Metaphase I arrest of starfish oocytes induced via the MAP kinase pathway is released by an increase of intracellular pH

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Summary

Reinitiation of meiosis in oocytes usually occurs as a two-step process during which release from the prophase block is followed by an arrest in metaphase of the first or second meiotic division [metaphase I (MI) or metaphase II (MII)]. The mechanism of MI arrest in meiosis is poorly understood, although it is a widely observed phenomenon in invertebrates. The blockage of fully grown starfish oocytes in prophase of meiosis I is released by the hormone 1-methyladenine. It has been believed that meiosis of starfish oocytes proceeds completely without MI or MII arrest, even when fertilization does not occur. Here we show that MI arrest of starfish oocytes occurs in the ovary after germinal vesicle breakdown. This arrest is maintained both by the Mos/MEK/MAP kinase pathway and the blockage of an increase of intracellular pH in the ovary before spawning. Immediately after spawning into seawater, activation of Na^+/H^+ antiporters via a heterotrimeric G protein coupling to a 1-methyladenine receptor in the oocyte leads to an intracellular pH increase that can overcome the MI arrest even in the presence of active MAP kinase.

Key words: MAP kinase, Meiosis, Metaphase arrest, Na^+/H^+ antiporter, Starfish

Introduction

In most animals, fully grown oocytes are arrested at prophase of meiosis I and resume meiosis when triggered by external stimuli, such as hormones (Masui and Clarke, 1979). In invertebrates, oocytes of insects and ascidians are arrested at metaphase I (MI) and meiosis reinitiation is triggered by fertilization, although the mechanism of MI arrest is unknown. In vertebrates, maturing oocytes progress through meiosis I, and then arrest in metaphase II (MII) until fertilization (Colas and Guerrier, 1995). The cytoplasmic activity responsible for MII arrest is termed cytostatic factor (CSF) (Masui, 1996). CSF is generated by the activated Mos/MEK/MAP kinase (MAPK) cascade (Gotoh and Nishida, 1995; Singh and Arlinghaus, 1997; Gebauer and Richter, 1997; Sagata, 1997). The protein kinase $p90^{Rsk}$ is a target of MAPK causing CSF arrest (Bhatt and Ferrell, 1999; Gross et al., 1999), and is involved in the inhibition of the anaphase-promoting complex (APC) which is an E3 ubiquitin ligase targeting cyclin B (Maller et al., 2002; Sagata, 1997). Also, the APC inhibitor Emi1 blocks the APC activation and prevents mitotic exit in CSF-arrested oocytes (Reimann and Jackson, 2002). Fertilization causes a transient increase in cytoplasmic calcium concentration, leading to the activation of calmodulin-dependent protein kinase II (Lorca et al., 1993), followed by APC activation, cyclin B destruction and completion of meiosis.

Meiosis reinitiation in starfish oocytes is induced by the hormonal stimulation of 1-methyladenine (1-MA) (Kanatani et al., 1969). The receptor of 1-MA coupling to the hetero trimeric G protein mediates the activation of phosphatidylinositol 3-kinase (PI 3-kinase) and Akt, which results in the activation of Cdc2 kinase and cyclin B complex, inducing germinal vesicle breakdown (GVBD) (Chiba et al., 1993; Jaffe et al., 1993; Nakano et al., 1999; Sadler and Ruderman, 1998; Okumura et al., 2002). MAPK in starfish oocytes is activated after GVBD by a newly synthesized starfish homolog of Mos functioning as a MAPK kinase kinase (MEK kinase) (Tachibana et al., 2000). MAPK activity decreases after the second polar body formation, when fertilization occurs during meiosis (Tachibana et al., 1997).

The standard procedure in experiments involves starfish oocytes being isolated from the ovary, placed in seawater (SW), and then treated with 1-MA. These oocytes proceed completely through meiosis I and II without metaphase arrest. However, this situation is rather artificial, since oocytes are naturally stimulated by 1-MA in the ovary. In this study, to induce natural spawning, we injected 1-MA into the body cavity of female starfish and found that a MI arrest, which was maintained by the MAPK pathway, occurred in the ovary. Release of the MI arrest was induced by an intracellular pH (pHi) increase when the oocyte was spawned into SW.

