**Int. J. Dev. Biol. 58:** 315-323 (2014)
doi: 10.1387/ijdb.130333yi

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

# Egg activation in physiologically polyspermic newt eggs: involvement of $IP_3$ receptor, PLCγ, 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) γ 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 PLCγ, 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, PLCγ*

## 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 ($[Ca^{2+}]_i$) 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, 2012; Tarin, 2000). 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

*Abbreviations used in this paper:* CS, citrate synthase; ER, endoplasmic reticulum; $IP_3$ receptor, inositol 1,4,5-triphosphate receptor; PLCγ, phospholipase Cγ.

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*Accepted:* 5 March 2014. *Final, author-corrected PDF published online:* 30 September 2014. *Edited by:* Makoto Asashima.

ISSN: Online 1696-3547, Print 0214-6282

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 $[Ca^{2+}]_i$ 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 (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.

## 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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

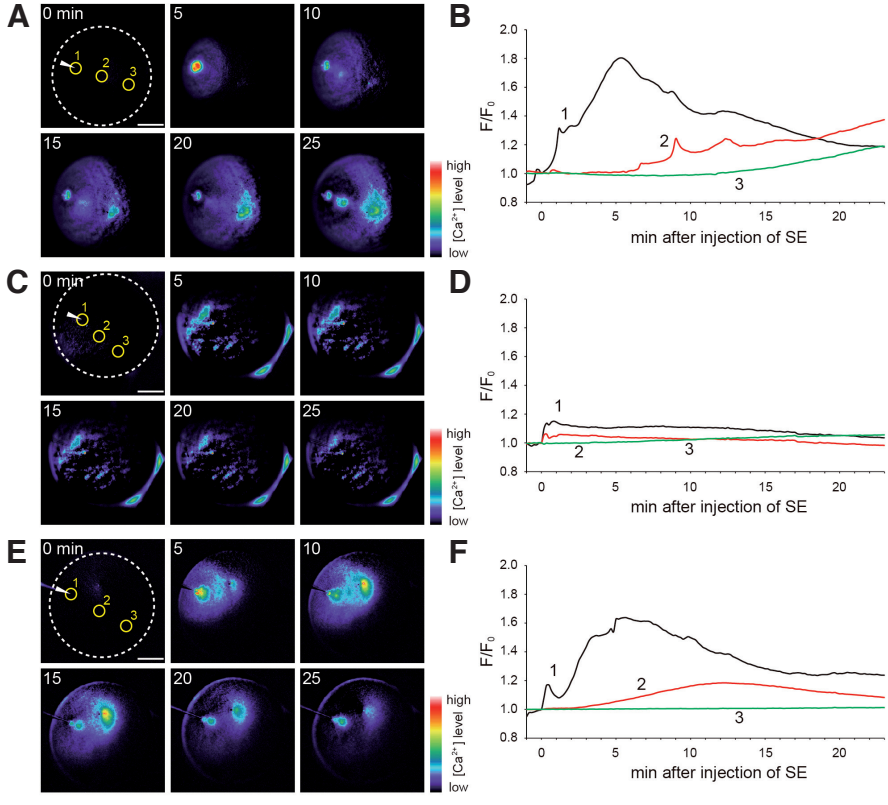


**Fig. 1. $[Ca^{2+}]i$ increases induced by the sperm factor.** *Typical increases in $[Ca^{2+}]_i$ in the DMSO (1%)-injected eggs* **(A,B)** *and nocodazole-injected eggs* **(C,D)** *(10 $\mu$M);* **(E,F)** *(2 $\mu$M) after injection of the sperm extract (SE, 28 ng protein) at the site (circle 1).* **(B,D,F)** *Changes in $[Ca^{2+}]_i$ 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.*

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).

CS in both the sperm extract and the egg cytosol was associated with the $IP_3$ receptor (Fig. 2 B-a, b), indicating that the complex with CS bound the ER containing the $IP_3$ receptor. The amount of the $IP_3$ 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 $IP_3$ receptor in the egg cytoplasm. The interaction between the $IP_3$ 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. 2 C,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 $IP_3$ 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.

