The Ca²⁺ increase by the sperm factor in physiologically polyspermic newt fertilization: Its signaling mechanism in egg cytoplasm and the species-specificity

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ABSTRACT

The newt, Cynops pyrrhogaster, exhibits physiological polyspermic fertilization, in which several sperm enter an egg before egg activation. An intracellular Ca²⁺ increase occurs as a Ca²⁺ wave at each sperm entry site in the polyspermic egg. Some Ca²⁺ waves are preceded by a transient spike-like Ca²⁺ increase, probably caused by a trypptic protease in the sperm acrosome at the contact of sperm on the egg surface. The following Ca²⁺ wave was induced by a sperm factor derived from sperm cytoplasm after sperm–egg membrane fusion. The Ca²⁺ increase in the isolated, cell-free cytoplasm indicates that the endoplasmic reticulum is the major Ca²⁺ store for the Ca²⁺ wave. We previously demonstrated that citrate synthase in the sperm cytoplasm is a major Ca²⁺ store for the Ca²⁺ wave. We now found that the activation by the sperm factor for egg activation in newt fertilization. In the present study, we found that the activation by the sperm factor as well as by fertilizing sperm was prevented by an inhibitor of citrate synthase, palmitoyl CoA, and that an injection of acetyl-CoA or oxaloacetate caused egg activation, indicating that the citrate synthase activity is necessary for egg activation at fertilization. In the frog, Xenopus laevis, which exhibits monospermic fertilization, we were unable to activate the eggs with either the homologous sperm extract or the Cynops sperm extract, indicating that Xenopus sperm lack the sperm factor for egg activation and that their eggs are insensitive to the newt sperm factor. The mechanism of egg activation in the monospermic frog eggs is quite different from that in the physiological polyspermy of newt eggs.

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Introduction

Fertilization leads to the activation of cell division in early embryonic development. The timing of egg activation during fertilization is critical for normal development: activation prior to sperm entry can cause parthenogenesis, while delayed activation can cause pathological polyspermy (Elison, 1986; Iwao, 2000a,b). While egg activation elicits several blocks to polyspermy at fertilization, amphibians contain two groups exhibiting very different blocks. One is a block prior to sperm–egg fusion, operating in monospermic eggs in frogs (Cross and Elison, 1980) and some primitive urodeles (Iwao, 1989; Iwao and Jaffe, 1989). The other is a block in egg cytoplasm after sperm entry, operating in physiologically polyspermic eggs in other urodeles (Iwao and Elison, 1990; Iwao and Katagiri, 1982, Iwao et al., 1993, Iwao et al., 2002). In physiologically polyspermic fertilization, several sperm enter an egg at normal fertilization, but only a single sperm nucleus ultimately participates in embryonic development, while the other sperm nuclei degenerate before cleavage. Among vertebrates, both urodeles and birds exhibit physiological polyspermy (Perry, 1987; Waddington et al., 1998). Although the mechanisms of egg activation have been well investigated in monospermic mamma-
increase, since the injection of cyclic-AMP ribose causes no Ca²⁺ increase in Cynops eggs (Yamamoto et al., 2001).

A testis-specific phospholipase C zeta (PLCζ) in mammals has been proposed as the molecule that triggers the repetitive Ca²⁺ increase (Ca²⁺ oscillation) and is the most probable candidate for the sperm factor in mammals (Saunders et al., 2002; Ito et al., 2008). The injection of a Cynops sperm extract into unfertilized eggs causes a Ca²⁺ increase that corresponds to that at fertilization (Yamamoto et al., 2001, Harada et al., 2007). We previously highly purified a Cynops sperm factor from the Cynops sperm extract capable of triggering egg activation and identified the Cynops sperm factor as the 45-kDa citrate synthase derived from sperm (Harada et al., 2007). The injection of citrate synthase-mRNA into unfertilized eggs triggered egg activation accompanied by a Ca²⁺ increase. However, the citrate synthase in the Cynops sperm factor is quite different from the PLCζ that is known as the candidate sperm factor in mammalian fertilization. Although citrate synthase is an enzyme that produces citrate from acetyl-CoA and oxaloacetate in mitochondria as a part of the Krebs cycle, we found that the enzymatic activity of citrate synthase in Cynops eggs is unclear.

In the monospermic fertilization of the frog, Xenopus laevis, egg activation can be induced by the external treatment with Arg–Gly–Asp (RGD)-containing peptides, which are known ligands for integrins (Iwao and Fujimura, 1996) or a KTE peptides (Shilling et al., 1998). The protease purified from the newt sperm or cathepsin B activates Xenopus eggs when applied externally (Iwao et al., 1994, Iwao and Masui, 1995; Mizote et al., 1999). A single-transmembrane protein, Xenopus uropilakin III (xUPIII), is localized to the lipid/membrane rafts on the egg surface and is involved in sperm–egg interaction and egg activation (Sakakibara et al., 2005). In addition, 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 γ, 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 is transmitted when the sperm binds to the egg surface (Sato, 2008). In contrast, a very small number of Cynops eggs were activated by the external treatment with the sperm protease or RGD-containing peptides (Iwao et al., 1994; Iwao, 2000a,b). However, it was reported that Xenopus sperm extract contains a factor for triggering Ca²⁺ oscillations in mouse eggs (Dong et al., 2000) and that in frog, Bufo arenarum, egg activation can be induced not only by external treatment with the sperm extract but also by its injection into the eggs (Bonilla et al., 2008). Thus, the mechanism for egg activation among amphibian species remains controversial.

