Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 306 (2007) 797–808
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Characterization of a sperm factor for egg activation at fertilization of the newt *Cynops pyrrhogaster*

Yuichirou Harada [a], Tamami Matsumoto [a], Shino Hirahara [a], Akira Nakashima [a], Shuichi Ueno [a], Shoji Oda [b], Shunichi Miyazaki [c], Yasuhiro Iwao [a,*]

[a] *Laboratory of Molecular Developmental Biology, Department of Applied Molecular Biosciences, Graduate School of Medicine, Yamaguchi University, 753-8512 Yamaguchi, Japan*
[b] *Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, 277-0882 Chiba, Japan*
[c] *Department of Physiology, Tokyo Women's Medical University School of Medicine, Shinjuku-ku, 162-8666 Tokyo, Japan*

Received for publication 12 October 2006; revised 17 April 2007; accepted 17 April 2007
Available online 21 April 2007

Abstract

Eggs of the newt, *Cynops pyrrhogaster*, arrested at the second meiotic metaphase are activated by sperm at fertilization and then complete meiosis to initiate development. We highly purified a sperm factor for egg activation from a sperm extract with several chromatographies. The purified fraction containing only a 45 kDa protein induced egg activation accompanied by an intracellular Ca^{2+} increase when injected into unfertilized eggs. Although injection of mouse phospholipase C (PLC) ζ-mRNA caused a Ca^{2+} increase and egg activation, partial amino acid sequences of the 45 kDa protein were homologous to those of *Xenopus* citrate synthase, but not to PLCs. An anti-porcine citrate synthase antibody recognized the 45 kDa protein both in the purified fraction and in the sperm extract. Treatment with the anti-citrate synthase antibody reduced the egg-activation activity in the sperm extract. Injection of porcine citrate synthase or mRNA of *Xenopus* citrate synthase induced a Ca^{2+} increase and caused egg activation. A large amount of the 45 kDa protein was localized in two lines elongated from the neck to the middle piece of sperm. These results indicate that the 45 kDa protein is a major component of the sperm factor for egg activation at newt fertilization.

Keywords: Amphibian; Fertilization; Egg activation; Citrate synthase; PLCζ

Introduction

For the normal initiation of animal development, the timing of egg activation must be precisely controlled during fertilization. A fertilizing sperm provides a signal for egg activation at the time of sperm–egg binding or fusion. It has been demonstrated in many species that egg activation is caused by a Ca^{2+} increase in the egg cytoplasm (Stricker, 1999; Miyazaki, 2006; Whitaker, 2006). Since the intracellular Ca^{2+} increase is not only necessary, but is also sufficient, for egg activation in many species, it is important to understand the mechanism of egg activation by a fertilizing sperm as it triggers the intracellular Ca^{2+} increase at fertilization.

In vertebrates, two different mechanisms have been proposed for the Ca^{2+} elevation in egg activation (Iwao, 2000a,b). One is that an agonist (ligand) of the sperm membrane binds to a receptor on the egg membrane to transmit a signal for releasing Ca^{2+} through an inositol 1,4,5-trisphosphate (IP3) receptor on the endoplasmic reticulum. In the anuran frog, *Xenopus laevis*, we demonstrated that an intracellular Ca^{2+} increase is induced by external treatment with Arg–Gly–Asp (RGD)-containing peptides (Iwao and Fujimura, 1996), which are known ligands for integrins on the plasma membrane. A protein of the metalloprotease/disintegrin/cysteine-rich (MDC) family (xMDC16) on *Xenopus* sperm membranes is known to be involved in sperm–egg binding (Shilling et al., 1997) and external application of a peptide containing its disintegrin domain, a KTE amino acid sequence, induces egg activation (Shilling et al., 1998). In addition, a protease purified from the newt sperm activates *Xenopus* eggs when applied externally (Iwao et al.,

* Corresponding author. Fax: +81 83 933 5713.
E-mail address: iwao@yamaguchi-u.ac.jp (Y. Iwao).

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.04.019

1994, 1995; Mizote et al., 1999). A protease, cathepsin B, which has similar substrate specificity to the newt sperm protease, causes egg activation accompanied by the intracellular Ca^{2+} elevation. Recently, we found that a single-transmembrane protein, *Xenopus* uroplakin III (xUPIII), is localized to the lipid/membrane rafts and exposed on the egg surface and that xUPIII is involved in sperm–egg interaction as well as subsequent Src-dependent intracellular events of egg activation (Sakakibara et al., 2005). Furthermore, cathepsin B causes digestion of xUPIII on the egg membrane to induce egg activation accompanied by tyrosine phosphorylation of egg-raft-associated Src kinase, phospholipase Cγ, and xUPIII itself (Sato et al., 1999, 2003; Mahbub Hasan et al., 2005). Thus, in frog fertilization, it is most likely that the signal for egg activation can be transmitted when the sperm binds to the egg surface (Sato et al., 2006). However, in urodele newt, *Cynops pyrrhogaster*, the eggs are resistant to the treatment with RGD-containing peptides (Iwao, 2000a), and a very small percentage of *Cynops* eggs are activated by external treatment with the sperm protease (Iwao et al., 1994), suggesting the involvement of a different mechanism of egg activation in newt fertilization. The signal transduction through the egg receptor on the egg membrane may occur in the activation of invertebrate eggs (Townley et al., 2006), but not in that of mammalian eggs (Miyazaki, 2006; Whitaker, 2006).

Another possible mechanism has been proposed for mammalian fertilization in which a soluble sperm factor for egg activation enters into the egg cytoplasm after sperm–egg fusion. The injection of cytosolic sperm extracts into unfertilized eggs has been shown to trigger Ca^{2+} oscillation, which is a series of increases in the intracellular Ca^{2+} concentration, in both hamster (Swann, 1990; Parrington et al., 1996) and mouse eggs (Oda et al., 1999), as well as in several invertebrates (Stricker, 1999; Whitaker, 2006) including ascidians (Kyozuka et al., 1998). Recent studies of mammals strongly support the soluble factor model for Ca^{2+} oscillation (Swann and Parrington, 1999). A novel isotype of phosphoinositide-specific PLCζ was isolated and found to be expressed in spermatids (Saunders et al., 2002). Injection of not only PLCζ mRNA, but its recombinant protein, generates Ca^{2+} oscillation in mouse eggs (Saunders et al., 2002; Kouchi et al., 2004). It is estimated that a single mammalian sperm contains sufficient PLCζ protein to induce Ca^{2+} oscillation in a fertilized egg (Saunders et al., 2002; Fujimoto et al., 2004; Yoda et al., 2004). Since PLCζ is present in the sperm extracts obtained from various mammalian sperm (Saunders et al., 2002; Kurokawa et al., 2005; Fujimoto et al., 2004), it is probably the active component in the sperm extracts for the Ca^{2+} oscillation that leads to egg activation in mammalian fertilization. PLCζ may also be involved in egg activation in other vertebrates because it was reported that a chicken homologue of PLCζ (Coward et al., 2005), and sperm extracts from a fish (Coward et al., 2003) and a frog (Dong et al., 2000), can each cause Ca^{2+} oscillation in mouse eggs. This has yet to be established, however, because no detailed characterization of the active molecule in the sperm extracts in non-mammalian vertebrates has been performed.

A fertilizing *Cynops* sperm causes a wave-like increase in the intracellular Ca^{2+} concentration in the egg cytoplasm at fertilization (Yamamoto et al., 1999, 2001). An initial brief Ca^{2+} increase occurs followed by a Ca^{2+} wave that spreads at a velocity of 5.0–6.0 μm/s for about 40 min after fertilization. Injection of sperm extract into unfertilized *Cynops* eggs induces a wave-like Ca^{2+} increase similar to that at fertilization, which results in egg activation: resumption of meiosis, degradation of cyclin B and Mos, and DNA replication followed by abortive cleavage (Yamamoto et al., 2001). This Ca^{2+} increase is both necessary and sufficient for egg activation in *Cynops* eggs because the disruption of this Ca^{2+} increase by injection of the Ca^{2+} chelator, BAPTA, inhibits all activation events at fertilization (Yamamoto et al., 2001) and ionophore A23187 caused egg activation with a Ca^{2+} increase (Yamamoto et al., 1999). The Ca^{2+} increase in *Cynops* eggs is probably induced by a release of Ca^{2+} from an intracellular Ca^{2+} store within the endoplasmic reticulum, via IP3 receptors, since injection of IP3, but not cyclic-ADP ribose, causes a Ca^{2+} increase (Yamamoto et al., 2001). Injection of heparin, an inhibitor of IP3 receptors, prevents Ca^{2+} waves at fertilization in *Cynops* eggs. The sperm factor for egg activation in *Cynops* sperm extract is a heat-labile protein(s) having a molecular weight more than 10 kDa. Although a single newt sperm seems to contain a sufficient amount of sperm factor to activate an egg, its molecular characteristics remain to be investigated.

In the present study, we highly purified from a newt sperm extract a 45 kDa protein that shows egg-activation activity and shares characteristics with a citrate synthase, and we also observed that a large amount of citrate synthase is localized in fibrous structures in the sperm. Porcine citrate synthase as well as mRNA of *Xenopus* citrate synthase induced egg activation, and treatment of the sperm extract with anti-porcine citrate synthase antibody decreased its egg-activation activity. Our findings strongly suggest that this 45 kDa protein is one of the major components of the sperm factor for egg activation at newt fertilization.

