Egg activation in physiologically polyspermic newt eggs: involvement of IP$_3$ receptor, PLC$\gamma$, and microtubules in calcium wave induction

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ABSTRACT The egg of the polyspermic newt is activated by Ca$^{2+}$ waves induced by several sperm at fertilization. A major component of the sperm factor for egg activation is the sperm-specific citrate synthase (CS), which is introduced into the egg cytoplasm after sperm-egg fusion. We tried to clarify the mechanism for sperm-specific CS to induce [Ca$^{2+}$]$_i$ increase in egg cytoplasm. The injection of the sperm factor into the unfertilized egg induces a [Ca$^{2+}$]$_i$ increase that propagates over the whole egg surface as a Ca$^{2+}$ wave. The propagation of the Ca$^{2+}$ wave is inhibited by depolymerization of microtubules in the egg cytoplasm. The sperm-specific CS is highly phosphorylated and binds the component containing microtubules and the IP$_3$ receptor. The sperm CS localized in the midpiece region was dispersed in the egg cytoplasm, but most of the CS accumulates at the sperm entry site and is distributed in association with the microtubules around the midpiece region and the nucleus. Phospholipase C (PLC)$\gamma$ in egg cytoplasm also accumulates around the midpiece region in association with the sperm CS. Thus, CS at the initiation site of the Ca$^{2+}$ wave forms a complex of microtubules and endoplasmic reticulum (ER) with the IP$_3$ receptor, in addition to PLC$\gamma$, indicating close involvement of those complexes in Ca$^{2+}$ releases from the ER by the sperm factor.

KEY WORDS: Ca$^{2+}$ wave, microtubule, citrate synthase, IP$_3$ receptor, PLC$\gamma$

Introduction

A sperm provides a signal for egg activation, and initiates the developmental program, into an unfertilized egg at fertilization. Unfertilized eggs of most vertebrates are arrested in the meiotic metaphase II (MII). They complete meiosis with degradation of cyclin B and c-Mos when the eggs are activated by an increase in intracellular the Ca$^{2+}$ concentration ([Ca$^{2+}$]) at fertilization. The Ca$^{2+}$ increase is the most important and primary trigger for egg activation in both monospermic and physiologically polyspermic eggs (Iwao, 2000a, b; Wong and Wessel, 2006). Most animals exhibit monospermy in which several blocks to polyspermy prevent extra sperm from entering the egg before sperm-egg fusion. In such cases, the egg must detect the arrival and entry of the first sperm, and then undergo activation to rapidly initiate blocking mechanisms against polyspermy. The fertilizing sperm provides an activation signal on the egg membrane, which rapidly propagates through the entire egg (Iwao, 2012). In contrast, physiologically polyspermic eggs have no block to polyspermy before the fusion with sperm (Iwao, 2000a, b; Wong and Wessel, 2006). Since slower egg activation allows the entry of more than one sperm in polyspermic species, the signal for egg activation in physiological polyspermic species is different from that in monospermic species. Nevertheless, only one sperm nucleus is ultimately allowed to contact the egg nucleus at syngamy to form a diploid zygote nucleus, while the extra sperm nuclei undergo degeneration, thus ensuring embryonic development with a diploid configuration (Iwao et al., 1985, 1993, 2002; Iwao and Elinson, 1990).

A Ca$^{2+}$ wave spreads in the egg cytoplasm in response to each sperm entry at physiologically polyspermic fertilization of the newt, Cynops pyrrhogaster (Harada et al., 2011). The Ca$^{2+}$ wave is, in some cases, preceded by an initial spike-like Ca$^{2+}$ increase (Yamamoto et al., 2001; Harada et al., 2011). The Ca$^{2+}$ wave induced by...
each sperm propagates at most in one-quarter of the egg surface. Since 2–20 sperm enter an egg at normal fertilization, the increase in [Ca\(^{2+}\)] continues for 30–40 min after the initial fertilization. The multiple Ca\(^{2+}\) waves induced by all fertilizing sperm are necessary for complete activation of physiologically polyspermic newt eggs. The initial spike-like Ca\(^{2+}\) rise at Cynops fertilization is induced by the sperm protease on the egg surface, but this is insufficient for egg activation (Harada et al., 2011). When a sperm factor for egg activation in a sperm-soluble component is introduced into an egg, a Ca\(^{2+}\) wave is induced by the sperm factor and then the egg undergoes activation (Yamamoto et al., 2001; Harada et al., 2007). The major component of the sperm factor for egg activation is the sperm-specific citrate synthase (CS, 45 kDa), which is slightly heavier than those observed in heart tissue and in unfertilized eggs (43 kDa) (Harada et al., 2007). Most CS in sperm is distributed in the midpiece region, from which point the sperm CS is exposed to the egg cytoplasm soon after sperm entry. A major Ca\(^{2+}\) store in egg cytoplasm is the endoplasmic reticulum (ER) containing the inositol 1,4,5-triphosphate (IP\(_3\)) receptor, 1,4,5-triphosphate (IP\(_3\)) receptor (Yamamoto et al., 2001), which forms large clusters in the egg cytoplasm (Harada et al., 2011). Adequate conformation of the ER is necessary for propagation of the Ca\(^{2+}\) wave.