In the present study, we demonstrated that each sperm induced a Ca²⁺ wave at its entry site, and that ER functions as the major Ca²⁺ store in response to the sperm factor in physiologically polyspermic newt fertilization. We found that the enzymatic activity of citrate synthase in the sperm extract is necessary for Cynops egg activation at fertilization and that it is possible that the acetyl-CoA and/or oxaloacetate, produced by citrate synthase, cause egg activation with the Ca²⁺ increase. The Xenopus sperm extract did not contain the sperm factor for egg activation, and Xenopus eggs were insensitive to the injection of the sperm extract. We suggest that the mechanism of the Ca²⁺ increases is quite different between physiologically polyspermic and monospermic fertilization.

**Materials and methods**

**Preparation of eggs and sperm extracts**

Sexually mature newts, C. pyrrhogaster, were purchased from dealers. To induce ovulation, the female was injected with 80 IU of human chorionic gonadotropin (HCG) (ASKA Pharmaceutical) every 2 days. Unfertilized eggs were obtained by squeezing the abdomen of the females. The jelly layers were removed with 1.5% sodium thiglycolate (pH 9.5), followed by thorough washing with Steinbergs’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. Insemination of unfertilized eggs was performed according to the method described by Yamamoto et al. (1999).

The frogs, X. laevis, were purchased from dealers and maintained in our laboratory. To obtain mature unfertilized eggs, the female was injected with 375 IU HCG in the dorsal lymph sac and kept at 18 °C for 14 h. The jelly layers were removed with 3% cysteine–HCl (pH 8.5), followed by through washing with 100 mM NaCl and 50 mM Tris–HCl (pH 7.0). The dejellied eggs were kept in De Boer’s solution (110.3 mM NaCl, 1.3 mM KCl, 1.3 mM CaCl₂, 5.7 mM Tris–HCl, pH 7.4) at 18 °C and used within 2 h.

Mature newt sperm obtained by squeezing the males were suspended in DB. 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 NaCl–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 s, 5 times; US500, Nissei) to disrupt sperm plasma membranes, and then centrifuged (10,000–g, 20 min, 4 °C). The supernatant was collected as a sperm “cytoplasmic” extract and stored at −80 °C until use. To obtain a sperm extract containing sperm protease activities, the mature sperm suspended in DB were frozen and thawed at −80 °C for 6–12 h, and then centrifuged (10,000×g, 20 min, 4 °C). The supernatant was collected as a sperm “protease” extract.

Mature Xenopus sperm was obtained by mincing a piece of testis in 33% DB, and then these sperm extracts were obtained as described above. The 45 kDa protein of sperm factor from the newt sperm extract was purified as described previously (Harada et al., 2007).

**Microinjection**

The dejellied eggs were washed with NKP solution (120 mM NaCl, 7.5 mM KCl, 10 mM NaH₂PO₄ and NaH₂PO₄ (pH 7.0), 4% Polyvinylpyrrolidone) and then transferred to injection buffer (IB: 5.0 mM KCl, 1.0 mM EGTA, 50 mM NaH₂PO₄ and NaH₂PO₄ (pH 7.0), 4% polyvinylpyrrolidone). The microinjection was carried out with a glass micropipette having a tip diameter of 20–30 μm in IB. Each newt egg was injected with 33 nl of samples, porcine citrate synthase (C3260, SIGMA), palmitoyl CoA (P9716, SIGMA), or butyryl CoA (B1508, SIGMA). After injection, the injected Cynops eggs were kept in IB 5 min and then incubated in healing buffer (HB: 5.0 mM KCl, 1.0 mM CaCl₂, 50 mM NaH₂PO₄ and NaH₂PO₄ (pH 7.0), 4% polyvinylpyrrolidone) 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 4 h after injection. The injected Xenopus eggs were kept in IB until the egg activation was determined, because Xenopus eggs are sensitive to Ca²⁺ in extracellular medium. The activation of Xenopus eggs was judged by the cortical contraction of eggs 30 min after injection in IB.

**Measurement of change in intracellular Ca²⁺ concentration**

A Ca²⁺-sensitive fluorescent dye, Oregon green 488 BAPTA-1 dextran 10,000 MW (2 mM, Molecular Probes) was injected into the dejellied eggs in IB (final concentration in the egg, 20 μM) and then kept 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. The 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) every 10 s.
The fluorescence intensity was measured by imaging software (Image J, NIH).

**Measurement of the enzymatic activity of citrate synthase**

The measurement of the enzymatic activity of citrate synthase was performed according to the method of Srere (1969). The reaction mixtures (1 mM 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) in 1 M Tris–HCl (pH 8.1), 50 μl; 10 mM acetyl-CoA, 15 μl; purchased porcine citrate synthase or samples, 25 μl; and H2O, 385 μl) were added to a cuvette and then the absorbance at 412 nm was measured every 10 s for 3 min. The reaction of citrate synthase was started by addition of 25 μl of 10 mM oxaloacetate in 0.1 M Tris–HCl (pH 8.1) to the cuvette. The absorbance at 412 nm was measured every 10 s for 3–5 min. The enzymatic activity of citrate synthase was estimated by the linear increase in absorbance for at least 2–3 min.