Materials and methods

Preparation of eggs, sperm extracts and mRNA

Sexually mature newts, *C. pyrrhogaster*, were collected near Yamaguchi, Japan or purchased from dealers. To induce ovulation, the female was injected 80 IU of human chorionic gonadotropin (HCG, ASUKA Pharmaceutical) every 2 days. Unfertilized eggs were obtained by squeezing the abdomen of the females. The jelly layers were removed with 1.5% sodium thioglycolate (pH 9.5) followed by thorough washing with Steinberg's solution (SB: 58.0 mM NaCl, 0.67 mM KCl, 0.34 mM $CaCl_2$, 0.85 mM $MgSO_4$, 4.6 mM Tris–HCl, pH 7.4). The dejellied eggs were kept in SB more than 2 h before use to remove the artificially activated eggs. Mature sperm were obtained by squeezing the males and suspended in De Boer's solution (DB: 110.3 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, 5.7 mM Tris–HCl, pH 7.4). After washing by centrifugation (350×*g*, 20 min, 4 °C), the precipitated sperm were suspended in intracellular-like medium (ICM: 120 mM KCl, 0.1 mM EGTA, 10 mM Na-β-glycerophosphate, 0.2 mM PMSF, 1 mM DTT, 20 mM HEPES–NaOH, pH 7.5). The sperm suspension was sonicated on ice (50 W, 15 s, 5 times; US50, Nissei, Tokyo) to disrupt sperm plasma membranes and then centrifuged (10,000×*g*, 20 min, 4). The supernatant was collected as a sperm extract and stored −80 °C until use. A cDNA fragment of *X. laevis* citrate synthase (CS, Gene Bank:

BC046571) was isolated by PCR of a *Xenopus* oocyte cDNA library by using *Eco*RI site-containing 5′ primer 5′-CCGGAATTCATGTCGCTCATTAGCGCTGG-3′ and 3′ primer 5′-CCGGAATTCTCAGCCAGACTTTGACCGAC-3′ (termed CS fragment). To construct an myc-tagged CS (Myc-CS), the *Eco*RI-cut CS fragment was subcloned into the *Eco*RI-cut pT7G (UKII+)-3xMyc (N-terminally tagged with three consecutive Myc epitopes). Myc-CS construct was cut singly at the *Not*I site, located downstream of the poly (A) tail, and then transcribed *in vitro* by using the MEGA Script T7 Kit (Ambion) with Cap analog (NEB).

Microinjection and measurement of change in intracellular Ca^{2+} concentration

The dejellied eggs were washed with NKP solution (120 mM NaCl, 7.5 mM KCl, 50 mM Na_2HPO_4 and NaH_2PO_4, 4% polyvinylpyrrolidinone, pH 7.0) and then transferred to injection buffer (IB: 5.0 mM KCl, 1.0 mM EGTA, 50 mM Na_2HPO_4 and NaH_2PO_4, 4% polyvinylpyrrolidinone, pH 7.0). The microinjection was carried out with a glass micropipette having a tip diameter of 20–30 μm. Each egg was injected 33 nl of the sperm extract, porcine citrate synthase (C3260, SIGMA) in ICM, or mRNA of *Xenopus* citrate synthase in DEPC-treated water. The injected eggs were kept in IB 5 min and then incubated in healing buffer (HB: 5.0 mM KCl, 1.0 mM $CaCl_2$, 50 mM Na_2HPO_4, and NaH_2PO_4, 4% polyvinylpyrrolidinone, pH 7.0) 5 min to enhance wound healing. The injected eggs were transferred to SB, and the activation of eggs was judged by the emission of the second polar body 3–4 h after injection. In some experiments, the eggs were injected with mouse PLCζ mRNA which was made according to the methods of Yoda et al. (2004).

To monitor the change of intracellular Ca^{2+} concentration, a Ca^{2+}-sensitive fluorescent dye, Oregon green 488 BAPTA-1 dextran 10,000 MW (Molecular Probes, 2 mM in 109 mM KCl, 5 mM Tris–HCl, pH 7.3) was injected into the dejellied eggs in IB (final concentration in the egg was 20 μM) and then kept in IB and HB each 5 min. The dye-injected eggs were washed with NKP solution and IB and then were injected with 33 nl of each sample in IB. Fluorescent images of the injected egg were taken with a highly sensitive CCD camera (Cool SNAP, ROPER SCIENTIFIC) and software operating the camera (Meta Cam, ROPER SCIENTIFIC) every 10 s. Fluorescence intensity was measured by imaging software (Scion Image, Scion Corp). Each fluorescence image (F) was subtracted by the image before injection or the image with the lowest fluorescence intensity (F_0). The changes in fluorescence intensity were represented as the ratio (F/F_0) after injection.

Purification of the sperm factor for egg activation

To determine whether lectins were able to precipitate a sperm factor for egg activation, the sperm extract was treated with various lectins (LECTIN KIT I, Vector Laboratories). Each lectin dissolved in buffer (2 mg/ml in 0.1 mM $CaCl_2$, 10 mM HEPES–NaOH, pH 7.5) was mixed with the sperm extract (1:10). After 3 h on ice, the mixtures were centrifuged (10,000×*g*, 20 min, 4 °C), and then the precipitates were resuspended with 10 μl of ICM. They were injected into unfertilized eggs to determine the egg-activation activity.

To purify the sperm factor, the sperm extract was dialyzed against A-buffer (10 mM KCl, 0.1 mM EGTA, 20 mM HEPES–KOH, pH 7.5) and then centrifuged (100,000×*g*, 60 min, 4 °C) to remove membrane fractions. The sperm extract was absorbed to a Hi-Trap Blue column (1 ml, Amersham Pharmacia) equilibrating with A-buffer. The absorbed proteins were eluted with 1.5 M KCl in A-buffer. The eluted fractions containing the sperm factor were dialyzed against A-buffer. The fractions were absorbed to a soy been agglutinin (SBA) agarose column (4 ml, Vector Laboratories) and then the absorbed proteins were eluted with 1.5 M KCl in A-buffer. The eluted fractions containing the sperm factor were dialyzed against A-buffer. Finally, the SBA agarose column-eluted fraction was absorbed to an anion-exchange column, a Hi-Trap Q column (Amersham Biosciences) or a Resource Q column (Pharmacia Biotech). The absorbed proteins were eluted by a gradient of KCl concentration (0.01–0.5 M) in A-buffer. The sperm factor for egg activation was recovered in 0.23–0.28 M KCl fractions.

To observe the behavior of the nuclei in the eggs injected with the sperm factor, the eggs were fixed in 100% methanol (−30 °C) 4 h after injection. The nuclei were observed under a laser-scanning confocal microscope (LSM510, Carl Zeiss) according to the methods described previously (Iwao et al., 2002).

The proteins in the purified sperm factor were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gels were stained with Coomasie Brilliant Blue (CBB) or silver staining kit (silver stain II kit wako, Wako pure chemical industries). For immunoblot, the proteins separated by SDS–PAGE were electrically transferred onto PVDF membranes with a semi-dray electroblotting system (AE-6675, ATTO). After blocking with TTBS (155 mM NaCl, 0.1% Tween-20, 100 mM Tris–HCl, pH 7.4) containing 5% nonfat dry milk for 1 h, the membrane was incubated with anti-porcine citrate synthase rabbit polyclonal antibody (1:1000 dilution, NE040/7S, Nordic Immunological Laboratories B.V.). Immunocomplexes were detected by a horseradish peroxidase-conjugated anti-rabbit IgG goat antibody (A6667, SIGMA) and ECL plus Western blotting detection system (RPN 2132, Amersham Biosciences).

The amino acid sequence analysis of an N-terminus in the purified protein was performed by Edman method. The inner amino acid sequence was analyzed with peptide fragments after trypsin digestion. The amino acid sequence analyses were performed by “Shimadzu Corp”.

Immunofluorescence microscopy

The sperm were suspended in 10% DB and then air-dried on the slide glass. The sperm were fixed with 3.5% formaldehyde in PBS (0.9% NaCl, 10 mM Na_2HPO_4 and NaH_2PO_4, pH 7.5) at room temperature for 30 min. After washing with PBS, the sperm were post-fixed with 100% methanol at −30 °C for 30 min. After washing with PBS for 5 min 3 times, the samples were blocked by PBS containing 1% bovine serum albumin (BSA) in a moist chamber. The samples were treated with the anti-porcine citrate synthase rabbit polyclonal antibody against the whole molecule (1:100 dilution, NE040/7S, Nordic Immunological Laboratories B.V.) in the moist chamber at 4 °C overnight. After washing with TTBS for 5 min 3 times, the samples were treated with anti-rabbit IgG goat antibody conjugated with Alexa 546 (1:400 dilution, A-11010, Molecular Probes) for 30 min at room temperature. After washing with TTBS for 5 min 5 times, some samples were treated with anti-α-tubulin monoclonal antibody conjugated with FITC (1:200 dilution, F2168, SIGMA) at 4 °C overnight. The samples were mounted with slowfade antifade kit (S-2828, Molecular Probes). In some experiments, the sperm were incubated with 1 μM MitoTracker Green FM (M-7514, Molecular Probes) at room temperature for 1 h and then fixed and immunostained as described above. Sperm were observed under the laser-scanning microscope.