High enzymatic activity in sperm-specific CS seems to be necessary for the Ca\(^{2+}\) increase (Harada et al., 2011). Although both acetyl CoA and oxaloacetate induce egg activation accompanied by a Ca\(^{2+}\) increase, the detailed mechanisms for the Ca\(^{2+}\) increase in egg cytoplasm are unknown. In this connection, in the protozoa Tetrahymena, CS has been found to form 14 nm filaments in the cytoplasm (Numata et al., 1985; Numata, 1996). Further, a dephosphorylated form of CS in the 14 nm filament protein is involved in oral morphogenesis and pronuclear behavior during fertilization (Numata et al., 1985; Kojima and Numata, 2002) and is associated with the HSP60 protein (Takeda et al., 2001).

In the present study, we tried to determine what kinds of molecules interact with the sperm factor, the sperm-specific CS, to induce the Ca\(^{2+}\) increase in the egg cytoplasm, since Ca\(^{2+}\) stores are associated with cytoskeletons in egg cytoplasm (Harada et al., 2011). We found that microtubules in the egg cytoplasm are necessary for the propagation of the Ca\(^{2+}\) wave induced by the sperm factor. The highly phosphorylated sperm CS bound the component containing tubulin and the IP\(_3\) receptor in addition to the association of phospholipase C (PLC) \(\gamma\) in the egg cytoplasm. We suggest that the sperm CS in the egg cytoplasm forms a complex of microtubules, the ER with the IP\(_3\) receptor, and PLC\(\gamma\), which are involved in the Ca\(^{2+}\) increase by the sperm factor at polyspermic newt fertilization.

**Fig. 1.** [Ca\(^{2+}\)] increases induced by the sperm factor.

Typical increases in [Ca\(^{2+}\)] in the DMSO (1%)-injected eggs (A,B) and nocodazole-injected eggs (C,D) (10 \(\mu\)M; (E,F) (2 \(\mu\)M) after injection of the sperm extract (SE, 28 ng protein) at the site (circle 1). (B,D,F) Changes in [Ca\(^{2+}\)] levels measured in the circles in A, C, and E, respectively. Figures on the top left show time (min) after injection. Scale bar, 0.5 mm.

**Results**

**Microtubules are necessary for propagation of the Ca\(^{2+}\) wave induced by the sperm factor**

To elucidate the role of microtubules in the sperm factor-induced propagation of the Ca\(^{2+}\) wave in the egg cytoplasm, the unfertilized eggs which had been injected with a microtubule-depolymerizing reagent, nocodazole, were injected with a sperm extract containing the sperm factor (28 ng, protein). In the control egg injected with DMSO, an increase in [Ca\(^{2+}\)] was initiated at the site of injection and propagated over the whole surface of the egg as a Ca\(^{2+}\) wave at a velocity of 4.0 \(\mu\)m/sec (Fig. 1 A,B). In the eggs which had been injected with nocodazole (final concentration in egg cytoplasm, 10 \(\mu\)M), a very small increase in [Ca\(^{2+}\)] was induced around the injection site of the sperm extract, but it did not propagate in the egg cytoplasm (Fig. 1 C,D). In the egg injected with 2 \(\mu\)M nocodazole, a Ca\(^{2+}\) wave was initiated at the injection site and propagated over about a half of the egg surface, but did not reach the opposite side (Fig. 1 E,F). The increase of [Ca\(^{2+}\)] in the nocodazole-injected eggs was significantly smaller than those in the DMSO-injected eggs, but the propagation rate of Ca\(^{2+}\) wave (4.6 \(\mu\)m/sec) was similar to that in the control egg. These results indicate that polymerized microtubules in the egg cytoplasm are necessary for the propagation of the Ca\(^{2+}\) wave at egg activation by the sperm factor.

