Egg activation in physiological polyspermy

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Abstract

Fertilization is indispensable not only for restoring diploid genomes but also for the initiation of early embryonic cell cycles in sexual reproduction. While most animals exhibit monospermy, which is ensured by polyspermy blocks to prevent the entry of extra sperm into the egg at fertilization, several animals exhibit physiological polyspermy, in which the entry of several sperm is permitted but only one sperm nucleus participates in the formation of a zygote nucleus. Polyspermy requires that the sperm transmit the egg activation signal more slowly, thus allowing the egg to accept several sperm. An increase in intracellular Ca^{2+} concentration induced by the fertilizing sperm is both necessary and sufficient for egg activation in polyspermy. Multiple small Ca^{2+} waves induced by several fertilizing sperm result in a long-lasting Ca^{2+} rise, which is a characteristic of polyspermic amphibian eggs. We introduced a novel soluble sperm factor for egg activation, sperm-specific citrate synthase, into polyspermic newt eggs to cause Ca^{2+} waves. Citrate synthase may perform dual functions: as an enzyme in mitochondria and as a Ca^{2+} -inducing factor in egg cytoplasm. We also discuss the close relationship between the mode of fertilization and the Ca^{2+} rise at egg activation and consider changes in this process through evolution in vertebrates.

Reproduction (2012) **144** 11–22

Introduction

Fertilization is essential for sexual reproduction in both animals and plants. It gives rise to several reactions important for embryonic development: mixing of the male and female genomes to restore diploid configuration, initiation of rapid cell cycles for early embryonic development, and activation of the synthesis of substances necessary for future morphogenesis. In this context, a haploid egg nucleus must fuse to a single haploid sperm nucleus to avoid syngamy with multiple sperm nuclei, which may lead to aneuploidy and arrested development (Elinson 1986, Iwao 2000a, Wong & Wessel 2006). Most animals exhibit monospermy in which several blocks to polyspermy prevent extra sperm from entering the egg before sperm–egg fusion. First, the number of sperm reaching the egg surface is reduced during passage through egg investments, jelly layers, and vitelline envelopes or through the female reproductive tracts, uterus, and oviducts. Although the probability of multiple sperm entries decreases at fertilization, the eggs are still at risk from polyspermy. Soon after the entry of the first sperm into the egg, the egg's plasma membrane quickly changes to initiate a fast block to polyspermy by, for example, eliciting a positive-going fertilization potential to prevent fusion of a second sperm (Elinson 1986, Iwao 2000a, Wong & Wessel 2006). The egg investments contribute by delaying the arrival of the

second sperm, which helps the egg prepare the block to polyspermy. After the temporal fast block, permanent blocks are achieved by, for example, the formation of a fertilization envelope mediated by exocytosis of cortical granules to prevent sperm penetration completely. Although the structures and the molecules involved in these polyspermy blocks vary among animal species (Wong & Wessel 2006), each is accomplished by eggs that can prevent the entry of extra sperm before their fusion with the egg membrane. However, in each case, the egg must detect the arrival and entry of the first sperm through its membrane and then undergo activation to rapidly initiate block mechanisms against polyspermy. The fertilizing sperm must provide an activation signal in the egg cytoplasm, which rapidly propagates through the entire egg cytoplasm.

Interestingly, there are some species in both invertebrates and vertebrates whose eggs normally accept more than one sperm in the egg cytoplasm at fertilization: physiological polyspermy has been observed in ctenophora, elasmobranchs, urodele amphibians, reptiles, and birds (Elinson 1986, Iwao 2000a, Wong & Wessel 2006, Snook *et al.* 2011). In contrast to those of monospermic species, physiologically polyspermic eggs produce no block to polyspermy before the membrane fusion with extra sperm, although the number of sperm reaching the eggs may be limited by jelly layers in urodeles or oviducts in birds (Iwao 2000a,b, Wong & Wessel 2006). As slower

egg activation may allow the entry of several sperm in polyspermic species, their signals for egg activation may be different from those in monospermic species. However, it should be noted that even in these polyspermic eggs, only one sperm nucleus is ultimately allowed to contact the egg nucleus at syngamy to form a diploid zygote nucleus, while the other sperm nuclei undergo degeneration, thus ensuring embryonic development with a diploid configuration.

An analysis of the molecular mechanisms of egg activation in amphibians, as well as the mode of fertilization, may provide insights into the evolution of vertebrate fertilization systems. Most anurans, frogs and toads, exhibit monospermy, while most urodeles, newts and salamanders, exhibit physiological polyspermy. In addition, the monospermic salamander, *Hynobius nebulosus* (Iwao 1989), as well as the occasionally polyspermic frog, *Discoglossus pictus* (Talevi 1989), exhibits intermediate characteristics that may help to elucidate the evolution of egg activation in the physiological polyspermy of vertebrates. Comparing egg activation mechanisms in physiological polyspermic eggs and monospermic species reveals that polyspermic eggs display unique fertilization Ca^{2+} signals that are very different to monospermic species. Such different fertilization Ca^{2+} signals seem to be mediated by a new type of sperm factor that is different from mammalian or monospermic amphibian species. Here, we analyze egg activation in physiological polyspermic species and compare it with that in monospermic species, with particular interest in the Ca^{2+} rise at fertilization and in a novel sperm factor for egg activation, citrate synthase, in polyspermic urodeles. In addition, such variations in the mechanisms responsible for sperm-triggered Ca^{2+} increases among amphibians raise questions on how the transition between monospermy and polyspermy might have occurred during vertebrate evolution. We also discuss the variation in the mode of fertilization and the Ca^{2+} rise at egg activation as a means of tracing the transition between monospermy and polyspermy during vertebrate evolution.