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 ($Mn^{2+}$–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

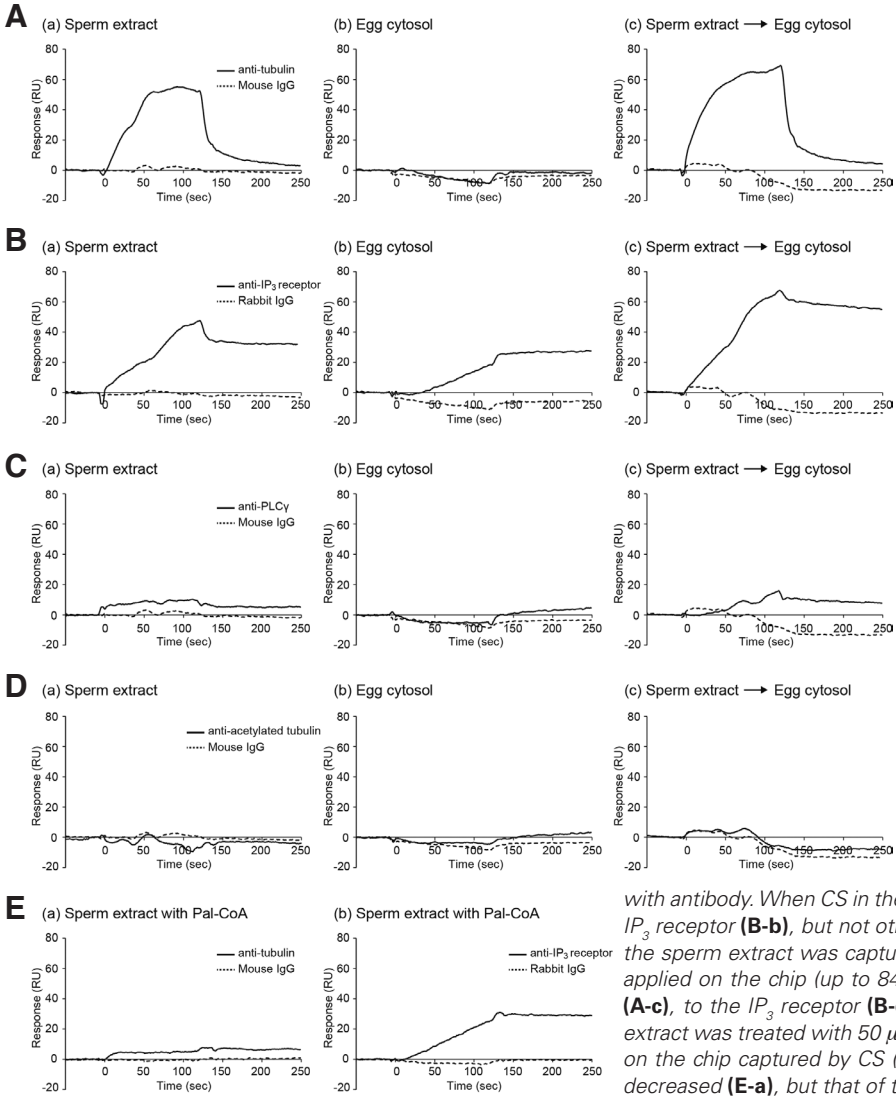


**Fig. 2. Detection of binding molecules to citrate synthase by surface plasmon resonance (SPR).** *After approximately 17 ng of an anti-CS antibody was immobilized on the sensor chip surface, 100 μl of sperm extract and egg cytosol was introduced to the chip surface at a flow rate of 30 μl/min. Then, 70 μl of each antibody (10 μg/ml) was applied on the chip surface for 2 min. Each sensorgram shows a relative response in RU after background subtraction. When CS in the sperm extract was captured on the chip (45.2±1.3 RU, mean±SEM, n=6), co-binding of tubulin* **(A-a)** *and the $IP_3$ receptor* **(B-a)** *was detected with each antibody, but not with acetylated tubulin* **(D-a)** *and non-specific IgG. The binding of PLCγ* **(C-a)** *was slightly detected with antibody. When CS in the egg cytosol was captured (81.3±7.3 RU, n=6), only the $IP_3$ receptor* **(B-b)**, *but not other molecule bound to CS* **(A-b, C-b, D-b)**. *When CS in the sperm extract was captured (36.7±3.2 RU, n=6), and then the egg cytosol was applied on the chip (up to 84.5±5.2 RU, n=6), increased binding of each to tubulin* **(A-c)**, *to the $IP_3$ receptor* **(B-c)**, *and to PLCγ* **(C-c)** *was observed. When the sperm extract was treated with 50 μM palmitoyl-CoA (Pal-CoA) for 10 min, and then applied on the chip captured by CS (43.8±RU, n 4), the binding of tubulin was significantly decreased* **(E-a)**, *but that of the $IP_3$ receptor remained relatively unchanged* **(E-b)**.

