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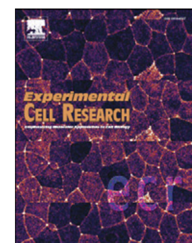

Research Article

Two-stage dependence for 1-methyladenine induced reinitiation of meiotic maturation in starfish oocytes

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ABSTRACT

The maturation hormone 1-methyladenine (1-MA) causes meiotic resumption of prophase arrested immature starfish oocytes. Continuous exposure to ≥0.5 μM 1-MA causes germinal vesicle breakdown (GVBD) in ~20 min, but oocytes pretreated for >30 min with a subthreshold dose of 1-MA undergo GVBD much faster (~10 min) when they are exposed to 1 μM 1-MA. Furthermore, a very low subthreshold 1-MA suffices to start the maturation process: oocytes exposed to 0.005 μM 1-MA for up to 10 min followed by 1 μM 1-MA is equivalent to continuous exposure to 1 μM 1-MA. These dose and timing relationships indicate that there is a two-stage dependence on 1-MA. A possible explanation for this dependence is that there are two processes involved: an initial process that is triggered by a low dose of 1-MA, and a second process that cannot start until the first process is completed and is stimulated by a higher dose of 1-MA. These subthreshold 1-MA effects on GVBD timing are not directly coupled to changes in calcium physiology that also occur during maturation. Subthreshold 1-MA was found to cause a transient accumulation of Cdc2/cyclin B into the nucleus. The two-stage dependence indicates that there are unsuspected features in this well-studied pathway leading to GVBD. In the animal, this hormone dependence may help to synchronize maturation throughout all parts of the ovary.
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Introduction

In higher animals, fully grown oocytes in the ovary are arrested at the G2/M transition of meiosis I. In most invertebrates and all vertebrates, these oocytes are "immature" in the sense that they do not develop normally if they are fertilized [1]. Endocrine signalling initiated from the nervous system induces changes in the ovary related to ovulation/spawning including oocyte maturation, during which meiosis is re-initiated and physiological changes occur in preparation for fertilization.

Many of the molecules involved in meiosis re-initiation have been found to be involved in the G2/M transition of somatic cells [2]. The pathway from binding of maturation hormone to nuclear envelope breakdown is relatively simple and many of the components of the pathway have been identified [3,4]. In the future, maturation could be among the first processes to be thoroughly understood at the level of the cell in terms of protein amounts, rate constants, protein locations, modules, and other similar quantities and system properties.

Studies with starfish oocytes began with the discovery that boiled extracts of the radial nerve injected into the coelomic cavity induces

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Abbreviations: 1-MA, 1-methyladenine; GVBD, germinal vesicle breakdown; IP_3, inositol 1,4,5 trisphosphate
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spawning [5]. Kanatani and co-workers [6] elucidated features of how the radial nerve factor acts on the ovary. The radial nerve factor causes breakdown of the interior tissue structure (follicles) which is required for the ovarian wall muscles to be able to expel the oocytes during spawning. Furthermore, the radial nerve factor acts on the ovarian tissues (later shown to be the follicle cells) to produce a second factor that acts directly on the oocytes to induce maturation [7]. This second factor, the "maturation hormone," was identified as 1-methyladenine (1-MA) [8].

Isolated starfish oocytes undergo maturation relatively rapidly and synchronously in response to 1-MA. The oocytes can be isolated as pure populations that are very well suited for biochemical studies. The oocytes can also be microinjected and their optical clarity makes them excellent specimens for light microscopy. These experimental advantages made possible many studies of intracellular mechanism. It was by using starfish oocytes that the H1 kinase assay for *Cdc2* was developed [9], the *Cdc2* inhibitor roscovitine was discovered [10], and *Cdc2*/cyclin B was identified as a one to one complex [11]. It is possible that all of the regulatory molecules leading to GVBD have been identified with the exception of the 1-MA receptor. It has been proposed that the pathway consists of the following: 1-MA receptor activates G-protein βγ subunits, which activate PI3 kinase, which activates *Akt*, which inactivates Myt1, which trips the *Myt1*/*Cdc25* positive feedback loop causing activation of *Cdc2*/cyclin B, which phosphorylates targets in the nucleus to cause GVBD [3].

The framework of the pathway of starfish oocyte maturation is to a large extent established. However, there are still many features, particularly related to dynamics, that are not well understood. We report here a two-stage dependence on 1-MA that indicates the existence of unsuspected features in this well-studied pathway.

Materials and methods

Animals

North American west coast bat stars (*Asterina miniata,* also known as *Patiria miniata*) were collected either by Karl Menard (Bodega Marine Laboratory, Bodega Bay, CA) in northern California or by Pat Leahy (Kerckhoff Marine Laboratory, Corona del Mar, CA) in southern California. For long-term storage, the starfish were kept in running sea water tanks in the Marine Resources Facility at the Marine Biological Laboratory at Woods Hole, MA. For short term use (4–8 weeks), starfish were kept in a recirculating tank with artificial sea water (Tropic Marin; Wartenberg, Germany) at the University of Connecticut at 15–17 °C.

Oocyte isolation

Since subthreshold concentrations of 1-MA change the GVBD timing (see Results), we changed from a rapid oocyte isolation method used previously [12] to a slightly modified, longer method in order to minimize exposure to 1-MA during oocyte isolation [13]. Using a razor blade, a single radial cut (1.5–2 cm in length) on the ventral side between two arms was made and a piece of ovary was removed by curved forceps. The ovary piece was transferred to ~15 ml calcium-free sea water (440 mM NaCl, 9 mM KCl, 23 mM $MgCl_2$, 26 mM $MgSO_4$, 2 mM $NaHCO_3$) for 15 min, then replaced with fresh calcium free sea water for another 15 min (all at room temperature). During the incubation, the calcium inside the ovary diffuses out through the permeable ovary walls. This causes the follicles to fall apart and the follicle cells to detach from the oocytes, after which the ovary wall muscle can be stimulated to squeeze out the oocytes as in normal spawning. The calcium depleted ovary was transferred to a 35 mm petri dish with 3 ml sea water with 10 μM acetylcholine (Sigma Chemical; St. Louis, MO; 10 mM stock solution in water, stored at 4 °C) which results in expulsion of the oocytes.

