-arrested oocytes of *C. lacteu*s that had been treated with a 25 μM PD98059 prior to insemination, 17/19 (89%) completed disassembly of their clusters by 2 hours post-fertilization (Fig. 4B).

As post-GVBD oocytes of various animals have high MPF activity before insemination and diminished MPF after fertilization (Murray and Hunt, 1993; Kishimoto, 1999; Maller et al., 2001), the effects of roscovitine, a relatively specific inhibitor of MPF (Meijer et al., 1997), were assessed in DiI-loaded, mature specimens to determine if roscovitine-treated oocytes also disassemble their ER clusters in a manner resembling fertilization. In 21/24 *C. lacteus* specimens, a

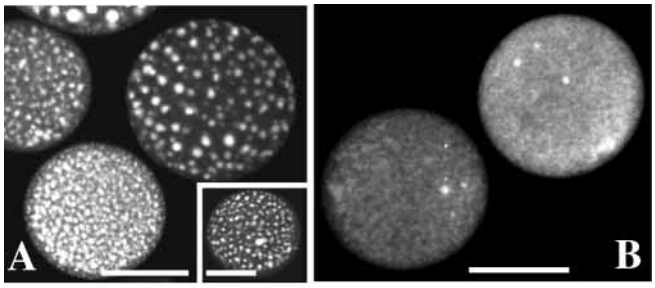

Fig. 3. (A) ER clusters continue to form during 5-HT-induced oocyte maturation in 20 μM curcumin or 20 μM SB-202190 (inset). Large oocyte in upper right of A is from *C. lacteus;* others are from *Cerebratulus* sp. (B) SP600125 fails to block 5-HT-induced GVBD in *Cerebratulus* sp. but does prevent ER clusters formation. Scale bars: 50 μm.

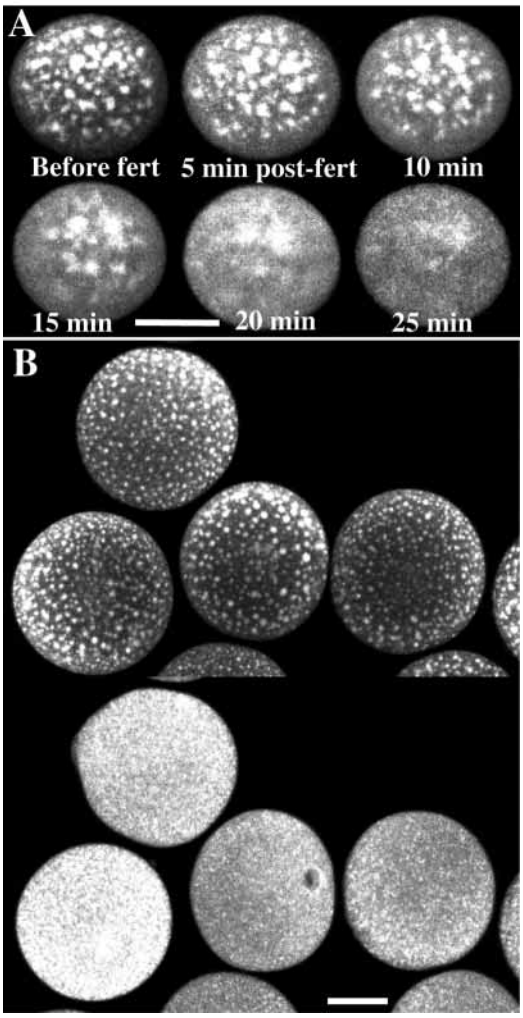

Fig. 4. (A) Post-fertilization loss of ER clusters in a DiI-loaded *Cerebratulus* sp. oocyte. (B) ER clusters that are present in mature oocytes of *Cerebratulus lacteus* before fertilization in 25 μM PD98059 (top) continue to be disassembled by 2 hours post-fertilization (bottom). Scale bars: 50 μm.

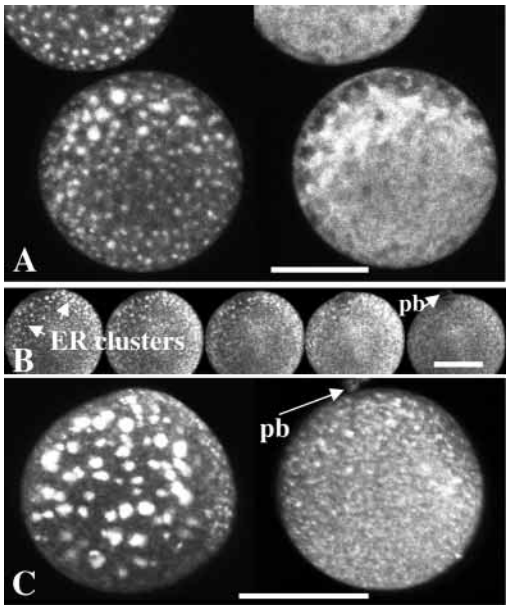

Fig. 5. (A) Left: two *Cerebratulus lacteus* oocytes with ER clusters before roscovitine addition. Right: 60 minutes after roscovitine treatment. (B) Roscovitine-induced disassembly of ER clusters and polar body (pb) formation in *C. lacteus*. Timing from left to right: before roscovitine, and 5 minutes, 10 minutes, 15 minutes and 30 minutes post-treatment. (C, right) A *Cerebratulus* sp. oocyte that spontaneously progressed through metaphase I arrest and formed polar bodies (pb). Note lack of ER clusters compared with metaphase-I-arrested cohort (left). Scale bars: 50 μm.

