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Hormone-induced cortical maturation ensures the slow block to polyspermy and does not couple with meiotic maturation in starfish

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ABSTRACT

Meiotic progression in starfish oocytes is reinitiated by a maturation-inducing hormone called 1-methyladenine (1-MeAde). In addition to meiotic maturation, 1-MeAde induces cortical maturation in which cortical granules become competent to discharge in response to fusion of a single sperm, which results in the formation of the fertilization envelope. We found that subthreshold concentrations of 1-MeAde induce cortical maturation without germinal vesicle breakdown (GVBD). During cortical maturation, the IP3 sensitivity of calcium stores was increased as well as during meiotic maturation. When oocytes were exposed with 1-MeAde only on a hemisphere of oocytes, the IP3 sensitivity of the cortical region was increased only in the exposed hemisphere, suggesting that signals and components involved in cortical maturation do not readily spread in the cytoplasm. Although a specific inhibitor of phosphatidylinositol-3 kinase, LY294002 blocked both GVBD and cortical maturation, a Cdc2 kinase inhibitor, roscovitine did not block cortical maturation. Inhibition of Akt activation by injecting the competitors for Akt phosphorylation and membrane recruitment also blocked cortical maturation. These results suggest that the signaling pathway leading to Akt activation is common in cortical maturation and meiotic maturation, and Cdc2 activation was not required for cortical maturation.

Introduction

In most animals, prevention of entry of multiple sperm to a fertilizing egg is crucial for normal embryonic development to occur. For this purpose, eggs have been developed the mechanisms to prevent fusion of extra sperm to the egg plasma membrane (Gardner and Evans, 2006; Wong and Wessel, 2006). Cortical granules (CGs) underlying the plasma membrane, once exocytosed, release their contents to modify irreversibly an extracellular matrix surrounding eggs (Kay and Shapiro, 1987; Hirohashi and Lennarz, 1998; Haley and Wessel, 1999; Dean, 2004), resulting in a permanent block to polyspermy. CG exocytosis is initiated at site of sperm entry and transmitted through the entire surface in response to calcium propagation (Zucker and Steinhardt, 1979; Stricker, 1999). An increase in intracellular calcium is a prominent factor controlling CG exocytosis. Calcium permeable channels on the plasma membrane (Churchill et al., 2003) and on the intracellular organelles (Miyazaki et al., 1992; Galione et al., 1993) are responsible for intracellular changes in spacio-temporal Ca^{2+} dynamics (Lechleiter and Clapham, 1992; Oda et al., 1999; Deguchi et al., 2000). In addition to Ca^{2+}, many molecules that have been proposed to play key roles in regulated exocytosis in excitable cells are also found in eggs (Conner et al., 1997; Cuellar-Mata et al., 2000; Conner and Wessel, 2001; Leguia and Wessel, 2004; Leguia et al., 2006).

In immature oocytes, CGs are unable to discharge by stimulation of Ca^{2+} in mouse (Ducibella et al., 1993), frog (Machaca and Haun, 2002; El-Jouni et al., 2005), sea urchin (Wessel et al., 2002), starfish (Chiba and Hoshi, 1989), and presumably many other vertebrates and invertebrates. During transition from immature oocytes to fertilizable maturing oocytes/matured eggs, CGs obtain their exocytotic competence (cortical maturation) concomitantly with progression of meiotic maturation. The term "meiotic maturation" is used to refer to the process following the release of oocyte from prophase arrest (Masui and Clarke, 1979). In sea urchin, translocation of CGs from the ooplasm to the cortex during meiosis reinitiation is accounted for cortical maturation (Wessel et al., 2002). However, a study with electron microscopy presented the evidence that CGs already localize in the cortex (and subcortex) of starfish immature oocytes, although orientation of the ellipsoid CGs may change after maturation (Longo et al., 1995).

We found that exocytotic competence of CGs can be induced in prophase-arrested oocytes by a meiotic maturation-inducing hormone, 1-MeAde at subthreshold concentrations. Our data suggested

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that neither CG translocation nor MPF activation is requisite for this process. Alternatively, the phospholipid signaling pathways that include PI3K and Akt activation are required. The signaling of cortical maturation does not diffuse through the cytoplasm, rather it associates tightly with the cortical region where the hormone is added. We propose that non-diffusible factors contribute to the establishment of exocytotic competence.