The oocytes were transferred by pasteur pipette to a scintillation vial containing 15 ml sea water and swirled. After settling (~5 min), the oocytes were transferred by pasteur pipette to a 60-mm (polystyrene) petri dish containing ~10 ml sea water. Full size, GV intact oocytes were then separated from damaged and spontaneously maturing oocytes using a dissecting scope (25× final magnification) with bottom illumination. Several hundred oocytes were sorted and transferred to a second 60-mm petri dish and were stored in an 18 °C incubator. Experiments described here were done in the afternoon using oocytes isolated in the morning, or sometimes on the following day. Oocytes can be stored for at least several days with <10% mortality rate. GVBD timing and 1-MA dose response was similar to that on the day of isolation, though after 2–3 days, there sometimes was a two fold increase in 1-MA sensitivity and a shortening of the GVBD timing by several minutes.

1-MA experiments

Natural sea water (obtained at the Marine Resources Center of the MBL at Woods Hole) was used to keep the oocytes and for experiments. 1-MA was obtained from either Spectrum Chemicals and Laboratory Products (Gardena, CA) or Sigma Chemical (St. Louis, MO). A stock solution of 1 mM 1-MA in water was stored at 4 °C for several months; this was checked against stocks stored at −20 °C and found to have the same potency. For experiments, a more dilute stock solution of 10 μM was made in sea water, then further dilutions were made from this stock. For the 1-MA dose dependence experiments, a serial two fold dilution from 1 μM was made then 0.24 ml of each concentration was transferred into individual wells of a 96 well plate (Nunc; Rochester, NY; untreated polystyrene flat bottom, #12-565-210). For this and other timing experiments, 12–20 oocytes were transferred into each well in ~4 μl by a pipetman. GVBD timing was monitored in the wells using a dissecting scope at 40× magnification or an inverted microscope with a 10× objective lens. The time when 50% of the oocytes had undergone GVBD was recorded as the time of GVBD.

The temperature dependence of GVBD timing was determined using the temperature gradient of a PCR machine (Mastercycler Gradient; Eppendorf, Westbury, NY). The 11 rows were programmed at 1 °C intervals. Oocytes were put into 0.65 ml Eppendorf tubes and monitored every minute in the bottom of the tube using a dissecting scope. For determination of the hormone dependent period, oocytes were put into a 60 mm dish with 5 ml sea water + 1 μM 1-MA. At the various times, ~12 oocytes were removed by pipetman in ~4 μl and transferred to a test tube (12×75 mm, borosilicate glass, Fisher 14-961-26) filled with sea water (6.2 ml) and allowed to settle the length of the test tube.

IP_3, βγ, and cyclin B-GFP experiments

Quantitative injections were done using front loaded needles with a mercury droplet to allow for fine control of pressure [14].

For the IP_3 experiments, oocytes were first injected with a 1% oocyte volume (30 pl) of 1 mM Ca green 10-kDa dextran (Molecular Probes, Eugene, OR) (final concentration 10 μM). IP_3 was injected as 1% oocyte volume of 1 μM IP_3 (Calbiochem; San Diego, CA) (final concentration 0.01 μM). Simultaneous with the IP_3 injection, Ca-Green fluorescence was imaged with a 20× 0.5 N.A. objective on a Zeiss upright microscope. In the first set of experiments, the fluorescence was monitored using a photomultiplier tube connected to a chart recorder [15] and were done at 18–20 °C, while in the subthreshold 1-MA experiments, the fluorescence was imaged with a confocal microscope (BioRad MRC600; Hercules, CA) at room temperature.

Injection of βγ protein purified from bovine retina at 1.8 μM or above (final concentration) caused GVBD to occur with the same timing as application of 1 μM 1-MA [16], so it became of interest to determine the effect of subthreshold βγ on GVBD timing. The original βγ preparation was unavailable, so recombinant bovine G protein βγ (wild type β1-his6γ2) produced in Sf9 cells and concentrated to 3.5 μg/μl (78 μM) was obtained from Tohru Kozasa (University of Illinois at Chicago) [17]. An inactive β1 subunit mutant C68S (3.4 μg/μl) was injected as control; at the highest concentrations it did not cause GVBD. 0.6 μM βγ and above was sufficient to cause GVBD, but the timing of GVBD was 20–30 min slower than 1-MA (data not shown), which made it much more difficult to find a relationship between subthreshold βγ and GVBD timing.

Cyclin B-GFP was expressed by injection of mRNA as described previously and imaged on the confocal microscope with a 40× 1.2 N.A. objective lens [12].

Results

Variation in response to 1-MA

The North American west coast starfish *Asterina miniata* was used for these studies. When 1-MA is added to isolated oocytes, GVBD occurs after approximately 20 min. The timing is quite uniform for any given group of oocytes; usually, >90% undergo GVBD within 2–3 min of each other, so this system should be well suited for determining the timing and order of activation events in the maturation pathway. However, in our experiments, the timing of GVBD (i.e., when 50% of the oocytes undergo GVBD) varied significantly from day to day and even hour to hour. There seemed to be environmental factors or conditions that affect GVBD timing and other features of the response to 1-MA. In addition, the oocytes from different individual animals seemed to undergo GVBD with different timing. We found it necessary to try to understand and control this variation.

One environmental factor that affects GVBD timing is temperature. GVBD timing was fastest between ~22 and ~26 °C (Fig. 1). This is also the range where GVBD timing is least sensitive to temperature change, so experiments were done routinely in this convenient temperature range. GVBD timing was more sensitive to higher than lower temperature, so higher temperatures were particularly avoided.

