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Calcium signaling and the MAPK cascade are required for sperm activation in *Caenorhabditis elegans*

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ABSTRACT

In nematode, sperm activation (or spermiogenesis), a process in which the symmetric and non-motile spermatids transform into polarized and crawling spermatozoa, is critical for sperm cells to acquire fertilizing competence. SPE-8 dependent and SPE-8 independent pathways function redundantly during sperm activation in both males and hermaphrodites of *Caenorhabditis elegans*. However, the downstream signaling for both pathways remains unclear. Here we show that calcium signaling and the MAPK cascade are required for both SPE-8 dependent and SPE-8 independent sperm activation, implying that both pathways share common downstream signaling components during sperm activation. We demonstrate that activation of the MAPK cascade is sufficient to activate spermatids derived from either wild-type or *spe-8* group mutant males and that activation of the MAPK cascade bypasses the requirement of calcium signal to induce sperm activation, indicating that the MAPK cascade functions downstream of or parallel with the calcium signaling during sperm activation. Interestingly, the persistent activation of MAPK in activated spermatozoa inhibits Major Sperm Protein (MSP)-based cytoskeleton dynamics. We demonstrate that MAPK plays dual roles in promoting pseudopod extension during sperm activation but also blocking the MSP-based, amoeboid motility of the spermatozoa. Thus, though nematode sperm are crawling cells, morphologically distinct from flagellated sperm, and the molecular machinery for motility of amoeboid and flagellated sperm is different, both types of sperm might utilize conserved signaling pathways to modulate sperm maturation.

1. Introduction

In the nematode *Caenorhabditis elegans*, spermatids are generated in both males and self-fertile hermaphrodites [1]. Within hermaphrodites, the gonad initially generates sperm before switching over to the exclusive production of oocytes during larval to adult transition. The first ovulated oocyte pushes the stored spermatids into the spermatheca [2], where spermatids are exposed to the hermaphrodite-derived sperm activator and activated into spermatozoa rapidly [3]. The SPE-8 group proteins (including three transmembrane proteins SPE-12 [4], SPE-19 [5], and SPE-29 [6] and two cytoplasmic proteins SPE-8 and SPE-27 [7]) play a central role during hermaphrodite self-sperm activation. Mutations in *spe-8* group genes cause hermaphrodite sperm activation defect and therefore self-sterility. However, spermatids of *spe-8* group mutant hermaphrodites could be activated by male-derived TRY-5 (trypsin-like serine protease), a seminal fluid component secreted by male vas deferens, indicating that TRY-5-induced sperm activation is a SPE-8 pathway independent process [8]. The phenomenon that male-derived sperm activators activate *spe-8* group mutant hermaphrodite self-sperm is known as sperm trans-activation [9]. The time and place of male sperm activation are distinguished from those of hermaphrodite sperm activation [3]. Male sperm activation occurs after inseminated into hermaphrodite uterus and is mediated by TRY-5 in a SPE-8 independent manner [8]. During a typical cross, male spermatids are preferentially used to fertilize oocytes promoting outcross progeny. Spermatids can also be activated in vitro by various factors, including an ionophore (monensin) [10], proteases (Pronase and trypsin), a weak base (triethanolamine/TEA) [11] and Calmodulin inhibitors (TFP, CPZ and W7) [9]. However, the physiological relevance of these in vitro sperm activators remains uncertain.