**Close association of citrate synthase with microtubules and the IP\(_3\) receptor in sperm and egg cytoplasm**

To clarify the role of microtubules in egg activation by the sperm factor, we tried to determine whether tubulins (microtubules) bound CS in sperm or in the egg cytoplasm. CS in a sperm extract or an
egg extract was bound on a Biacore sensor chip on which anti-CS antibody had been captured. The molecules associated with CS on the sensor chip were analyzed by monitoring the further binding of specific antibodies with surface plasmon resonance (SPR) analysis (Biacore system). We found that CS in the sperm extract was associated with tubulin (Fig. 2A-a), but no significant binding was detected CS in the egg cytosol (Fig. 2A-b). The binding signal for tubulins slightly increased when the egg cytosol containing a large amount of tubulin was added to the chip on which the sperm extract had been bound (Fig. 2A-c), indicating that some parts of sperm CS retain the capacity to bind tubulin. No association of tubulin with the sperm CS was detected when the sperm extract was first exposed to an inhibitor of CS, palmitoyl-CoA (50 μM, Fig. 2E-a). The enzymatic activity of CS seems to be involved in the interaction between the sperm CS and tubulin, probably microtubules. The interaction of sperm CS with tubulin was also determined by immunoprecipitation of the mixture of sperm and egg extracts with an anti-CS antibody (Fig. 3A). A large amount of tubulin bound to the sperm CS in the egg extract, but no significant amount of tubulin was co-precipitated in the egg extract (Fig. 3A).

Indeed, the apparent molecular weight of the sperm-specific CS (45 kDa) was larger than that of CS in unfertilized eggs or heart (43 kDa) when determined by ordinary SDS-PAGE (Fig. 3C upper lanes, Harada et al., 2007). When we analyzed a CS cDNA clone obtained from a newt testis, the deduced amino acid sequence (52 kDa) showed a mitochondrial targeting sequence prior to the N-terminus of the newt CS (Fig. 3B). The newt CS exhibited similarity to Xenopus laevis CS with 87% identity at the amino acid level. The larger sperm CS probably undergoes modification such as phosphorylation. When CS was analyzed by phosphate-affinity SDS-PAGE (Mn–Phos-tag SDS-PAGE) for detection of a mobility shift in phosphoproteins, the sperm CS was detected as the P2 level (Fig. 3C middle lanes). About a half of the sperm CS was detected a down-shifted state (P0 level) after the treatment by λ-phosphatase.

CSs in both the sperm extract and the egg cytosol was associated with the IP3 receptor (Fig. 2B-a, b), indicating that the complex with CS bound the ER containing the IP3 receptor. The amount of the IP3 receptor associated with CS significantly increased when the egg cytosol was added to the chip on which the sperm extract had been bound (Fig. 2B-c), indicating that the sperm CS can bind the complex with the IP3 receptor in the egg cytoplasm. The interaction between the IP3 receptor and the sperm CS was not affected by the treatment with palmitoyl-CoA (50 μM, Fig. 2E-b). In contrast, there was no significant binding of CS with either PLCγ, or acetylated tubulin in the complex with CS in the sperm extract or the egg extract (Fig. 2C D). However, a slight amount of PLCγ was detected by the addition of the egg cytosol on the chip after the binding of the sperm extract (Fig. 2C-c). These results indicate that sperm CS participates in the formation of the complex between microtubules and the IP3 receptor-containing components in the egg cytoplasm, possibly with a small amount of PLCγ. Thus, the properties of CS in the sperm are quite different from those in the egg cytoplasm.
indicating that the sperm CS is highly phosphorylated. Some CS in unfertilized eggs also exhibited a slightly lighter state (P1 level) and the P0 level (Fig. 3C middle lanes). These results indicate that the sperm CS is highly phosphorylated, and the egg cytoplasm contains lower phosphorylated and non-phosphorylated CS. Since several phosphorylation sites were predicted in newt CS (Fig. 3B asterisks), the difference in mobility shift is probably due to the difference in the number of phosphorylation sites.