Another environmental factor that affects GVBD timing is tonicity. While testing microscope chamber conditions, we found that GVBD was significantly slower in open shallow depression slides compared to 96 well plates, test tubes or petri dishes, and that this effect could be eliminated by preventing evaporation from the depression slide. This is consistent with previous observations on oocytes from the Japanese starfish *Asterina pectinifera*—a change of 50 milliosmols had a detectable effect on the threshold dose for 1-MA, and a change of 100 milliosmols caused the threshold dose to decrease by 2-fold [18].

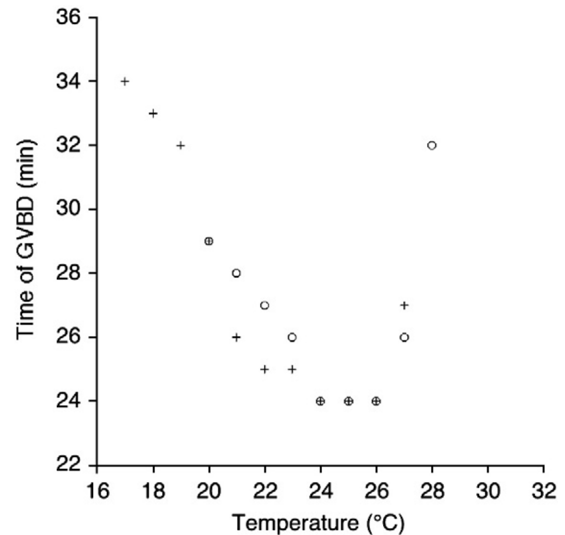

Fig. 1 – Temperature dependence of 1-MA induced GVBD. Oocytes were put into pre-equilibrated tubes in the wells of a temperature gradient PCR machine programmed to 1 °C steps. Every minute, the tubes were removed individually for a few seconds to be inspected in a dissecting scope for GVBD. Two experimental runs were done in order to span a greater temperature range. Similar results were obtained on another batch of oocytes. In the range from ~22 to 26 °C, GVBD was rapid and more stable to temperature changes, so experiments were done in this range.

The oocyte isolation procedure also affected the 1-MA response. In the ovary, each oocyte is surrounded by a layer of follicle cells. During natural spawning, these cells make and release 1-MA in response to the neural hormone, gonad stimulating substance (GSS) [19]. To work with oocytes outside of the ovary, the follicle cells are removed by treatment with calcium-free sea water. The oocyte isolation process invariably results in the production of some 1-MA and depending on how the isolation is done as well as the species of starfish, a fraction of oocytes always undergoes spontaneous maturation [20]. We found that exposure to subthreshold concentrations of 1-MA causes GVBD to occur much faster in response to 1 μM 1-MA (described in detail in the next section). We switched from our previous isolation procedure to a more involved procedure which reduced exposure to subthreshold 1-MA (see Materials and methods); after switching to this procedure, there was significantly less day to day variation in GVBD timing.

Dose response characteristics have not been as well documented in *A. miniata* as in the other starfish *A. pectinifera* and the European *Marthasterias glacialis*. Above a certain concentration (0.25–0.5 μM), GVBD occurred in 100% of the oocytes at a minimal time (i.e., the timing of GVBD did not become faster with higher concentrations). In the "threshold" dose range (0.125–0.25 μM), the synchrony was poor, the percentage that underwent GVBD

was often under 100% and the average GVBD time was longer, up to 60 min. The timing and fraction of GVBD in closely spaced threshold range of concentrations was observed (Fig. 2). In "subthreshold" doses (0.05–0.125 µM and below), none of the oocytes underwent GVBD even after overnight incubation.

Oocytes from different animals have different GVBD timing and sensitivity to 1-MA. For instance, among six animals tested within a short period, the GVBD timing was 15, 16, 19, 20, 22 and 24 min. In contrast, the sensitivity to 1-MA varied less, in the oocytes from one animal, the threshold dose was 0.06 µM and in the oocytes from the other animals, the threshold dose was 0.012 M. There may also be longer term variations; in previous seasons, the average GVBD timing was a few minutes longer, and the oocytes were ~2 times less sensitive to 1-MA. This may be a difference in a group of animals (where they were collected, etc.), or possibly to changes in isolation, storage or experimental conditions.

Effects of subthreshold 1-MA

GVBD in response to 1 µM 1-MA was significantly faster when oocytes were pre-treated with a subthreshold concentration of 1-MA. For example, the following data were collected for a batch of oocytes that underwent GVBD at 25 min in 1 µM 1-MA and which had a subthreshold concentration (i.e., 0% GVBD in 1 h or more) of 0.1 µM. When these oocytes were pre-treated for 60 min with 0.1 µM 1-MA then put into 1 µM 1-MA, GVBD occurred in 11 min. 30–45 min pretreatment with subthreshold 1-MA produced a maximum decrease in GVBD timing (Fig. 3A). When oocytes were kept in subthreshold 1-MA for a day, they still underwent rapid GVBD when exposed to 1 µM 1-MA. To test how long the subthreshold exposure affected the oocytes, oocytes were treated with subthreshold 1-MA for 1 h, then put back into sea water. The effect on GVBD timing wore off gradually over a period of about 5 h (Fig. 3B).

The dose dependence for a 1-h treatment with subthreshold 1-MA was tested (Fig. 3C). Concentrations from about 1 nM to about 5 nM had an increasing effect on GVBD timing, then a 10-fold range of concentration, from 5 nM to 50 nM 1-MA, had the same effect on GVBD timing. Above this, GVBD timing was reduced even further but then was difficult characterize because a fraction of oocytes underwent GVBD in the threshold concentrations.

A somewhat more complicated protocol may give the most insight into the subthreshold effects. The following data were collected for a batch of oocytes that underwent GVBD at 24 min in 1 µM 1-MA. The oocytes were exposed to 0.01 µM 1-MA for varying amounts of time then changed to 1 µM 1-MA. For subthreshold exposures of 10 min or less, GVBD still occurred at 24 min; in other words, the sum of the subthreshold and suprathreshold exposure times was 24 min (Fig. 4). This shows that a subthreshold dose suffices for the first part of the maturation pathway. When the subthreshold exposure was longer than 10 min, the interval between 1 µM 1-MA addition and GVBD was a constant interval (in this case, 14 min). For longer subthreshold exposures the time interval between 1-MA addition and GVBD decreased (just beginning at 16 min in this experiment) and reached a minimum value (Fig. 3A).