Materials and methods

Animals and oocytes

Starfish (*Asterina pectinifera*) were collected on the Pacific coast of Honshu, Japan, and kept in laboratory aquaria supplied with circulating SW at 15°C. To induce spawning, 6 ml of SW containing 0.1 mM 1-MA was injected into the body cavity of a female animal using a 22 gauge needle. In some experiments, 8 mg radial nerve/ml (as a source of gonad stimulating substance; GSS) (Kanatani et al., 1969) was used to induce spawning. To induce synchronous GVBD in the ovary, 1-MA or GSS was injected into several points of the body cavity.

Microinjection into starfish oocytes and quantification of injection volumes were done as previously described (Chiba et al., 1993; Chiba et al., 1999a). All experiments were done at 20°C unless otherwise indicated.

Immunofluorescence microscopy

Oocytes were washed several times with cold calcium-free SW. The oocytes were then treated with extraction buffer containing detergent (Shirai et al., 1990) and fixed with 100% methanol for 1 hour at –20°C. After fixation, they were transferred to PBS-T (phosphate-buffered saline/0.05% Tween 20) and left to stand for 5 minutes. They were then incubated with a mouse monoclonal antibody against α-tubulin (Amersham Corp., Buckinghamshire, England) for 50 minutes, washed with PBS-T, then stained with a FITC-conjugated goat anti-mouse IgG antibody (Tago, Burlingame, CA) for 40 minutes. DNA was stained with DAPI (Sigma) for 30 minutes, and washed with PBS-T.

Determination of pHi with BCECF-dextran

A dextran (10 kDa) conjugate of 2′, 7′-bis[2-carboxyethyl]-5-[and-6]-carboxyfluorescein (BCECF) (Molecular Probes) was dissolved at 2 mM in 100 mM potassium aspartate and 20 mM Hepes at pH 7.2. The volume injected was 2% of the total oocyte volume. To estimate pHi, an inverted light microscope (DMIRB; Leica) was connected by an adapter tube to a HiSCA CCD camera (C6790) of the ARGUS/HiSCA image processing system (Hamamatsu Photonics K. K.). Excitation light from a xenon lamp was alternated between 450 and 490 nm under computer control (C6789; Hamamatsu Photonics K. K.). The emitted light passed through a dichroic beam splitter at 510 nm and through a 515- to 560-nm emission filter (Leica). The ratios of the emission intensities at 490/450 nm were calculated using the ARGUS/HiSCA image processing system. Model intracellular medium containing 300 mM glycine, 175 mM KCl, 185 mM mannitol, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM Hepes, and 25 mM Pipes, adjusted to the indicated pH with KOH and 100 μM digitonin to permeabilize the oocytes, was used for calibration.

Preparation of the oocyte homogenate and supernatant

The cell-free preparation (oocyte supernatant) was made as previously described (Chiba et al., 1999b). Briefly, oocytes (1 ml) just undergoing GVBD were washed twice in 10 ml of ice-cold buffer P (150 mM glycine, 100 mM EGTA, 200 mM Hepes buffer, pH 7.0). After the oocytes were sedimented by gravity, buffer P was removed as completely as possible. Then the oocytes were transferred to a net of 60 μm mesh in the neck of a microtube (1.5 ml) and pressed onto the net with the cap of the tube. When the tube was centrifuged at 1400 ***g*** for 3 seconds, oocytes were homogenized by passage through the net. The homogenate was centrifuged at 20,000 ***g*** for 15 minutes at 0°C. The supernatant was transferred to a microtube and frozen by immersion in liquid nitrogen. Before use, the frozen supernatant was thawed at 15°C, and kept on ice.