**Centrifugation of unfertilized eggs and collection of cytoplasm**

Unfertilized, dejellied eggs were washed with Ca2+-free solution (25 mM NaCl, 0.45 mM KCl, 10 mM MgCl2, 2 mM EGTA, 5 mM Tris–HCl pH 7.4) and floated on the interface between Ca2+-free solution and 40% Ficoll (GE Healthcare) solution in a centrifuged tube, and then centrifuged (3000×g, 30 min, 4 °C) to stratify the eggs without breaking the plasma membrane. Lipid, clear cytoplasm, and yolk and pigment granules were sedimented to be stratified from the animal to the vegetal pole (cf., Iwao et al., 2005). The droplet of clear cytoplasm was collected into mineral oil on a glass-based dish with a glass micropipette having a tip diameter of 20–30 μm. The cytoplasmic droplets were stored on ice until use. In some cases, the isolated cytoplasm was further centrifuged (5000–20,000×g, 30 min, 4 °C) to separate endoplasmic reticulum (ER).

To measure the change in Ca2+ concentration in the isolated cytoplasm, 20 μM Oregon green 488 BAPTA-1 dextran 10,000 MW was mixed with the cytoplasm, and then fluorescence images were taken by an inverted fluorescence microscope (Nikon, ECLIPSE Ti-S) and a highly sensitive CCD camera (Nikon, DS-QiMc-U2) with NIS-Elements imaging software. For observation of cortical ER in dejellied eggs, the eggs were treated with 2.5 μM DiOC6(3) 1 min in DB and then twice washed with DB.

**Results**

**Intracellular Ca2+ increase at physiologically polyspermic fertilization of newt eggs**

In the physiologically polyspermic fertilization of the newt, C. pyrrhogaster, it remained unclear whether each fertilizing sperm causes a Ca2+ increase at egg activation. We observed the first spike-like increase of [Ca2+]i at the animal hemisphere around a sperm entry site, about 3 min after insemination (Fig. 1A, arrow 1), followed by a long-lasting Ca2+ increase which peaked about 8 min after initiation, and continued for about 20 min thereafter (Fig. 1B, line 1). The wave-like Ca2+ increase, having a velocity of 5.1 ± 1.0 μm/s (mean ± SEM, n = 3), was propagated toward the egg cortex in about one-quarter of the animal hemisphere. In the same egg, the second and the third Ca2+ increases occurred on the animal hemisphere about 6 min 30 s and 7 min after insemination, respectively (Fig. 1A, arrows 2 and 3). Their increases were not associated with apparent spike-like rises but peaked about 10 min after initiation, and continued for about 20 min (Fig. 1B, lines 1 and 2). Each Ca2+ wave was propagated in roughly each quarter of the animal hemisphere. These results indicate that each fertilizing sperm causes a relatively small Ca2+ wave in the egg cytoplasm, in some cases, preceded by the short, spike-like Ca2+ increase at egg activation.

To investigate how the fertilizing sperm causes the Ca2+ increase in egg cytoplasm, we first tried to induce it by an external treatment with sperm acrosomal protease, since a tryptic acrosomal protease of the newt sperm can induce activation in the eggs of frog, Xenopus, accompanied by the Ca2+ increase (Mizote et al., 1999). When the sperm extract containing the tryptic protease activity was externally applied on the surface of dejellied, pigmented eggs (Fig. 2A), no Ca2+ increase was detected (Fig. 2F). To reveal smaller Ca2+ increases in the egg cytoplasm, the eggs were centrifuged on a Ficoll–cushion without breaking the egg plasma membrane. They were then stratified into a lipid layer, translucent cytoplasm, and the mass of pigment granules and yolk granules (Fig. 2B). In the translucent cytoplasm, a small, transient Ca2+ increase lasting about 2 min was elicited soon after the treatment with the sperm protease extract (Fig. 2C and G), but no subsequent Ca2+ wave and egg activation occurred. The transient Ca2+ increase was induced in Ca2+-free solution (Fig. 2H), indicating a Ca2+ release from the intracellular Ca2+ stores. In addition, the Ca2+ increase by the sperm protease extract was inhibited in the presence of the trypsin inhibitor, aprotinin (Fig. 2I). These results indicate that the initial spike-like Ca2+ increase at newt fertilization is caused by the sperm tryptic protease at the binding of the sperm on the egg surface but is insufficient for inducing the Ca2+ wave to cause egg activation. The insensitivity against...
the Ca\(^{2+}\) increase in the cortex is probably due to the lack of cortical ER as a Ca\(^{2+}\) store in newt eggs (Fig. 2D), in contrast to the well-developed cortical ER in \textit{Xenopus} eggs (Fig. 2E).