Egg activation responses at physiological polyspermy Ca^{2+} rises during fertilization

In both amniote and anamniote vertebrates, physiological polyspermy is found in species with internal fertilization and yolky eggs, such as cartilaginous fishes, urodele amphibians, reptiles, and birds (Elinson 1986, Iwao 2000a, Wong & Wessel 2006, Snook *et al.* 2011). Fertilizing sperm must provide a signal to trigger the initiation of development, i.e. egg activation. An increase in free Ca^{2+} concentration in the egg cytoplasm ($[\text{Ca}^{2+}]_i$) induced by the fertilizing sperm is the most important factor for egg activation in both monospermic and physiologically polyspermic species (Fig. 1;

Whitaker 2006). In the monospermic frog, *Xenopus laevis*, a transient and large increase in $[\text{Ca}^{2+}]_i$ induced by a single sperm spread into the entire egg cytoplasm as a Ca^{2+} wave (Fig. 1A and D; Nuccitelli *et al.* 1993, Fontanilla & Nuccitelli 1998), whereas a slow rise in $[\text{Ca}^{2+}]_i$ is reported in the polyspermy of the newt, *Cynops pyrrhogaster*, as detected by Ca^{2+} -sensitive photoprotein (Yoshimoto & Hiramoto 1991, Yamamoto *et al.* 1999) and a locally propagative change in $[\text{Ca}^{2+}]_i$ was observed by a Ca^{2+} -sensitive electrode in *Pleurodeles waltl* (Grandin & Charbonneau 1992). The detailed changes in $[\text{Ca}^{2+}]_i$ at polyspermy have been investigated in *Cynops* eggs by a Ca^{2+} -sensitive fluorescence dye (Harada *et al.* 2011). An initial Ca^{2+} rise at a sperm entry site propagates as a Ca^{2+} wave in the egg cytoplasm (Fig. 1B). The peak level of the Ca^{2+} rise is estimated to be 0.15 μM in *Pleurodeles* (Grandin & Charbonneau 1992). Although the precise level of $[\text{Ca}^{2+}]_i$ has yet to be determined in *Cynops* eggs, the peak level is much lower than that in *Xenopus* eggs: about 1.2 μM in the cortex (Fontanilla & Nuccitelli 1998). The Ca^{2+} wave at *Cynops* fertilization, in some cases, is preceded by an initial spike-like Ca^{2+} rise (Fig. 1E). Each Ca^{2+} wave initiated from the sperm entry site spreads in the egg cytoplasm but does not reach the opposite side of the egg. The observed velocity of 5.1 $\mu\text{m/s}$ for the Ca^{2+} wave in *Cynops* eggs is slightly slower than that observed in the cortex of *Xenopus* eggs (8.9 $\mu\text{m/s}$) but similar to that in the center (5.7 $\mu\text{m/s}$; Fontanilla & Nuccitelli 1998). The slower Ca^{2+} waves are probably due to a lack of endoplasmic reticulum (ER) as intracellular Ca^{2+} stores in the cortex of the *Cynops* eggs (Fig. 2; Harada *et al.* 2011). The Ca^{2+} wave induced by a single sperm propagates in one-eighth to one-quarter of the egg surface and multiple Ca^{2+} waves occur 10–15 min after the first sperm entry. The relatively high $[\text{Ca}^{2+}]_i$ is maintained for 30–40 min after fertilization. Thus, several sperm must enter to increase $[\text{Ca}^{2+}]_i$ over the entire egg. Usually, 2–20 sperm enter a *Cynops* egg and then initiate egg activation (Iwao *et al.* 1985, 1993). The multiple Ca^{2+} waves induced by all fertilizing sperm are probably necessary for complete activation of physiologically polyspermic eggs. In the polyspermy of the frog, *Discoglossus*, an increase in $[\text{Ca}^{2+}]_i$ (0.4–1.3 μM) lasts for 50 min after fertilization, and a Ca^{2+} wave probably propagates toward the entire egg cortex from the sperm entry sites restricted in an animal dimple (Nuccitelli *et al.* 1988). Several spike-like depolarizations in response to each sperm entry are, however, preceded before a long-lasting depolarization mediated by the opening of Ca^{2+} -activated Cl^- channels, as described below (Talevi 1989). This suggests that a nonpropagative small Ca^{2+} rise induced by each sperm entry occurs in advance of the major Ca^{2+} wave. The changes in $[\text{Ca}^{2+}]_i$ at fertilization of other polyspermic species remain unknown.