Table 2. Timing of ER cluster disassembly versus duration of Ca^{2+} oscillations (*Cerebratulus* sp.)

Specimen	Time at which ER clusters are fully disassembled (minutes post-fertilization)	Duration of Ca^{2+} oscillations (minutes post-fertilization)
Female 1	20.7±1.9 (*n*=6)	22.3±5.3 (*n*=6)
Female 2	26.7±3.5 (*n*=8)	32.0±8.9 (*n*=8)

Several oocytes from two female specimens of *Cerebratulus* sp. were injected with either DiI to monitor ER clusters or CGDex/RBDex for Ca^{2+} dynamics (note ER morphology and Ca^{2+} signaling were not examined within the same oocyte, as the double injections required to load DiI and the Ca^{2+}-sensitive probes tended to reduce oocyte viability compared with singly injected specimens). Timelapse confocal microscopy was begun, and sperm were subsequently added to the specimen dish within ~30-60 minutes of injection of the fluorescent probe.

n, number of oocytes.

marked restructuring of the clusters routinely occurred in such unfertilized specimens by 1 hour after the addition of 50 μM roscovitine (Fig. 5A,B). ER clusters were also disassembled in all 12 unfertilized oocytes of *Cerebratulus* sp. that had been treated with roscovitine. In addition, immature oocytes of *C. lacteus* that had been arrested at prophase I by roscovitine continued to retain their GV and failed to form ER clusters after being treated with 5-HT (data not shown), suggesting that roscovitine can also block maturation and ER cluster formation by keeping MPF levels low in prophase-arrested specimens.

Consistent with a roscovitine-induced drop in MPF activity that is presumably necessary to drive mature oocytes from metaphase arrest through the completion of meiosis I, mature oocytes that had been treated with roscovitine not only disassembled their ER clusters but also formed a polar body (Fig. 5B). Similarly, as noted previously (Stricker et al., 2001), a few unfertilized oocytes that had not been treated with roscovitine or any agent other than natural seawater continued through metaphase I for some undetermined reason and made polar bodies. In *Cerebratulus* sp., such spontaneously progressing specimens that presumably lowered their MPF activities before completing meiosis I also lacked prominent ER clusters in all 13 cases examined (Fig. 5C).

To verify that MPF activity decreases after fertilization, Rb phosphorylation assays were performed on fertilized *C. lacteus* oocytes. Such assays indicated a substantial drop in MPF activity occurred after fertilization, as opposed to the lack of a marked decline in metaphase-I-arrested specimens that were not inseminated (Fig. 6A,B). As noted for the Rb signal, fertilization also caused an apparent decrease in ERK1/2 activity, based on phospho-MAPK western blots and phospho-ELK-1 phosphorylation assays (Fig. 6C). However, in both

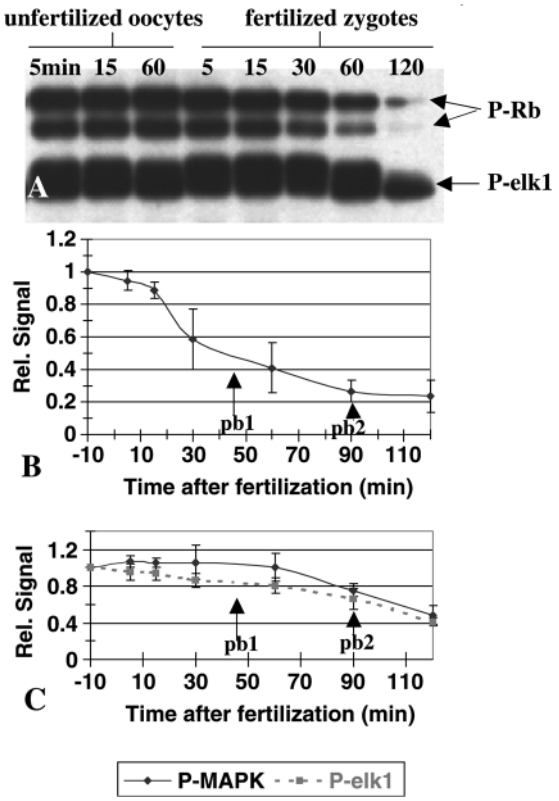

Fig. 6. (A) Typical blot of post-fertilization MPF and MAPK declines, as measured by Rb phosphorylation (upper two rows) and ELK-1 phosphorylation (lowest row), respectively. No decrease is observed in uninseminated metaphase-I-arrested controls. (B) MPF activity in 10 fertilization runs subjected to Rb phosphorylation assays. (C) MAPK activity decreases in fertilization runs assayed by phospho-ERK1/2 western blots ('P-MAPK', n=8) or ELK-1 phosphorylations ('P-ELK-1'; n=8). Bars indicate standard errors. pb1, typical onset of polar body 1 formation; pb2, typical onset of polar body 2 formation.