To determine whether the sperm cytoplasmic components induce the spike-like Ca\(^{2+}\) increase, the sperm cytoplasmic extract was injected into the unfertilized egg while monitoring the Ca\(^{2+}\) level. When a large amount of sperm extract without the protease activity was injected into the egg cytoplasm, the Ca\(^{2+}\) level increased slowly about 45 s after the injection, peaked about 15 min after initiation, and continued for about 30 min (Fig. 3A and B). A Ca\(^{2+}\) wave having a velocity of 6.2 ± 0.4 μm/s (n = 3) was propagated through nearly the whole egg cortex and the eggs underwent activation. The Ca\(^{2+}\) wave induced by the injection of sperm cytoplasmic extract was quite similar to that induced by the fertilizing sperm, but the initial spike-like increase was absent. In addition, when the sperm cytoplasmic extract was injected into the translucent cytoplasm in the stratified eggs by centrifugation at 3000×g, a Ca\(^{2+}\) increase was initiated at the injection site about 1 min after the injection without the spike-like increase, and then propagated through the whole translucent cytoplasm with a velocity of about 2.4 ± 0.1 μm/s (n = 4) (Fig. 3C and D). Thus, the stratified eggs were still capable of a Ca\(^{2+}\) increase in the clear cytoplasm in response to the sperm extract.

To investigate the role of cytoplasmic organelles in the Ca\(^{2+}\) increase triggered by the sperm extract, we withdrew the translucent cytoplasm of the eggs, which had been centrifuged at 3000×g for 30 min, using a fine micro-capillary, and collected them on a glass-based petri-dish covered by mineral oil. After mixing in a Ca\(^{2+}\)-sensitive fluorescence dye, the sperm cytoplasmic extract was added into the isolated cytoplasm (300–500 nl) (Fig. 4A and C). The Ca\(^{2+}\) increase was initiated soon after the addition of the sperm extract, and then propagated at a velocity of about 1.9 μm/s through the entire cytoplasm. When the isolated cytoplasm was further centrifuged at 5000×g for 30 min, the sperm extract induced a Ca\(^{2+}\) increase in the 5000×g supernatant (Fig. 4D), but not in the 5000×g precipitate. When the isolated cytoplasm was centrifuged at 20,000×g for 30 min, no Ca\(^{2+}\) increase was triggered by the sperm extract in the 20,000×g supernatant (Fig. 4E). The Ca\(^{2+}\) increase was induced by each addition of the sperm extract in the 20,000×g precipitated fraction (Fig. 4B and F), but their propagation over the cytoplasm was not uniform. In

Fig. 2. The animal view of the unfertilized \textit{Cynops} egg before centrifugation (A) or after centrifugation at 3,000 g for 30 min (B), and a schematic cross section of the centrifuged egg. AP, animal pole; C, translucent cytoplasm; L, lipid layer; Y, pigment and yolk granules. (C) A typical intracellular Ca\(^{2+}\) increase at the site of treatment by the sperm protease extract, showing a local, small Ca\(^{2+}\) increase on the translucent cytoplasm. Figures on the top left, showing time (seconds) after injection. C, Translucent cytoplasm; L, lipid layer; Y, yolk and pigment granules. Cortical endoplasmic reticulum (ER) in \textit{Cynops} eggs (D) and \textit{Xenopus} eggs (E), showing lack of cortical ER in \textit{Cynops} eggs. (F–I) Typical intracellular Ca\(^{2+}\) increases at the site of treatment by the sperm protease extract in non-centrifuged egg (F), in the translucent cytoplasm of the centrifuged eggs in Steinberg’s solution with 0.34 mM Ca\(^{2+}\) (G, yellow circle in C) or in Ca\(^{2+}\)-free Steinberg’s solution with 10 mM EGTA (H). No intracellular Ca\(^{2+}\) increase was observed by the sperm protease extract which had been treated by aprotinin (220 μg/ml) (I). Scale bars, 0.5 mm in A and C, 10 μm in D and F.
addition, a Ca\(^{2+}\) increase induced by inositol 1,4,5-trisphosphosphate (IP\(_3\)) occurred in the 20,000×g precipitate, but not in the 20,000×g supernatant, indicating precipitation of ERs by centrifugation at 20,000×g (Fig. 4G and H). Thus, ERs also seem to exist as large complexes in the cytoplasm. These results suggest that ERs in the inner cytoplasm, rather than cortical ERs, are required to trigger a Ca\(^{2+}\) increase by the sperm cytoplasmic extract.

Roles of sperm citrate synthase in the intracellular Ca\(^{2+}\) increase at egg activation