Our previous studies have demonstrated that the labile zinc, enriched in both male and hermaphrodite reproductive tracts, has the capacity to induce sperm activation dependent on SPE-8 pathway [12]. The exogenous zinc induces the mobilization of intracellular zinc and the release of vesicular zinc into the extracellular space during sperm activation. The exogenously applied zinc or the exocytosed zinc from activating sperm, like the zinc released synaptically from the glutamatergic neurons of mammalian cerebral cortex [13], might enter the same or other sperm cells via voltage-gated zinc or calcium channels. This would

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then function as an autocrine or paracrine signal to trigger a chain of molecular events required for sperm activation. In the glutamatergic neurons, the elevation of zinc levels around the neuron terminals activates phospholipase C (PLC), and then the activated PLC cleaves the phosphatidyl inositol bisphosphate (PIP_2) generating the diacyl glycerol (DAG) and inositol 1,4,5 phosphate (IP_3) [13]. IP_3 binds to inositol 1,4,5-trisphosphate receptor (IP_3R) and triggers the release of calcium from endoplasmic reticulum (ER). The hydrophobic DAG is tethered to the inner leaflet of the plasma membrane and recruits protein kinase C (PKC). Recruited PKC is then activated by the increased calcium ions in the cytoplasm [14,15]. This results in a series of cellular responses by stimulation of various calcium-sensitive proteins for signal transduction [16]. The intracellular calcium signaling induced by the changes of extracellular zinc triggers the activation of MAPK and MEK/ERK-dependent activation of Na^+/H^+ exchanger, leading to cell proliferation [17–19]. ERK1/2 and p38 MAPK are identified as the regulators of human sperm motility and acrosome reaction [20,21]. Intracellular calcium signaling is also critical for nematode sperm activation [22] and the inhibition of Calmodulin, a calcium-binding messenger protein, directly triggers *C. elegans* sperm activation [9]. However, whether calcium signaling and the MAPK cascade are involved during zinc-induced sperm activation in nematode remains to be elucidated.

Here, we show that the activity of PLC and IP_3R regulating the intracellular calcium signaling is essential for *C. elegans* sperm activation. Furthermore, the activity of MEK/ERK is also required during sperm activation and the activation of JNK/p38 is sufficient to trigger sperm activation, bypassing the requirement of upstream signaling. Thus, though nematode sperm are morphologically distinct from flagellated sperm, both types of sperm might utilize evolutionarily conserved signaling cascades (such as the MAPK cascade and calcium signaling) during sperm motility acquisition. These observations indicate that some signaling pathways regulating sperm maturation appear to be broadly conserved though sperm cells from different species are under strong selection pressure during evolution.

2. Material and methods

2.1. Chemicals and reagents

The following reagents were used in this study: Pronase (Calbiochem); AEBSF, aprotinin, bestatin, E-64, and pepstatin A (Pierce Biotechnology); trypsin, U73343, U73122, 2-APB (2-aminoethoxydiphenyl borate), PD169316, FR180204, and Anisomycin (Sigma-Aldrich); U0126 (Invitrogen); and FM 1-43 (Molecular Probes). Stock solutions of 10 mM U73122 or U73343, 100 mM 2-APB, 50 mM U0126, 5 mM PD169316, 4 M AEBSF or PMSF, 1 M aprotinin, 1 M bestatin, 1 M E-64, 1 M pepstatin A, 200 mM Anisomycin and 50 mM FR180204 were stored at −80 °C. All stock solutions were prepared in dimethylsulfoxide (DMSO). Prior to treatment, aliquots of each drug or peptide were thawed at room temperature and diluted with sperm medium (SM) buffer (50 mM Hepes, 1 mM $MgSO_4$, 25 mM KCl, 45 mM NaCl, 5 mM $CaCl_2$, 10 mg/mL PVP, pH 7.0) or calcium-free SM buffer (50 mM Hepes, 25 mM KCl, 45 mM NaCl, 5 mM EGTA, 10 mg/mL PVP, pH 7.0). For the Control group, sperm cells were treated with the same final concentration of DMSO.

2.2. Worm strains

C. elegans strains were grown on nematode growth medium (NGM) plates at 20 °C according to standard protocols [23]. All strains were derived from Bristol N2 strains and obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. To increase the frequency of males, *him-5(e1490)* males were used as a source of male spermatids in our experiments. Strains used in this study were as follows: *spe-8(hc50)*I, *spe-8(hc53)*I, *spe-12(hc76)*I, *spe-27(it110)*IV, *spe-29(it127)*IV, and *him-5(e1490)*V.