Materials and methods

Animal collection and gamete handling

Starfish *Asterina pectinifera* were collected from Tokyo Bay, Tokyo; Otsuchi Bay, Iwate; Futtsu Port and Choushi Port, Chiba; and Amakusa, Kagoshima, Japan depending on their breeding seasons and kept in a closed-circulation natural seawater tank maintained at 15–20 °C. A piece of ovary was obtained by dissecting females, washed with ice-cold calcium-free seawater (CFSW) and teased by scissors to release oocytes into CFSW. A gentle agitation in CFSW allowed the follicle cells to separate from oocytes and after several washes with fresh CFSW, follicle cell-free immature oocytes were obtained. They were replaced immediately in artificial seawater (ASW) and kept at 15–20 °C. Sperm were collected as undiluted semen by dissecting testis in a microtube and stored on ice.

Micromanipulations

Microinjection, cytoplasmic transfer (Kishimoto, 1986), and enucleation (Miyazaki et al., 2000) were performed as reported earlier. These two methods were combined to transfer GV contents into the cytoplasm of a host GV-intact oocyte. Briefly, a fine-tip microneedle equipped with a microinjection apparatus (Narishige) was inserted into GV of an intact oocyte and then sucked up until all of the GV contents were removed from oocyte under the microscope (Zeiss). The GV contents were thereafter injected entirely into the cytoplasm of another oocyte. By this method, whole GV contents obtained from a single oocyte could be transferred into a host GV oocyte. To expose a hemisphere of oocytes with 1-MeAde, follicle-free GV oocytes were placed between coverslips bridged with ~200 μm double sticky tape, positioned on the bottom of the chamber (near the double sticky tape) using a micropipette, and aligned side by side with neighboring oocytes. This chamber was filled with ASW containing 0.03 μM 1-MeAde and 0.01% Nile blue for 1 h, then rinsed with ASW.

Ca^{2+} imaging and pH_i measurement

To measure mobilization of cytoplasmic free Ca^{2+} in a whole cell, Fura 2-dextran conjugate (Molecular probes) was microinjected and Fura 2 signals were detected under a fluorescence microscope (UV filter set) mounted with a CCD camera. The total fluorescence intensity acquired from a whole area of oocyte was recorded. For calcium imaging, calcium green-dextran (Molecular probes) was preloaded in the cytoplasm. These oocytes that had been replaced in spacer-bridged coverslips were set on a fluorescence microscope (excitation/emission wavelength of 490/515–560 nm), the images captured by a CCD camera and analyzed on the ARGUS/HiSCA 7.1.1 image analyzing system (Hamamatsu Photonics K. K.). For pH_i measurements (Harada et al., 2003), oocytes loaded with BCECF-dextran were analyzed by the ARGUS/HiSCA system with excitation/emission wavelength pairs of 490/515–560 nm and 450/515–560 nm. The BCECF signals were represented as the ratio of 490/450 nm.

Construction and expression of Akt PH-domain with GFP

Nucleotides encoding GFP was extracted from pEGFP-C1vector (Clontech) and subcloned into pCal-n vector (Stratagene, pCal-n-GFP). The PH domain (19–124 a.a.) of starfish *A. pectinifera* Akt (accession # AB060291) was amplified by pfu-polymerase (Stratagene) with a primer set of 5′-gcggatccgtggtcaaagaaggatgggtcagc-3′ (forward) and 5′-gcggatcctccactacatctcgtattgctgc-3′ (reverse) from cDNAs prepared from starfish ovary. A PCR product was subcloned into pCal-n-GFP (pCal-n-AktPH-GFP). Each construct was transformed into BL21-CodonPlus(DE3) cells and cultured in 100 ml LB broth at 28 °C for 8 h, transferred in 1L LB broth and cultivated at 16 °C for overnight. Thereafter, cells were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), incubated at 16 °C for 20 h. The methods for cell extraction and protein purification were followed according to the manufacture's protocol. Affinity-purified proteins were concentrated by a microcon-50 (Millipore) apparatus with several washes of injection buffer (50 mM pipes, 150 mM KCl, 0.1 mM EGTA, pH 6.8). For protein purification, all procedures were performed at 0–4 °C and the purified proteins stored at −80 °C.

The Akt peptide, HQFEKFSYS^{477}GDKGGL (Akt-S477) and the phosphorylated Akt peptide (Akt-S477P) were dissolved in injection buffer (100 mM potassium aspartate, 20 mM HEPES, pH 7.0) at the final concentration of 100 μg/μl as stock solutions. Oocytes were injected with 5% volume of these stock solutions (final at 5 μg/μl), incubated for 1 h at 20 °C and then treated with 1-MeAde.