SDS-PAGE and immunoblot analysis

The cell-free preparation was boiled for 5 minutes in sample buffer, and separated by electrophoresis on a 10% SDS-polyacrylamide gel, and the proteins were transferred to a PVDF transfer membrane (Millipore, Bedford, MA). The membrane was blocked with PBS-T containing 5% skim milk, and incubated with an anti-starfish cyclin B antibody at 1:1000 for 1 hour at room temperature. After the membrane was washed with PBS-T, it was incubated with a horseradish-peroxidase-conjugated goat anti-rabbit antibody (1:1000) for 1 hour. After the membrane was washed, the bound antibody was detected using a chemiluminescent substrate (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) and a LAS-1000 Luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

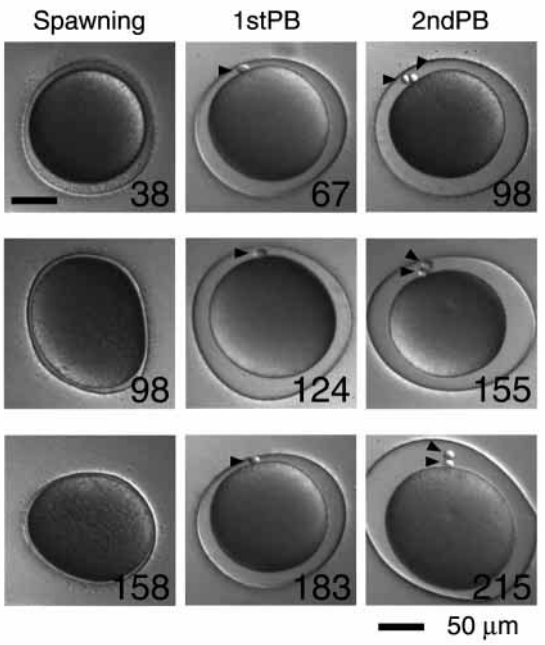

Fig. 1. Extrusion of polar bodies after spawning. Photographs were taken at the indicated times (minutes) after the injection of 1-MA into the body cavity of the recipient animal. (Top panels) Immediately after spawning (35 minutes after the injection of 1-MA into the body cavity), the oocyte was inseminated. The first and second polar bodies (arrowheads: 1stPB and 2ndPB) were formed after the elevation of the fertilization envelope, indicating that they were extruded after fertilization or spawning. (Middle and bottom panels) Oocytes spawned into SW at 95 or 155 minutes after the injection of 1-MA were inseminated. Similarly, polar bodies were extruded after spawning. Scale bar: 50 μm.

Results and discussion

First polar body formation is blocked in the ovary

Spawning started 30-50 minutes after the injection of 1-MA or GSS into the starfish body cavity, and continued for over 3 hours. Released oocytes did not have germinal vesicles, indicating that GVBD had occurred in the ovary before spawning. Indeed, we could see that all oocytes were undergoing GVBD in the ovary when we dissected the animals and isolated oocytes from them 40 minutes after the injection of 1-MA into the body cavity.

When 1-MA is applied to oocytes isolated from animals without 1-MA injection, GVBD and first polar body formation usually occur 20 and 70 minutes after 1-MA treatment, respectively. We expected that the timing of GVBD of oocytes in the ovary of 1-MA-injected animals would be similar. However, to our surprise, oocytes spawned from animals that had been injected with 1-MA 158 minutes previously had not formed the first polar bodies when they were observed immediately after spawning. These oocytes formed first polar bodies 183 minutes after 1-MA injection (about 30 minutes after spawning: Fig. 1, bottom panels). Similar results were obtained whenever oocytes were examined just after spawning (Fig. 1, 38 or 98 minutes after 1-MA injection). Thus, the first polar body formation was blocked in the ovary, while GVBD proceeded normally.