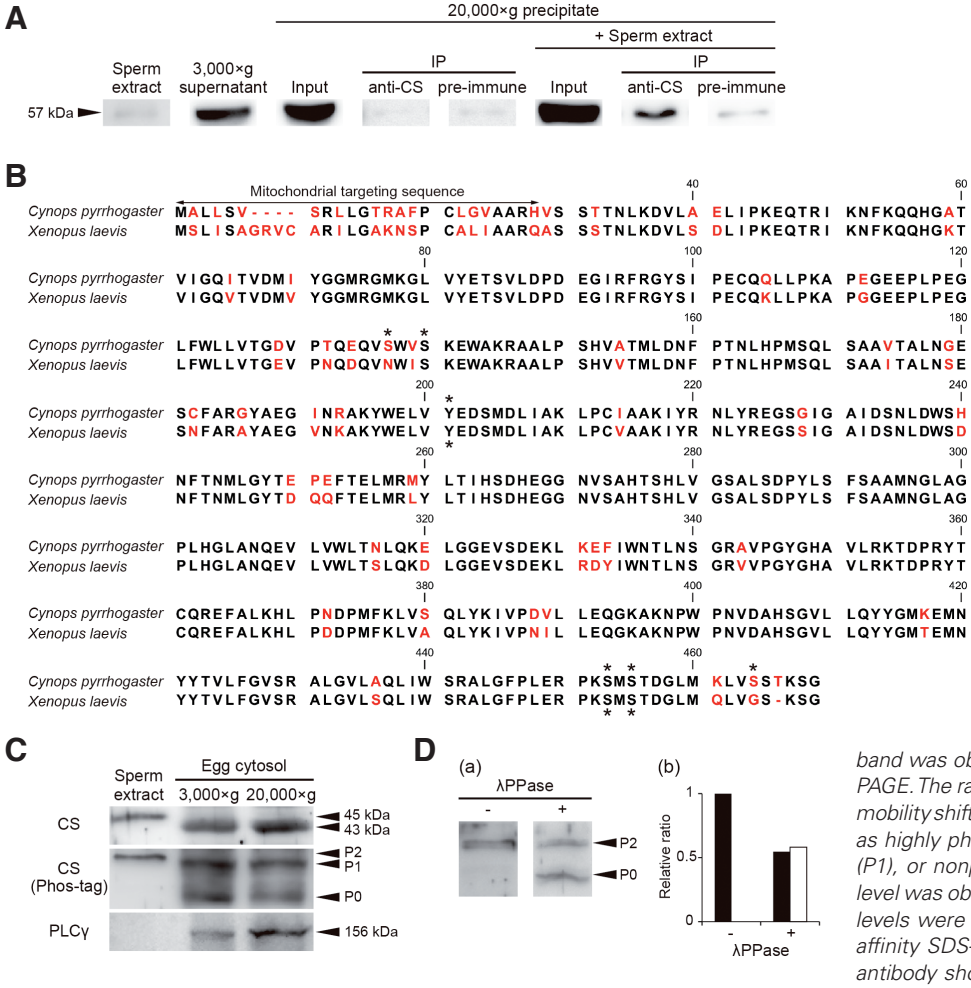


**Fig. 3. Phosphorylated sperm citrate synthase is associated with tubulin. (A)** *Association of α-tubulin with CS. Western blots with anti-α-tubulin antibody in the sperm extract and the egg cytosols (3,000 g supernatant and 20,000 g precipitate), showing a large amount of tubulin (57 kDa) in the 3,000 g supernatant and 20,000 g precipitate, but a very small amount in the sperm extract. The 20,000 g precipitate or 20,000 g precipitate which had been mixed with the sperm extract was immunoprecipitated with anti-CS antibody (IP), and then analyzed by immunoblotting using anti-α-tubulin antibody. This showed that CS in the mixture of 20,000 g precipitate and the sperm extract was associated with tubulin.* **(B)** *Sequence alignment between CS of the newt,* Cynops pyrrhogaster *and the frog,* Xenopus laevis. *The amino acid identity of the newt against the frog CS is 87 . The asterisks show the predicted phosphorylation sites (predictions score 0.9 by NetPhos 2.0 Server).* **(C)** *Western blots with anti-CS antibody after ordinary SDS-PAGE (upper lanes) and phosphate-affinity SDS-PAGE (middle lanes) of the sperm extract and the egg cytosol (3,000×g supernatant and 20,000 g precipitate). A 45 kDa band was observed in the sperm extract, but a 43 kDa band was observed in the egg cytosols with ordinary SDS-PAGE. The rates of phosphorylation were determined by the mobility shift corresponding to the phosphorylation, indicated as highly phosphorylated (P2), moderately phosphorylated (P1), or nonphosphorylated (P0) states, respectively. A P2 level was observed in the sperm extract, but only P1 and P0 levels were observed in the egg cytosols with phosphate-affinity SDS-PAGE (Phos-tag). Western blot with anti-PLCγ antibody showed a 156 kDa band in the egg cytosols, but not in the sperm extract (lower lanes).* **(D)** *The sperm extracts before the treatment (λPPase -) and after the treatment (λPPase +) with λ-phosphatase were separated by phosphate-affinity SDS-PAGE followed by Western blot with anti-CS antibody (a). The relative phosphorylated state is shown for P2 (closed bars) and P0 (open bar) down-shifted state (P0) following the λ-phosphatase treatment (b).*

(Fig. 3D), 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 PLCγ in fertilized eggs*

To determine the role of sperm CS in egg activation at fertilization, we examined the distribution of CS and microtubules both 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

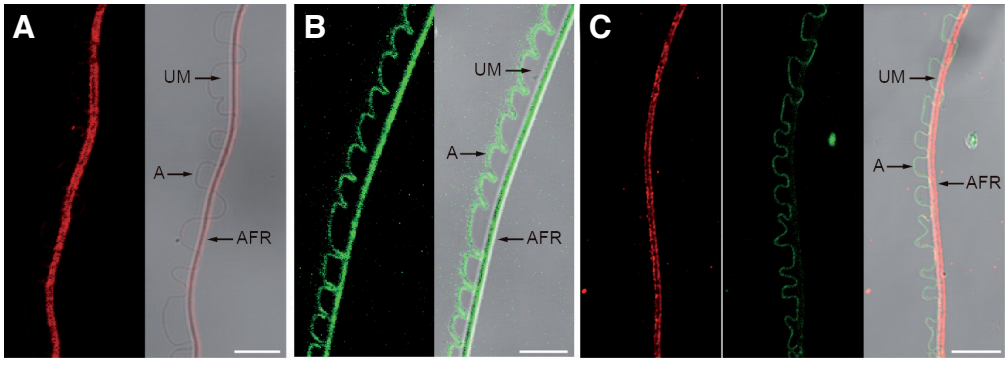


**Fig. 4. Localization of citrate synthase and tubulin in newt sperm.** *Fixed sperm immunostained with anti-CS antibody* **(A)** *(red) and anti-α-tubulin antibody* **(B)** *(green).* **(C)** *Sperm double-stained with anti-CS antibody (red) followed by anti-α-tubulin antibody (green). CS was localized in two lines in the axial fiber with microtubules, but not in the axoneme. Tubulin was not detected in axial fiber with double staining* **(C)**. *A, axoneme; AFR, axial fiber region; UM, undulating membrane. Scale bar, 10 μm.*