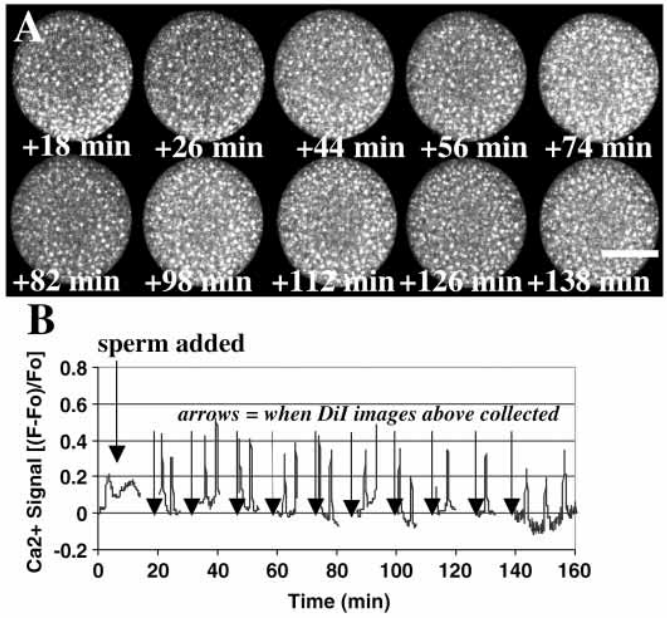

Fig. 7. (A) Colchicine delays fertilization-induced disassembly of ER clusters (A) and prolongs Ca^{2+} oscillations (B). Arrows in B indicate timepoints when Ca^{2+} imaging was discontinued to obtain a z-series of DiI fluorescence. Scale bar: 50 μm. Representative of three doubly injected specimens.

cases, MAPK activity diminished at a much slower rate than that observed for MPF, with apparent $t_{1/2}$ declines in activity registering ~105-115 minutes for MAPK versus ~45 minutes for MPF.

Given that colchicine can maintain high MPF levels apparently by inhibiting cyclin degradation (Hunt et al., 1992), metaphase-I-arrested oocytes of *C. lacteus* were checked for ER reorganizations after insemination in colchicine that had been either microinjected to a final concentration of ~300 μM or simply included at that dosage in the ASW bath. Although considerable variability in ER dynamics was evident across both colchicine-injected and colchicine-bathed specimens, an overall trend towards the sustained presence of ER clusters after fertilization in colchicine could nevertheless be discerned. Thus, 80% of the colchicine-treated mature oocytes (n=40) exhibited ER clusters before fertilization, but only 50% of those cluster-containing specimens fully reorganized their ER by 2 hours post-fertilization in colchicine, as opposed to the 80-90% levels of cluster disassembly that was typically observed in control fertilizations or in inseminations involving MAPK inhibitors. In fact, some oocytes that were treated with colchicine during fertilization retained their ER clusters for at least 2.5 hours while undergoing prolonged fertilization-induced Ca^{2+} oscillations (Fig. 7), which in turn confirmed that fertilization had indeed occurred but failed to reorganize the ER in a timely fashion (see next section).

Fertilization-induced Ca^{2+} dynamics in oocytes with or without altered MAPK or MPF levels

Previous analyses of fertilization in *C. lacteus* revealed that metaphase-I-arrested oocytes normally undergo oscillations that typically last ~60-80 minutes and thus end at a time that coincides with the post-fertilization disassembly of ER clusters (Stricker, 1996b; Stricker et al., 1998). Alternatively, it has been shown that insemination of prophase-arrested specimens, which lacked ER clusters to begin with, triggered only one or a few irregularly arranged, non-wavelike Ca^{2+} transients that typically subsided by 25 minutes post-insemination (Stricker et al., 1998).

Similarly, in this study, multiple Ca^{2+} elevations could be observed in only 6/34 (18%) of the prophase-arrested *C. lacteus* oocytes that were inseminated prior to GVBD, and even in these six, the transients were more irregular and ephemeral than normal oscillations (Fig. 8; Table 3). Mature oocytes of *Cerebratulus* sp. exhibited multiple Ca^{2+} oscillations at fertilization, although the spiking was of higher frequency and typically less protracted than in *C. lacteus* (Fig. 9). Such Ca^{2+} oscillations were nevertheless characteristic of normal development, as specimens displaying these Ca^{2+} responses underwent proper cleavage (Fig. 9, inset). Moreover, the relatively short duration of the fertilization-induced Ca^{2+} oscillations in *Cerebratulus* sp. matched the comparatively rapid post-insemination disassembly of ER clusters displayed by this species (Table 2).

As noted for ascidians (Levasseur and McDougall, 2000),

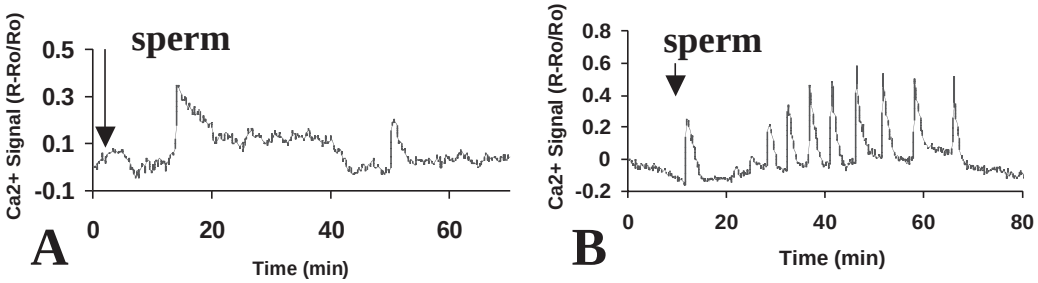

Fig. 8. (A,B) Typical fertilization-induced Ca^{2+} response of immature, prophase-arrested (A) and mature, metaphase-I-arrested (B) oocyte of *C. lacteus*. In general, polar body 1 and 2 in *C. lacteus* form at ~45 and 90 minutes post-insemination, respectively.