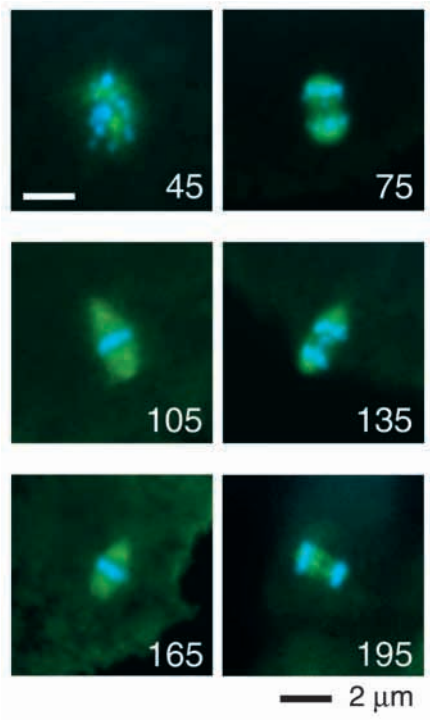

Fig. 2. MI arrest in the ovaries of stimulated animals. (Left column) Oocytes spawned into SW at 45, 105 and 165 minutes after the injection of 1-MA into the body cavity of the animal were immediately collected and treated with extraction buffer, followed by fixation and staining with DAPI and an anti-tubulin antibody to visualize chromosomes (blue) and tubulin (green). (Right column) Oocytes incubated in normal SW for 30 minutes after spawning were similarly treated and stained at 75, 135, and 195 minutes. Representative figures are shown. (Top row) Prometaphase at 45 minutes and anaphase at 75 minutes. (Middle row) Metaphase at 105 minutes and anaphase at 135 minutes. (Bottom row) Metaphase at 165 minutes and anaphase at 195 minutes. Scale bar: 2 μm.

MI arrest occurs in the ovaries of stimulated animals

To determine the stage of oocytes arrested in the ovary after GVBD, the oocytes were stained with DAPI and an anti-microtubule antibody. As shown in Fig. 2, all oocytes were at prometaphase 45 minutes after the injection of 1-MA into the body cavity. At 105 minutes, 57±20% (mean ± s.e.m., n=4) of the oocytes were at metaphase (Fig. 2, 105), and the remaining oocytes were at prometaphase. At 165 minutes, 72±11% (mean ± s.e.m., n=3) of the oocytes were at metaphase (Fig. 2, 165), and the rest were at prometaphase. Thus, MI arrest occurred in the ovaries of stimulated animals. Since some oocytes were at prometaphase even at 165 minutes, the progression from prometaphase to metaphase appears to be retarded in the ovary. After spawning, MI arrest was released (Fig. 2, 75, 135 and 195 minute samples). Extrusion of the first and second polar bodies then occurred, without additional arrest (Fig. 1). It is well known that optimal development of starfish embryos occurs when oocytes are fertilized between GVBD and the first polar body formation, since protection against polyspermy is gradually lost after the first polar body formation (Meijer and Guerrier, 1984). Thus, the occurrence of MI arrest before spawning ensures that all maturing oocytes can be fertilized before the first polar body extrusion, even if the spawning period is relatively long.

Na^+/H^+ antiporter is activated by 1-MA

Na^+/H^+ antiporters (Na^+/H^+ exchangers: NHEs) are a family of plasma membrane proteins that catalyze the electroneutral exchange of intracellular H^+ for extracellular Na^+ (Counillon and Pouyssegur, 2000). They play a central role in a variety of cell functions, including regulation of intracellular pH, cell volume and cell growth (Dibrov and Fliegel, 1998; Fliegel et al., 1998). They are activated by stimuli such as hormones, growth factors and osmotic shrinkage. In starfish, there are contradictory reports showing that 1-MA induces a pHi increase (Johnson and Epel, 1982) or not (Peaucellier et al., 1988). These discrepancies may be due to differences of the species of the animals or the methods used to measure pHi. In the present study using a dextran (10 kDa)-conjugate of BCECF, we clearly found that 1-MA treatment in normal SW resulted in an increase in pHi of about 0.4 pH units (Fig. 3A). It is well known that the absence of extracellular Na^+ inhibits the Na^+/H^+ antiporter-induced pHi increase. As expected, in zero-Na^+ artificial SW, the pHi increase of 1-MA-treated oocytes was blocked (Fig. 3A). Also, 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), an inhibitor of Na^+/H^+ antiporters, blocked the pHi increase induced by 1-MA in SW (Fig. 3A).

When G protein βγ subunits (Gβγ) were injected into oocytes without 1-MA, the pHi increase as well as GVBD occurred (Fig. 3B), indicating that G protein coupling to the 1-MA receptor (Tadenuma et al., 1992) is involved in the activation of Na^+/H^+ antiporters. An effector of Gβγ is PI 3-kinase (Nakano et al., 1999; Sadler and Ruderman, 1998), and a specific inhibitor of PI 3-kinase, LY294002, inhibited the 1-MA-induced pHi elevation (Fig. 3C), supporting the hypothesis that the Na^+/H^+ antiporter functions downstream of PI 3-kinase in the signaling pathway of 1-MA-induced starfish oocyte maturation.