Co-localization of citrate synthase with microtubules, the IP$_3$ receptor, and PLC$_{g}$ in fertilized eggs

To determine the role of sperm CS in egg activation at fertilization, we examined the distribution of CS and microtubules in the sperm and the fertilized eggs using immunocytochemistry. A large amount of CS was localized in the axial fiber in the midpiece region of sperm, but not in the axoneme (Fig. 4A). By contrast, α-tubulin was observed in both the axial fiber and the axoneme.
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When the sperm were treated with the anti-CS antibody primarily, followed by treatment with the anti-α-tubulin antibody, tubulin was mainly observed in the axoneme, while CS was detected in the axoneme, but not in axoneme (Fig. 4C, Harada et al., 2007). These results indicated that since tubulin in the axoneme was closely associated with CS, the binding of the antibody against tubulin is prevented by the anti-CS antibody. Thus, some CS in the sperm seems to be closely associated with microtubules in the axial fiber.

In the egg 1 hour after fertilization, CS was highly accumulated around a sperm entry site near the egg cortex (Fig. 5A) and along the sperm under the egg cortex (Fig. 5A, B). CS was distributed in the condensed sperm nucleus (Fig. 5A, D) and was dispersed in the midpiece region (Fig. 5B). However, CS was not associated with stable acetylated tubulin in the axoneme of incorporated sperm (Fig. 5C). Thus, CS was co-localized with microtubules (tubulin) and nuclei in the fertilized eggs. The IP$_3$ receptor was also accumulated in the sperm nucleus and around the center of the sperm aster (centrosome) in the fertilized eggs (Fig. 6A). The IP$_3$ receptor was co-localized with CS near the incorporated sperm nucleus (Fig. 6B), indicating that a close interaction of CS with the ER containing the IP$_3$ receptor in the egg cytoplasm. Most PLC$_\gamma$ was distributed in a sperm entry site (Fig. 5D) and in the midpiece region of sperm (Fig. 5D, E). PLC$_\gamma$ was co-localized with CS around the sperm aster. In addition, the IP$_3$ receptor was co-localized with PLC$_\gamma$ around the center of sperm aster (Fig. 6C, D).

Since PLC$_\gamma$ was present in the egg cytoplasm, but not in the sperm (Fig. 3C lower lanes), PLC$_\gamma$ derived from egg cytoplasm was accumulated around the incorporated sperm.

**Discussion**

In physiologically polyspermic newt eggs, the injection of sperm-soluble cytoplasm, a sperm factor, into the unfertilized egg causes a Ca$^{2+}$ wave and subsequent egg activation (Yamamoto et al., 2001; Harada et al., 2007). The major component of the sperm factor for egg activation has been identified as the sperm-specific CS which is introduced into egg cytoplasm at sperm-egg fusion (Harada et al., 2007, 2011). The Ca$^{2+}$ increase in egg cytoplasm is induced by IP$_3$, but not by cADP-ribose in newt eggs (Yamamoto et al., 2001). Since the Ca$^{2+}$ increase in a 20,000 g precipitate of egg cytoplasm is induced by IP$_3$, but not by cADP-ribose in newt eggs (Yamamoto et al., 2001), the sperm CS releases Ca$^{2+}$ from the ER which consists of large complexes with cytoskeletons in the inner egg cytoplasm. However, the role of cytoskeletons in the Ca$^{2+}$ wave induced by the sperm factor remains unknown. First, we investigated the role of microtubules in unfertilized eggs for the initiation and propagation of the Ca$^{2+}$ wave by the sperm factor. We found that nocodazole, a depolymerizing agent of microtubules, inhibited the propagation of the Ca$^{2+}$ wave by the sperm factor. When the eggs were treated with a higher concentration of nocodazole, a very small amount of Ca$^{2+}$ increase was induced by the sperm factor without subsequent propagation, indicating that microtubules in the egg cytoplasm are necessary for maintenance of the propagation of the Ca$^{2+}$ wave. To clarify the role of microtubules in sperm-induced Ca$^{2+}$ increase, it is...
worth to investigate the change in the co-localization of molecules in nocodazole-treated eggs.

The association of sperm CS with tubulins was confirmed by both SPR analysis (Biacore) and immunoprecipitation. In the present study, we found a large amount of CS localized in the axial fiber in the midpiece region of the sperm in association with α-tubulin by staining each antibody independently (Fig. 4B). When the sperm were treated with the anti-CS antibody first, followed by treatment with the anti-α-tubulin antibody, tubulin was hard to detect in the axial fiber region (Fig. 4C, Harada et al., 2007). These results suggest that tubulin in the axial fiber is closely associated with CS, so that the binding of the antibody against tubulin is prevented by the anti-CS antibody, indicating a close association of CS with microtubules in the axial fiber. In addition, the CS complexes in both sperm and the egg cytoplasm contain IP₃ receptors responsible for Ca²⁺ release from the ER. These results strongly suggest that the sperm CS facilitates the formation of large complexes between microtubules and the ER with IP₃ receptors in the inner egg cytoplasm, which is responsible for the induction of the Ca²⁺ wave at newt fertilization (Fig. 7).