Thus, a very low concentration of 1-MA is sufficient to start maturation, but the high, "supra-threshold" concentration is required to complete it. Another way to characterize these results is that that subthreshold 1-MA brings the oocyte to a stable intermediate stage, and that suprathreshold 1-MA is required to progress to GVBD.

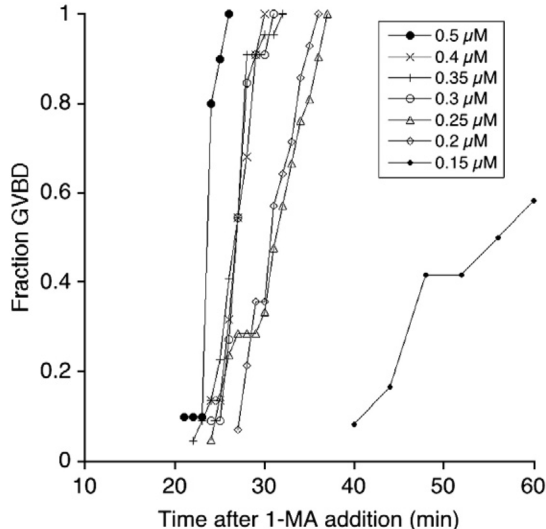

Fig. 2 – GVBD timing for threshold doses of 1-MA. The GVBD timing and fraction for intermediate, "threshold" doses was characterized. For this batch of oocytes, 0.5 µM was a suprathreshold dose which caused rapid GVBD in 100% of oocytes, while 0.1 µM was a subthreshold dose which failed to cause any oocytes to undergo GVBD within 1 h. The concentration was varied in 0.05 µM increments, from 0.4 µM down to 0.1 µM. Symbols: large filled circle 0.5 µM, diagonal cross 0.4 µM, vertical cross 0.35 µM, open circle 0.3 µM, open triangle 0.25 µM, open diamond 0.2 µM, small filled circle 0.15 µM. In 0.15 µM, 8/12 oocytes had undergone GVBD at 72 min, then 8/12 at 2 h. In 0.1 µM, 0/18 had undergone GVBD after 2 h.

Hormone dependent period

With respect to the timing of hormone requirements, suprathreshold 1-MA is not required to be present for the *last* part of the maturation pathway [21]. The exposure time for suprathreshold 1-MA that is sufficient to cause GVBD is called the "hormone dependent period" (HDP). Since cdc2 is thought to undergo an essentially irreversible activation during the G2/M transition (e.g. [22]), it is possible that the end of the HDP is closely related to the timing of cdc2 activation.

For *A. miniata* oocytes, GVBD was 24.7 ± 2.0 min (n = 6 animals; std dev), the HDP was 18.8 ± 1.5 min, and the interval between the HDP and GVBD was 5.9 ± 0.7 min. There are occasional animals whose oocytes undergo GVBD in >30 min; if these are included, GVBD was 30.6 ± 8.6 min (n = 10), the HDP was 20 ± 7.8 min and the interval between HDP and GVBD was 6.3 ± 1.0 min. The standard deviation for the interval between HDP and GVBD is much smaller than that for the HDP. This indicates that the best way to characterise the 1-MA requirement for *A. miniata* oocytes is that suprathreshold 1-MA must be present until ~6 min before GVBD.

For a batch of oocytes that underwent GVBD at 23 min in 1 µM 1-MA, the HDP was 16 min. In this same batch of oocytes, subthreshold 1-MA for 10–12 min followed by 1 µM 1-MA

resulted in GVBD at 23 min. Thus, it appears that these oocytes required a low concentration of 1-MA (5–10 nM) for 11 min, a higher concentration of 1-MA (0.5 μM) between 11 min and 16 min, and then was independent of 1-MA afterwards, with GVBD at 23 min.

The oocytes of *A. pectinifera*, *M. glacialis*, and *A. miniata* resemble each other in size, appearance, and GVBD timing. However, the HDP for *A. miniata* (~14 min for GVBD at 20 min) is significantly longer than the 7.5 min in *M. glacialis* [21], and 8 min in *A. pectinifera* (e.g., [23]), though this was more recently reported to be 14 min [24]. *A. miniata* oocytes also appear to be less sensitive to 1-MA. In *M. glacialis* and *A. pectinifera*, 0.06 μM 1-MA and 0.1 μM, respectively, are sufficient to cause 100% GVBD [23,25] whereas 0.25–0.5 μM is sufficient for *A. miniata*. Possibly, these differences could be explained by a higher K_{off} for 1-MA binding to its receptor in *A. miniata*, which would lead to lower affinity and faster reversibility of 1-MA receptor activation.