Figure 1 Ca^{2+} rises and formation of zygote nucleus in vertebrate eggs. (A) A Ca^{2+} wave in an egg of the monospermic frog, *Xenopus*, spreading in the entire egg cytoplasm from the entry site of a single sperm. The transient Ca^{2+} increase peaks 1–3 min after fertilization and continued for about 15 min (D). The sperm pronucleus develops a large aster and makes contact with an egg pronucleus to form a zygote nucleus. (B) Multiple Ca^{2+} waves in an egg of the polyspermic newt, *Cynops*, showing each Ca^{2+} wave propagating in about one-eighth to one-quarter of the egg cytoplasm. Some Ca^{2+} waves are preceded by a spike-like increase (arrow). As each Ca^{2+} wave propagates for about 15–20 min, the total Ca^{2+} rise continues for about 40 min (E). The sperm pronucleus in the animal hemisphere develops larger asters and the nucleus nearest to the egg pronucleus likely makes contact with it to form a zygote nucleus. Other accessory sperm pronuclei undergo degeneration without participating in cell division. (C) Ca^{2+} oscillations in a monospermic mouse egg. The first Ca^{2+} wave is induced at the sperm entry site followed by repetitive Ca^{2+} waves. The Ca^{2+} rises continue every 10–15 min for 2 h after fertilization (F). The sperm pronucleus develops an aster to form a zygote nucleus with an egg pronucleus.

Mechanisms of sperm-triggered Ca^{2+} release in polyspermic eggs

The rise in $[\text{Ca}^{2+}]_i$ induced by the fertilizing sperm is both necessary and sufficient for egg activation in physiologically polyspermic urodeles. Prevention of the Ca^{2+} rise at fertilization by a Ca^{2+} chelator inhibits resumption of meiosis (Yamamoto *et al.* 1999), and an artificial increase in $[\text{Ca}^{2+}]_i$ from intracellular Ca^{2+} stores caused egg activation (Charbonneau & Picheral 1983, Iwao & Masui 1995, Yamamoto *et al.* 1999). Although the eggs of both monospermic frogs and the monospermic *Hynobius* salamander are activated by pricking with a fine needle to bring about a small Ca^{2+} influx, the eggs of most polyspermic urodeles, except for *Pleurodeles* (Aimar & Labrousse 1975), are insensitive to pricking and less sensitive to a Ca^{2+} ionophore (Iwao & Masui 1995, Iwao 2000b), corresponding to a lack of ER as a Ca^{2+} -propagating system in the egg cortex of newt eggs. Inositol-1,4,5-trisphosphate (IP_3) receptors on the

ER are closely involved in the Ca^{2+} rises at polyspermy. Injection of IP_3 into the eggs or the isolated egg cytoplasm induces a Ca^{2+} rise, and injection of heparin to inhibit IP_3 receptors prevents Ca^{2+} waves at fertilization (Yamamoto *et al.* 2001, Harada *et al.* 2011). The expression of exogenous phospholipase C (PLC) activates *Cynops* eggs with an accompanying Ca^{2+} rise (Harada *et al.* 2007). Thus, the Ca^{2+} waves in *Cynops* eggs are probably induced by the propagative Ca^{2+} release acting on IP_3 receptors directly or through IP_3 production (Fig. 2), but ryanodine receptors are unlikely to be involved in the Ca^{2+} rise (Yamamoto *et al.* 2001). However, the mechanism of Ca^{2+} rise is probably different from that in monospermic *Xenopus* eggs with a single Ca^{2+} wave propagating whole egg cytoplasm.

Responses in egg activation

The Ca^{2+} rise at fertilization causes egg activation, which is characterized by a series of morphological and

Figure 2 Schematic potential models of the signaling in egg activation of the monospermic frog, *Xenopus* (A), or of the polyspermic newt, *Cynops* (B). In *Xenopus*, the protease activity on the sperm, in association with sperm surface glycoprotein (SGP) and ADAM16, may cleave an egg receptor, Uroplakin III (UPIII), and then the activated Src kinase (Src) stimulates phospholipase Cγ (PLCγ). Inositol 1,4,5-trisphosphate (IP₃) from phosphatidylinositol 4,5-bisphosphate (PIP₂) induces a local Ca²⁺ increase in the egg cortex. The cortical Ca²⁺ increase seems to propagate through the cortical endoplasmic reticulum (ER), which is abundant in the *Xenopus* egg, as a Ca²⁺ wave. In *Cynops*, the sperm protease activity induces a small nonpropagative Ca²⁺ rise and then sperm-specific citrate synthase introduced from each sperm induces each Ca²⁺ wave. While the Ca²⁺ increase from the inner ER with cytoskeletons, or from mitochondria, may be caused by oxaloacetate, or by acetyl-CoA, respectively, another molecule in association with cytoskeletons may be involved in the course of Ca²⁺ rise induced by sperm citrate synthase.

biochemical changes in the egg. In monospermic species, frogs, and *Hynobius* salamanders, the Ca rise opens Ca²⁺-activated Cl⁻ channels on the egg plasma membrane to produce a rapid (<1 s) depolarization of the membrane and a positive-going fertilization potential, which prevents the entry of other sperm, as a fast block to polyspermy (Cross & Elinson 1980, Iwao 1989, Iwao & Jaffe 1989). In polyspermic urodeles, the eggs of *Pleurodeles* and *Ambystoma mexicanum* display no electrical responses to sperm entry (Charbonneau *et al.* 1983), but *Cynops* eggs elicit small hyperpolarizations probably mediated by Na⁺ channels in response to each sperm entry (Iwao 1985). The polyspermic urodele eggs lack the ability to produce a positive-going fertilization potential, i.e. Ca²⁺-activated Cl⁻ channels. In addition, the entry of *Cynops* sperm into the eggs is not affected by the positive membrane potential, indicating a lack of the fast electrical block to polyspermy on the egg plasma membrane (Iwao & Jaffe 1989). On the other hand, polyspermic *Discoglossus* eggs elicit a positive-going fertilization potential mediated by Cl⁻ channels (Talevi *et al.* 1985, Nuccitelli *et al.* 1988), but they do not block subsequent sperm entries, as sperm penetration is independent of the egg's membrane potential (Talevi 1989). Brief depolarizations

occur in response to each sperm entry into the periphery of the dimple (Talevi & Campanella 1988), indicating a small Ca²⁺ rise induced by each sperm entry, as described above. Although there have been few studies on the electrical responses in other polyspermic vertebrate species, in the polyspermy of the tcnophore, *Beroe ovata*, each sperm induces a Na⁺-dependent depolarization, lasting about 60 s, preceded by an action potential (Goudeau & Goudeau 1993), suggesting that there is no electrical regulation of sperm-egg fusion that prevents polyspermy.