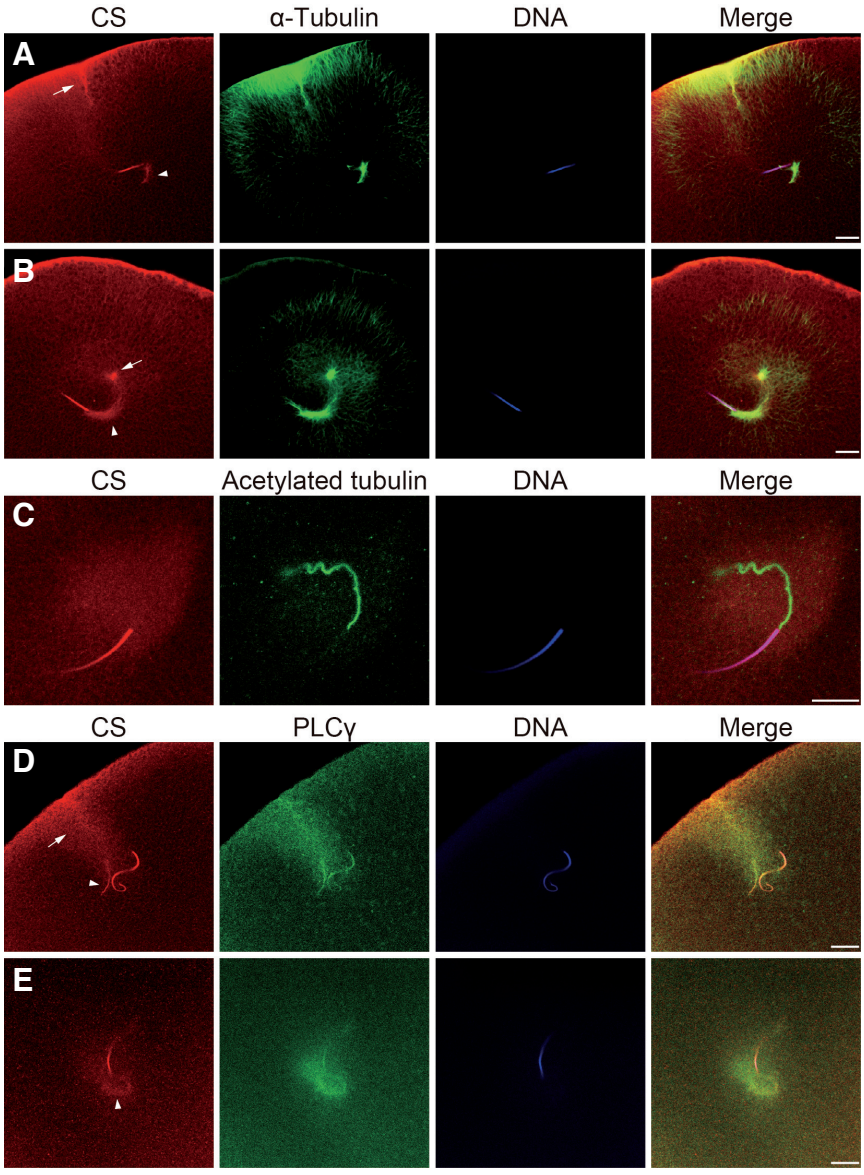


**Fig. 5. Confocal fluorescence micrographs showing the localization of citrate synthase in fertilized eggs.** *Eggs were fixed with FGX solution* **(A–C)** *or with FG solution* **(D,E)** *(see Materials and Methods).* **(A,B)** *Localization of CS (red) and α-tubulin (green) in the eggs 1 h after fertilization, showing CS localized around the sperm entry site near the egg surface (A, arrow) associated with microtubules, and accumulation of CS both in the swelling sperm nucleus, in the dispersing midpiece (arrowheads), and in the sperm aster (B, arrow).* **(C)** *Localization of CS (red) and acetylated tubulin (green) in the eggs 1 h after fertilization, showing accumulation of CS around the sperm entry site, but not in the incorporated sperm flagellum containing acetylated tubulin.* **(D,E)** *Localization of CS (red) and PLCγ (green) in the eggs 1 h after fertilization, showing accumulation of CS around the sperm entry site (D, arrow) and the dispersing midpiece (arrowheads) in association with PLCγ. No fluorescence was observed in the eggs treated with non-specific antibody. Scale bar, 50 μm.*

(Fig. 4B). 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 axial fiber region, but not in axoneme (Fig. 4C, Harada *et al.*, 2007). These results indicated that since tubulin in the axial fiber 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. 5 A,B). CS was distributed in the condensed sperm nucleus (Fig. 5 A,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 PLCγ was distributed in a sperm entry site (Fig. 5D) and in the midpiece region of sperm (Fig. 5 D,E). PLCγ was co-localized with CS around the sperm aster. In addition, the $IP_3$ receptor was co-localized with PLCγ around the center of sperm aster (Fig. 6 C,D). Since PLCγ was present in the egg cytoplasm, but not in the sperm (Fig. 3C lower lanes), PLCγ 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 $IP_3$ or the sperm factor (Harada *et al.*, 2011), 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 $IP_3$ receptors responsible for $Ca^{2+}$ release from the ER. These results strongly suggest that the sperm CS facilitates the formation of large complexes between microtubules and the ER with $IP_3$ receptors in the inner egg cytoplasm, which is responsible for the induction of the $Ca^{2+}$ 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 $Ca^{2+}$ 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 $IP_3$ receptors within the ER patches higher sensitivity to $IP_3$ in mature *Xenopus* oocytes (Sun *et al.*, 2011). Thus, microtubules in the newt eggs seem be necessary for ER-clustering to promote the propagative $Ca^{2+}$ release for the $Ca^{2+}$ wave. The transport of the ER along the microtubules probably contributes to the spatial regulation of intracellular $Ca^{2+}$ signaling at fertilization. Interestingly, since an inhibitor of CS, palmitoyl-CoA, abolished the interaction between CS and tubulins (microtubules), but not that between CS and $IP_3$ 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 $Ca^{2+}$ 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 $Ca^{2+}$ increase through sensitization of the $IP_3$ receptors on the ER to induce $Ca^{2+}$ releases (Missiaen *et al.*, 1997). The local increase of $Ca^{2+}$ probably causes a successive $Ca^{2+}$ release from the neighboring ER by directly stimulating the $IP_3$ 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 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 $[Ca^{2+}]_i$ increase to produce a $Ca^{2+}$ wave at newt egg activation (Fig. 7). Further investigation is necessary, however, to determine the interaction of those molecules in the $Ca^{2+}$ 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