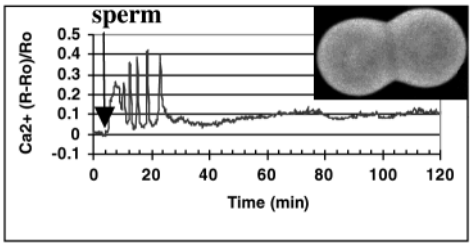

Fig. 9. Normal fertilization-induced Ca^{2+} oscillations in *Cerebratulus* sp. (Inset) two-cell stage that developed at 2.5 hours post-fertilization after generating oscillations shown in graph. In general, polar body 1 and 2 in *Cerebratulus* sp. form at ~20 and 45 minutes post-insemination, respectively.

essentially normal fertilization-induced Ca^{2+} signals were produced by mature *C. lacteus* oocytes that were inseminated in solutions of PD98059 or U0126 (Table 3; Fig. 10A-C). Similarly, fertilizations of 5-HT-treated *Cerebratulus* sp. oocytes in 20 μM U0126 continued to exhibit repetitive Ca^{2+} oscillations (data not shown).

Repetitive Ca^{2+} waves also occurred in four out of seven oocytes that were obtained from two *Cerebratulus* sp. females prior to being matured in 5-HT and fertilized in 20 μM SP600125 (Fig. 11). In two of those four cases, the oscillations appeared essentially normal in amplitude, frequency and overall duration (compare Fig. 9 with Fig. 11A). Alternatively, the other two oocytes displayed an irregular pattern of Ca^{2+} spiking (Fig. 11B). In all 21 cohort oocytes that were examined from these two particular females, no ER clusters formed after 5-HT-induced GVBD in SP600125-containing ASW. Thus, although further analyses are needed to determine unambiguously the effects of SP600125 on Ca^{2+} signaling patterns, at least some form of repetitive Ca^{2+} spiking was evident in four mature specimens that presumably lacked ER clusters, based on the ER morphology observed in DiI-loaded cohorts.

On the other hand, mature oocytes of *C. lacteus* that had been pre-incubated in roscovitine generally failed to produce multiple Ca^{2+} elevations after being inseminated, as only 6/29 (21%) displayed any sort of repetitive Ca^{2+} spiking, and even in these 6 cases, the oscillations were more irregular and ephemeral than normal (Fig. 12A). Similarly, the addition of roscovitine to the specimen dish while fertilization-induced Ca^{2+} oscillations were under way prematurely terminated the Ca^{2+} oscillations (Fig. 12B). Conversely, treatment of mature oocytes with 300 μM colchicine, which tended to maintain ER clusters in inseminated specimens, yielded a substantially longer series of fertilization-induced Ca^{2+} oscillations (Table 2) in 19/33 (58%) of the *C. lacteus* oocytes that exhibited repetitive Ca^{2+} transients (Fig. 7B).

Table 3. Fertilization-induced Ca^{2+} signaling in mature oocytes (*Cerebratulus lacteus*)

Treatment	% displaying Ca^{2+} oscillations after insemination (number of oocytes; number of females)	Time at which fertilization-induced Ca^{2+} oscillations cease* (minutes post-insemination)
Control fertilizations	80.0 (25; 3)	64.5±6.7 (14; 4)
25 μM PD98059	77.1 (35; 4)	69.6±13.6 (26; 4)
300 μM colchicine	57.6 (33; 3)	134±26.0 (8; 3)

*Results are mean±s.d. (number of oocyes; number of females)

Oocytes from several ripe females were injected with CGDex/RBDex and subsequently imaged in the absence of inhibitors (control fertilizations) or in the presence of either PD98059 to block ERK1/2 MAPKs or colchicine (presumably to block cyclin degradation and thereby maintain elevated MPF levels). Not all of the specimens that displayed oscillations were monitored until their oscillations ceased (hence the discrepancy in sample sizes).

DISCUSSION

ERK1/2 MAPKs are not required for ER reorganizations or repetitive Ca^{2+} signaling

Using alternative methods to inhibit and trigger GVBD, this study corroborated a previous analysis of spontaneously maturing oocytes of *Cerebratulus lacteus*, which showed that immature oocytes lack ER clusters and normal Ca^{2+} signaling at fertilization, whereas mature oocytes possess ER clusters and generate multiple Ca^{2+} waves for about an hour post-fertilization, at which time the ER clusters disassemble (Stricker et al., 1998). The current study also expanded on these findings by presenting data from two other nemertean species that verified the overall trend of ER clusters developing during maturation and subsequently disassembling after fertilization.