The pHi increase induced by 1-MA is not required for the induction of GVBD

While GVBD of maturing oocytes in zero-Na^+ artificial SW occurred 20 minutes after 1-MA addition, polar body formation was blocked (Fig. 4). Thus, the pHi increase induced by 1-MA was not required for the induction of GVBD, but appeared to be required for the extrusion of polar bodies. As mentioned above, polar body formation was blocked in the ovaries of stimulated animals, suggesting that the pHi increase of oocytes in the ovary is also blocked.

The pHi increase of oocytes is blocked in the ovary, causing MI arrest

To test whether the MI arrest during starfish oocyte maturation in the ovary is dependent on the inhibition of a rise in pHi, BCECF was microinjected into the oocytes in the ovaries of animals stimulated by 1-MA. This experiment, however, did not succeed, since the packed oocytes in the ovary were very fragile. Therefore, we isolated the ovaries from stimulated animals and transferred them to zero-Na^+ artificial SW without 1-MA. Then, the oocytes arrested at MI in the ovaries were isolated in zero-Na^+ artificial SW to block a possible pHi increase, and injected with BCECF. As shown in Fig. 5A, when the zero-Na^+ artificial SW was replaced by normal SW containing Na^+ ions, a significant rise of pHi occurred, suggesting that pHi of maturing oocytes in the ovaries is kept at low level.

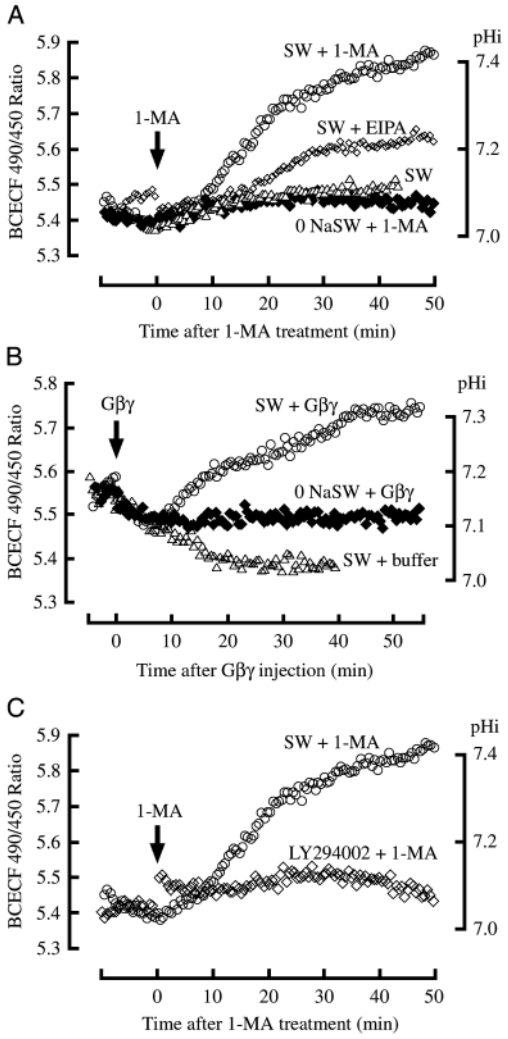

Fig. 3. An increase of pHi during oocyte maturation induced by Na^+/H^+ antiporters coupling to 1-MA signal transduction. (A) Representative traces of pHi before and after 1-MA treatments. Fluorescence ratios of BCECF-dextran were measured every 30 seconds using a single oocyte before and after 1-MA treatments. Symbols represent oocytes treated with 1 μM 1-MA in normal SW (open circles, *n*=40), or in zero-Na^+ artificial SW (0 NaSW) (closed diamonds, *n*=25) containing 480 mM choline chloride, 55 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM KCl, 2.5 mM $KHCO_3$, pH 8.0 adjusted with KOH, or in normal SW with 0.6 mM EIPA (open diamonds, *n*=22). Open triangles represent oocytes in normal seawater without 1-MA treatment (*n*=11). (B) Representative traces of pHi after Gβγ (0.6 μM) microinjection. Gβγ purified from bovine brain was stored in 0.6% Na^+ cholate, 100 mM NaCl, 20 mM Tris-HCl, pH 8.0, and microinjected as previously described (Chiba et al., 1993). Fluorescence ratios of a single oocyte before and after Gβγ microinjection were measured. Symbols represent oocytes microinjected with Gβγ in normal SW (open circles, *n*=22) or in zero-Na^+ artificial SW (closed diamonds, *n*=6), or microinjected in normal SW with the buffer used for Gβγ (open triangles, *n*=8). (C) The fluorescence ratios of a single oocyte in artificial SW with (open diamonds, *n*=14) or without (open circles, *n*=40) 0.1 mM LY294002 were measured before and after 1-MA treatment.