Monospermic frog eggs undergo cortical granule exocytosis to transform the vitelline envelope into the fertilization envelope through which the extra sperm cannot penetrate (Hedrick 2008). The eggs of urodele amphibian, including the monospermic *Hynobius* (Iwao 1989), have no cortical granules, indicating the absence of a formed fertilization envelope. Cortical contraction, characterized by apparent movement of cortical pigments toward the animal pole, does not occur in response to the Ca²⁺ rise at newt egg activation, but small accumulations of cortical pigments at sperm entry sites are visible on the animal hemisphere (Iwao 2000a). The cell cycle of unfertilized newt eggs is arrested at the second meiotic metaphase with a high activity of

M phase-promoting factor (MPF; cdc2 kinase (cdk1), and cyclin B) maintained by c-Mos (Iwao & Masui 1995, Sakamoto *et al.* 1998, Vaur *et al.* 2004, Pelczar *et al.* 2007). The amount of cyclin B in the unfertilized egg is approximately one-fourth in cleaving eggs but is primarily distributed in the cortex of the animal hemisphere and chromosomes (Sakamoto *et al.* 1998, Iwao *et al.* 2002). Cyclin B as well as c-Mos disappeared soon after fertilization when the sperm asters expand through the egg cytoplasm (Yamamoto *et al.* 2001, Iwao *et al.* 2002, Pelczar *et al.* 2007) and then the activity of both cdc2 kinase and MAPK decreases (Iwao *et al.* 1993, Sakamoto *et al.* 1998, Pelczar *et al.* 2007). Degradation of MPF might occur downstream of the Ca^{2+} rises at fertilization through a calcineurin/CaMKII/APC cascade alike in *Xenopus* eggs (Nishiyama *et al.* 2007). Inhibition of protein synthesis causes resumption of meiosis in *Cynops* eggs (Iwao & Masui 1995), indicating that inhibition of short-lived proteins such as cyclin B, for example, is involved in egg activation downstream of the Ca^{2+} rises. Following the emission of the second polar body at the animal pole, the fertilized egg undergoes S phase of the first mitotic cell cycle.

A cytoplasmic block to polyspermy in physiologically polyspermic eggs

In previous studies on the physiological polyspermy of newt eggs, all incorporated sperm form sperm pronuclei concomitantly with spreading sperm asters from each sperm centriole (Fankhauser 1948, Iwao *et al.* 1985, 1993, 1997, 2002). Only one principal sperm pronucleus, probably nearest to the egg nucleus and with the largest sperm aster, makes contact with an egg pronucleus to form a zygote nucleus in the center of the animal hemisphere. The γ -tubulin predominantly distributed in the animal hemisphere is heavily concentrated in the centrosome in the principal sperm pronucleus to promote microtubule polymerization (Iwao *et al.* 2002). However, it remains unknown how the sperm pronucleus makes contact with the egg nucleus. Sperm pronuclei enter S phase, but the progress in DNA synthesis is faster in the principal sperm pronucleus and the egg pronucleus than in the accessory sperm pronuclei (Wakimoto 1979, Iwao *et al.* 1993). The zygote nucleus, therefore, enters M phase to form a bipolar spindle, earlier than the accessory sperm nuclei (Iwao & Elinson 1990, Iwao *et al.* 1993, 2002). Separation of the centrosome never occurs in the accessory sperm nuclei that undergo degeneration (pycnosis) before the first cleavage. The failure of progress in the nuclear cycle and the subsequent nuclear degeneration are probably caused by insufficient exposure of MPF (cdc2 kinase) activity in the accessory sperm pronuclei in the periphery of the egg (Iwao & Elinson 1990, Iwao *et al.* 2002). MPF activity in the

animal hemisphere is higher than that in the vegetal hemisphere at M phase (Iwao *et al.* 1993), and more active cdc2 kinase is localized in the animal hemisphere than in the vegetal hemisphere. Cyclin B1 is highly accumulated in the zygote nucleus but not in the accessory sperm nuclei (Iwao *et al.* 2002). The degeneration of both centrosomes and chromosomes in the accessory sperm nuclei appears to be caused through a process similar to that of apoptosis in somatic cells, but the molecular mechanisms remain to be investigated. Thus, a very slow block to polyspermy in egg cytoplasm is accomplished in physiologically polyspermic eggs.

In polyspermic bird eggs, accessory sperm nuclei enter into M phase at the first cleavage but do not induce the extra cleavage furrow and undergo degeneration in the margin of a blastodisc (Harper 1904, Perry 1987, Waddington *et al.* 1998). In eggs of the polyspermic ctenophore, *Beroë*, a single sperm nucleus is selected to form the zygote nucleus in the egg cytoplasm (Carré & Sardet 1984, Houliston *et al.* 1993, Rouvière *et al.* 1994). The molecular mechanisms for suppression of accessory sperm nuclei, however, remain unknown in those species.