It has been shown that microtubules play an important role in the configuration of the ER in egg cytoplasm. The ER membrane accumulates in the region of the microtubule array surrounding the sperm nucleus and the sperm aster (centriole) in sea urchin eggs (Jaffe and Terasaki, 1993). In hippocampal neurons, large ER vesicles that are capable of taking up and releasing Ca²⁺ move along the dendrite microtubules, probably by a motor protein of kinesin (Bannai et al., 2004; Mikoshiba, 2007). It is also known that the remodeling of the ER into large ER patches gives IP₃ receptors within the ER patches higher sensitivity to IP₃ in mature Xenopus oocytes (Sun et al., 2011). Thus, microtubules in the newt eggs seem be necessary for ER-clustering to promote the propagative Ca²⁺ release for the Ca²⁺ wave. The transport of the ER along the microtubules probably contributes to the spatial regulation of intracellular Ca²⁺ signaling at fertilization. Interestingly, since an inhibitor of CS, palmityloyl-CoA, abolished the interaction between CS and tubulins (microtubules), but not that between CS and IP₃ receptors (ER), CS with enzymatic activity may mediate the binding of microtubules with ER-clusters in the egg cytoplasm.

The role played by CS in the initiation of the Ca²⁺ increase at fertilization may involve its production of acetyl CoA from the citrate that is abundant in the egg cytoplasm (Harada et al., 2011). The injection of acetyl CoA might induce a Ca²⁺ increase through sensitization of the IP₃ receptors on the ER to induce Ca²⁺ releases (Missiaen et al., 1997). The local increase of Ca²⁺ probably causes a successive Ca²⁺ release from the neighboring ER by directly stimulating the IP₃ receptors or through activation of PLC (Iwao, 2012). In this connection, β-tubulin has been shown to bind Src homology 2 (SH2) domains (Itoh et al., 1996) and pleckstrin homology (PH) domains of PLCγ1 to promote its activity (Chang et al., 2005). Interestingly, the present study found that PLCγ is accumulated around the midpiece region of the sperm incorporated into the egg. Since the binding between CS and PLCγ was slightly observed by the addition of the egg cytosol on the SPR-chip after the binding of the sperm extract, it is possible that PLCγ around the sperm was derived from egg cytoplasm. The CS/tubulin complex may play important roles in the assembly of PLCγ into the complexes with microtubules and the ER in the egg cytoplasm. Thus, the midpiece region of the fertilizing sperm seems to contribute to the formation of a center for the [Ca²⁺] wave at newt egg activation (Fig. 7). Further investigation is necessary, however, to determine the interaction of those molecules in the Ca²⁺ increase at newt fertilization.

The CS in sperm cytoplasm, but not in egg cytoplasm, is associated with the component consisting of microtubules. The apparent molecular weight of the sperm CS (45 kDa) was larger than that of CS in unfertilized eggs or heart (43 kDa) in ordinary SDS-PAGE (Fig. 3 and Harada et al., 2007). The signal sequence at the N-terminus region of the newt

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**Fig. 6. Confocal fluorescence micrographs showing the localization of the IP₃ receptor in fertilized eggs fixed with FG solution.**