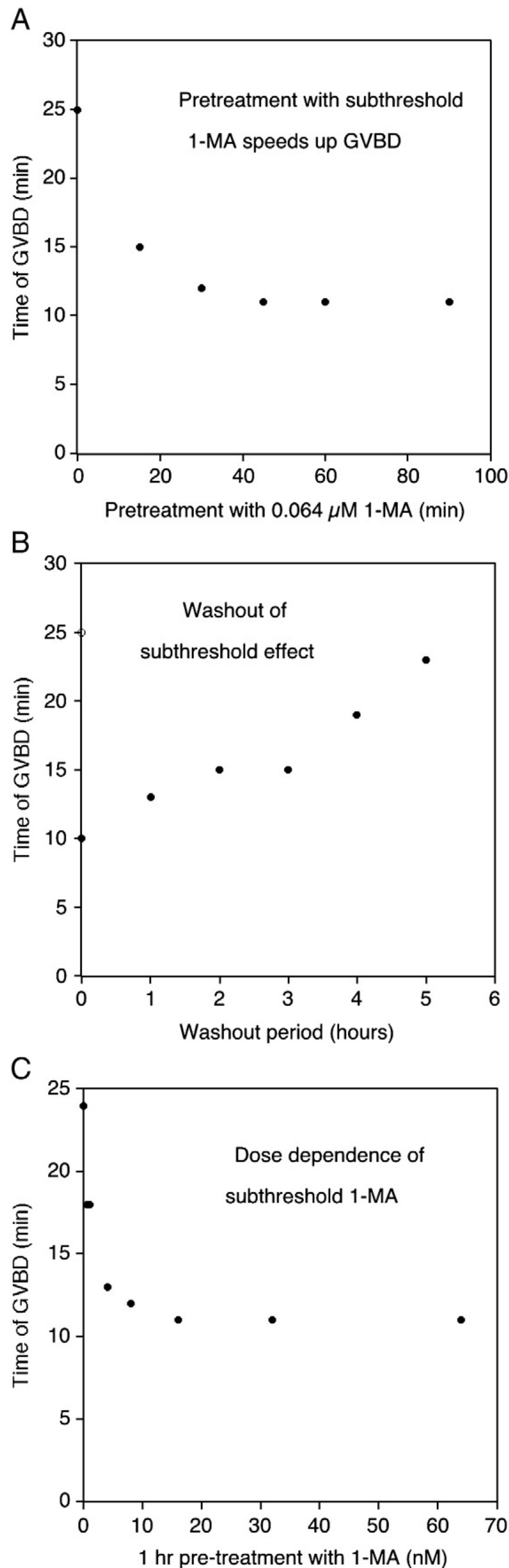

Fig. 3 – Effects of subthreshold 1-MA on GVBD timing. (A) Time dependence. This batch of oocytes underwent GVBD in 1 μM 1-MA at 25 min and the highest subthreshold dose (i.e., 0 GVBD in 1 h) was 0.06 μM. Oocytes were put into 0.05 μM for varying amounts of time then into 1 μM 1-MA and the timing of GVBD induced by 1 μM 1-MA was determined. The pre-treatment with subthreshold 1-MA significantly speeds up the timing of GVBD. In this experiment, the maximum speed up is induced by pre-treatment for 30 min or longer. In another batch of oocytes, pre-treatment for ~45 min or longer was required. (B) Washout of the subthreshold effect on GVBD timing. The same batch of oocytes as in Fig. 3A were used. Oocytes were put into 0.06 μM 1-MA for 1 h, then put into a large volume of sea water to wash out the 1-MA. After varying lengths of wash out periods, the oocytes were put into 1 μM 1-MA and the timing of GVBD was determined. Without subthreshold exposure, GVBD occurred at 25 min (open circle). In this experiment, the speed up of GVBD timing caused by subthreshold 1-MA had worn off by 5 h after washout. In another batch of oocytes, the effect had worn off by 4 h. (C) Dose dependence of the subthreshold effect on GVBD timing. For this batch of oocytes, 1 μM 1-MA caused GVBD at 24 min, and the highest subthreshold dose was 0.06 μM. The oocytes were put into varying amounts of subthreshold 1-MA for 1 h, then put into 1 μM 1-MA and the timing of GVBD was determined. A wide range of subthreshold doses, from 64 nM down to 4 nM, had a similar effect on GVBD timing.

Maturation-related changes in calcium physiology

In addition to the meiotic reduction in chromosome number, 1-MA induces several cytoplasmic changes that are primarily related to the establishment of normal fertilization. We investigated whether subthreshold 1-MA induces two of the cytoplasmic changes that occur during maturation.

IP_3 is very likely to be the second messenger which causes intracellular Ca^{2+} release at fertilization [26]. Injection of IP_3 into starfish oocytes causes Ca^{2+} release, but ~100 times as much IP_3 is required in immature oocytes to stimulate a comparable Ca release as in matured oocytes [15]. Using caged IP_3 and simultaneous monitoring of cytoplasmic calcium levels, the sensitivity of intracellular calcium release to IP_3 was found to increase primarily in the cortex just around the time of GVBD in *A. pectinifera* and *A. auranciacus* [27]. We used direct injection of IP_3 at different

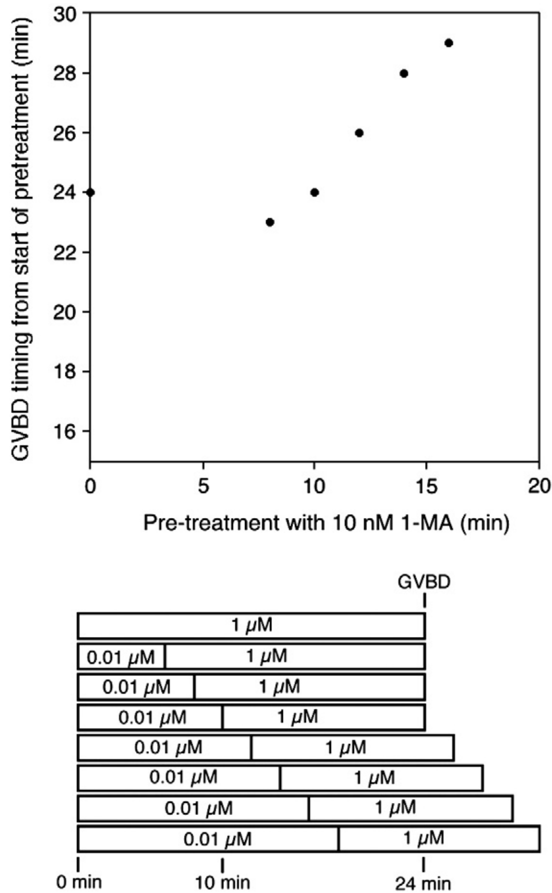

Fig. 4 – Two-stage dependence on 1-MA. This batch of oocytes underwent GVBD at 24 min, and the highest subthreshold dose was 0.125 μM. Oocytes were put into a low dose of 1-MA (10 nM) for varying lengths of time then into the suprathreshold dose of 1 μM 1-MA. If the first period (of low 1-MA) was 10 min or shorter, the sum of the first and second periods was the same as GVBD caused by continuous exposure to the suprathreshold dose. When the first period exceeded 10 min by up to 6 min, the sum of the first and second periods was lengthened by the same amount of time. These results are shown in schematically in the bottom panel.