Mechanism of egg activation in physiological polyspermy

Comparison of the mechanisms of Ca^{2+} release in mono vs polyspermic eggs

There must be a specific mechanism by which a fertilizing sperm transmits the initial signal for the Ca^{2+} rise at physiological polyspermy. As mentioned earlier, faster egg activation is probably characteristic of monospermic species, but not of physiologically polyspermic species, so that the mechanisms underlying egg activation must be different between those species. To elucidate this process, we compared the signaling mechanisms in egg activation between monospermic and polyspermic species in vertebrates. There are at least two major models to account for the $[\text{Ca}^{2+}]_i$ increase at vertebrate fertilization (Fig. 2; Iwao 2000b, Nomikos *et al.* 2012). One is that a sperm binds to a receptor on the egg plasma membrane and then stimulates a signal transduction pathway causing the Ca^{2+} rise. Another is that a sperm introduces factors into the egg, such as Ca^{2+} , PLC, or other agents, that can induce the Ca^{2+} rise. In any case, a fertilizing sperm must stimulate a propagative $[\text{Ca}^{2+}]_i$ increase in the egg cytoplasm to produce the Ca^{2+} wave.

In monospermic *Xenopus* eggs, a Ca^{2+} rise is induced by external application of peptides containing RGD residues acting as ligands for integrins (Iwao & Fujimura 1996) or peptides from the disintegrin domain of xMDC16 (Shilling *et al.* 1998). Protease activity against a peptide containing GRR residues also induces activation in *Xenopus* eggs (Iwao *et al.* 1994, Mizote *et al.* 1999). In *Xenopus* fertilization, a protein tyrosine

kinase, Src kinase, localized in membrane microdomains (membrane rafts) of unfertilized eggs, is phosphorylated and then the activated Src kinase stimulates IP_3 production through $PLC\gamma$ (Fig. 2A; Sato *et al.* 1999, 2003). Sperm induces transient phosphorylation of Uroplakin III (UPIII) on the egg membrane dependent on Src kinase (Mahbub Hasan *et al.* 2005, 2007, Sakakibara *et al.* 2005). It has been postulated that the sperm protease associated with sperm surface glycoprotein (SGP, Nagai *et al.* 2009) cleaves UPIII to activate Src kinase. UPIII seems to serve as a primary sperm receptor for the Ca^{2+} release from cortical ER (Fig. 2A). In the monospermy of the primitive jawless fish lamprey, a positive-going fertilization potential blocks sperm–egg fusion, but not egg activation (Kobayashi *et al.* 1994), indicating the involvement of a receptor on the egg membrane in the signaling pathway for egg activation. Monospermic species without the fast polyspermy block, however, seem to possess a different system for egg activation. Bony fishes exhibit monospermy without the fast electrical block to polyspermy on the egg membrane (Nuccitelli 1980). Their monospermy is ensured by the micropyle on the chorion (vitelline envelope) to limit the number of sperm reaching the egg plasma membrane (Hart 1990, Iwamatsu 2000). Bony fish sperm contain a factor for activation of homologous eggs (Iwamatsu & Ohta 1974) or for inducing a Ca^{2+} rise in mouse eggs or sea urchin egg homogenates (Coward *et al.* 2003), but $PLC\zeta$ is unlikely involved in the sperm-induced activation of fish eggs (Coward *et al.* 2011). Monospermic mammalian eggs also lack a fast electrical block to polyspermy (Jaffe *et al.* 1983, Gianaroli *et al.* 1994, Kline & Stewart-Savage 1994). Ca^{2+} oscillations induced by sperm, however, appear to play a role in the slow membrane block to polyspermy in mammalian fertilization (Gardner *et al.* 2007). A soluble sperm factor for egg activation enters into the egg cytoplasm after sperm–egg fusion in mammals (Swann 1990, Parrington *et al.* 1996, Oda *et al.* 1999). A sperm-specific $PLC\zeta$ generates Ca^{2+} oscillation in mouse eggs (Fig. 1C and F; Saunders *et al.* 2002, Kouchi *et al.* 2004) and a single sperm contains sufficient $PLC\zeta$ protein to induce Ca^{2+} oscillation (Saunders *et al.* 2002, Yoda *et al.* 2004), indicating that the major active component for Ca^{2+} oscillations is $PLC\zeta$ in mammalian fertilization. Thus, the signal transduction for egg activation around the sperm binding to the egg membrane may play a role in the fast electrical block to polyspermy in monospermic species.