Interestingly, the pHi of oocytes arrested at MI (approx. pH 6.7; Fig. 5A, time 0) was lower than that of oocytes from non-stimulated animals in normal SW (approx. pH 7.1; Fig. 3A, time 0). Similarly, low pHi was observed when immature oocytes were isolated from ovaries treated with zero-Na^+ artificial SW (approx. pH 6.6; Fig. 5B, time 0). Since zero-Na^+ artificial SW treatment by itself did not cause a significant decrease of the pHi in either immature (Fig. 5B, time 60) or maturing oocytes (Fig. 5C, time 65), the low pHi of oocytes in the ovaries is not artificially induced by zero-Na^+ artificial SW, but is related to the physiological condition. Instead, pHi of oocytes from non-stimulated animals in normal SW (approx. pH 7.1; Fig. 3A, time 0) seems to be artificially induced, since a small rise of pHi occurred when the zero-Na^+ artificial SW was replaced by normal SW (Fig. 5B, time 10). Although we do not know the mechanism of the pHi increase of ovarian oocytes induced by normal SW, such pHi elevation may prevent detection of the pHi rise induced by 1-MA in some species of starfish.

In another series of experiments, we isolated the ovaries from stimulated animals 180 minutes after 1-MA injection and transferred them directly to normal SW without 1-MA, followed by a rapid injection of BCECF. As shown in Fig. 5D, the pHi increased with time after removal of the ovary from the animal. Thus, in the ovary, the pHi elevation of oocytes undergoing GVBD is blocked, which causes MI arrest. Also, it is likely that the Na^+/H^+ antiporters in the ovaries of stimulated animals are maintained in an activated state even after isolation of the oocytes in zero-Na^+ artificial SW without 1-MA. When Na^+ is added, the pre-activated Na^+/H^+ antiporters start working. A similar situation may occur in vivo; in the ovary, the concentration of Na^+ may be low, and the MI arrest may be released by the Na^+ in SW immediately after spawning. Another possibility is that the Na^+/H^+ antiporters of oocytes in the ovary may be inhibited

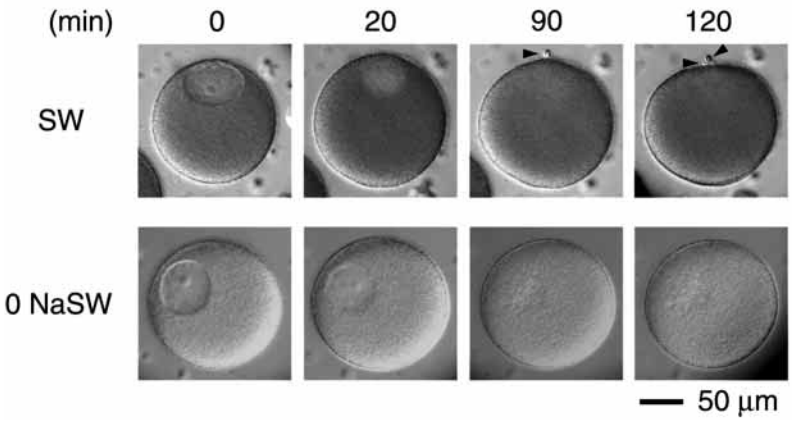

Fig. 4. Inhibition of polar body formation in zero-Na^+ artificial SW after 1-MA addition. GVBD occurred 20 minutes after 1-MA addition both in normal SW and zero-Na^+ artificial SW. In normal SW, the first and second polar body (arrowheads) were extruded 70-90 minutes and 100-120 minutes after 1-MA addition, respectively. In zero-Na^+ artificial SW, polar bodies were not extruded.

