Chapter 15
Egg Activation in Polyspermy: Its Molecular Mechanisms and Evolution in Vertebrates

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Abstract In amphibians, most urodeles (newts) exhibit polyspermy physiologically, but primitive urodeles (*Hynobius*) and anurans (frogs) exhibit monospermy. Several fertilizing sperm induce multiple small Ca^{2+} waves in the polyspermic egg, but a single large Ca^{2+} wave occurs in the monospermic egg. The Ca^{2+} waves in newt eggs are caused by a sperm-specific citrate synthase localized outside the mitochondria. The single Ca^{2+} wave at monospermy is necessary for eliciting a fast block to polyspermy, whereas the small multiple Ca^{2+} waves provide slower egg activation to permit the entry of several sperm at polyspermy. Physiological polyspermy seems to be evolved in association with the increase in size of eggs in urodeles, reptiles, and birds laying larger yolky eggs. The sperm factor (citrate synthase) operating in slower egg activation in polyspermic eggs is already prepared in the monospermic urodele *Hynobius*. We have focused on comparative studies in fertilization among amphibians to understand the role of egg activation in establishment of polyspermy with discussion of the evolution in vertebrates.

Keywords Amphibians • Ca^{2+} wave • Citrate synthase • IP3 receptor • Polyspermy block

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H. Sawada et al. (eds.), *Sexual Reproduction in Animals and Plants*,
DOI 10.1007/978-4-431-54589-7_15, © The Author(s) 2014

15.1 Introduction

Fertilization is indispensable for sexual reproduction in animals. Both sperm and eggs are highly specialized to ensure development with the diploid genome. We have focused on the molecular mechanism of fertilization in amphibians, which are one of the best models for studying fertilization, in particular, for understanding the evolution in egg activation and polyspermy blocks in vertebrates. There are two different types of fertilization in vertebrates: monospermy, in which only one sperm penetrates into the egg, and physiological polyspermy, in which several sperm enter the egg at normal fertilization. Fertilization in the ancestral vertebrates seems to be monospermic, because not only deuterostome invertebrates such as sea urchins and ascidians, but also most fishes, including a primitive fish, the lamprey, exhibit monospermy (Iwao 2012). Amphibians consist of three groups: anurans (frogs and toads), urodeles (newts and salamanders), and caecilians (limbless amphibians) (Iwao 2000a). Although there is little information on fertilization of caecilians, most anurans exhibit external and monospermic fertilization (Table 15.1). Only one sperm is incorporated into the egg, and other sperm are prevented from entering the fertilized egg. Several blocks to polyspermy operate to exclude the extra sperm outside the egg plasma membrane. In contrast, most urodeles exhibit internal fertilization and the female stores the sperm in a spermatheca near the cloaca (Akiyama et al. 2011). The eggs are inseminated by a small number of sperm released from the spermatheca just before oviposition. Although several sperm enter a physiologically polyspermic urodele egg, development with the diploid genome is ensured by the

Table 15.1 Characteristics of fertilization in amphibians

Species	Mode of fertilization	Ca^{2+} wave	Positive fertilization potential	Fast polyspermy block	Sperm citrate synthase	Size of egg (diameter in mm)
Anurans						
Discoglossus pictus	External occasional polyspermy	Multiple[a]	+	–	ND	1.6
Xenopus laevis	External monospermy	Single	+	+	–	1.2
Bufo japonicus	External monospermy	Single[a]	+	+	ND	1.8
Urodeles						
Hynobius nebulosus	External monospermy	Single[a]	+	+	+	2.4
Andrias japonicus	External polyspermy	ND	ND	–	ND	5.0
Cynops pyrrhogaster	Internal polyspermy	Multiple	–	–	+	2.3

ND not determined

[a]Based on the transient opening Ca^{2+}-activated Cl^- channels

intracellular block to polyspermy in egg cytoplasm (Fankhauser 1948; Iwao and Elinson 1990; Iwao et al. 1993, 2002). Only one sperm nucleus forms a zygote nucleus with the egg nucleus, and the other extra sperm nuclei degenerate before cleavage. However, the most primitive *Hynobius* salamanders exhibit external fertilization and monospermy as with anurans (Iwao 1989). Thus, comparative studies in fertilization among amphibians will provide better understanding of the role of egg activation in establishment of polyspermy during vertebrate evolution.