Citrate synthase as a novel sperm factor for newt egg activation

In polyspermic *Cynops* eggs, a small and nonpropagative Ca^{2+} rise is induced by the tryptic acrosomal protease (Harada *et al.* 2011), but only a small number of eggs are activated by treatment with the sperm protease (Iwao *et al.* 1994). The initial spike-like Ca^{2+} rise at *Cynops*

fertilization seems to be induced by the sperm protease at the binding of the sperm on the egg surface but is insufficient for inducing the propagative Ca^{2+} wave necessary for egg activation (Fig. 2B). The injection of sperm soluble components, a sperm factor, into the egg causes a Ca^{2+} wave and egg activation (Yamamoto *et al.* 2001). The Ca^{2+} wave induced by the sperm factor has a velocity of 6.2 $\mu m/s$, which is similar to those induced by the fertilizing sperm, and it triggers a complete activation, including resumption of meiosis, degradation of cyclin B and c-Mos, and DNA replication followed by abortive cleavage due to the lack of sperm centrioles. The sperm factor for egg activation is highly purified and characterized (Harada *et al.* 2007), which reveals that citrate synthase in the sperm cytoplasm induces the Ca^{2+} wave that causes the egg activation (Fig. 2B). The sperm lack sufficient PLC activity to induce egg activation because no Ca^{2+} rise occurs in *Xenopus* eggs by injection of the *Cynops* sperm extract. The sperm-specific citrate synthase (45 kDa) is slightly heavier than those observed in heart tissue and in unfertilized eggs (43 kDa). The Ca^{2+} rise is induced by the injection of not only porcine citrate synthase but also citrate synthase mRNA into unfertilized eggs (Harada *et al.* 2007). The egg-activating activity in the sperm extract was reduced by the treatment with anti-citrate synthase antibody. Most citrate synthase is distributed as a fibrous structure from the neck to the middle piece outside the mitochondria. The sperm citrate synthase can be exposed to egg cytoplasm soon after sperm entry because all sperm components, including the middle piece and the tail, are incorporated into the egg (Picheral 1977, Iwao 2000a). It is unknown whether a single newt sperm can induce sufficient egg activation activity in the newt egg. In *Cynops* fertilization, 2–20 sperm enter an egg (Iwao *et al.* 1985, 1993), indicating that at least a double sperm entry is required for full activation. A sperm extract containing the cytoplasm equivalent of a single sperm is able to activate about 20% of the egg, corresponding well to the proportionately low level of citrate synthase (2 pg) and its enzymatic activity in a single sperm (Yamamoto *et al.* 2001, Harada *et al.* 2007, 2011). The multiple Ca^{2+} increases induced by all fertilizing sperm must be necessary for complete activation of polyspermic *Cynops* eggs. The lower level of egg activation activity induced by a single sperm will delay the initiation of egg activation until several sperm enter the same egg. Thus, the sperm-specific citrate synthase appears to represent a novel and major sperm factor for egg activation in physiologically polyspermic newt eggs.

A Ca^{2+} rise by citrate synthase

It is important to understand the mechanism by which citrate synthase, derived from fertilizing sperm, can induce a Ca^{2+} rise in the egg cytoplasm. The ER containing IP_3 receptor in unfertilized *Cynops* eggs has been observed to form large clusters, probably with

cytoskeletons, in the inner egg cytoplasm (Fig. 2B; Harada *et al.* 2011). A local and spike-like Ca^{2+} rise is induced by each injection of the sperm extract into an isolated ER-rich fraction. Although adequate conformation of the ER must be necessary for the formation of Ca^{2+} waves, Ca^{2+} is mainly released from the ER rather than from mitochondria in response to sperm citrate synthase. The ability to induce multiple Ca^{2+} rises in the heavy ER clusters corresponds well with the multiple Ca^{2+} waves through the inner ER at egg activation and is somewhat analogous to that induced by the mouse sperm factor, a PLC ζ -induced Ca^{2+} mobilization by hydrolyzing internal phospholipid stores (Yu *et al.* 2012). The purified sperm factor shows high enzymatic activity for citrate synthase and the inhibition of its activity prevents egg activation not only by the sperm extract but also by the fertilizing sperm (Harada *et al.* 2011), indicating a central role for the enzymatic activity in the Ca^{2+} rises (Fig. 2B). Citrate synthase produces citrate from acetyl-CoA and oxaloacetate in the mitochondrial tricarboxylic acid (TCA) cycle but might inversely cleave the citrate, which is abundant in the egg cytoplasm, to produce acetyl-CoA and oxaloacetate (Sreere 1992). Both acetyl-CoA and oxaloacetate have sufficient activity to induce Ca^{2+} waves and egg activation in *Cynops* eggs, while citrate has not (Harada *et al.* 2011). It is reported that acetyl-CoA sensitizes the IP_3 receptors on the ER to induce Ca^{2+} releases (Missiaen *et al.* 1997) and that oxaloacetate induces the Ca^{2+} release from mitochondria (Leikin *et al.* 1993). As *Cynops* eggs are more sensitive to acetyl-CoA than to oxaloacetate, the acetyl-CoA associated with the sperm citrate synthase seems to be a major signal for the Ca^{2+} release from IP_3 receptors on the ER at fertilization (Fig. 2B). However, the detailed mechanisms remain to be investigated. On the other hand, it is possible that sperm citrate synthase interacts with some other molecules involved in Ca^{2+} signaling in the egg cytoplasm. In this connection, the treatment of *Cynops* eggs with D_2O enhances microtubule polymerization to form numerous small cytoasters in the egg cortex and causes egg activation (Iwao & Masui 1995), suggesting at least some role for cytoskeletal filaments in the egg activation process. Interestingly, in the protozoa *Tetrahymena*, citrate synthase displays dual functions: as an active enzyme in mitochondria and as a cytoskeleton protein for 14 nm filaments (Numata *et al.* 1985, Numata 1996). The 14 nm filament protein, a dephosphorylated form of citrate synthase, is involved in oral morphogenesis and pronuclear behavior during fertilization (Numata *et al.* 1985, Kojima & Numata 2002) and associated with the HSP60 protein (Takeda *et al.* 2001). Citrate synthase in newts may also play dual roles: as a mitochondrial enzyme and as a sperm factor for egg activation. Indeed, it is well known that some active enzymes, such as lactate dehydrogenase, have been recruited, unchanged, to an extra role as structural protein crystallins in the lens of the eye (Wistow *et al.* 1987, Tomarev & Piatigorsky 1996).