To analyze possible regulatory mechanisms related to ER cluster formation and disassembly, immature oocytes were incubated in MAPK inhibitors and assayed for ER clusters after 5-HT-induced GVBD. In all species, inhibitors of ERK1/2 signaling failed to prevent the formation of ER clusters even at inhibitor concentrations that significantly reduced MAPK activity. Accordingly, oocytes that were fertilized in the presence of MAPK inhibitors continued to display normal Ca^{2+} dynamics and ER restructurings. Collectively, such findings indicate that active ERK1/2 MAPKs are not required for either

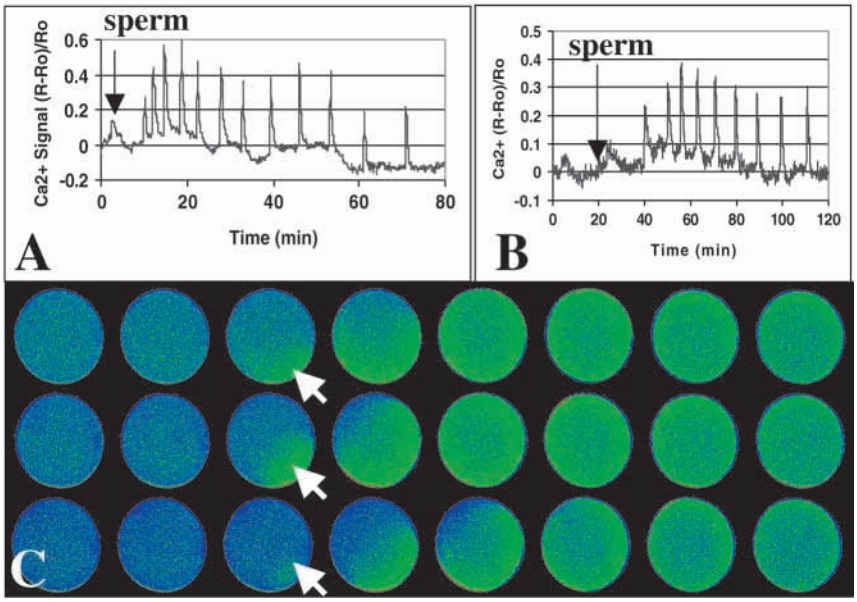

Fig. 10. (A,B) Essentially normal fertilization-induced Ca^{2+} response of *Cerebratulus lacteus* in 25 μM PD98059 (A) or 20 μM U0126 (B). (C) Three consecutive fertilization-induced Ca^{2+} waves (arrows) in PD98059-treated oocyte (images between waves have been deleted). Scale bar: 50 μm.

ER reorganizations or fertilization-induced Ca^{2+} signaling. To our knowledge, this is the first report of the effects of the MAPK signaling pathway on ER structure in maturing or fertilized oocytes.

A comparable lack of effect was also observed in oocytes treated with either the p38 inhibitor SB-202190, or curcumin, which supposedly targets JNK MAPKs. Even though such inhibitors could block spontaneous GVBD, thereby demonstrating their ability to cross the oolemma, they did not routinely alter ER structure relative to controls. However, in tests employing another JNK inhibitor, SP600125, oocytes of *Cerebratulus* sp. completed 5-HT-induced GVBD but failed to display ER clusters. Exactly why SP600125 blocked cluster formation, while another putative JNK antagonist, curcumin, did not, remains unknown. Perhaps, either curcumin or SP600125 targeted other substrates in addition to JNK that the other putative JNK inhibitor did not affect, based on the findings that supposedly specific kinase inhibitors often show some nonspecificity when tested against a broad spectrum of substrates (Davies et al., 2000).

The loss of ER clusters in response to SP600125 may also pertain to the results of a previous study in which inhibition of the proteasome by MG-132 stabilized ER clusters following fertilization of mouse oocytes (FitzHarris et al., 2003). Such results were attributed to the ability of MG-132 to block the post-fertilization decrease in MPF activity, given that either MG-132 or cyclin B overexpression maintained elevated MPF levels and ER clusters in experimentally treated zygotes, whereas a roscovitine-triggered inactivation of MPF caused cluster disassembly (FitzHarris et al., 2003). However, MG-132 is also known to activate JNK in various somatic cells (Meriin et al., 1998; Nakayama et al., 2001). Hence, the regulation of ER structure by activated JNK, either as part of the MPF signaling pathway or as a redundant modulator in parallel to MPF activity, remains a possibility.

MPF levels apparently modulate repetitive Ca^{2+} signaling and cell-cycle-dependent reorganizations in ER structure

Based on the apparently non-essential nature of ERK1/2 MAPK signaling in ER reorganizations, the possible effects of MPF were assessed both in normally maturing specimens and in aberrant cases of development. By monitoring ER and Ca^{2+} dynamics in *Cerebratulus* sp. where the first cell cycle is accelerated compared that of *C. lacteus*, it could be shown that ER cluster disassembly and the cessation of fertilization-induced Ca^{2+} oscillations coincided with each other. However, instead of the ~60 minute timing that was displayed by *C. lacteus*, ER disassembly and oscillation cessation in *Cerebratulus* sp. were completed by ~30 minutes post-insemination, indicating that ER cluster disassembly occurs relative to progression in the first cell cycle, rather than absolute time after fertilization.