(A) Localization of the IP₃ receptor (red) and α-tubulin (green) in the eggs 45 min after fertilization, showing the IP₃ receptor localized in the sperm nucleus (arrowhead) and in the midpiece (arrow). (B) Localization of the IP₃ receptor (red) and CS (green) in the eggs 45 min after fertilization, showing accumulation of the IP₃ receptor around the sperm nucleus (arrowhead) with CS. (C,D) Localization of the IP₃ receptor (red) and PLCγ (green) in the eggs 1 h after fertilization, showing PLCγ accumulated around the midpiece region (arrowheads). Scale bar, 50 μm.
Cynops CS is slightly shorter than that of the frog Xenopus (Fig. 3B), but contains a putative mitochondrial targeting signal (MTS) with conserved basic residues (Cheng et al., 2009). It was reported that the human CS gene is a single nuclear gene which transcribes into two mRNA variants, isoform a (CSa) and b (CSb), by alternative splicing (Cheng et al., 2009). Since the amino acid sequence of CS expressed in the newt testis was not significantly different than those in the eggs, some modifications probably occur in the sperm CS after transcription. An analysis of CS in the electrophoresis with Phos-tag indicated that all sperm CS (P2 level) is highly phosphorylated. In contrast, about half of the egg CS (P0 level) was not phosphorylated at all, while the other half (P1 level) was probably phosphorylated to some extent. Since the treatment of sperm CS by λ- phosphatase shifted half of the P2 level into the P0 level, the major modification in the sperm CS is phosphorylation. However, it may be difficult for phosphatase to access the sperm CS in larger complexes. Indeed, there are several predicted phosphorylation sites: five serine and one tyrosine residues in the newt CS (Fig. 3B). Interestingly, it has been reported in the protozoa Tetrahymena that CS displays dual functions: as an active enzyme in mitochondria and as a cytoskeleton protein for 14 nm filaments (Numata et al., 1985; Numata, 1996) which is involved in oral morphogenesis and pronuclear behavior during fertilization (Numata et al., 1985). The dephosphorylated CS forms filaments accompanied by decreased enzymatic activity (Kojima and Numata, 2002). Thus, the alteration in phosphorylation states may cause changes in the properties of CS in the newt sperm, such as binding to microtubules and ER with IP3 receptors. The function of phosphorylated CS at fertilization should be investigated in both sperm and eggs.

We previously reported that CS was mainly localized in an axial fiber of newt sperm in the midpiece region (Harada et al., 2007). When the sperm enter the egg cytoplasm, all sperm components including, a tail region, were incorporated into the egg cytoplasm (see Fig. 5). CS in the midpiece region was dispersed in the egg cytoplasm, but some CS was localized in the condensed sperm nucleus. CS was localized in center of the sperm aster (centrosome). Although it is difficult to distinguish CS derived from sperm from that in unfertilized eggs by immunofluorescence microscopy, CS around the sperm nucleus and the midpiece region is probably derived from sperm. CS around nucleus might accumulate PLCγ in association with microtubules. Since some phosphorylation of CS occurs in the egg cytoplasm, it would be worthwhile to investigate the interaction between the egg CS and microtubules during cleavage and embryonic development.

In summary, the present results demonstrate that microtubules in the egg cytoplasm are necessary for the propagation of the Ca2+ wave induced by the sperm factor, CS, at newt fertilization. The highly phosphorylated CS in sperm is able to bind to the large complexes containing microtubules and the ER with the IP3 receptors. The sperm CS which binds tubulins seems to be involved in promotion of ER-clustering and to closely associate with the IP3 receptors and PLCγ to induce the Ca2+ release.

**Materials and Methods**

**Preparation of egg cytosol and sperm extract**

Sexually mature news (Cynops pyrhogaster) were purchased from dealers. To induce ovulation, the female was injected with 80 IU of human chorionic gonadotropin (HCG) (ASKA Pharmaceutical) every two days. Unfertilized eggs were obtained by squeezing the abdomen of the female. 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 CaCl2, 0.85 mM MgSO4, 4.6 mM Tris-HCl, pH 7.4). The dejellied eggs were kept in SB more than 2 h to remove the artificially activated eggs. Unfertilized, dejellied eggs were washed twice with GV buffer (100 mM sucrose, 100 mM KCl, 50 mM MgSO4, 25 mM Tris-HCl, pH 7.4), and then with GV buffer containing 1% protease inhibitor cocktail (25955-11, Nacalai tesque). The eggs were homogenized in GV buffer containing 1% protease inhibitor cocktail (5 μl/egg). To remove yolk granules, the homogenate was centrifuged (3,000 g, 30 min, 4°C), and the supernatant was collected as an egg cytosol and stored at –80°C until use. In some cases, the egg cytosol was further centrifuged (20,000 g, 30 min, 4°C) to separate endoplasmic reticulum (ER). Mature newt sperm obtained by squeezing the abdomen of males were suspended in De Boer’s solution (DB: 110.3 mM NaCl, 1.3 mM KCl, 1.3 mM CaCl2, 5.7 mM Tris-HCl, pH 7.4). After washing by centrifugation (350 g, 20 min, 4°C), the precipitated sperm were resuspended in intracellular-like medium (ICM: 120 mM KCl, 100 μM EGTA, 10 mM Na-β-glycerophosphate, 0.2 mM PMSF, 1 mM DTT, 20 mM HEPES-KOH, pH 7.5). The sperm suspension was sonicated on ice (50 W, 15 sec, 5 times, Nissei) to disrupt sperm plasma membranes, and then centrifuged (10,000 g, 20 min, 4°C). The supernatant was collected as a sperm extract and stored at –80°C until use. All animal experimentation was performed based on guidelines approved by Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan.