We have also recently demonstrated that SGP on the sperm membrane has a bifunctional role in sperm binding to both the vitelline envelope and the egg plasma membrane at *Xenopus* fertilization (Nagai *et al.* 2009, Kubo *et al.* 2010). Further investigation of the function of citrate synthase, not only in fertilization but also in early embryonic development, will provide insight into Ca^{2+} signaling and cell cycle regulation.

Specificity and variations in the sperm factor in vertebrates

Egg activation by the *Cynops* sperm factor, citrate synthase, is specific for homologous eggs. *Xenopus* eggs are insensitive not only to the *Cynops* sperm factor but also to the homologous sperm extract without citrate synthase activity (Harada *et al.* 2011). These results indicating lack of Ca^{2+} release-inducing factor in *Xenopus* sperm cytoplasm supports the notion that the activation of *Xenopus* eggs is mediated by the membrane receptor. *Xenopus* sperm, however, contain a small amount of heat-stable activity to activate *Cynops* eggs, but which is quite different from that induced by citrate synthase. In this connection, it is reported that a *Xenopus* sperm extract contained a factor for triggering Ca^{2+} oscillations in mouse eggs (Dong *et al.* 2000) and that the injection of several *Xenopus* sperm into a homologous egg or a sperm-borne protein (PAWP) caused egg activation (Aarabi *et al.* 2010). The sperm of the monospermic frog, *Bufo arenarum*, contained two different types of activity, causing activation by injection into the egg or by external treatment (Bonilla *et al.* 2008). Further characterization of those factors will be necessary to clarify their roles in egg activation at frog fertilization.

The changes in $[\text{Ca}^{2+}]_i$ have not been determined at polyspermy in other vertebrates, but an artificial increase in $[\text{Ca}^{2+}]_i$ in a bird blastodisc causes egg activation accompanied by pronucleus formation (Mizushima *et al.* 2007, 2009). The injection of a sperm extract, but not of somatic cell extracts, induces egg activation dependent on intracellular Ca^{2+} activity (Mizushima *et al.* 2009). In addition, the injection of chicken sperm extract into mouse eggs induces Ca^{2+} rises and initiates embryo development (Dong *et al.* 2000, Kim & Gye 2003). As bird PLC ζ causes activation in quail eggs (Mizushima *et al.* 2009) and triggers Ca^{2+} oscillations in mouse eggs (Coward *et al.* 2005), PLC ζ seems to be a potent sperm factor for egg activation in polyspermic birds, but further investigation for the factor like citrate synthase will be important.

Perspectives in egg activation during evolution of vertebrates

A single Ca^{2+} rise in monospermy

A review of the variations in egg activation systems, and particularly the Ca^{2+} rise at fertilization in living

vertebrates (Fig. 3), may elucidate the evolutionary history of egg activation concomitant with the acquisition of polyspermy. In vertebrates, most fishes exhibit monospermy, except for cartilaginous fishes (elasmobranchs) such as sharks and chimaera (Hart 1990). In the primitive jawless fishes (Agnathans), the lampreys exhibit monospermy with a positive-going fertilization potential mediated by Ca^{2+} -activated Cl^- channels (Kobayashi *et al.* 1994). The pattern of fertilization potential alike in anuran eggs indicates a transient Ca^{2+} rise at lamprey fertilization. In the bony fish (Teleosts), Medaka (*Oryzias latipes*), a transient Ca^{2+} wave is induced by a fertilizing sperm from its entry site (Gilkey *et al.* 1978). In frogs, monospermy is ensured by a positive-going fertilization potential mediated by the propagative opening of Ca^{2+} -activated Cl^- channels (Kline & Nuccitelli 1985) preceded by a transient Ca^{2+} wave. In monospermic salamanders (urodeles), *Hynobius* eggs open Ca^{2+} -activated Cl^- channels to produce a positive-going fertilization potential (Iwao 1989), indicating a transient Ca^{2+} wave at egg activation. Thus, the transient and large Ca^{2+} wave induced by a single sperm entry is probably characteristic of monospermic eggs in vertebrates, implying that the ancestor of vertebrates exhibited monospermy with a single and long-lasting Ca^{2+} wave.

Multiple Ca^{2+} rises in polyspermy

In anamniotes, multiple Ca^{2+} waves (rises) are required for egg activation in the polyspermic eggs of