Measurement of oocyte stiffness

Measurements of the stiffness of oocytes were performed as reported earlier (Yoneda, 1989). Briefly, oocytes were dejellyed by a treatment with 0.04% actinase for 5 min, rinsed with ASW, kept at 20 °C for 1 h before use. A single oocyte was compressed between a pair of plates that were set vertically in the seawater-filled chamber. The top plate is attached with a flexible glass fiber working as a bending balance. The bottom plate acts as a stage for mounting the oocyte. The oocyte was compressed by lifting the bottom plate until the top plate begins bending, by which the oocyte was given a constant force. Under this condition, 1-MeAde was added to the chamber. The distance between two plates was monitored by a microscope equipped with an eyepiece micrometer. Relative changes of the stiffness (ΔS_t) is shown as:

$$\Delta S_t = (Z_t / Z_0)$$

where Z_0 represents an initial diameter of oocyte with zero force of compression. Z_t represents an equilibrium thickness (diameter) of oocyte reached under the constant compression as a function of time t.

Bioassays

For assay of hormone-induced induction of GVBD, follicle-free GV oocytes (~50 oocytes) were placed in a 96-well culture dish and filled with 100 μl ASW. An equal volume of 2 μM 1-MeAde was added and incubated for 1 h. Percent GVBD was scored under the microscope. For the assay of cortical maturation, GV oocytes were treated with 0.03 μM 1-MeAde for 1 h, thereafter 1/10 volume of 10 μM ionomycin added. After 20-min incubation, %FE formation was scored. Assay for polyspermy block was carried out as follow. Oocytes were inseminated with sperm (final at 10^6–10^8 cells/ml) for 30 min followed by three washes with ASW to remove excess unfertilized sperm, treated with 0.04% actinase for 20 min followed by three washes, then fixed with 4% formaldehyde for 15 min. Sperm nuclei localizing in the cytoplasm of oocyte were stained with 2 μg/ml DAPI for 30 min, and scored under the fluorescent microscope with a UV filter set. Bioassays were performed at 20 °C, unless otherwise described.

Results

Cortical maturation can be introduced independently from meiotic maturation

It has been shown that a certain period (more than 10 min) of 1-MeAde treatment is required for induction of germinal vesicle breakdown (GVBD) of prophase-arrested starfish oocytes. The period necessary for induction of GVBD by a sufficient amount (such as 1 μM 1-MeAde) is called the 'hormone dependent period' (Guerrier and Doree, 1975). Similarly, we determined the period of 1-MeAde treatment that is necessary to induce cortical maturation (Fig. 1A). At given time points, the oocytes treated with 1-MeAde were washed with seawater, incubated for 1 h then treated with ionomycin. In this assay, the hormone dependent period for cortical maturation was calculated as an average of 8.0±1.0 min (3 experiments), whereas the hormone dependent period for GVBD was 14.0±1.0 min (3 experiments). Next, we used a subthreshold concentration of 1-MeAde (~0.03 μM) in which no GVBD occurs for 48 h. We found that cortical maturation was induced in ~100% of oocytes by 0.03 μM 1-MeAde within 1 h (Fig. 1B). For these experimental conditions, induction of cortical maturation was highly reproducible throughout the entire spawning season. To determine when the cortical maturation occurs, the oocytes treated with 0.03 μM 1-MeAde for different lengths of time were immediately placed in seawater containing ionomycin. We found that the time-course of the establishment of cortical maturation is quite similar to that of the hormone dependent period for cortical maturation, suggesting that a persistent hormonal stimulus is required for the progression of cortical maturation (Fig. 1C).

To investigate whether CM oocytes acquired the block to polyspermy, CM oocytes were inseminated with relatively high concentrations of sperm. When oocytes were inseminated with sperm at the concentration of 10^8 cells/ml, the numbers of sperm found in the oocyte cytoplasm was 1.5±0.17 (n=10) in CM oocytes and 12.3±1.49 (n=10) in GV oocytes (Fig. 1H). There was no morphological difference in the structure of the FE, once formed, between CM oocytes and GVBD-triggered maturing oocytes (GVBD oocytes) (Figs. 1E and F). However, the kinetics of FE formation was slower in CM oocytes than in