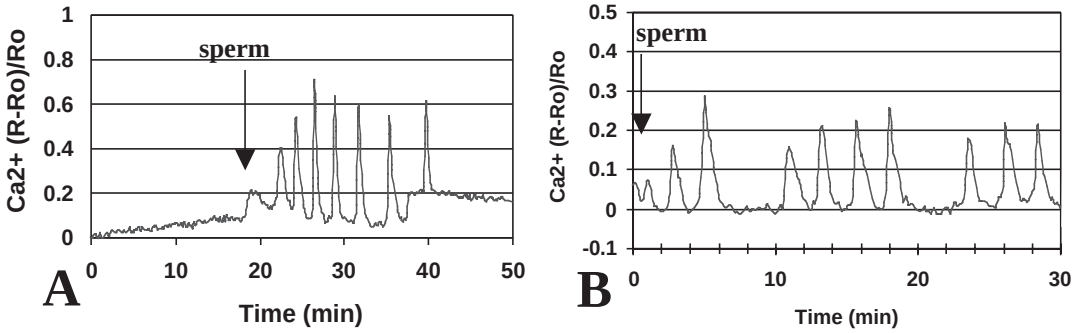

Fig. 11. (A,B) Two examples of Ca^{2+} oscillations of mature *Cerebratulus* sp. oocytes fertilized in ASW solution of SP600125. (A) Essentially normal oscillations that were characteristic of two out of four specimens showing repetitive Ca^{2+} elevations after fertilization (compare with Fig. 9 for normal oscillations in a control specimen). (B) Abnormal repetitive spiking that was characteristic of the other two oocytes displaying multiple fertilization-induced Ca^{2+} rises.

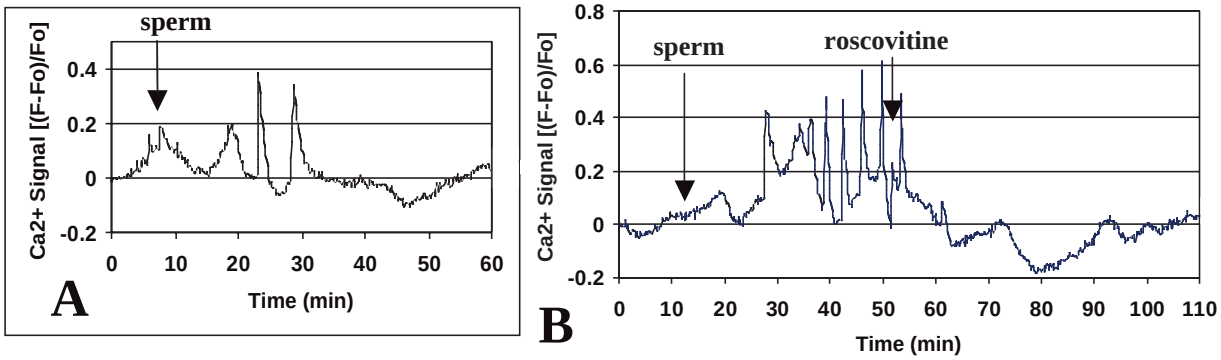

Fig. 12. (A) 50 μM roscovitine pre-treatment prevents normal fertilization-induced Ca^{2+} oscillations in *C. lacteus*. (B) When added during the oscillations, 50 μM roscovitine causes premature termination of Ca^{2+} response.

In particular, ER disassembly appeared to track the drop in MPF levels that drives oocytes from metaphase arrest to polar body formation (Sagata, 1996; Sagata, 1997). Accordingly, in idiopathic cases where nemertean oocytes failed to stop at metaphase I and for some undetermined reason continued to make polar bodies, such spontaneously progressing specimens lacked prominent ER clusters after polar body production and thus mimicked the events normally triggered by fertilization. In addition, whether MPF activity actually increases in maturing *C. lacteus* oocytes that form ER clusters and drops in fertilized specimens that undergo ER cluster disassembly was monitored using an Rb fusion protein as a substrate for active MPF. Such activity assays demonstrated an apparent pre-GVBD rise in MPF as well as a post-fertilization drop, and similar results were also obtained by using phospho-specific antibodies against cdc2 epitopes (anti-phospho-Y15 and anti-phospho-T161) to track phosphorylation states indicative of inactive and active MPF (data not shown).

Exactly why no clear increase in MPF activity was observed before the second meiotic division remains to be determined. In any case, an overall trend of MPF activity rising before GVBD and falling after fertilization was nevertheless evident. Moreover, P-MAPK and ELK-1 assays showed that compared with the MPF drop, a much slower decrease in MAPK activity occurred during the completion of meiosis. Such a continued elevation in MAPK activity while MPF levels fall has been noted for other species (Verlhac et al., 1994; McDougall and Levasseur, 1998), and presumably functions to prevent an intervening S phase from occurring between meiosis I and II (Tachibana et al., 2000).

In order to assess whether MPF levels can affect ER structure and Ca^{2+} signaling, mature specimens were treated with roscovitine and were found to disassemble their ER clusters in a manner similar to that demonstrated for mouse oocytes (FitzHarris et al., 2003). Moreover, as noted in other species (Deng and Shen, 2000; Levasseur and McDougall, 2000; Gordo et al., 2002), nemertean oocytes failed to produce normal Ca^{2+} oscillations if pre-treated with roscovitine, and applications of roscovitine after fertilization-induced Ca^{2+} oscillations had already started prematurely terminated the oscillatory response as shown for mice (Deng and Shen, 2000). This suggests that ER structure and Ca^{2+} signaling were altered by a roscovitine-induced inhibition of MPF, but whether MPF was indeed the target of the action of the drug, or the only target affected, remains to be determined.