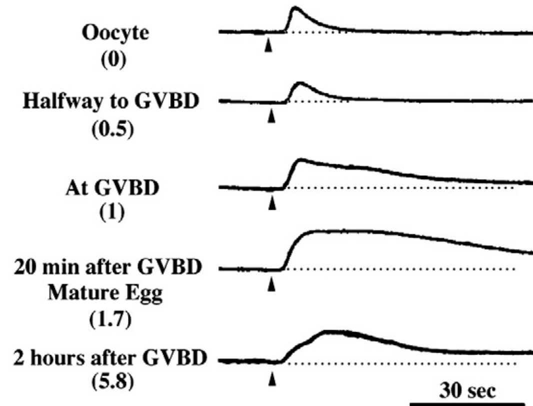

Fig. 5 – Time course for the increase in IP_3-induced calcium release during maturation. Immature oocytes were pre-injected with the fluorescent calcium indicator Ca-Green dextran in order to monitor IP_3 induced calcium release. IP_3 was injected to a final concentration of 0.01 μM at various times during maturation while Ca-Green fluorescence was simultaneously monitored. Each record is the change in Ca-Green fluorescence over time for a single oocyte. The arrowhead represents the time of IP_3 injection and the dotted line is an extension of the baseline. Injection times were normalized to the time of GVBD of uninjected oocytes in the injection chamber. (Upper panel) chart recordings of Ca-Green fluorescence following IP_3 injection (indicated by arrows). (Lower panel) Values for the area under the curve of Ca-Green fluorescence for a 1-min period after the beginning of the IP_3-induced Ca^{2+} rise are shown (mean ± standard; normalized by dividing by the area under the baseline). The increase in calcium release begins at GVBD and is maximal soon afterwards. Experiments were done at 18–20 °C.

times during maturation and found similarly that the IP_3 sensitivity increases just after GVBD (Fig. 5, Table 1). The peak IP_3 sensitivity was reached several minutes after GVBD and lasted for over an hour before decreasing.

To determine if subthreshold 1-MA induces this change in IP_3 sensitivity, oocytes were incubated in subthreshold 1-MA for 1 h then injected with IP_3 (the subthreshold concentration we used was the highest concentration that did not cause any oocytes to undergo GVBD). The amount of Ca^{2+} release was indistinguishable from that of immature oocytes (Table 2). This shows that subthreshold 1-MA is not sufficient to cause this change that normally occurs during maturation.

The numerous cortical granules underlying the plasma membrane acquire the ability to undergo calcium stimulated exocytosis during maturation [28]. When the sperm initiates increase in calcium, the exocytosed contents of the cortical granules modify the extracellular matrix leading to elevation of the fertilization envelope. It was recently found that subthreshold 1-MA causes this cortical granule change in the Japanese starfish *A. pectinifera* [24]. To assay for this, the ionophore ionomycin was added to artificially increase intracellular Ca^{2+} and then the presence or absence of a fertilization envelope was determined 20 min later.

We verified that ionomycin causes a large increase in cytosolic Ca^{2+} in immature and mature oocytes (data not shown). To determine the effects of subthreshold 1-MA on cortical granule

Table 1 – Time course for the increase in IP_3-induced calcium release during maturation. Data from the same set of experiments as Fig. 5 were analyzed. Values for the area under the curve of Ca-Green fluorescence for a 1-min period after the beginning of the IP_3-induced Ca^{2+} rise are shown (mean ± standard deviation; normalized by dividing by the area under the baseline). The increase in calcium release begins at GVBD and is maximal soon afterwards.

Time after 1-MA addition (normalized to time of GVBD)	Ca-Green fluorescence after IP3 injection
0 (oocyte)	0.03 ± 0.03 (n = 11)
0.31–0.8	0.06 ± 0.03 (n = 11)
0.81–1.1 (~GVBD)	0.21 ± 0.11 (n = 18)
1.3–2.2 (mature)	0.53 ± 0.15 (n = 21)
2.4–4.8	0.45 ± 0.19 (n = 24)
5.0–10	0.27 ± 0.13 (n = 13)

Table 2 – IP_3 sensitive calcium release in subthreshold 1-MA. Similarly to Fig. 5 and Table 1, immature oocytes were injected with Ca Green dextran to monitor IP_3-induced calcium release, though instead of a photomultiplier, a confocal microscope was used to image the fluorescence. Oocytes were put into 1 μM 1-MA to induce GVBD or into subthreshold 1-MA for 1 h. The average fluorescence in a small region near the cortex was measured. The peak value after injection was normalized to the average of the five frames just preceding the injection.

	Fold increase in Ca-Green fluorescence after IP3 injection
Immature	1.06 ± 0.03 (n = 5)
GVBD (<2 min after GVBD)	1.32 ± 0.04 (n = 4)
Mature (>10 min after GVBD)	1.52 ± 0.04 (n = 4)
Subthreshold (0.05 μM for 1 h)	1.05 ± 0.05 (n = 5)