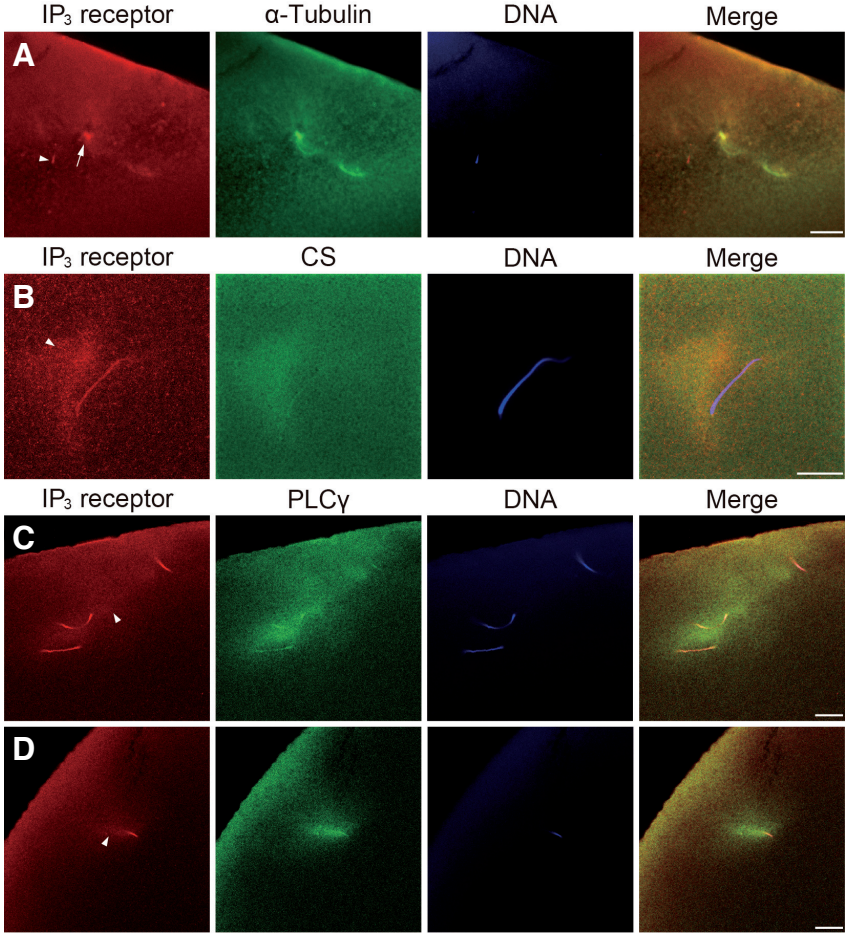


**Fig. 6. Confocal fluorescence micrographs showing the localization of the $IP_3$ receptor in fertilized eggs fixed with FG solution. (A)** *Localization of the $IP_3$ receptor (red) and α-tubulin (green) in the eggs 45 min after fertilization, showing the $IP_3$ receptor localized in the sperm nucleus (arrowhead) and in the midpiece (arrow).* **(B)** *Localization of the $IP_3$ receptor (red) and CS (green) in the eggs 45 min after fertilization, showing accumulation of the $IP_3$ receptor around the sperm nucleus (arrowhead) with CS.* **(C,D)** *Localization of the $IP_3$ receptor (red) and PLCγ (green) in the eggs 1 h after fertilization, showing PLCγ accumulated around the midpiece region (arrowheads). Scale bar, 50 μm.*