We previously demonstrated that *Cynops* eggs were activated by the sperm factor, citrate synthase, in a highly purified sperm extract (Harada et al., 2007). As described above, the ERs in the egg cytoplasm are necessary for the sperm factor-induced Ca\(^{2+}\) increase. To determine whether the enzymatic activity of citrate synthase is involved in egg activation, we analyzed the citrate synthase activities in the crude sperm extract and the purified 45-kDa sperm citrate synthase (Harada et al., 2007). The crude sperm extract equivalent to 10⁷ sperm/ml and the purified sperm citrate synthase equivalent to 10⁷ sperm/ml exhibited 3.7 U/ml and 3.0 U/ml of the citrate synthase activity, respectively. In the presence of an inhibitor of citrate synthase, palmitoyl CoA (50 μM), the citrate synthase activity in these samples was completely inhibited. When the crude sperm extract (2.43 U/ml), which had been mixed with 0.5 μM or 50 μM palmitoyl CoA, was injected, the rate of egg activation was significantly reduced 67% or 27%, respectively (Fig. 5A). However, the egg activation was not completely inhibited even by 50 μM palmitoyl CoA. In addition, about 20% of the eggs were activated by an injection of palmitoyl CoA alone (0.5–50 μM). Since it was reported that palmitoyl CoA induces a [Ca\(^{2+}\)] increase (Chini and Dousa, 1996; Fitzsimmons et al., 1997), some eggs were probably activated by the [Ca\(^{2+}\)] increase induced by palmitoyl CoA, even when the citrate synthase activity was completely inhibited. In contrast, butyryl CoA (50 μM) without the inhibitory activity against citrate synthase did not affect the egg activation activity in the sperm extract (Fig. 5A). In
addition, when 50 μM palmitoyl CoA was injected into unfertilized eggs, and then a large amount of porcine citrate synthase (33.3 U/ml) was injected, 88% (38/43) of eggs underwent activation. It was estimated in this case that a small amount of citrate synthase activity (53 mU/ml) remained in the egg cytoplasm enough for inducing egg activation. Furthermore, when 50 μM palmitoyl CoA was injected into unfertilized eggs, and then the eggs were inseminated, several sperm entry sites, the accumulation of pigment, appeared on the egg surface within 15 min after insemination, but egg activation was inhibited. Therefore, the onset of the first cleave was delayed for more than 2 h in 84% (21/25) of palmitoyl CoA-injected eggs in comparison with distilled water-injected eggs. Thus, palmitoyl CoA significantly

Fig. 4. (A) A typical intracellular Ca2+ increase at the site (circle 1) injected with the cytoplasmic sperm extract equivalent to 400 sperm into the isolated translucent cytoplasm (3000×g), showing a Ca2+ wave with the velocity of the Ca2+ wave was 1.9 μm/s (B). Figures on the top left, showing time (seconds) after injection. Scale bar, 0.5 mm. (B) A typical intracellular Ca2+ increases at the site injected with the cytoplasmic sperm extract equivalent to 400 sperm into the centrifuged cytoplasm (20,000×g precipitate), showing the Ca2+ increase in response to each injection of sperm extract every 1 min (arrows in F). Figures on the top left, showing time (seconds) after injection. Scale bar, 0.5 mm. (C-H) Typical intracellular Ca2+ increases at the site injected with the sperm extract (400 sperm) in isolated cytoplasm (C, circles 1 and 2 in A), 5000×g supernatant (D), 20,000×g supernatant (E), and 20,000×g precipitate (F, circle 1 in B). (G and H) Typical intracellular Ca2+ increases at the site injected with IP3 (10 μM in cytoplasm) in 20,000×g supernatant (G) and 20,000×g precipitate (H), showing the Ca2+ increase in the precipitate.
inhibited egg activation without affecting fertilization process. These results indicate that the enzymatic activity of citrate synthase in the fertilizing sperm is closely involved in egg activation at newt fertilization.

Although citrate synthase produces citrate from acetyl-CoA and oxaloacetate in the mitochondrial TCA cycle, an injection of trisodium citrate did not cause significant egg activation (Fig. 5B). Citrate synthase inversely cleaves citrate into acetyl-CoA and oxaloacetate (Srere, 1992). Since acetyl-CoA potentiates Ca\(^{2+}\) release from the IP3 receptor (Missiaen et al., 1997) and oxaloacetate induces Ca\(^{2+}\) release from mitochondria (Leikin et al., 1993), we investigated whether these reaction products of citrate synthase can induce egg activation. The injection of 1 μM acetyl-CoA (final concentration in the egg) activated 47% of the eggs (Fig. 5C), and this was found to be the optimal concentration for egg activation. In addition, an injection of 100 μM or 1000 μM oxaloacetate (final concentration in the egg) activated 41% or 71% of the injected eggs, respectively (Fig. 5D). Further, the egg activation by oxaloacetate was induced in a dose-dependent manner. Pronucleus formation after the resumption of meiosis was confirmed in the eggs activated by acetyl-CoA or oxaloacetate 4–5 h after injection. The injection of acetyl-CoA (1 μM) induced a Ca\(^{2+}\) increase which was initiated at the injection
site and spread through the entire egg cortex of the animal hemisphere as a Ca\textsuperscript{2+} wave (Fig. 6A). The relatively high [Ca\textsuperscript{2+}], continued in the animal hemisphere for about 15 min after the injection (Fig. 6B). When injected with oxaloacetate (1 mM), a Ca\textsuperscript{2+} increase spread from the injection site through about one-quarter of the egg cortex of the animal hemisphere (Fig. 6C and D). Thus, it is possible that the acetyl-CoA and/or oxaloacetate produced by the sperm citrate synthase causes the observed Ca\textsuperscript{2+} increase in fertilized newt eggs.

The species specificity of sperm factors for egg activation in amphibians

To investigate the species specificity of sperm factors for egg activation among amphibians, we injected sperm extract from the frog, *Xenopus*, equivalent to 330 sperm, into *Xenopus* eggs, but observed no egg activation (Fig. 7A). This suggests that *Xenopus* sperm lack the cytoplasmic sperm factor responsible for activation in homologous eggs. In contrast, when the newt, *Cynops*, sperm extract, equivalent to 1 or 330 sperm, was injected into *Cynops* eggs, 24% or 78% of the injected eggs were activated, respectively (Fig. 7B), suggesting that a *Cynops* egg is activated by the sperm factor introduced by several sperm at fertilization.