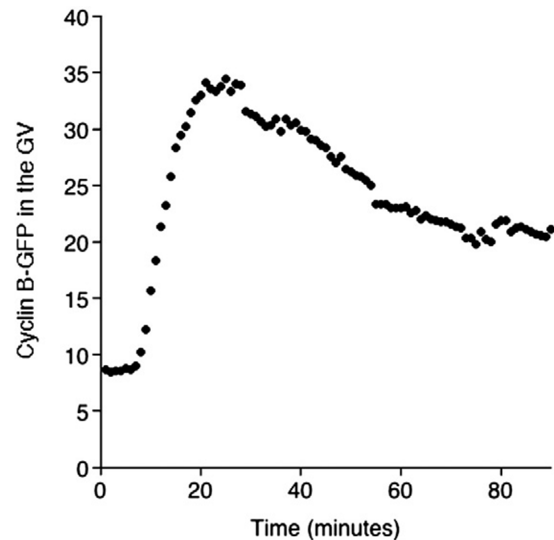

Fig. 7 – Transient entry of Cdc2/cyclin B-GFP into the GV in subthreshold 1-MA. Cyclin B-GFP was expressed in an immature oocyte by mRNA injection. As described earlier [12], cyclin B-GFP labels "aggregates" in the cytoplasm. The oocyte was put into 0.025 μM 1-MA (the highest subthreshold concentration for this batch of oocytes) and then imaged at 1 min intervals using a confocal microscope. By 8 min, the aggregates had disappeared. Cyclin B-GFP in the GV began to increase within a few minutes, reached a plateau level in the nucleus then decreased slowly (see Supplemental Movie: transient.mov). The time course of cyclin B-GFP fluorescence in the GV is shown in the lower panel; the fluorescence begins to increase very soon after the aggregates had disappeared then reaches a plateau level at ~20 min.

exocytosis in *A. miniata*, oocytes were incubated in a subthreshold dilution series of 1-MA for 1 h. Oocytes in 64 nM 1-MA followed by ionomycin resulted in fertilization envelope elevation comparable to that of mature oocytes; lower subthreshold doses (32 nM and 16 nM 1-MA) followed by ionomycin resulted in a partial elevation of the fertilization envelope, while 8 nM was indistinguishable from untreated oocytes (Fig. 6). The same batch of oocytes was tested for GVBD timing after addition of 1 μM 1-MA. As in the experiment shown in Fig. 5, 1 h pre-treatment with 8 nM and 32 nM 1-MA had the same effect on GVBD timing. Thus, only a high subthreshold concentration of 1-MA causes the cortical granule change, while much lower subthreshold concentrations affect GVBD timing.

Transient nuclear accumulation of Cdc2/cyclin B in subthreshold 1-MA

During maturation, Cdc2/cyclin B moves from the cytoplasm into the GV where it phosphorylates targets and causes GVBD [29]. Cyclin B-GFP, which binds tightly to excess free Cdc2, was used to image the dynamics of Cdc2/cyclin B in living oocytes [12]. Cdc2/cyclin B-GFP aggregates were present in the cytoplasm of immature oocytes. These aggregates dispersed after 1-MA (1 μM) treatment and a few minutes later, Cdc2/cyclin B-GFP began to accumulate in the GV. The nuclear accumulation began midway between 1-MA addition and GVBD, and Cdc2/cyclin B-GFP continued to increase in the GV until GVBD.

In subthreshold 1-MA, the aggregates of Cdc2/cyclin B-GFP dispersed, followed soon after by the beginning of Cdc2/cyclin B-

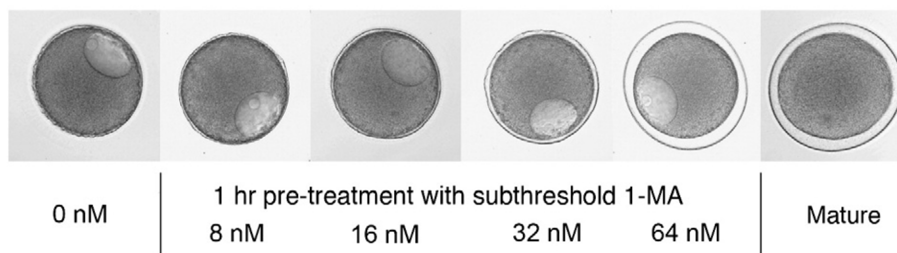

Fig. 6 – Cortical maturation in subthreshold 1-MA. During maturation, the cortical granules acquire the ability to undergo exocytosis in response to calcium. This change can be assayed by fertilization envelope elevation in response to ionomycin (1 μM for 20 min). Ionomycin caused fertilization envelope elevation in mature oocytes (far right), and very little elevation in immature oocytes (far left). The other images show the response to ionomycin after 1 h in various subthreshold concentrations of 1-MA. Oocytes in 64 nM 1-MA do not undergo GVBD but nevertheless undergo cortical maturation, as reported by Hirohashi et al. (2008). Oocytes in 8 nM 1-MA are indistinguishable from immature oocytes. The suprathreshold dose of 1 μM 1-MA was added to a parallel batch of subthreshold treated oocytes (similar to the experiment shown in Fig. 3C). The timing of GVBD was as follows: 64 nM—10 min, 32 nM—12 min, 16 nM—13 min, 8 nM—13 min, 0 nM—17 min. Since 8 nM 1-MA causes a significant speed up of GVBD without causing cortical maturation, the subthreshold 1-MA effect on GVBD timing is not tightly coupled with cortical maturation.

GFP accumulation into the nucleus (Fig. 7; see also Supplemental movie). Cyclin B-GFP fluorescence increased in the GV until about 20 min, remained at a high level for several minutes then slowly decreased. By 90 min, aggregates had re-appeared in the cytoplasm. In other experiments, the aggregates were monitored by microscopy after 1 μM 1-MA addition; as soon as the aggregates began to disappear, the 1-MA was thoroughly washed out of the chamber. As in subthreshold 1-MA, Cdc2-cyclin B-GFP increased to a plateau level in the GV then decreased, and GVBD did not occur. If the 1-MA was washed out a few minutes after the aggregates began to disappear, GVBD did occur. These experiments show that the Cdc2/cyclin B nuclear accumulation phase of maturation can occur transiently without causing GVBD.

Discussion

Variation of response to 1-MA

We characterised and identified some environmental factors that are major sources of variability of GVBD timing. The effect of temperature was determined, and we found that subthreshold 1-MA generated during the isolation procedure speeds up GVBD timing. By controlling the temperature, tonicity [18], and exposure to subthreshold 1-MA, the GVBD timing is stable on most days.