Conversely, in an attempt to maintain elevated MPF levels for a protracted period, oocytes were inseminated in colchicine. Colchicine is not as specific a blocker of the fertilization-induced drop in MPF, as other treatments that have been used (Levasseur and McDougall, 2000; FitzHarris et al., 2003). Nevertheless, microtubule-depolymerizing agents such as colchicine delay cleavage and/or prolong Ca^{2+} signaling in various animals (Hunt et al., 1992; Keating et al., 1994; Jones et al., 1995; Stricker, 1995; Gordo et al., 2002), and this effect may result from a maintenance of MPF activity owing to reduced cyclin B degradation (Whitfield et al., 1990; Hunt et al., 1992). Accordingly, during fertilization of nemertean oocytes, colchicine-treated specimens typically generated Ca^{2+} oscillations for 1 hour longer than did controls, and the ER clusters remained evident during such prolonged oscillations. This in turn suggests MPF levels affect both ER reorganizations and fertilization-induced Ca^{2+} signals, but exactly how MPF might exert its putative effect on the ER remains unknown.

What are the relationships between ER structure, MPF levels and Ca^{2+} signaling patterns?

Given the apparent linkage between ER clusters, MPF levels and fertilization-induced Ca^{2+} oscillations, the clusters could be strictly required to produce a normal Ca^{2+} response at fertilization, or they may be dispensable for repetitive Ca^{2+} spiking in at least some mature oocytes. Preliminary results from fertilizations of *Cerebratulus* sp. oocytes in SP600125 tend to support the latter conclusion, because in some cases repetitive Ca^{2+} spiking could still be observed in the presence of SP600125 – a drug that, in parallel studies, proved to be highly effective in preventing the formation of ER clusters. Such findings suggest either that the clusters are not involved at all in producing a repetitive Ca^{2+} response at fertilization, or that they indeed facilitate normal Ca^{2+} signaling, but their absence can be compensated for by redundant mechanisms that do not rely on ER aggregates. It should be noted, however, that of the seven specimens that were treated with SP600125 prior to fertilization, only two displayed what could be classified as fully normal Ca^{2+} oscillations. Thus, it remains possible that ER clusters are indeed generally required for producing a completely normal Ca^{2+} response at fertilization. Accordingly,

this unresolved question is to be investigated further in subsequent analyses that aim to identify the exact targets of SP600125, while clearly defining the patterns of ER reorganizations and Ca^{2+} dynamics in oocytes that retain their viability after being doubly injected with DiI and Calcium Green.

Should ER clusters turn out to be involved in mounting a normal Ca^{2+} response at fertilization, two logical questions to address are by what means are ER clusters formed, and how might such structures facilitate the production and transmission of repetitive Ca^{2+} waves at fertilization. As for how clusters form, microtubules do not appear to be essential for ER cluster maintenance, based on the continued presence of these aggregates in mature oocytes that had been treated with colchicine. Accordingly, recent analyses of *Xenopus* oocyte extracts reveal that an F-actin/myosin-V network is capable of rapidly repositioning ER components (Wollert et al., 2002), indicating that such a non-microtubular-based system could also play a role in reorganizing the ER of nemertean oocytes.

As to how ER clusters might facilitate Ca^{2+} signaling, similar appearing structures in the ER of vertebrate oocytes possess numerous Ins(1,4,5)P_3 receptors that regulate Ca^{2+} release from cisternal stores (Shiraishi et al., 1995; Mehlmann et al., 1996; Kume et al., 1997; Fissore et al., 1999; Terasaki et al., 2001). In vertebrate oocytes, the predominant isoform is a type 1 Ins(1,4,5)P_3 receptor (Miyazaki et al., 1993; Parys and Bezprozvanny, 1995; Fissore et al., 1999; Kline et al., 1999; Oda et al., 1999; Goud et al., 2002), although the ER clusters of mice may also possess type 2 Ins(1,4,5)P_3 receptors (Fissore et al., 1999). Thus, the ER clusters of nemertean oocytes may facilitate Ca^{2+} release, owing to the increased density and/or specific types of Ins(1,4,5)P_3 receptors that are present in such aggregates.

ER clusters of nemerteans could also aid in the global propagation of Ca^{2+} waves by helping to integrate isolated 'elementary Ca^{2+} signals' (Bootman and Berridge, 1995; Berridge, 1997). Such signals are elicited as localized 'puffs' (Bootman et al., 2001) that spread ~6-7 μm, based on measurements of *Xenopus* oocytes (Marchant and Parker, 2001). This value in turn corresponds well to the inter-cluster distance observed in nemertean oocytes (Stricker et al., 1998), suggesting that ER clusters may be positioned in a manner to aid global wave propagation.

Comparative biology of ER dynamics and Ca^{2+} signaling: patterns displayed by these protostome worms have parallels in other animal groups

Based on classical embryological studies and more recent molecular data (Winnepenninckx et al., 1995; Turbeville, 2002), nemerteans clearly constitute a phylum of protostome worms. As such, nemerteans represent the only non-deuterostome group of animals that has been investigated for both ER structure and Ca^{2+} signaling patterns during oocyte maturation and fertilization. However, in spite of the fact that nemerteans are evolutionarily far removed from mammals and other deuterostome animals, it is clear that the patterns observed here are not simply unique attributes of a highly aberrant group of invertebrates. ER aggregates also form during oocyte maturation in deuterostomes such as ascidians and mammals that undergo Ca^{2+} oscillations at fertilization (Speksnijder et al., 1993; Mehlmann et al., 1995; Kline et al., 1999). To our knowledge, neither the relationship between ER clusters and MPF activities nor the fate of the individual ER clusters after fertilization has been reported for ascidians, although the overall intracellular localizations of ER-rich cytoplasm are well known (Speksnijder et al., 1993; Dumollard and Sardet, 2001; Dumollard et al., 2002). In mouse oocytes, however, the cortical ER clusters of mature oocytes disassemble at ~1.5-3.5 hours post-insemination, and this disassembly is apparently caused by a drop in MPF levels (FitzHarris et al., 2003). Similarly, in *Xenopus*, which produces a single Ca^{2+} wave at fertilization (Fontanilla and Nuccitelli, 1998), ER clusters that are about 3-5 μm in diameter develop transiently at metaphase I and then again in MII-arrested specimens (Terasaki et al., 2001). Such clusters subsequently dissipate within a few minutes after MII-arrested oocytes generate a Ca^{2+} wave in response to prick activation (Terasaki et al., 2001). Accordingly, the high levels of MPF that occur at metaphase II arrest are dramatically reduced within ~10 min after prick activation of these oocytes (Watanabe et al., 1991).