Results

Purification of a sperm factor from newt sperm extract

We previously demonstrated that injection of a sperm extract into unfertilized newt eggs caused egg activation, accompanied by a wave-like increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and resumption of meiosis (Yamamoto et al., 2001). The propagative $[Ca^{2+}]_i$ increase by injection of the sperm extract was confirmed in unfertilized eggs that had been injected with a Ca^{2+}-sensitive fluorescence dye, Oregon Green 488 BAPTA-1 conjugated with dextran (MW 10,000) (Figs. 1A, B, D and E). Since newt eggs contain a large amount of pigment granules in the cortex of animal hemispheres which disturbed the excitation of fluorescence dyes and emission from them, we had some difficulty in obtaining detailed images of the $[Ca^{2+}]_i$ change at activation of the eggs in response to the injection of a small amount of sperm extract containing the cytoplasm equivalent to 400 sperm (Figs. 1A and D). However, we did clearly observe that the increase in $[Ca^{2+}]_i$ was initiated at the injection site soon after injection and then propagated through

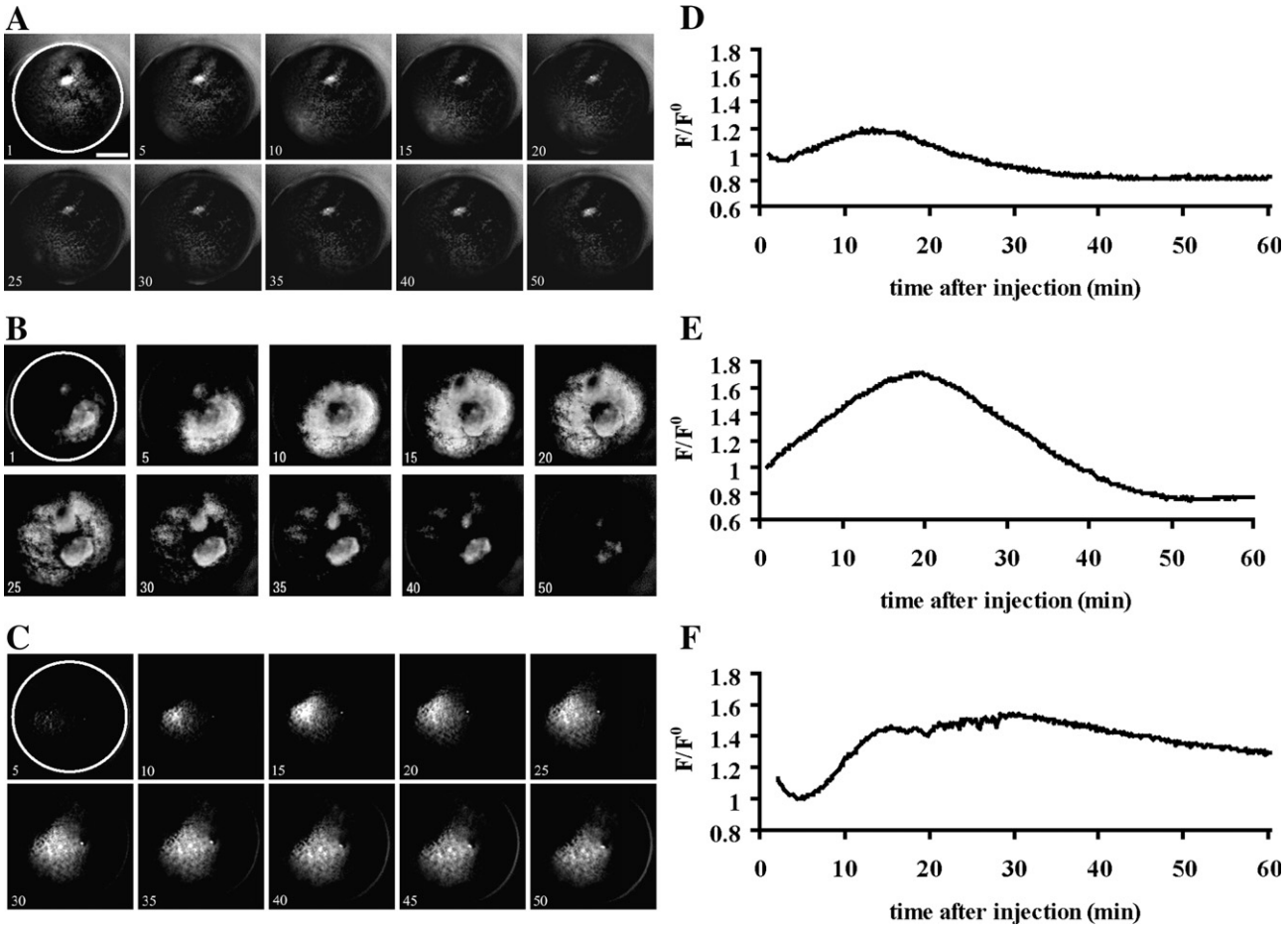

Fig. 1. A typical pattern of Ca^{2+} increase in the Oregon green-injected egg after injection of the sperm extract equivalent to 400 sperm (3.8 μg)/egg (A and D) or 1000 sperm (9.5 μg)/egg (B and E), or purified 45-kDa protein (0.36 ng/egg) (C and F). In panels A–C, each fluorescence image was subtracted by the image before injection. Each circle in the first image shows the outline of egg. Time after injection (min) is shown at lower left. In panels D–F, the eggs were injected with each sample at 0 min, and then the fluorescence images were taken every 10 s 1–5 min after injection, showing the ratio (F/F_0) between the fluorescence intensity before injection (F_0) and after injection (F). Scale bar, 0.5 mm.

the whole egg cytoplasm with the injection of a larger amount of sperm extract containing the cytoplasm equivalent to 1000 sperm (Figs. 1B and E). The increase in $[Ca^{2+}]_i$ reached a peak at around 20–25 min, but continued for 40–50 min after injection (Figs. 1D and E). The eggs that exhibited the Ca^{2+} increase underwent a resumption of meiosis and emission of the second polar body, and some underwent the abortive cleavage. In addition, at least 20% of the eggs (3/15) underwent activation by injection of the sperm extract containing cytoplasm equivalent to one sperm into an egg. These results indicate that the sperm extract contains a sperm factor that activates unfertilized newt eggs, accompanied by an intracellular Ca^{2+} increase.

To determine whether the sperm factor was associated with carbohydrate moieties, we treated sperm extracts with various lectins that recognize specific terminal carbohydrate chains. We examined the egg-activation activity in the precipitates with lectins by injection into unfertilized eggs (Fig. 2). Injection of the precipitate by soy bean agglutinin (SBA) induced activation in more than 90% of the eggs, whereas that of the precipitates by Dolichos biflorus agglutinin (DBA), peanut agglutinin (PNA), wheat germ agglutinin (WGA), Ulex europaeus agglutinin-1 (UEA-1), or concanavaline A (ConA) activated only 4–26% of the eggs. The egg-activation activity in the supernatant of the extract precipitated by SBA decreased significantly since 15% or 60% of the eggs ($n=20$) were activated by the injection of the

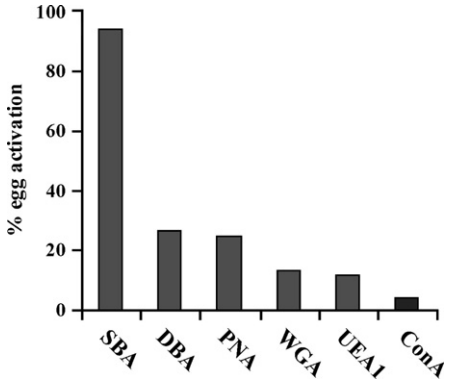

Fig. 2. The egg activation by injection of the sperm extract precipitated with various lectins, showing precipitation by SBA. The sperm extract (0.3 mg/ml) was treated with each lectin (0.2 mg/ml) and unfertilized eggs (about 20 eggs) were injected with 33 nl of the sperm extract that had been precipitated by each lectin.

supernatant by SBA or by the buffer-treated extract, respectively. No egg was activated by the injection with lectins only (data not shown). These results indicate that the sperm factor consists of a component(s) of a glycoprotein at least containing *N*-acetylgalactosamine at the sugar terminals.

To purify the active component of the sperm factor, we separated the sperm extract by several chromatographies: a Hi-Trap Blue column that binds some polysaccharides, an SBA–agarose column, and then an anion-exchange column, in that order. All activity for egg activation was bound to the Hi-Trap Blue column or the SBA–agarose column and then eluted by 1.5 M KCl. When the active fractions collected by the SBA–agarose column were applied on the anion-exchange column, most proteins were recovered in fraction Nos. 35–40 (0.23–0.28 KCl) (Fig. 3A). When the fractions (Nos. 35–40) were injected into the unfertilized eggs, 30–64% of the eggs were activated (Fig. 3B). A pronucleus formation after resumption of meiosis was confirmed in the eggs 4 h after the injection of the purified fractions (Fig. 3C). Although it was still difficult to measure the precise amount of egg-activation activity, we tried to estimate the rate of purification during chromatographies (Table 1). The specific activity of the final Hi-Trap Q fractions was approximately 22-fold greater than that of the initial crude sperm extract, but about 14% of the activity was recovered through these chromatographies. The injection of the purified fractions into the unfertilized eggs caused an increase in $[Ca^{2+}]_i$, which was initiated at the injection site, and then propagated in the animal hemisphere (Figs. 1C and F). The $[Ca^{2+}]_i$ increase reached a peak at about 20 min and then continued for about 40–50 min after the injection. Thus, the pattern of the $[Ca^{2+}]_i$ increase was similar to that induced by the crude sperm extract. We analyzed the components of the sperm factor purified by the anion-exchange chromatography using SDS–PAGE and detected a 45 kDa protein in the fractions containing the egg-activation activity (fraction Nos. 35–40) (Fig. 4A). The major band of 45 kDa protein was detected in the fractions of Nos. 35–40 with higher egg-activation activity after silver staining (Fig. 4B). These results strongly indicate that the 45 kDa protein is one of the major components of the sperm factor for egg activation in the sperm extract.