Since the injection of the *Cynops* sperm extract equivalent to 330 sperm failed to induce the activation of *Xenopus* eggs (Fig. 7C), they must be insensitive to the *Cynops* sperm factor. However, when a large amount of *Xenopus* sperm extract, equivalent to 330 sperm, was injected into *Cynops* eggs, 63% of the injected eggs were activated (Fig. 7D). We detected no citrate synthase activity in the *Xenopus* sperm extract (107 sperm/ml) and no citrate synthase protein was detected in the *Xenopus* sperm extract (Fig. 7D). Thus, the egg activation activity in *Xenopus* sperm must involve a heat-stable molecule(s) different from citrate synthase. These results support the notion that eggs of the newt, *Cynops*, are activated by the sperm factor, while those in the frog, *Xenopus*, are activated by a different mechanism involving signal from the egg surface.

**Discussion**

In the physiologically polyspermic eggs, it remained unknown whether each fertilizing sperm causes the Ca\textsuperscript{2+} increase at egg activation. The present study demonstrated that each fertilizing sperm elicited a Ca\textsuperscript{2+} wave at its fertilization site 3–7 min after insemination in physiologically polyspermic newt eggs. The duration of each Ca\textsuperscript{2+} wave was about 15–20 min and the total Ca\textsuperscript{2+} increase continued for about 30–40 min, which corresponded well with the Ca\textsuperscript{2+} increases reported previously throughout the eggs (Yamamoto et al., 1999). Since the Ca\textsuperscript{2+} wave induced by one sperm propagated in one-quarter to one-eighth of the egg surface, several sperm must enter to induce a Ca\textsuperscript{2+} increase through the entire egg cytoplasm. The relatively contained propagation by each sperm entry is consistent with the fact that the sperm cytoplasmic extract equivalent of a single sperm activated about 20% of the eggs (Fig. 7, Harada et al., 2007). Since 2–20 sperm enter an egg and then initiate egg activation in the physiologically polyspermic newt fertilization (Iwao, 2000a,b), the multiple Ca\textsuperscript{2+} increases induced by all fertilizing sperm seem to be necessary for egg activation. The egg must be activated after the entry of several sperm in physiologically polyspermic eggs. The slow activation that occurs after the first sperm entry seems to be necessary for the slow initiation of development in the physiologically polyspermic eggs. In contrast, the first fertilizing sperm induces a Ca\textsuperscript{2+} wave that propagates through the entire egg cytoplasm in the monospermic frog, *Xenopus* (Nuccitelli et al., 1993).

In recordings from the whole egg surface, we previously observed an early Ca\textsuperscript{2+} increase for about 30 s followed by a late Ca\textsuperscript{2+} increase that continued for 30–40 min (Yamamoto et al., 1999). In the present study, we found that some Ca\textsuperscript{2+} waves induced by the sperm were...
preceded by a spike-like Ca\(^{2+}\) increase at the sperm entry site (Fig. 1). The early spike-like Ca\(^{2+}\) increase is probably caused by a different mechanism from that for the long-lasting Ca\(^{2+}\) wave, because even in the injection of the large amount of sperm cytoplasmic extract into the unfertilized eggs, only a long-lasting Ca\(^{2+}\) wave was induced without a preceding spike-like Ca\(^{2+}\) increase in both non-stratified and stratified eggs (Fig. 3). In addition, the treatment of the eggs with the sperm protease extract caused a transient, non-propagative Ca\(^{2+}\) increase on the egg surface which was inhibited by the trypsin inhibitor. Thus, the spike-like rise at the initial phase of the Ca\(^{2+}\) increase is probably caused by the sperm tryptic protease localizing in the acrosome (Mizote et al., 1999) at the initial contact of the sperm with the egg plasma membrane. It should be noted that the Ca\(^{2+}\) increase induced by the sperm protease is insufficient for egg activation in newt eggs, although it is sufficient for the egg activation of the frog, *Xenopus* (Mizote et al., 1999). The insensitivity of the Ca\(^{2+}\) increase against the stimulations at the egg cortex by protease treatment or needle-pricking (Iwao, 2000a,b) is probably due to the lack of cortical ERs serving as Ca\(^{2+}\) stores in newt eggs. We found a complex of ERs in the inner cytoplasm of newt eggs, but fewer cortical ERs as compared with *Xenopus* eggs (Fig. 2). These results support our previous notions (Iwao, 2000a,b) that the frog sperm causes egg activation triggered by the sperm protease near the sperm–egg interface, whereas in the newt sperm, which contain the sperm acrosomal protease, the egg activation is mediated mainly by the sperm factor in the sperm cytoplasm, which is introduced into the egg cytoplasm after sperm–egg membrane fusion (Fig. 8).