2.3. Microscopy

Sperm were observed using differential interference contrast (DIC) microscopy. Samples were observed using an Axio Imager M2 (Carl Zeiss) equipped with a charge-coupled device (CCD) (Andor) under the control of MetaMorph software (Universal Imaging). Fluorescent imaging was obtained using a Leica HCS A confocal microscopy (Leica) controlled with LAS AF MATRIX M3 automation software. Images were processed using Image J and Photoshop (Adobe Systems).

2.4. In vitro sperm activation and inhibition assays

Virgin L4 males were placed in fresh NGM plates without hermaphrodites for 48–72 h at 20 °C. Spermatids were dissected from male gonads and released into a drop of SM buffer with or without activators (200 μg/mL Pronase, 1 mg/mL trypsin, 1 mM $ZnCl_2$ or $ZnSO_4$) at room temperature. Live sperm cells were maintained in chambers formed by mounting a 22 × 22-mm glass coverslip onto a glass slide with two parallel strips of two-sided sticky tape and examined with the Axio Imager M2 microscope (Carl Zeiss) equipped with a 100× DIC objective lens with appropriate filters. After 15 min exposure to the in vitro activators, sperm activation rate was scored based on the pseudopod extension. For AEBSF-induced sperm activation, spermatids were treated with 100 μM–20 mM AEBSF for 3 min as described above, and then the remnant AEBSF was washed out by perfusion with SM buffer lacking AEBSF.

For the inhibition of sperm activation, spermatids were bathed with various inhibitors at different concentrations in SM buffer for 10 min, then perfused with a mixture of inhibitor and activator, and finally scored for the rate of sperm activation. For each sperm inhibition assay, the toxicity of inhibitors was determined by rescuing the inhibitor-treated sperm with perfusion of activator-containing SM buffer.

2.5. MO fusion assay

To visualize the fusion of sperm specific membranous organelle (MO) with the plasma membrane, sperm cells were treated with 5 μg/mL FM 1-43 for 3 min. The lipophilic fluorescent dye FM 1-43, which partitions into the outer membrane leaflet of cells, can monitor the MO fusion during sperm activation. At the points of MO fusion, permanent membrane invaginations are generated, which allow FM 1-43 entering into the fused MO displaying a bright fluorescent puncta pattern at the rear edge of the cell body. For spermatids in which MO fusion did not occur, FM 1-43 distributed evenly on the plasma membrane of sperm cells. The images were captured using a confocal laser-scanning microscope (Leica).

2.6. Immunofluorescence

The antibody MAPK-YT (derived from rabbit, against the *dp*MPK-1 in *C. elegans* sperm, Sigma) was used at 1:100 dilution to detect the diphosphorylated MAPK using indirect immunofluorescence [24,25]. The MO was visualized with monoclonal antibody 1CB4 (derived from mouse, a gift from Steven W. L'Hernault, Emory University, Georgia) used at 1:200 dilution. To detect the ERK1/2 phosphorylation in spermatids or spermatozoa, we used the phospho-ERK1/2 (Y204, ImmunoWay) polyclonal antibody at 1:200 dilution.

Spermatids and spermatozoa were fixed with 4% paraformaldehyde and 0.01% glutaraldehyde in SM buffer at room temperature for 10 min. Fixed samples were permeabilized with 0.5% Triton X-100 in PBS. Blocked with 2% BSA in PBS at room temperature for 6 h, samples were incubated with the primary antibodies (diluted in PBS with 2% BSA) overnight at 4 °C. After being washed with PBS three times, samples were stained for 1 h with Alexa Fluor 488-conjugated goat anti-rabbit (for MAPK-YT antibody and phospho-ERK1/2 polyclonal antibody) or rhodamine-conjugated goat anti-mouse (for 1CB4 antibody)