15.2 Egg Activation at Physiologically Polyspermic Fertilization

The egg nucleus in unfertilized eggs of vertebrates is arrested at metaphase of the second meiotic division until the sperm breaks the attest at fertilization. Fertilization provides the sperm nucleus into the egg, as well as initiates its embryonic development, which is called egg activation. An increase in $[Ca^{2+}]_i$ in the egg cytoplasm induced by the fertilizing sperm is essential for egg activation in vertebrate fertilization (Iwao 2000b). In monospermic anurans, the fertilizing sperm induces a transient and single $[Ca^{2+}]_i$ increase that propagates from a sperm entry site toward the opposite site on the whole egg surface as a $[Ca^{2+}]_i$ wave (Fontanilla and Nuccitelli 1998). The $[Ca^{2+}]_i$ increase causes the opening of Cl^- channels on the egg plasma membrane to produce a positive fertilization potential within 1 s (Kline and Nuccitelli 1985), which prevents the entry of another sperm as a fast, but temporal, block to polyspermy (Cross and Elinson 1980; Iwao 1989; Iwao and Jaffe 1989). The Ca^{2+} wave then induces exocytosis of cortical granules, resulting in transformation of the vitelline coat into a fertilization coat (Hedrick 2008). The fertilization coat prevents extra sperm from reaching the fertilized egg, as a slow and permanent block to polyspermy. In the monospermic salamander *Hynobius nebulosus*, the eggs exhibit a large positive fertilization potential mediated by opening of Ca^{2+}-activated Cl^- channels (Iwao 1989). The fast and transient opening of Cl^- channels indicates a single Ca^{2+} wave at fertilization. Polyspermy is also prevented by a positive fertilization potential without formation of a fertilization coat in monospermic salamanders. Thus, the fast generation of a single Ca^{2+} wave soon after the entry of the first sperm is important for the accomplishment of the fast polyspermy block in monospermic species.

In contrast, in physiologically polyspermic newts, small and multiple increases of $[Ca^{2+}]_i$ occur at fertilization (Grandin and Charbonneau 1992; Yamamoto et al. 1999; Harada et al. 2011). The $[Ca^{2+}]_i$ increase at the sperm entry site propagates as a Ca^{2+} wave (Harada et al. 2011). The small Ca^{2+} wave is induced by each fertilizing sperm in the polyspermic egg. Because 2 to 20 sperm enter an egg at normal newt fertilization (Iwao et al. 1985), multiple Ca^{2+} waves induce $[Ca^{2+}]_i$ increase, lasting 30–40 min after the first sperm entry (Harada et al. 2011). The $[Ca^{2+}]_i$ increase induces no change (Charbonneau et al. 1983) or a very small hyperpolarization in

response to each sperm entry (Iwao 1985) in the egg membrane potential. Sperm entry into newt eggs is not sensitive to the positive membrane potential (Iwao and Jaffe 1989). No cortical granule is observed in urodele eggs, indicating lack of fertilization envelope formation (Iwao 2000a).

15.3 The Signaling Mechanism of $[Ca^{2+}]_i$ Increase Induced by the Fertilizing Sperm

The Ca^{2+} increase at fertilization is caused by the release of Ca^{2+} ions from the endoplasmic reticulum (ER), a major intracellular Ca^{2+} store in the egg cytoplasm (Fig. 15.1). Inositol 1,4,5-trisphosphate (IP3) generated by phospholipase C (PLC) opens Ca^{2+} channels of IP3 receptors on the ER. However, the mechanism for induction of $[Ca^{2+}]_i$ increase by the fertilizing sperm is quite different between monospermic and physiologically polyspermic eggs. In monospermic anurans, a sperm agonist (ligand) probably binds an egg receptor at contact between the sperm and egg membranes, and then a signal for stimulating IP3 production is transmitted into the egg cytoplasm. Indeed, *Xenopus* eggs are activated by external treatment with tryptic sperm protease (Iwao et al. 1994; Mizote et al. 1999), which can hydrolyze one of the candidates of egg receptors, uroplakin III (UP III), on the egg plasma