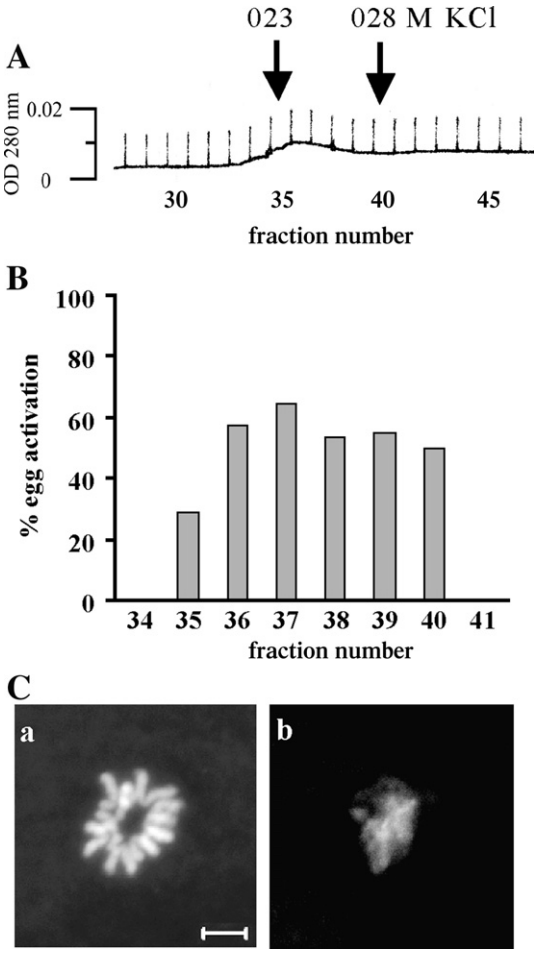

Fig. 3. The chromatography of the sperm extract by the anion-exchange column, showing the pattern of the eluted proteins in the fractions by a KCl gradient (A). Egg activation was determined by injection of each fraction into about 15 unfertilized eggs, showing higher egg-activation activity in the fractions Nos. 35–40 (B). An egg nucleus arrested at the second meiotic metaphase in unfertilized eggs before injection (C, a) and formation of a pronucleus in the activated egg 4 h after injection of the fraction No. 37 (C, b). Scale bar, 10 μm.

Table 1
Purification of the egg-activation activity in the sperm

Purification step	Total activity[a] (units × 10^3)	Total protein (μg)	Specific activity (units/μg)	Recovery (%)	Purification
Crude extract	1090.8	3090.0	353.0	100	1.0
Hi-Trap Blue column	175.7	265.0	663.2	16.1	1.9
SBA column	163.6	28.0	5843.6	15.0	16.6
Hi-Trap Q column	149.8	19.7	7612.0	13.7	21.6

[a] 1 unit: 25% of the eggs were activated when injected 33 nl/egg.

45 kDa protein homologous to citrate synthase localized in the middle piece of sperm

We analyzed both the N-terminus and inner amino acid sequences of the purified 45 kDa protein and identified 13 amino acids (VXXTTNLKDVLXXLITK, X; undetermined) and 9 amino acids (ELGGEVSDE), respectively (Fig. 5). We then examined the homology of the 45 kDa protein to other proteins for the successive eight amino acids (TTNLKDVL) of the N-terminus sequence and nine amino acids (ELGGEVSDE) of the inner sequence with a basic local alignment search tool (BLAST) and found that 88% of the amino acids were identical to those of *X. laevis* citrate synthase and the 45 kDa protein is most homologous to *X. laevis* citrate synthase (Fig. 5). Thus, this protein appears to be a glycoprotein with terminal carbohydrate chains containing *N*-acetylgalactosamine, according to its binding to SBA and DBA.

Since *X. laevis* citrate synthase is highly homologous to porcine citrate synthase (Fig. 5), we analyzed both the sperm extract and the purified sperm factor by immunoblotting with an antibody against the whole molecule of porcine citrate synthase. The antibody recognized the 45 kDa protein in both the sperm extract and the purified sperm factor (Fig. 4C), indicating that

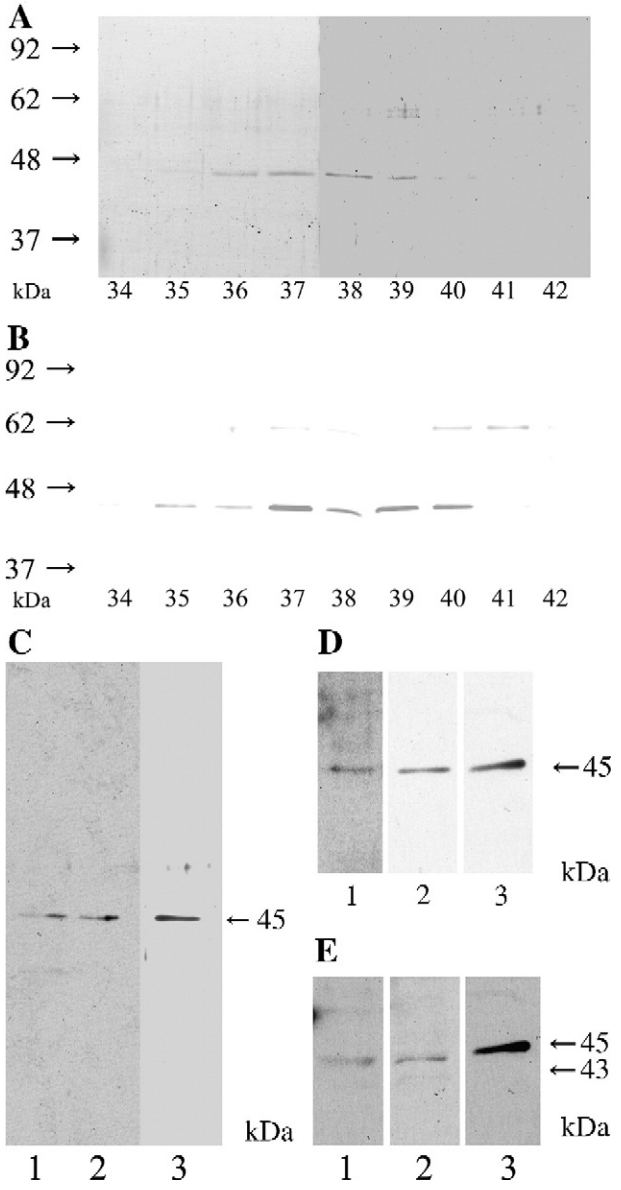

Fig. 4. SDS–PAGE of the fractions purified by the Hi-Trap Q column chromatography, showing a 45 kDa protein in CBB staining (A) and in silver staining (B). The amount of proteins applied was 3–14 μg/lane. (C) Immunoblot of the purified sperm factor with anti-porcine citrate synthase antibody, showing the recognition of the 45 kDa protein in the crude sperm extract (2.0 μg, lane 1), in the purified sperm factor of fraction No. 36 in Fig. 4 (0.03 μg, lane 2) and in porcine citrate synthase (0.9 μg, lane 3). (D) Immunoblot of the precipitate (lane 1) and the supernatant (lane 2) of the sperm factor treated by SBA, or the buffer-treated sperm extract (lane 3) with anti-porcine citrate synthase antibody, showing the precipitation of the 45 kDa protein and its decrease in the supernatant (about 38% of that in the buffer-treated sperm extract). (E) Immunoblot of the extracts obtained from heart (lane 1), unfertilized eggs (lane 2) and mature sperm (lane 3) of the newts with anti-porcine citrate synthase antibody, showing the 43 kDa protein in the heart and the unfertilized eggs, but the 45 kDa protein in the sperm.

this protein is a homologue of citrate synthase in newt sperm. According to the comparison on the immunoblots, it was estimated that the sperm extract obtained from a single sperm contained about 2 pg of 45 kDa protein (citrate synthase), whereas the injection of 22 pg or 230 pg of the purified 45 kDa protein was necessary for the activation of 30% or 67% of the eggs, respectively. The 45 kDa protein (citrate synthase) was detected in the precipitate of the sperm extract treated by SBA and its amount in the supernatant decreased significantly (Fig. 4D). Slightly light molecules (43 kDa) were, however, detected in heart and unfertilized eggs (Fig. 4E), suggesting the existence of a specific form (45 kDa) of citrate synthase for the sperm. Furthermore, when we tested the sperm extracts containing cytoplasm equivalent of 400 sperm with the antibody against the whole molecule of porcine citrate synthase for 20 min at room temperature and then injected this into an unfertilized egg, we observed that the rate of egg activation was significantly decreased in comparison with the treatment by non-specific rabbit IgG (Fig. 6A). These results indicate that the 45 kDa citrate synthase is involved in egg activation as the sperm factor. We then examined whether porcine citrate synthase was able to induce a $[Ca^{2+}]_i$ increase and egg activation in unfertilized eggs. We found that more than 70% of the eggs injected with citrate synthase (0.5 mU, 3.3 ng/egg) underwent activation, while boiled citrate synthase did not induce egg activation (Fig. 6B). Citrate synthase caused egg activation in a dose-dependent manner. We observed that citrate synthase induced an increase in $[Ca^{2+}]_i$ at the injection site and that the Ca^{2+} increase had spread over the entire animal hemisphere by about 35 min after injection (Figs. 7A and C). Furthermore, when mRNA of *X. laevis* citrate synthase was injected into unfertilized eggs, the eggs underwent activation (Fig. 8A). The injection of 2.5 μg/ml or 0.0025 μg/ml the citrate synthase mRNA (final concentration in egg cytoplasm) caused activation in 68% or 16% of the injected eggs, respectively. The injection of the citrate synthase mRNA induced an increase of $[Ca^{2+}]_i$ in the egg (Fig. 8B), but the $[Ca^{2+}]_i$ began to increase very slowly about 40 min after injection and then peaked at about 90 min after injection. Taken together, these results strongly support the notion that the 45 kDa citrate synthase is one of the major components of the sperm factor for newt egg activation.