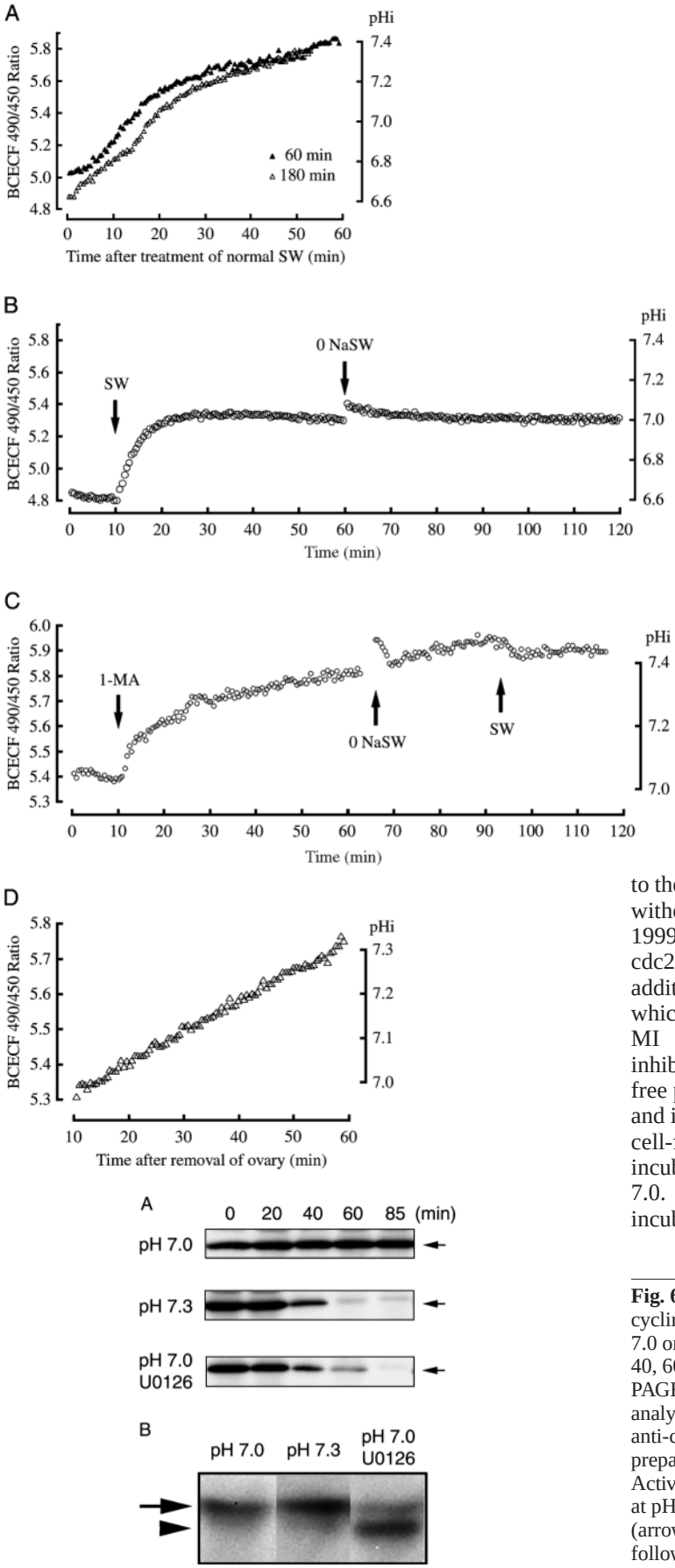

Fig. 5. Blockage of pHi increase of oocytes in the ovary of stimulated animals. (A) Ovaries were isolated from the stimulated animals 15 minutes before measurement of fluorescence ratios. They were immersed in zero-Na^+ artificial SW and dissociated oocytes were obtained. Oocytes were continuously treated with zero-Na^+ artificial SW for 15 minutes, during which time they were microinjected with BCECF. The zero-Na^+ artificial SW was replaced by normal SW 60 minutes or 180 minutes after injection of 1-MA into the body cavity of the animal, followed by measurement of fluorescence ratios. (B) Ovaries were isolated from non-stimulated animals and immersed in zero-Na^+ artificial SW. Then, oocytes in the ovaries were isolated and microinjected with BCECF. The zero-Na^+ artificial SW was replaced by normal SW at 10 minutes, and again replaced by zero-Na^+ artificial SW at 60 minutes. A small rise of pHi occurred when the zero-Na^+ artificial SW was replaced by normal SW. (C) The pHi increase of oocytes from non-stimulated animals was induced by 1-MA in normal SW, which was replaced by zero-Na^+ artificial SW without 1-MA at 65 minutes, and again replaced by normal SW at 93 minutes. High pHi induced by normal SW containing 1-MA was not affected by zero-Na^+ artificial SW. (D) Ovaries were isolated from the stimulated animals and immersed in normal SW. Immediately, oocytes in the ovaries were isolated and microinjected with BCECF, and the pHi of oocytes was measured. pHi increased with time after removal of the ovary from the animal.

by CO_2 as shown in sea urchin sperm in semen (Johnson et al., 1983).

MI arrest is maintained by the MAPK pathway and low pHi

Hormonal stimulation of starfish oocytes by 1-MA leads to the activation of the cdc2/cyclin B complex in the cytoplasm without the requirement for new protein synthesis (Kishimoto, 1999; Doree and Hunt, 2002). During metaphase, the cdc2/cyclin B complex is stable, but 50-60 minutes after 1-MA addition, cyclin B is suddenly degraded by the proteasome, which results in exit from metaphase. To determine whether MI arrest in the ovary is caused by the pHi-dependent inhibition of cyclin B degradation, we adjusted the pH of a cell-free preparation from