physiological polyspermic *Cynops* (urodeles). Multiple Ca^{2+} rises appear to occur in occasionally polyspermic eggs of the frog *Discoglossus* (anurans). The multiple Ca^{2+} waves caused by a sperm cytosolic factor, e.g. citrate synthase, may reflect an evolutionary adaptation related to a change in mode of fertilization, e.g. the acquisition of physiological polyspermy. The close relationships in the acquisition of polyspermy between internal fertilization, increased egg size, centrosome dynamics, etc. have been the subject of much discussion (Elinson 1986, Wong & Wessel 2006, Snook *et al.* 2011). Physiological polyspermy might arise in large eggs with external fertilization, as in the Japanese giant salamander, *Andrias japonicus*, the large eggs (5–8 mm in diameter) inseminated after oviposition undergo polyspermic fertilization (Iwao 2000a). The extra space in the egg cytoplasm of large eggs is one of the most important factors for eliminating accessory sperm nuclei in the same egg cytoplasm, according to the 'large egg model' (Elinson 1986). Although physiological polyspermy is required to ensure fertilization in large eggs (Harper 1904), a single sperm does not contain sufficient sperm factor to induce the Ca^{2+} rise required for activation in a large egg. Multiple Ca^{2+} rises appear to be necessary for complete activation of large eggs. It has been hypothesized that the cytological polyspermy block in physiological polyspermy represents a more ancient type (Wong & Wessel 2006) and that urodeles and anurans may have arisen from different origins (Elinson 1986). As monospermic anurans may have branched from

Figure 3 Variations in the mode of fertilization and Ca^{2+} rises in egg activation among vertebrates. poly, polyspermy; occ. poly, occasional polyspermy; mono, monospermy; single, a single Ca^{2+} rise at egg activation; multiple, multiple Ca^{2+} rises at egg activation; * Ca^{2+} rises presumed due to opening of Ca^{2+} -activated Cl^- channels.

urodeles during the beginning of the Mesozoic period (240 million years ago; Feller & Hedges 1998), around the period when the ancestor of mammals appeared, multiple Ca^{2+} rises capable of activating large polyspermic eggs might be a characteristic shared with species that possess polyspermic amniotic eggs. It will be important to determine whether multiple Ca^{2+} rises occur at physiological polyspermy in living amniotes, reptiles, and birds.

Ca^{2+} oscillations in monospermic mammals

Higher eutherian mammals, however, exhibit monospermy and their sperm-specific PLC ζ induce repetitive Ca^{2+} rises (oscillations) at egg activation (Fig. 1C and F; Swann *et al.* 2006, Nomikos *et al.* 2012). The fertilization of primitive mammals can be very instructive. The monotrematous platypus, *Ornithorhynchus anatinus*, lays large yolky eggs (about 4 mm in diameter) that undergo meroblastic cleavage in blastodiscs (Hughes & Hall 1998). The platypus eggs are polyspermic and several sperm probably enter into a blastodisc ($41 \times 368 \mu\text{m}$; Gatenby & Hill 1924). In the small marsupial mammal, *Sminthopsis crassicaudata*, the relatively small egg (about $120 \mu\text{m}$ in diameter) contained a yolk mass in the center and some eggs are polyspermic (Breed & Leigh 1990). Thus, the decrease in egg size and yolk content is closely associated with the change in the mode of fertilization, from polyspermy to monospermy, in mammals. Although the changes in $[\text{Ca}^{2+}]_i$ at fertilization have not been reported in those primitive mammals, it may be that the ancestor of mammals exhibited polyspermy, which is required for repetitive Ca^{2+} waves and the induction of egg activation. Higher eutherians have small eggs (about $100 \mu\text{m}$ in diameter) without a yolk in the egg cytoplasm, but multiple Ca^{2+} rises lasting more than 1 h are still necessary for complete activation of mouse eggs (Ozil 1990, Ducibella *et al.* 2002), indicating that the first Ca^{2+} rise by the entry of a single sperm is insufficient for egg activation. It may be the case that Ca^{2+} oscillations in the relatively small eutherian eggs might be functioning in place of multiple Ca^{2+} waves of the ancestral polyspermic eggs. It should be, however, mentioned that the monospermic eggs in invertebrates exhibit Ca^{2+} oscillations during fertilization (Stricker 1999, Dumollard *et al.* 2002) and multiple Ca^{2+} rises might be associated with longer period in completion of meiosis after fertilization.

Conclusions

We have described the mechanism of the induction of multiple Ca^{2+} waves required for egg activation in polyspermic eggs. The review of the variations in the Ca^{2+} rise at egg activation among vertebrates suggests

the Ca^{2+} rise concomitant with the transition in the mode of fertilization between monospermy and polyspermy. The sperm cytosolic factors for egg activation may have played a role in the evolution of slower activation in polyspermic eggs and may promote the reproductive isolation necessary for speciation in vertebrates. Interestingly, mouse sperm having the potent sperm factor PLC ζ , as well as sperm of the monospermic salamander, *Hynobius*, contain a large amount of citrate synthase outside the mitochondria (Iwao & Harada 2011, unpublished observations). As reliable fertilization mechanisms are indispensable for sexual reproduction, any abrupt change in egg activation, such as that induced by an alternative sperm factor, would not likely be selected for during evolution. As some species among vertebrates may still possess intermediate characteristics of these sperm factors, future research should attempt to clarify the role of citrate synthase in egg activation of amniotic vertebrates. A comprehensive phylogenetic analysis of the sperm factors for egg activation will help to elucidate their roles in vertebrate evolution and may uncover previously overlooked mechanisms in reproductive and early developmental systems.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

Funding

This work was supported in part by a grant-in-aid for scientific research on innovative areas from MEXT (22112518, 24112712).

Acknowledgements

The authors dedicate this paper to late Prof. Chiaki Katagiri for his valuable advice and words of encouragement over the years. They also thank Yuichiro Harada and Shuichi Ueno for critical reading of the manuscript and Tomoyo Ueno for her help on preparing the manuscript.

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Received 23 March 2012

First decision 23 April 2012

Accepted 23 May 2012