We confirmed that the velocity of Ca\(^{2+}\) waves (about 6.2 \(\mu\)m/s) in eggs injected with the sperm cytoplasmic extract is quite similar to that in the fertilized eggs (about 5.1 \(\mu\)m/s). When the eggs were stratified by centrifugation to more sensitively monitor the Ca\(^{2+}\) level, it was evident that the Ca\(^{2+}\) wave was induced by injection of the sperm cytoplasmic extract propagated in the translucent cytoplasm (Fig. 3) or in the isolated cytoplasm (Fig. 4) with a velocity of about 2.4 \(\mu\)m/s or about 1.9 \(\mu\)m/s, respectively. This rate is somewhat slower than that in the fertilized eggs, which probably reflects changes in the conformation of the ERs after centrifugation. While previous groups have studied Ca\(^{2+}\) increases in isolated egg cytoplasm from sea urchin eggs (Galione et al., 1993; Galione et al., 1997) and frog eggs (Tokmakov et al., 2001), our approach allows collection of the cytoplasm without any addition of a buffer solution, which makes possible an analysis of the mechanism of the Ca\(^{2+}\) wave in the egg cytoplasm in vitro.

We previously demonstrated that a Ca\(^{2+}\) increase could be induced by the injection of IP\(_3\), but not by cADP-ribose (Yamamoto et al., 2001), indicating that IP\(_3\) receptors on the ER are responsible for the Ca\(^{2+}\) wave at newt fertilization. We have found that ERs form large complexes in the egg cytoplasm, because they were precipitated at 20,000\(\times\)g and the IP\(_3\)-induced Ca\(^{2+}\) increase was elicited only in the precipitated fraction. The narrow and unevenly propagated Ca\(^{2+}\) increase was induced in the 20,000\(\times\)g precipitate in response to each injection of the sperm cytoplasmic extract, indicating that adequate conformation of the ER is necessary for the formation of Ca\(^{2+}\) waves in the egg cytoplasm. Thus, our cell-free system clearly demonstrated that the sperm factor directly induced the release of Ca\(^{2+}\) from organelles in the egg cytoplasm. The observed multiple Ca\(^{2+}\) increases in response to each injection of the sperm cytoplasmic extract into the isolated egg cytoplasm corresponded well with the multiple Ca\(^{2+}\) waves induced by each fertilizing sperm in the polyspermic egg.

We previously demonstrated that the sperm factor for the activation of newt eggs is sperm citrate synthase, which was introduced into the egg cytoplasm (Harada et al., 2007). The present findings suggest that the sperm factor induces each Ca\(^{2+}\) wave via a Ca\(^{2+}\) release from the ERs in the egg cytoplasm. The purified sperm factor contained high enzymatic activity from the citrate synthase, and the egg activation by the sperm factor was inhibited by an inhibitor of citrate synthase, palmitoyl CoA (50 \(\mu\)M), but not by butyryl CoA at the same concentration (Fig. 3A). Since the activity of

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**Fig. 8.** Schematic model of egg-activation signaling involving the sperm protease activity and the sperm factor, citrate synthase in the newt, *Cynops* and the frog, *Xenopus* fertilization. In the membrane of unfertilized eggs, the protease activity on the sperm may interact with an egg receptor to induce a local Ca\(^{2+}\) increase in egg cortex. The cortical Ca\(^{2+}\) increase seems to propagate through cortical endoplasmic reticulum (C-ER) as a Ca\(^{2+}\) wave in the *Xenopus* egg, but not in the *Cynops* egg. In the *Cynops* egg, citrate synthase released from each sperm can induce each Ca\(^{2+}\) wave. The Ca\(^{2+}\) increase from mitochondria and from ER might be caused by oxaloacetate and by acetyl-CoA, respectively. Signal transduction events downstream of citrate synthase remains to be investigated.
citrate synthase is inhibited by palmitoyl CoA (IC50 = 900 µM), but not by butyryl CoA (IC50 = 900 µM) (Lai et al., 1993), the inhibition of egg activation by palmitoyl CoA have a high correlation with the inhibition of citrate synthase activity, rather than with nonspecific action of fatty acyl CoA derivatives. In addition, the addition of a large amount of citrate synthase to the palmitoyl CoA-injected eggs caused egg activation. Thus, the egg activation activity in the sperm factor seems to be closely related to its enzymatic activity of citrate synthase.

Since we found that only the expression of citrate synthase by the fertilizing sperm was inhibited by palmitoyl CoA, the activity of citrate synthase in the fertilizing sperm is probably necessary for the Ca2+ release in egg cytoplasm at newt fertilization.

Citrate synthase produces citrate from acetyl-CoA and oxaloacetate in the mitochondrial TCA cycle, but citrate synthase might inversely cleave citrate to produce acetyl-CoA and oxaloacetate (Sere, 1992). Indeed, we demonstrated that both acetyl-CoA and oxaloacetate had sufficient activity to induce egg activation, while citrate did not. Acetyl-CoA produced by sperm citrate synthase in egg cytoplasm might sensitize IP3 receptors on the ER, since acetyl-CoA has been reported to sensitize IP3 receptor (Missiaen et al., 1997). On the other hand, oxaloacetate might induce the Ca2+ increase, since it induces the Ca2+ release from mitochondria (Leikin et al., 1993). Thus, it is possible that the Ca2+ increase at newt fertilization is mediated by acetyl-CoA as well as oxaloacetate produced by the sperm citrate synthase (Fig. 8). To confirm this, future studies should measure the amount of those reaction products in the egg cytoplasm at fertilization. Furthermore, sperm citrate synthase might interact with some of the molecules involved in Ca2+ signaling in the egg cytoplasm. Indeed, in Tetrahymena, citrate synthase forms a 14-nm filament and associates with HSP60 protein (Takeda et al., 2001). Future studies should also try to identify the molecules that interact with citrate synthase to induce the Ca2+ increase in the egg cytoplasm.