To investigate the localization of citrate synthase in newt sperm, we immunostained the sperm with anti-porcine citrate synthase antibody. A large amount of citrate synthase was localized in the anterior two-thirds of the tail region of the sperm (Figs. 9A and B), which corresponds to the neck and the middle piece, but not in the principal piece (posterior one-third of the tail region). Citrate synthase was observed neither in the acrosome nor in the nucleus. No fluorescence was observed in the sperm stained with non-specific rabbit IgG (Fig. 9C). When the sperm were double-stained with the anti-porcine citrate synthase antibody and anti-α-tubulin antibody, microtubules were observed in the axoneme, and citrate synthase forming two lines was seen to be elongated in the axial fiber region, but not in axoneme (Fig. 9C). Citrate synthase was not distributed in the undulating membranes connecting the axoneme and the axial fiber region. The sperm were treated with MitoTracker Green, which preferentially stains mitochondria, and were then immunostained with the anti-porcine citrate synthase antibody (Fig. 9D). The mitochondria were arrayed along one side of the middle piece, whereas the citrate synthase was distributed in the axial fiber adjacent to the undulating membranes as well as in

Fig. 5. Identity and homology of alignment of a 45-kDa protein against porcine citrate synthase (upper low, GenBank accession no. M21197) and *Xenopus* citrate synthase (middle low, GenBank accession no. BC046571), showing close relation of partial amino acid sequences of the purified 45-kDa protein (lower low). X, undetermined. Amino acid identities (E-values in BLAST) of TTNLKDVL or ELGGEVSDE against *Xenopus* citrate synthase were 88% (1.7) and 89% (0.25), respectively.

the distribution area of mitochondria, indicating that some part of citrate synthase does not co-localize with the mitochondria. These results suggest that the extra-mitochondrial citrate synthase forms the fibrous structures in the middle piece, and most probably in the axial fiber (Figs. 9D and E).

PLCζ was able to activate newt eggs

Since it was previously demonstrated that a sperm-specific phospholipase C, PLCζ, induces Ca^{2+} oscillation in mouse eggs (Saunders et al., 2002; Yoda et al., 2004), we investigated whether mouse PLCζ can activate newt eggs. We injected 2.6 μg/ml or 0.026 μg/ml PLCζ mRNA into unfertilized eggs (final concentration in egg cytoplasm) and observed activation in 95% (39/41) or 7% (2/30) of the injected eggs, respectively. Furthermore, the injection of PLCζ mRNA induced an increase of $[Ca^{2+}]_i$ in newt eggs (Figs. 7B and D). The $[Ca^{2+}]_i$ began to increase slowly about 10 min after the injection and then peaked at about 50–60 min after the injection. These results indicate that PLCζ can activate newt eggs and is accompanied by a $[Ca^{2+}]_i$ increase.

Discussion

In the present study, we highly purified the sperm factor for egg activation from a newt sperm extract by several chromatographies to characterize the molecule that induces the Ca^{2+} increase at fertilization. We obtained the fraction containing egg-activation activity in the final purification by ion-exchange chromatography. The eggs injected with the fraction underwent not only a Ca^{2+} increase, but resumption of meiosis followed by pronucleus formation. We demonstrated that a 45 kDa protein detected in the fraction is a major component of the sperm factor in the sperm extract, which is consistent with the previous results that the egg-activation activity in the newt sperm extract is a heat-labile protein with a molecular weight of greater than 10 kDa (Yamamoto et al., 2001). This 45 kDa protein appeared to share homology with citrate synthase according to an analysis of partial amino acid sequences and because it was recognized by the anti-porcine citrate synthase antibody. Furthermore, injection of porcine citrate synthase (45 kDa) caused egg activation with Ca^{2+} elevation, whereas treatment of the sperm extract with anti-porcine citrate synthase antibody greatly reduced its egg-activation activity. Finally, the injection of *Xenopus* citrate synthase mRNA into unfertilized eggs induced egg activation accompanied by a Ca^{2+} increase. These findings strongly support the notion that citrate synthase is one of the major components of the sperm factor for egg activation at fertilization in newt eggs. It was estimated that a single newt sperm contained about 2 pg of the citrate synthase (45 kDa), but more than 10–100 fold larger amount of the protein seems to be necessary for the activation of 30–67% of the eggs injected by the purified 45 kDa protein. In addition, the sperm extract containing the cytoplasm equivalent to a single sperm was able to activate about 20% of the eggs. Since 2–20 sperm enter an egg and initiate egg activation in the physiologically polyspermic newt fertilization (Iwao,

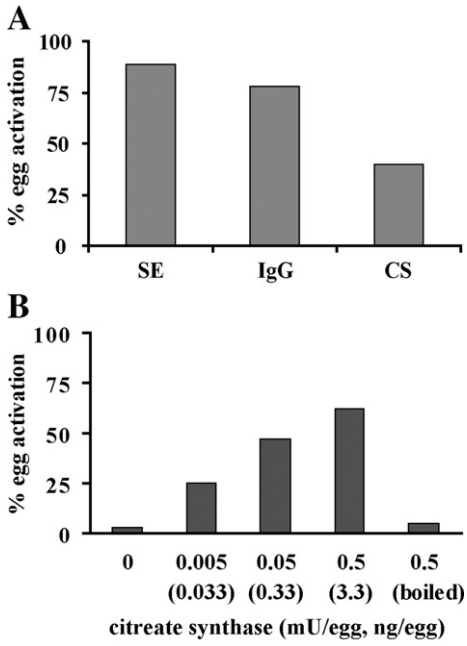

Fig. 6. (A) The inhibition of egg activation by the treatment of sperm extract with anti-porcine citrate synthase antibody. The sperm extract containing cytoplasm equivalent to 400 sperm was treated with the anti-porcine citrate synthase antibody (1:100 dilution, 70 μg/ml) or non-specific rabbit IgG (100 μg/ml) for 20 min and then injected into unfertilized eggs (about 25 eggs), showing the decrease of egg activation in the eggs injected with the antibody-treated extract (CS), compared with the non-specific IgG-treated extract (IgG) or non-treated extract (SE). (B) Injection of porcine citrate synthase into unfertilized newt eggs, showing egg activation in a dose-dependent manner. Boiled sample (0.5 mU, 3.3 ng/egg) was heated at 95 °C for 10 min. Each sample was injected into about 20 eggs.

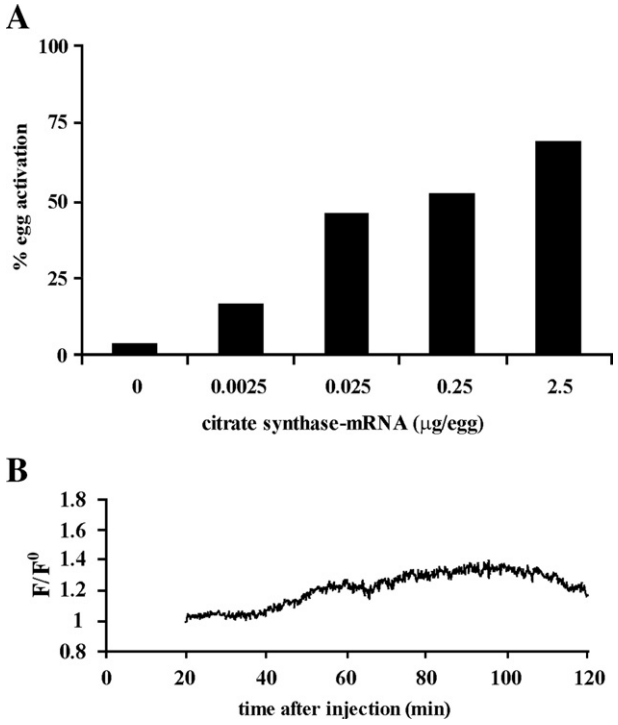

Fig. 8. (A) Injection of *Xenopus* citrate synthase mRNA into unfertilized eggs, showing egg activation in a dose-dependent manner. Each sample was injected into about 20 eggs. (B) A typical pattern of Ca^{2+} increase in the Oregon green-injected egg after injection of the *Xenopus* citrate synthase mRNA (2.5 μg/egg). The egg was injected with the mRNA at 0 min, and then the fluorescence images were taken every 10 s, showing the ratio (F/F_0) between the fluorescence intensity before injection (F_0) and after injection (F).

2000a,b), at least double sperm seem to contain enough activity to activate the egg. The decrease of the activity in the sperm extract or the purified protein might be due to that all the 45 kDa protein was unable to be extracted from the sperm and/or that some activity was lost during the purification steps.