**Microinjection**

The dejellied eggs were washed with NKP solution (120 mM NaCl, 7.5 mM KCl, 10 mM NaH2PO4 and NaHPO4 pH 7.0, 4% polyvinylpyrrolidinone) and then transferred to injection buffer (IB: 5.0 mM KCl, 1.0 mM EGTA, 50 mM NaH2PO4 and NaHPO4 pH 7.0, 4% polyvinylpyrrolidinone). The microinjection was carried out with a glass micropipette having a tip di-
ameter of 20-30 μm in IB. Each newt egg was injected with 5 or 33 nl of nocodazole (in DMSO, M1404, Sigma) and a Ca²⁺-sensitive fluorescent dye, Oregon green 488 BAPTA-1 dextran 10,000 MW (2 mM, O6798, Molecular Probes). After injection, the injected eggs were kept in IB 5 min and then incubated in healing buffer (HB: 5.0 mM KCl, 1.0 mM CaCl₂, 50 mM Na₂HPO₄, and NaH₂PO₄, pH 7.0, 4% polyvinylpyrrolidone) 5 min to enhance wound healing. The injected eggs were transferred to SB, and then were injected with 33 nl of sperm extract. The fluorescent images of the injected egg were taken with a highly sensitive CCD camera (ORCA-flash2.8, Hamamatsu photons) and software operating the camera (MetaMorph, Molecular Devices) every 10 sec. The fluorescence intensity was measured by MetaMorph.

**Antibodies**

The following antibodies were used for Biacore system, immunoprecipitation, Western blotting, and immunocytochemistry: anti-porcine citrate synthase rabbit polyclonal antibody (NE0407/5, Nordic Immunological Laboratories B.V.), anti-PLCγ1 mouse monoclonal antibody (05-163, Millipore), anti-αᵢ receptor rabbit polyclonal antibody (07-1210, Millipore), anti-acetylated tubulin mouse monoclonal antibody (T7451, Sigma), anti-α tubulin mouse monoclonal antibody conjugated with FITC (F2168, Sigma), anti-α tubulin mouse monoclonal antibody (T6199, Sigma), and anti-β tubulin mouse monoclonal antibody (T4026, Sigma).

**Analysis of molecular interactions with Biacore system**

The binding assay using Biacore J (GE Healthcare) was done according to the method described previously (Johnsson et al., 1991). Anti-CS antibody was immobilized on a surface of sensor chip (CM5, research grade) by amino-coupling; briefly, activation of carboxyl groups on the chip surface with N-hydroxysuccinimide and N-ethyl-N'-[3-dimethylaminopropyl] carbodiimide hydrochloride, coupling of 180 μl of 60 μg/ml anti-CS antibody in 10 mM sodium acetate buffer (pH 5.0), and then blocking of excess carboxyl groups by 1.0 M ethanolamine. In this way, about 17.2 ng of anti-CS antibody was immobilized on the sensor chip. The sperm extract and the egg cytosol was introduced to the sensor chip surface at a flow rate of 30 μl/min at 25°C with HBS-EP (150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20, 10 mM HEPES-NaOH, pH 7.4), and then each antibody was applied on the sensor chip surface. The sensor chip was washed with 30 μl of 10 mM glycine-HCl (pH 1.7) to regenerate the chip. To make sure that the binding is specific one, it monitored also the samples with non-specific antibody in HBS-EP. The background was subtracted by the blank run, using BIA evaluation 4.1 software.

**Immunoprecipitation and Western blot**

Agarose-beads conjugated with protein A were suspended in HNTG buffer (150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 20 mM HEPES-NaOH, pH 7.5) and washed twice by centrifugation. The sperm extract and/or egg cytosol was mixed with anti-CS antibody and incubated overnight at 4°C. The mixture was incubated with the agarose-beads for 1 h at 4°C with constant shaking. The agarose-beads were washed three times with HNTG buffer by centrifugation. The immunocomplexes were extracted from the agarose-beads by the treatment of a sample buffer for SDS-PAGE (95°C, 5 min), and analyzed by Western blot with anti-α tubulin antibody (1:1,000 dilution). The signals were detected with anti-mouse IgG rabbit antibody, HRP-conjugated (019-1792, Wako) and enhanced chemiluminescence system (ECL plus, RPN2132, GE Healthcare). The sperm extract dialyzed against PBS was treated with 1,000 U/50 μl λ-phosphatase (P0753S, New England Biolabs) (30°C, 24 h). The sperm extract or the egg cytosol was analyzed by Western blot with anti-CS antibody (1:1,000 dilution) or anti-PLCγ1 antibody (1:1,000 dilution). Species-specific secondary antibodies were used for detection with ECL plus system.