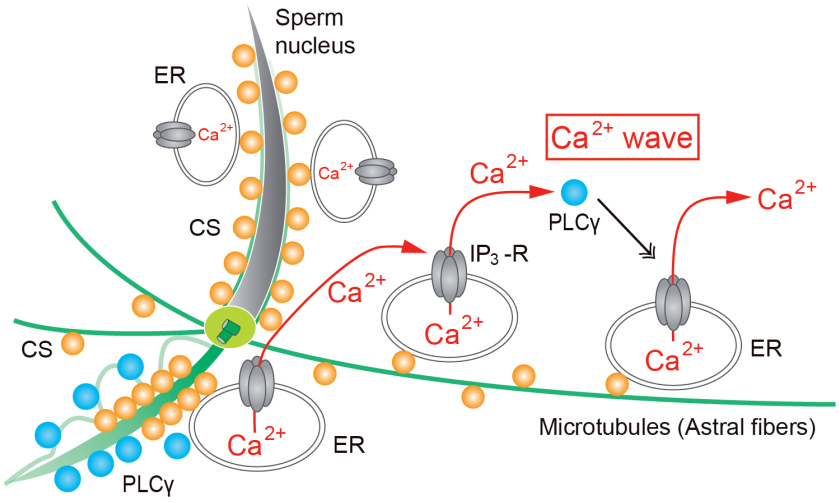


**Fig. 7. A schematic model of egg activation by the sperm-specific citrate synthase at newt fertilization.** *The sperm CS with tubulins in the midpiece is dispersed into the egg cytoplasm. The phosphorylated sperm CS associates with the microtubules around the sperm nucleus and the midpiece, and then accumulates to the ER containing the $IP_3$ receptor and PLCγ to initiate the $[Ca^{2+}]_i$ increase. The $[Ca^{2+}]_i$ increase then propagates through the ER with microtubules as a $Ca^{2+}$ wave by sequential activation of PLCγ to stimulate the $IP_3$ receptors.*

*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 $IP_3$ 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 $Ca^{2+}$ 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 $IP_3$ receptors. The sperm CS which binds tubulins seems to be involved in promotion of ER-clustering and to closely associate with the $IP_3$ receptors and PLCγ to induce the $Ca^{2+}$ release.

## Materials and Methods

### *Preparation of egg cytosol and sperm extract*

Sexually mature newts (*Cynops pyrrhogaster*) 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 $CaCl_2$, 0.85 mM $MgSO_4$, 4.6 mM Tris-HCl, pH 7.4). The dejellied eggs were kept in SB more than 2 h to remove the artificially activated eggs. Unfertilized, dejellied eggs were washed twice with GV buffer (100 mM sucrose, 100 mM KCl, 50 mM $MgSO_4$, 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, 4ºC), and the supernatant was collected as an egg cytosol and stored at –80ºC until use. In some cases, the egg cytosol was further centrifuged (20,000 g, 30 min, 4ºC) 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 $CaCl_2$, 5.7 mM Tris-HCl, pH 7.4). After washing by centrifugation (350 g, 20 min, 4ºC), 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 $Na_2HPO_4$ and $NaH_2PO_4$, pH 7.0, 4% polyvinylpyrrolidinone) and then transferred to injection buffer (IB: 5.0 mM KCl, 1.0 mM EGTA, 50 mM $Na_2HPO_4$ and $NaH_2PO_4$, 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 $Ca^{2+}$-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 $CaCl_2$, 50 mM $Na_2HPO_4$ and $NaH_2PO_4$, pH 7.0, 4% polyvinylpyrrolidinone) 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 photonics) 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 (NE040/7S, Nordic Immunological Laboratories B.V.), anti-PLCγ1 mouse monoclonal antibody (05-163, Millipore), anti-$IP_3$ receptor rabbit polyclonal antibody (07-1210, Millipore), anti-acetylated tubulin mouse monoclonal antibody (T7451, SIGMA), anti-α-tubulin 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*-hydroxysuccimide 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 with 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 $Na_2HPO_4$ 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 (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 $MgCl_2$, 5 mM EGTA, 80 mM PIPES-KOH, pH 6.8) or FGX fixation solution (FG 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 $NaBH_4$ 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-$IP_3$ 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 bezonate; 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).*

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