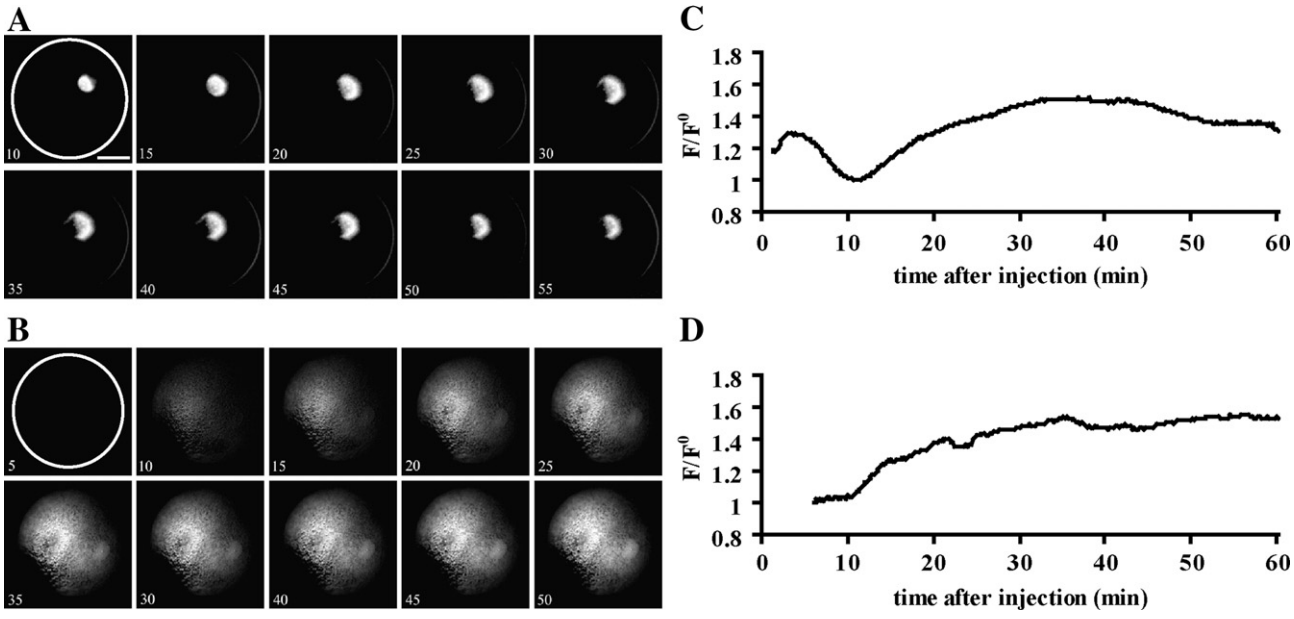

Fig. 7. A typical pattern of Ca^{2+} increase in the Oregon green-injected egg after injection of the porcine citrate synthase (3.3 ng/egg) (A and C), or mouse PLCζ mRNA (8.6 ng/egg) (B and D). In panels A and B, each circle in the first image shows the outline of egg. Time after injection (min) is shown at lower left. In panels C and D, the eggs were injected with each sample at 0 min, and then the fluorescence images were taken every 10 s 1–5 min after injection, showing the ratio (F/F_0) between the fluorescence intensity before injection (F_0) and after injection (F). Scale bar, 0.5 mm.

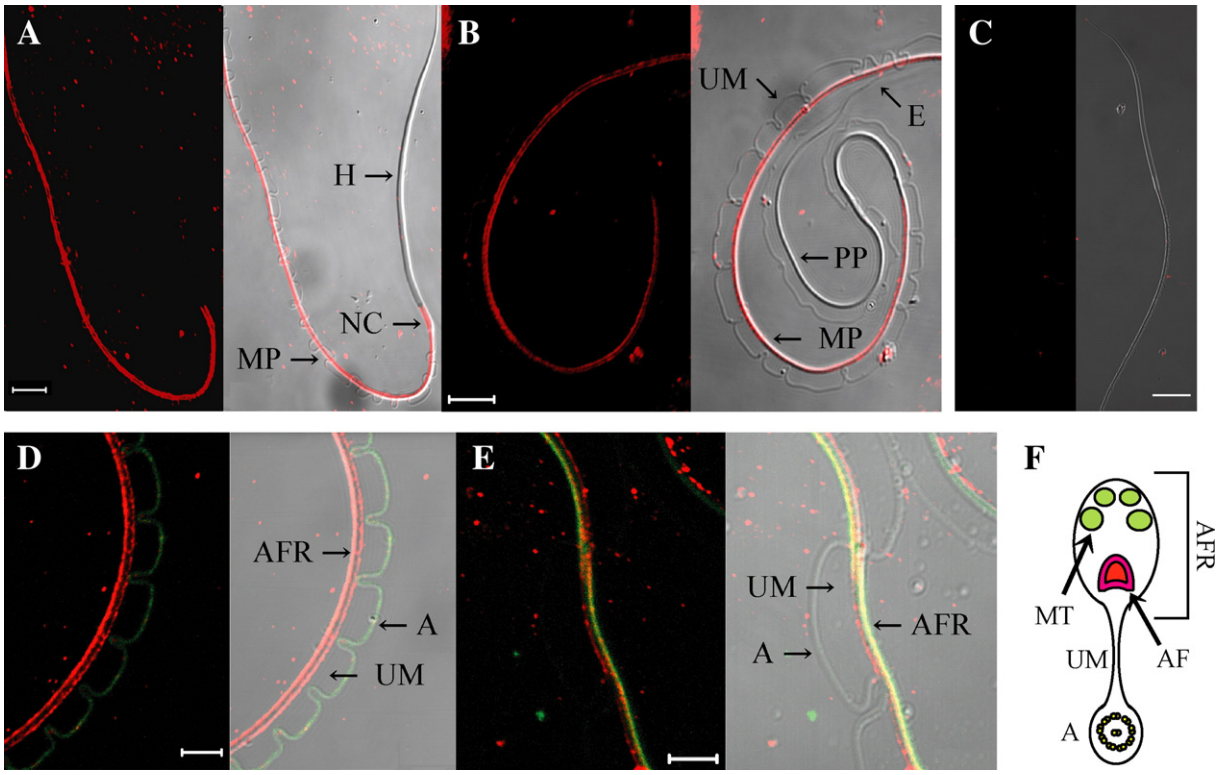

Fig. 9. Localization of citrate synthase, α-tubulin and mitochondria in newt sperm. The fixed sperm were immunostained with anti-citrate synthase antibody (red) (A and B) or with non-specific rabbit IgG (red) (C). The sperm were immunostained with anti-porcine citrate synthase antibody (red) and anti-α-tubulin antibody (green) (C) or with anti-porcine citrate synthase antibody (red) after staining with MitoTracker Green (green) (D). Citrate synthase localized in two lines at neck and axial fiber in middle piece (A), but not in principal piece (B) and axoneme (D). The fibrous citrate synthase close to the undulating membrane did not co-localize with mitochondria (E). (F) A schematic cross-section of the middle piece. A; axoneme, AF; axial fiber, AFR; axial fiber region, E; end of tail, H; head, NC; neck, MP; middle piece, MT; mitochondria, PP; principal piece. Scale bars in panels A and B (10 μm), in panel C (20 μm), in panels D and E (5 μm).

It was previously demonstrated that a phospholipase C (PLC) isoform, PLCζ, triggers Ca^{2+} oscillation in mouse eggs (Saunders et al., 2002; Kouchi et al., 2004) and that PLCζ is a strong candidate as a Ca^{2+} oscillation-inducing protein in mammalian sperm (Miyazaki, 2006; Swann et al., 2006). PLCζ may also be involved in egg activation in other vertebrates since a chicken homologue of PLCζ (Coward et al., 2005) and an extract from chicken sperm (Coward et al., 2003) have been shown to cause Ca^{2+} oscillations in mouse eggs. In the present study, we showed that injection of mouse PLCζ mRNA induced Ca^{2+} elevation in newt eggs, which is consistent with the activation of newt eggs by IP3 injection (Yamamoto et al., 2001). It is, however, unlikely that PLCζ functions as the sperm factor in the newt sperm extract. The properties of the purified 45 kDa protein are quite different from those of PLCζ. Mammalian PLCζ (74 kDa) is much heavier than the 45 kDa protein (Saunders et al., 2002), and the partial amino acid sequences of the 45 kDa protein do not exhibit homology with PLCs. In this connection, a hamster sperm extract that probably contained PLCζ was found to induce a Ca^{2+} increase in newt eggs, while a newt sperm extract did not cause significant Ca^{2+} oscillation in mouse eggs (Iwao and Oda, unpublished data), indicating that the newt sperm extract did not possess sufficient PLCζ activity for egg activation. While it is possible that some other molecules closely associated with the 45 kDa protein, but which was not detected by silver staining on SDS–PAGE, were involved in the egg activation, the 45 kDa protein remains the most likely candidate as the sperm factor for activation at newt fertilization because the injection of *Xenopus* citrate synthase mRNA can induce egg activation. In this connection, we sought to identify the full amino acid sequence of this 45 kDa protein in newt sperm to characterize its molecular feature in detail and to determine whether injection of its mRNA or the protein expressed *in vitro* into newt eggs could induce a Ca^{2+} increase similar to that at fertilization.