To clarify the species specificity of sperm factors in amphibian fertilization, the sperm cytoplasmic extracts obtained from the sperm of the monospermic frog, R. laevis, and the physiologically polyspermic newt, C. pyrrhogaster, were reciprocally injected into the heterogeneous eggs. We confirmed that Cynops sperm had enough of the sperm factor to activate homologous eggs, but Xenopus sperm had no activity to activate the homologous eggs. In Cynops egg activation, larger amount of the sperm factor seems to be necessary for the activation, so that the sperm extract containing the cytoplasm equivalent to a single sperm was able to activate about 25% of the eggs. However, since 2–20 sperm enter an egg and initiate egg activation in the physiologically polyspermy newt fertilization (Fig. 1 and Iwao, 2000a,b), fertilizing sperm seem to provide enough activity to activate the egg. The decrease of the activity in the sperm extract might occur during preparation and all the citrate synthase was possibly unable to be extracted from the sperm (Harada et al., 2007).

The Xenopus sperm, however, contained a small amount of egg activation activity against Cynops eggs, but the activity was heat-stable and no citrate synthase activity was detected in the Xenopus extract. The egg activation activity in Xenopus sperm seems to be quite different from the Cynops sperm factor. In addition, Xenopus eggs were insensitive to the Cynops sperm factor. Thus, the mechanism of activation in Xenopus eggs is probably quite different from that in newt eggs, supporting our notion that Xenopus eggs are activated by signal transduction at the binding of the agonist on the sperm membrane to the receptor on egg membrane at sperm–egg binding and/or fusion (Iwao, 2000a,b). In Xenopus fertilization, ligands for integrins on the plasma membrane, such as Arg–Gly–Asp (RGD)-containing peptides (Iwao and Fujimura, 1996) or KTE-containing peptides (Shilling et al., 1998), induce egg activation when applied externally. In addition, a sperm trypsin protease and cathepsin B cause egg activation accompanied by an intracellular Ca2+ increase (Mizote et al., 1999). Interestingly, it was recently reported that a Ca2+ increase in mouse eggs can be induced by polymers displaying EDC peptides from a fertilin β (ADAM2) disintegrin domain probably independently of integrin (Iwao and Fujimura, 1996). Thus, in Xenopus fertilization, it is most likely that the signal for egg activation is transmitted when the sperm binds and/or fuses to the egg membrane (Sato et al., 1999, 2003; Mahbub Hasan et al., 2005). Thus, in Xenopus fertilization, it is most likely that the signal for egg activation is transmitted when the sperm binds and/or fuses to the egg membrane (Sato et al., 2006). Although a Xenopus sperm extract can induce the Ca2+ oscillation when injected into mouse eggs (Dong et al., 2000), we found that the Xenopus sperm extract equivalent to 330 sperm fail to induce egg activation when injected into homologous eggs. The activation of mouse eggs by the Xenopus sperm extract might be induced by the egg-activating activity that activated newt eggs. It was recently reported in the frog, Bufo arenarum, that the sperm contained two distinct egg-activating factors: one causes egg activation as the sperm cytoplasmic factor and the other acts on the egg surface (Bonilla et al., 2008). In addition, it was reported that the injection of several sperm into a Xenopus egg caused egg activation (Aarabi et al., 2010). However, these factors may need to be further characterized to better compare among the Cynops sperm factors, including the role in egg activation at fertilization.

In Xenopus fertilization, monospermy is mainly ensured by a fast block on the egg membrane, followed by the formation of the fertilization envelope. The rapid Ca2+ increase in egg cortex after the first sperm entry seems to be necessary to produce the rapidly rising, positive-going fertilization potential, as the fast electrical block to polyspermy (Iwao, 2000a,b). The slower Ca2+ increase by the sperm factor after complete sperm–egg fusion is probably unsuitable for egg activation in monospermic species. On the other hand, since physiologically polyspermy Cynops eggs lack both the fast electrical polyspermy block and the slow block on the fertilization membrane (Iwao, 2000a,b), it is understandable that Cynops eggs undergo much slower egg activation induced by the Ca2+ increase at fertilization. The PLCζ in sperm is the most plausible candidate for a sperm factor in mammals (Saunders et al., 2002) and birds (Mizushima et al., 2009), and the injection of mRNA from PLCζ also caused egg activation in Cynops eggs (Harada et al., 2007). However, Xenopus sperm probably lacks sufficient PLC activity for egg activation, because no Ca2+ increase or egg activation was induced in Xenopus eggs following injection of the Cynops sperm extract. A trypsin protease (Mizote et al., 1999) and/or ligands for integrins (Iwao and Fujimura, 1996; Shilling et al., 1998) on the sperm membrane probably induce egg activation at Xenopus fertilization, but Cynops eggs are not activated by the sperm protease or those ligands. Thus, the egg activation-inducing factors are quite different among the amphibian species (Fig. 8). This variation in such factors among species might contribute to their reproductive isolation, which is necessary for evolution in amphibians. This may have been particularly important at the transition between monospermy in the anurans and physiological polyspermy in the urodèles. Future studies should compare the egg activation systems in other vertebrate species to further elucidate the evolution of fertilization systems in vertebrates.

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