Once this had been done, it was possible to better evaluate variability among oocytes from different animals, and we found some significant variation. Some factors that could contribute to this variation are genetic variations among individuals which result in different amounts or efficiencies of the regulatory molecules, the state of the animals when they were collected, or health of the animals in the aquarium or tank. It is possible that this variability will be useful experimentally. For instance, if it was found that GVBD timing varied directly with the amount of a particular molecular component, this would probably be an important clue about the functioning of the maturation pathway.

Two-stage dependence on 1-MA

Guerrier and Dorée found that GVBD will occur even if 1-MA is removed several minutes before GVBD [21]. The minimal length of time required to cause GVBD is called the "hormone dependent period". Nemoto [23] showed that the hormone dependent period can be broken into two or even several separate exposure intervals, as long as those intervals add up to the total hormone dependent period. For instance, if the hormone dependent period is 14 min, two 7-min exposures separated by 30 min will also cause GVBD. This indicates that the action of 1-MA is cumulative and that stable intermediate states can exist. A simple model, for instance, is that 1-MA induces the accumulation of a critical substance, and once this substance exceeds a threshold amount, the oocyte becomes committed to undergo GVBD.

We have now found several effects of subthreshold 1-MA on GVBD timing that should provide new starting points for investigations. We have focused on "two-stage dependence," in which a very low subthreshold concentration can start maturation while a ~50× fold higher concentration is required to finish it.

One possible explanation for the two-stage dependence is that the pathway is organized in such a way that there are two cumulative processes or "tasks" that must be completed before GVBD can occur. For instance, referring to the data shown in Fig. 6, low 1-MA could start a process which is complete at 10 min while a second process cannot be started until the first process is complete and requires high 1-MA. Incidentally, in experiments that used only high 1-MA, only one cumulative process would be apparent.

In order for two concentrations of 1-MA to affect two processes like this, there could be one 1-MA receptor that triggers the different processes at different levels of receptor occupancy, or there could be two forms of the 1-MA receptor that have different affinities and couple to different processes. There is some evidence for the first possibility in that radioactive 1-MA is reported to bind to a single class of binding site with a dissociation constant of 0.3 μM on isolated plasma membranes [30]. However, the 1-MA receptor has not been identified yet, so it is difficult to evaluate this further.

Intracellular processes stimulated by subthreshold 1-MA

The outlines of the intracellular processes during meiotic reinitiation are known in starfish oocytes, but the quantitative and kinetic features are not well understood, as well as whether the pathway has some modular organization. The unexpected subthreshold effects should be useful towards understanding these aspects better, and we began to characterize the intracellular events caused by subthreshold 1-MA with this aim.

In addition to GVBD, 1-MA causes physiological changes related to fertilization. In oocytes from *A. pectinifera*, the cortical granules acquire the ability to undergo Ca^{2+} stimulated exocytosis in subthreshold 1-MA [24], and we verified this in *A. miniata* oocytes. The intracellular process that causes the cortical granule change is not the identical with the process that causes the subthreshold effect on GVBD timing, because only high subthreshold 1-MA causes the cortical granule change while low subthreshold doses cause the GVBD timing change. We also found that an increase in IP_3 sensitive calcium release that occurs during normal maturation did not occur in subthreshold 1-MA; this physiological change may be downstream of *Cdc2* activation in *A. miniata*.

Cdc2 is thought to be part of a bistable positive feedback loop whereby *Cdc2* activates its activator (*Cdc25*) and inactivates its inactivator (*Wee1*/*Myt1*) [22,31]. We previously found that *Cdc2*/cyclin B is present in "aggregates" that disappear during maturation followed by accumulation of *Cdc2*/cyclin B in the GV [12]. The transient nuclear entry of *Cdc2*/cyclin B in subthreshold 1-MA indicates that nuclear accumulation can begin before the irreversible and full activation of *Cdc2*. The transient nuclear accumulation can be considered to be the response of a bistable system to a subthreshold stimulus that initially pushes the system towards the on state but is pushed back towards the off state.

Possible significance in vivo

The starfish ovaries (two per arm) are multi-lobed and fill up most of the coelomic cavity. During spawning, the ovary walls contract and push the oocytes along until they pass through the gonopore into the sea water. Oocytes are at the meiosis I metaphase stage as they exit the gonopore and undergo first polar body formation ~30 min later. Oocytes in meiosis I metaphase are at the optimal stage for fertilization and normal development, because fertilization before meiosis I metaphase or after first polar body results in

polyspermy and aborted development [32]. Spawning can take place over a few hours, and it was found that oocytes mature rapidly in the ovary but become arrested at meiosis I metaphase until they are spawned [33]. This arrest would prevent the oocytes from progressing past the optimal stage for fertilization before being spawned.

The two-stage dependence may have a related role. Natural spawning is initiated by the nervous system, which releases GSS (gonad stimulating substance, a relaxin-like peptide; [34]) into the coelom. The ovary wall is permeable to macromolecules, so GSS is thought to diffuse through it and bind to the follicle cells surrounding the oocytes. GSS then stimulates the follicle cells to synthesize and release 1-MA which then starts oocyte maturation [35]. Around the time of GVBD, the oocyte is thought to signal to the follicles to break down; the release of the jelly substance around the oocyte is then proposed to contact the ovarian muscles and cause them to contract and push the oocytes en masse towards the gonopore [36].

The lobes of the ovary are ~2 mm in diameter. A 5-kDa molecule with a diffusion coefficient of ~200 $\mu m^2/s$ has a characteristic time for diffusing 1 mm of 83 min. Thus there is a likelihood of large concentration gradients of GSS in the ovary, which could result in very asynchronous maturation. However, with the two-stage dependence on 1-MA, the oocytes in the interior could begin maturation even in the presence of a large gradient and then could complete maturation as the concentration increases in the interior. This would give rise to better synchrony of maturation. According to this idea, the M1 arrest caused by the ovarian environment halts those oocytes which mature faster so that the slower oocytes can catch up, and the two-stage dependence reduces the time difference between the faster and slower maturing oocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2010.05.031.

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