We demonstrated that a large amount of the 45 kDa citrate synthase was present as a fibrous structure in the middle piece of the newt sperm. Compared with the detailed, electron-microscopic observation of newt sperm by Picheral (1979), we observed a relatively fibrous citrate synthase localized from the neck to the middle piece, but not in the principal piece, in the undulating membrane, or in the axoneme with a 9+2 structure of microtubules. Citrate synthase in the middle piece was distributed in two lines: one in the distribution area of mitochondria and another in the axial fiber. Since all sperm components including the middle piece are incorporated into the egg at newt fertilization (Picheral, 1977; Iwao, 2000b), fibrous 45 kDa citrate synthase is exposed to egg cytoplasm soon after sperm entry, indicating that the 45 kDa citrate synthase can act as a sperm factor for egg activation at fertilization. Some part of citrate synthase was localized in the region where no mitochondria were detected by MitoTracker Green, indicating

that the citrate synthase was located outside the mitochondria. In addition, most of the 45 kDa protein in *Cynops* sperm lack some amino acid residues in its original amino terminus, known as a mitochondria-targeting sequence (Rosenkrantz et al., 1986). Interestingly, in the protozoa *Tetrahymena*, a 49 kDa protein has dual functions as a citrate synthase in mitochondria and as a cytoskeleton protein for 14 nm filaments in the cytoplasm (Numata et al., 1985; Numata, 1996). In the cytoplasm, the 14 nm filament protein is involved in both oral morphogenesis and in pronuclear behavior during fertilization (Numata et al., 1985). Likewise, the 45 kDa citrate synthase may also play dual roles in the newt sperm: as the mitochondrial enzyme and as the sperm factor for egg activation. Since the enzymatic form and the cytoskeletal form of the *Tetrahymena* 49 kDa protein are maintained by their phosphorylation (Kojima and Numata, 2002) and the citrate synthase (45 kDa) in the sperm is slightly heavier than those (43 kDa) in heart and unfertilized eggs, it would be useful to investigate whether the phosphorylation states of the 45 kDa citrate synthase regulate its egg-activation activity in the newt sperm.

While it is not known how the 45 kDa citrate synthase introduced from fertilizing sperm induces the Ca^{2+} increase, there are several possibilities. First, since mitochondrial citrate synthase is the initial enzyme of the tricarboxylic acid (TCA) cycle, which plays a central role in aerobic energy production, the increase of extra-mitochondrial citrate synthase in egg cytoplasm might change the $[Ca^{2+}]_i$ by disturbing the activity of the mitochondria, such as by affecting the electrical potentials between mitochondrial membranes, production of ATP or generation of reactive oxygen species (ROS), which could influence the spatio-temporal pattern of sperm-triggered Ca^{2+} waves (Dumollard et al., 2006). Indeed, amphibian eggs contain a large number of mitochondria; the *X. laevis* egg, for example, contains more than 10^7 mitochondria (Marinos, 1985). It has been suggested that, in sea urchin eggs, the mitochondria are involved in Ca^{2+} release during fertilization (Eisen and Reynolds, 1985; Girard et al., 1991). Mitochondria are known to be involved in the Ca^{2+} change during the propagation of Ca^{2+} wave in somatic cells (Hajnoczky et al., 2000; Rizzuto et al., 2000). In addition, mitochondrial ATP production seems to participate in the activity of a Ca^{2+} oscillation pacemaker in ascidian and mouse eggs (Dumollard et al., 2003, 2004). Ca^{2+}-releasing activity induced by IP3 is strengthened by oxidizable substrates, such as pyruvate and malate in *Xenopus* oocytes (Jouaville et al., 1995) and ATP^{4-} sensitizes IP3 receptor to IP3 (Mak et al., 1999). Furthermore, it was reported that treatment with H_2O_2 is able to activate Src kinase on the egg membrane followed by stimulation of PLCγ to produce IP3 (Sato et al., 2001). These previous findings suggested the value of investigating whether mitochondria is involved in a Ca^{2+} signaling induced by the sperm factor in newt eggs. Second, free citrate produced by citrate synthase in egg cytoplasm might influence other signaling molecules to cause a Ca^{2+} increase and egg activation. Since citrate is a weak Ca^{2+} chelator commonly found in the cytosol and the mitochondria, it may change the $[Ca^{2+}]_i$ through major Ca^{2+} stores in the egg cytoplasm, such as the endoplasmic reticulum, mitochondria or lysosomes (Whitaker, 2006). In addition, citrate is able to bind calmodulin to induce its conformational modifications (Neufeld et al., 1998) and to associate with Src homology 2 (SH2) domain in Src kinase (Shakespeare et al., 2000), which is required for the Ca^{2+} increase at egg activation in *Xenopus* (Glahn et al., 1999; Sato et al., 1999, 2003). Thus, the role of citrate ion and other weak organic acids in the Ca^{2+} increase at egg activation should be further investigated. Third, the 45 kDa citrate synthase may interact with some of the molecules involved in Ca^{2+} signaling in the egg cytoplasm. In *Tetrahymena*, citrate synthase forms 14 nm filament and associates with HSP60 protein (Takeda et al., 2001). Since some HSP proteins are reportedly involved in fertilization in mammals (Matwee et al., 2001) and amphibians (Coux and Cabada, 2006), it is important to determine the type of molecules that act synergistically with citrate synthase to induce the Ca^{2+} increase in the egg cytoplasm. In any cases, PLC(s) in egg cytoplasm would be stimulated to produce IP3 and then induce Ca^{2+} release through IP3 receptor (Yamamoto et al., 2001).

In the present study, we characterized citrate synthase (45 kDa protein) as the sperm factor for egg activation. However, the detailed molecular mechanisms responsible for the Ca^{2+} increase at egg activation have yet to be determined. We hope that the Ca^{2+} increase mediated by citrate synthase in sperm may serve as a useful model for examining details of the molecular mechanisms in egg activation at fertilization in vertebrates.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (to Y.I.).

References

Coux, G., Cabada, M.O., 2006. Characterization of *Bufo arenarum* oocyte plasma membrane proteins that interact with sperm. Biochem. Biophys. Res. Commun. 343, 326–333.

Coward, K., Campos-Mendoza, A., Larman, M., Hibbitt, O., McAndrew, B., Bromage, N., Parrington, J., 2003. Teleost fish spermatozoa contain a cytosolic protein factor that induces calcium release in sea urchin egg homogenates and triggers calcium oscillations when injected into mouse oocytes. Biochem. Biophys. Res. Commun. 305, 299–304.

Coward, K., Ponting, C.P., Chang, H.Y., Hibbitt, O., Savolainen, P., Jones, K.T., Parrington, J., 2005. Phospholipase Cζ, the trigger of egg activation in mammals, is present in a non-mammalian species. Reproduction 130, 157–163.

Dong, J.B., Tang, T.S., Sun, F.Z., 2000. *Xenopus* and chicken sperm contain a cytosolic soluble protein factor which can trigger calcium oscillations in mouse eggs. Biochem. Biophys. Res. Commun. 268, 947–951.

Dumollard, R., Hammar, K., Porterfield, M., Smith, P.J., Cibert, C., Rouviere, C., Sardet, C., 2003. Mitochondrial respiration and Ca^{2+} waves are linked during fertilization and meiosis completion. Development 130, 683–692.

Dumollard, R., Marangos, P., Fitzharris, G., Swann, K., Duchen, M., Carroll, J., 2004. Sperm-triggered $[Ca^{2+}]$ oscillations and Ca^{2+} homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. Development 131, 3057–3067.

Dumollard, R., Duchen, M., Sardet, C., 2006. Calcium signals and mitochondria at fertilisation. Semin. Cell Dev. Biol. 17, 314–323.

Eisen, A., Reynolds, G.T., 1985. Source and sinks for the calcium released during fertilization of single sea urchin eggs. J. Cell Biol. 100, 1522–1527.

Fujimoto, S., Yoshida, N., Fukui, T., Amanai, M., Isobe, T., Itagaki, C., Izumi, T., Perry, A.C., 2004. Mammalian phospholipase Cζ induces oocyte activation from the sperm perinuclear matrix. Dev. Biol. 274, 370–383.

Girard, J.P., Gillot, I., De Renzis, G., Payan, P., 1991. Calcium pools in sea urchin eggs: roles of endoplasmic reticulum and mitochondria in relation to fertilization. Cell Calcium 12, 289–299.

Glahn, D., Mark, S.D., Behr, R.K., Nuccitelli, R., 1999. Tyrosine kinase inhibitors block sperm-induced egg activation in *Xenopus laevis*. Dev. Biol. 205, 171–180.

Hajnoczky, G., Csordas, G., Madesh, M., Pacher, P., 2000. The machinery of local Ca^{2+} signalling between sarco-endoplasmic reticulum and mitochondria. J. Physiol. 529 (Pt. 1), 69–81.

Iwao, Y., 2000a. Mechanisms of egg activation and polyspermy block in amphibians and comparative aspects with fertilization in other vertebrates. Zool. Sci. 17, 699–709.

Iwao, Y., 2000b. Fertilization in amphibians. In: Tarin, J.J., Cano, A. (Eds.), Fertilization in Protozoa and Metazoan Animal. Springer-Verlag, Heidelberg, pp. 147–191.

Iwao, Y., Fujimura, T., 1996. Activation of *Xenopus* eggs by RGD-containing peptides accompanied by intracellular Ca^{2+} release. Dev. Biol. 177, 558–567.

Iwao, Y., Miki, A., Kobayashi, M., Onitake, K., 1994. Activation of *Xenopus* eggs by an extract of *Cynops* sperm. Dev. Growth Differ. 36, 469–479.

Iwao, Y., Kobayashi, M., Miki, A., Kubota, H.Y., Yoshimoto, Y., 1995. Activation of *Xenopus* egg by *Cynops* sperm extract is dependent upon both extra- and inter-cellular Ca activities. Zool. Sci. 12, 572–581.