Such findings generally fit the cell cycle-related changes in ER structure presented here and coincide well with recent demonstrations that elevated MPF levels sustain fertilization-induced Ca^{2+} oscillations in ascidian and mammalian oocytes (Deng and Shen, 2000; Levasseur and McDougall, 2000; Nixon et al., 2000; Gordo et al., 2002). However, unlike in nemerteans where the ER clusters disappear at about the time that fertilization-induced oscillations cease, the ER clusters of mouse zygotes disassemble a full 2 hours before the Ca^{2+} oscillations are terminated (FitzHarris et al., 2003).

Although the reasons for this discrepancy remain unknown, a differential timing of the downregulation of Ins(1,4,5)P_3 receptor functioning may play a role (Parrington et al., 1998; He et al., 1999; Brind et al., 2000; Jellerette et al., 2000). In mammals that possess a relatively protracted first cell cycle, the fertilization-induced downregulation of Ins(1,4,5)P_3 receptors may take longer to achieve than does the post-fertilization disassembly of ER clusters. Conversely, ER cluster disassembly and Ins(1,4,5)P_3 receptor downregulation may simply be more synchronized during the comparatively short first cell cycle of nemerteans. Alternatively, fertilization-induced Ca^{2+} oscillations cease in mammals at the time of pronuclear formation, presumably owing to the sequestration of either a soluble oscillogenic factor produced by the sperm or a critical downstream target required for Ca^{2+} oscillations (Jones et al., 1995; Kono et al., 1995; Day et al., 2000). However, the termination of Ca^{2+} signaling by nemertean pronuclei seems less likely, as fertilization-induced oscillations tend to cease before the second polar body is formed.

In any case, a model (Fig. 13) can be proposed for nemerteans. According to this model, prophase-arrested oocytes with low MPF levels lack ER clusters, whereas mature metaphase-I-arrested oocytes with high MPF levels assemble ER clusters that may facilitate the production of a normal fertilization-induced Ca^{2+} response. After fertilization, MPF activity is reduced, and the ER clusters are eventually disassembled between first and second polar body formation.

Although this model implies a tight correlation between MPF levels and the presence or absence of ER clusters, factors other than just MPF may also regulate ER organization. For example, a post-fertilization decrease in MAPK activity could act synergistically with the MPF decrease to cause disassembly

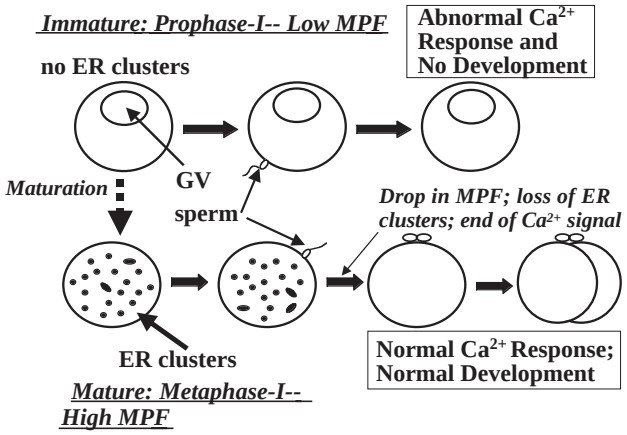

Fig. 13. General model of the interactions between ER structure, MPF levels and Ca^{2+} signaling during oocyte maturation and fertilization in nemerteans.

of ER clusters. Similarly, a simple increase in MPF levels is probably not always sufficient by itself to trigger ER cluster formation, as prominent ER aggregations have not been observed either in the later stages of meiosis or during early cleavages (Stricker et al., 1998) (S.A.S., unpublished).

Aside from being spawned externally into the sea prior to fertilization, as opposed to undergoing internal fertilization in the female reproductive tract, the oocytes of nemerteans and mammals differ in the arrest point that is attained prior to fertilization (metaphase I versus typically, metaphase II), the length of the first cell cycle (a few hours versus up to about a day), and the absence versus presence of follicle cells, respectively. Nevertheless, in spite of such marked differences in the oocytes of these two distantly related groups of animals, a general concordance can be seen in the relationship between MPF levels on the one hand and both ER structure and Ca^{2+} signaling patterns on the other. Moreover, based on currently available data, an overall trend of MPF levels affecting Ca^{2+} signals and/or ER structure may also exist for ascidians and frogs. Such findings, in turn, suggest that further investigations of other animal groups are warranted in order determine what roles MPF activities and ER structure might play in shaping fertilization-induced Ca^{2+} signals in general.

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