**Immunocytochemistry**

The sperm were suspended in 10% DB and then air-dried on the slide glass. The sperm were fixed with 3.6% formaldehyde in PBS (0.9% NaCl, 10 mM Na₂HPO₄ and NaH₂PO₄, 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 (5 min, three times), the samples were blocked by PBS with 1% bovine serum albumin (BSA) in a moist chamber. The samples were treated with the anti-CS antibody (1:100 dilution) in the moist chamber at 4°C overnight. After washing with TTBS (0.1% Tween-20, 155 mM NaCl, 100 mM Tris-HCl, pH 7.4) (5 min, three times), the samples were treated with anti-rabbit IgG goat antibody conjugated with Alexa546 (1:400 dilution, Molecular Probes) in PBS with 1% BSA for 30 min at room temperature. After washing with TTBS (5 min, three times), some samples were treated with anti-α tubulin antibody conjugated with FITC (1:100 dilution) at 4°C overnight. The samples were mounted with slowfade antifade kit (S2828, Molecular Probes).

Unfertilized eggs were inseminated with the sperm suspended in 10% SB. The eggs were dejellied with 1.5% sodium thioglycolate 30 min after insemination, and then washed with SB. Dejellied eggs were allowed to develop in 10% SB at 23°C. Fertilized eggs were fixed with FG fixation solution (3.6% formaldehyde, 0.25% glutaraldehyde, 1 mM MgCl₂, 5 mM EGTA, 80 mM PIPES-KOH, pH 6.8) or FGX fixation solution (FGX fixation solution with 0.2% Triton X-100) overnight at room temperature, and post-fixed with 100% methanol and stored at −30°C until use. Fixed eggs were reduced with 50 mM NaBH₄ in PBS overnight at room temperature. After washing with TBSN (0.1% NP-40, 155 mM NaCl, 100 mM Tris-HCl, pH 7.4) (90 min, three times), they were treated with anti-CS antibody (1:200 dilution) or anti-αᵢ receptor antibody (1:100 dilution) in TBSN with 2% BSA for 24 h at 4°C. After washing with TBSN (12 h, three times), they were treated with anti-rabbit IgG goat antibody conjugated with Alexa546 (1:400 dilution, A11010, Molecular Probes) in TBSN with 2% BSA for 24 h at 4°C. After thorough washing with TBSN (12 h, three times), they were treated with anti-PLCγ1 antibody (1:250 dilution), anti-α tubulin antibody (1:200 dilution), or anti-acetylated tubulin antibody (1:1,000 dilution) in TBSN with 2% BSA for 24 h at 4°C. After washing with TBSN (12 h, three times), they were treated with anti-mouse IgG goat antibody conjugated with Alexa633 (1:400 dilution, A21050, Molecular Probes) in TBSN with 2% BSA for 24 h at 4°C. After thorough washing with TBSN (12 h, three times), the specimens were stained with 0.5 μM SYTOX green (S7020, Molecular Probes) for 30 min and washed twice with TBSN, and were dehydrated with 100% methanol (90 min, three times) at room temperature, and stored at −30°C until observation. They were cleared with BA/BB solution (Benzyl alcohol: Benzyl benzate; 1:2) just before observation, and the images were taken with a confocal laser-scanning microscope (LSM510 Meta, Carl Zeiss).

**Cloning of Cynops citrate synthase cDNA**

Following isolation of total RNA from newt testis with Trizol reagent (15596-026, Invitrogen), RACE was performed using the GeneRacer kit (L1502-01, Invitrogen). Newt CS fragment was obtained by degenerate PCR against Xenopus laevis CS (GenBank, BC046571). The 5' and 3' RACE PCRs were carried out using gene specific primers to newt CS fragment. The RACE products were cloned into pGEM-T Easy vector (A1360, Promega) and sequenced with BigDye Terminator v3.1 reaction kit (4337454, Applied Biosystems). The full length sequence of newt CS was obtained (GenBank, AB818537). The prediction of phosphorylation sites in newt and X. laevis CS was performed by NetPhos 2.0 Server (CBS, Denmark).

**Acknowledgements**

*This work was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas from MEXT (22112518, 24112712).*

**References**


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