Iwao, Y., Murakawa, T., Yamaguchi, J., Yamashita, M., 2002. Localization of γ-tubulin and cyclin B during early cleavage in physiologically polyspermic newt eggs. Dev. Growth Differ. 44, 489–499.

Jouaville, L.S., Ichas, F., Holmuhamedov, E.L., Camacho, P., Lechleiter, J.D., 1995. Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes. Nature 377, 438–441.

Kojima, H., Numata, O., 2002. Enzymatic form and cytoskeletal form of bifunctional *Tetrahymena* 49 kDa protein is regulated by phosphorylation. Zool. Sci. 19, 37–42.

Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T., Miyazaki, S., 2004. Recombinant phospholipase Cζ has high Ca^{2+} sensitivity and induces Ca^{2+} oscillations in mouse eggs. J. Biol. Chem. 279, 10408–10412.

Kurokawa, M., Sato, K., Wu, H., He, C., Malcuit, C., Black, S.J., Fukami, K., Fissore, R.A., 2005. Functional, biochemical, and chromatographic characterization of the complete $[Ca^{2+}]_i$ oscillation-inducing activity of porcine sperm. Dev. Biol. 285, 376–392.

Kyozuka, K., Deguchi, R., Mohri, T., Miyazaki, S., 1998. Injection of sperm extract mimics spatiotemporal dynamics of Ca^{2+} responses and progression of meiosis at fertilization of ascidian oocytes. Development 125, 4099–4105.

Mahbub Hasan, A.K., Sato, K., Sakakibara, K., Ou, Z., Iwasaki, T., Ueda, Y., Fukami, Y., 2005. Uroplakin III, a novel Src substrate in *Xenopus* egg rafts, is a target for sperm protease essential for fertilization. Dev. Biol. 286, 483–492.

Mak, D.O., McBride, S., Foskett, J.K., 1999. ATP regulation of type 1 inositol 1,4,5-trisphosphate receptor channel gating by allosteric tuning of Ca^{2+} activation. J. Biol. Chem. 274, 22231–22237.

Marinos, E., 1985. The number of mitochondria in *Xenopus laevis* ovulated oocytes. Cell Differ. 16, 139–143.

Matwee, C., Kamaruddin, M., Betts, D.H., Basrur, P.K., King, W.A., 2001. The effects of antibodies to heat shock protein 70 in fertilization and embryo development. Mol. Hum. Reprod. 7, 829–837.

Miyazaki, S., 2006. Thirty years of calcium signals at fertilization. Semin. Cell Dev. Biol. 17, 233–243.

Mizote, A., Okamoto, S., Iwao, Y., 1999. Activation of *Xenopus* eggs by proteases: possible involvement of a sperm protease in fertilization. Dev. Biol. 208, 79–92.

Neufeld, T., Eisenstein, M., Muszkat, K.A., Fleminger, G., 1998. A citrate-binding site in calmodulin. J. Mol. Recognit. 11, 20–24.

Numata, O., 1996. Multifunctional proteins in *Tetrahymena*: 14-nm filament protein/citrate synthase and translation elongation factor-1 α. Int. Rev. Cytol. 164, 1–35.

Numata, O., Sugai, T., Watanabe, Y., 1985. Control of germ cell nuclear behaviour at fertilization by *Tetrahymena* intermediate filament protein. Nature 314, 192–194.

Oda, S., Deguchi, R., Mohri, T., Shikano, T., Nakanishi, S., Miyazaki, S., 1999. Spatiotemporal dynamics of the $[Ca^{2+}]_i$ rise induced by microinjection of sperm extract into mouse eggs: preferential induction of a Ca^{2+} wave from the cortex mediated by the inositol 1,4,5-trisphosphate receptor. Dev. Biol. 209, 172–185.

Parrington, J., Swann, K., Shevchenko, V.I., Sesay, A.K., Lai, F.A., 1996. Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. Nature 379, 364–368.

Picheral, B., 1977. La fecondation chez le triton Pleurodele: II. La penetration des spermatozoides et la reaction locale de l'oeuf. J. Ultrastruct. Res. 60, 181–202.

Picheral, B., 1979. Structural, comparative, and functional aspects of spermatozoon in urodeles. In: Fawcett, D.W., Bedford, J.M. (Eds.), The Spermatozoon. Urban & Schwarzenberg, Baltimore, pp. 267–287.

Rizzuto, R., Bernardi, P., Pozzan, T., 2000. Mitochondria as all-round players of the calcium game. J. Physiol. 529 (Pt. 1), 37–47.

Rosenkrantz, M., Alam, T., Kim, K.S., Clark, B.J., Srere, P.A., Guarente, L.P., 1986. Mitochondrial and nonmitochondrial citrate synthases in *Saccharomyces cerevisiae* are encoded by distinct homologous genes. Mol. Cell. Biol. 6, 4509–4515.

Sakakibara, K., Sato, K., Yoshino, K., Oshiro, N., Hirahara, S., Mahbub Hasan, A.K., Iwasaki, T., Ueda, Y., Iwao, Y., Yonezawa, K., Fukami, Y., 2005. Molecular identification and characterization of *Xenopus* egg uroplakin III, an egg raft-associated transmembrane protein that is tyrosine-phosphorylated upon fertilization. J. Biol. Chem. 280, 15029–15037.

Sato, K., Iwao, Y., Fujimura, T., Tamaki, I., Ogawa, K., Iwasaki, T., Tokmakov, A.A., Hatano, O., Fukami, Y., 1999. Evidence for the involvement of a Src-related tyrosine kinase in *Xenopus* egg activation. Dev. Biol. 209, 308–320.

Sato, K., Ogawa, K., Tokmakov, A.A., Iwasaki, T., Fukami, Y., 2001. Hydrogen peroxide induces Src family tyrosine kinase-dependent activation of *Xenopus* eggs. Dev. Growth Differ. 43, 55–72.

Sato, K., Tokmakov, A.A., He, C.L., Kurokawa, M., Iwasaki, T., Shirouzu, M., Fissore, R.A., Yokoyama, S., Fukami, Y., 2003. Reconstitution of Src-dependent phospholipase Cγ phosphorylation and transient calcium release by using membrane rafts and cell-free extracts from *Xenopus* eggs. J. Biol. Chem. 278, 38413–38420.

Sato, K., Fukami, Y., Stith, B.J., 2006. Signal transduction pathways leading to Ca^{2+} release in a vertebrate model system: lessons from *Xenopus* eggs. Semin. Cell Dev. Biol. 17, 285–292.

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLC ζ: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. Development 129, 3533–3544.

Shakespeare, W., Yang, M., Bohacek, R., Cerasoli, F., Stebbins, K., Sundaramoorthi, R., Azimioara, M., Vu, C., Pradeepan, S., Metcalf III, C., Haraldson, C., Merry, T., Dalgarno, D., Narula, S., Hatada, M., Lu, X., van Schravendijk, M.R., Adams, S., Violette, S., Smith, J., Guan, W., Bartlett, C., Herson, J., Iuliucci, J., Weigele, M., Sawyer, T., 2000. Structure-based design of an osteoclast-selective, nonpeptide src homology 2 inhibitor with in vivo antiresorptive activity. Proc. Natl. Acad. Sci. U. S. A. 97, 9373–9378.

Shilling, F.M., Kratzschmar, J., Cai, H., Weskamp, G., Gayko, U., Leibow, J., Myles, D.G., Nuccitelli, R., Blobel, C.P., 1997. Identification of metalloprotease/disintegrins in *Xenopus laevis* testis with a potential role in fertilization. Dev. Biol. 186, 155–164.

Shilling, F.M., Magie, C.R., Nuccitelli, R., 1998. Voltage-dependent activation of frog eggs by a sperm surface disintegrin peptide. Dev. Biol. 202, 113–124.

Stricker, S.A., 1999. Comparative biology of calcium signaling during fertilization and egg activation in animals. Dev. Biol. 211, 157–176.

Swann, K., 1990. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development 110, 1295–1302.

Swann, K., Parrington, J., 1999. Mechanism of Ca^{2+} release at fertilization in mammals. J. Exp. Zool. 285, 267–275.

Swann, K., Saunders, C.M., Rogers, N.T., Lai, F.A., 2006. PLCζ(zeta): a sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. Semin. Cell Dev. Biol. 17, 264–273.

Takeda, T., Yoshihama, I., Numata, O., 2001. Identification of *Tetrahymena* hsp60 as a 14-nm filament protein/citrate synthase-binding protein and its possible involvement in the oral apparatus formation. Genes Cells 6, 139–149.

Townley, I.K., Roux, M.M., Foltz, K.R., 2006. Signal transduction at fertilization: the Ca^{2+} release pathway in echinoderms and other invertebrate deuterostomes. Semin. Cell Dev. Biol. 17, 293–302.

Whitaker, M., 2006. Calcium at fertilization and in early development. Physiol. Rev. 86, 25–88.

Yamamoto, S., Yamashita, M., Iwao, Y., 1999. Rise of intracellular Ca^{2+} level causes the decrease of cyclin B1 and Mos in the newt eggs at fertilization. Mol. Reprod. Dev. 53, 341–349.

Yamamoto, S., Kubota, H.Y., Yoshimoto, Y., Iwao, Y., 2001. Injection of a sperm extract triggers egg activation in the newt *Cynops pyrrhogaster*. Dev. Biol. 230, 89–99.

Yoda, A., Oda, S., Shikano, T., Kouchi, Z., Awaji, T., Shirakawa, H., Kinoshita, K., Miyazaki, S., 2004. Ca^{2+} oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation. Dev. Biol. 268, 245–257.