oocytes undergoing GVBD to 7.0 or 7.3, and incubated the extract. As shown in Fig. 6A, cyclin B in the cell-free preparation was completely degraded after 60 minutes incubation at pH 7.3, while no degradation was observed at pH 7.0. Also, when the cell-free preparation at pH 7.0 was incubated with the MEK inhibitor U0126 to inhibit the MAPK

Fig. 6. Effects of pH and the MAPK pathway on the destruction of cyclin B in a cell-free preparation. (A) The cell-free preparation at pH 7.0 or 7.3 with or without U0126 (0.1 mM) was incubated for 0, 20, 40, 60 and 85 minutes. After incubation, sample buffer for SDS-PAGE was added to stop the reaction. Then, the samples were analyzed by 10% SDS-PAGE, followed by immunoblotting with an anti-cyclin B antibody. (B) The MAPK activity of the cell-free preparation at pH 7.0 or 7.3 with or without U0126 (0.1 mM). Activation of MAPK was inhibited by U0126. MAPK was activated at pH 7.0 and 7.3 (arrow), and inactivated at pH 7.0 with U0126 (arrowhead). Each sample was analyzed by 12.5% SDS-PAGE, followed by immunoblotting with an anti-MAPK (ERK1) antibody.

pathway, cyclin B was degraded after 60 minutes incubation. Activation of MAPK at pH 7.0 and 7.3, and inactivation of MAPK by U0126 at pH 7.0 were confirmed as shown in Fig. 6B. Thus, the MAPK pathway is required for establishing MI arrest at lower pH (<pH 7.0). At higher pH (>pH 7.3), MI arrest does not occur, although the MAPK is still activated. These results clearly show that MAPK cannot inhibit cyclin B degradation at higher pH.

MI arrest of starfish oocytes has not been reported previously, since starfish oocytes from non-stimulated animals are usually treated with normal SW containing 1-MA for induction of GVBD, which causes an increase of pHi before GVBD. At the lower pHi of maturing oocytes in the ovary, the MAPK pathway may inhibit the APC-dependent degradation of cyclin B. After spawning, cyclin B degradation is induced by the pHi increase while the MAPK pathway is still active, and is involved in the blockage of DNA synthesis during meiosis (Tachibana et al., 2000). Identification of the target of MAPK regulating the MI arrest in a pHi-dependent manner will be the next aim of our studies.

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