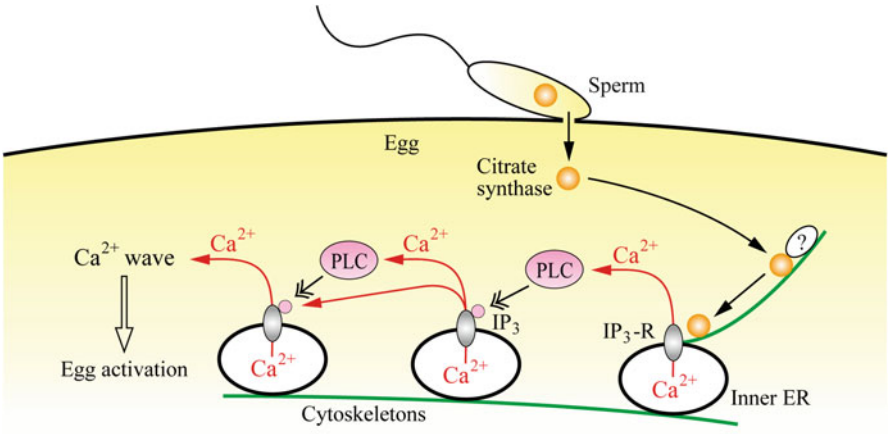

Fig. 15.1 Model of signaling for Ca^{2+} increase in a physiologically polyspermic newt egg. The sperm-specific citrate synthase is introduced from the sperm cytoplasm into the egg cytoplasm after sperm–egg fusion. Citrate synthase, in association with cytoskeletons, sensitizes the inositol-1,4,5-trisphosphate (IP3) receptor on the inner endoplasmic reticulum (*ER*) to release Ca^{2+} ions. The local Ca^{2+} increase propagates through the inner ER with cytoskeletons as a Ca^{2+} wave by the activation of phospholipase C (*PLC*) to produce IP3 or stimulation of IP3 receptors

membrane (Sakakibara et al. 2005; Sato et al. 2003). The cleavage of UP III induces activation of Src kinase and PLCγ to produce IP3 in egg cytoplasm (Mahbub Hasan et al. 2005, 2007; Ijiri et al. 2012). In addition, the $[Ca^{2+}]_i$ increase in *Xenopus* eggs is induced by external treatment of the egg with RGD-containing peptides (Iwao and Fujimura 1996) or KTE-containing peptides (Shilling et al. 1998), which binds integrins on the plasma membrane accompanied by activation of Src kinase (Sato et al. 1999). RGDS peptide also activates the eggs of monosperrmic *Hynobius* salamanders (Iwao, unpublished observations, 2012). Although the precise interaction between those molecules remains to be investigated, the initial Ca^{2+} release induced at the sperm entry site is propagated through further activation of PLCγ or direct sensitization of IP3 receptors on ER abundant in egg cortex, resulting in the formation of a single Ca^{2+} wave.

In polyspermic newt eggs, the signal for egg activation is provided from sperm cytoplasm after sperm and egg fusion (Fig. 15.1). Injection of an extract containing newt sperm cytoplasm into unfertilized eggs induces egg activation accompanied by a Ca^{2+} wave (Yamamoto et al. 2001; Harada et al. 2007, 2011). A sperm-specific form of citrate synthase is purified from the sperm extract as one of the major components of the sperm factor for egg activation (Harada et al. 2007). A large amount of citrate synthase is localized in the neck to the midpiece of newt sperm (Fig. 15.2A), but a smaller amount is also distributed under the plasma membrane around the nucleus (Fig. 15.2B). Injection of not only purified citrate synthase protein, but also mRNA of citrate synthase, induces egg activation with a Ca^{2+} increase (Harada et al. 2007). A single newt sperm contains about 2 pg citrate synthase, but injection of sperm cytoplasm equivalent to one sperm activates about 20 % of the eggs, indicating that the entry of at least two sperm is necessary for activating the egg. This estimation corresponds well to the observation that a small Ca^{2+} wave is induced by each sperm entry in the polyspermic newt egg (Harada et al. 2011). How does the sperm-specific citrate synthase induce the Ca^{2+} wave in the egg cytoplasm? In some cases, the Ca^{2+} wave is preceded by a small spike-like Ca^{2+} increase (Harada et al. 2011). The sperm tryptic protease seems to be involved in the small and nonpropagative Ca^{2+} increase, but this is insufficient for inducing the Ca^{2+} increase to cause egg activation, probably because of the lack of cortical ER in newt eggs. The inner ER forms a larger complex with some cytoskeletons and is required to trigger a Ca^{2+} wave by the sperm factor (Harada et al. 2011). Egg activation not only by injection of the sperm factor, but also by fertilizing sperm, is probably mediated by the enzymatic activity of sperm citrate synthase (Harada et al. 2011). Reactive substrates of citrate synthase, acetyl CoA and oxaloacetate, induce Ca^{2+} increase to cause egg activation, but citrate does not. The reverse reaction might occur in egg cytoplasm containing a large amount of citrate, and acetyl CoA might then sensitize IP3 receptors to release Ca^{2+}. Further investigation is, however, necessary for determining the exact changes of those substances at fertilization. Furthermore, it is possible that citrate synthase interacts with other molecules such as cytoskeletons (see Fig. 15.1; Iwao and Masui 1995). Investigations into the role of microtubules and microfilaments are important for clarifying the Ca^{2+}-signaling cascade by the sperm factor.

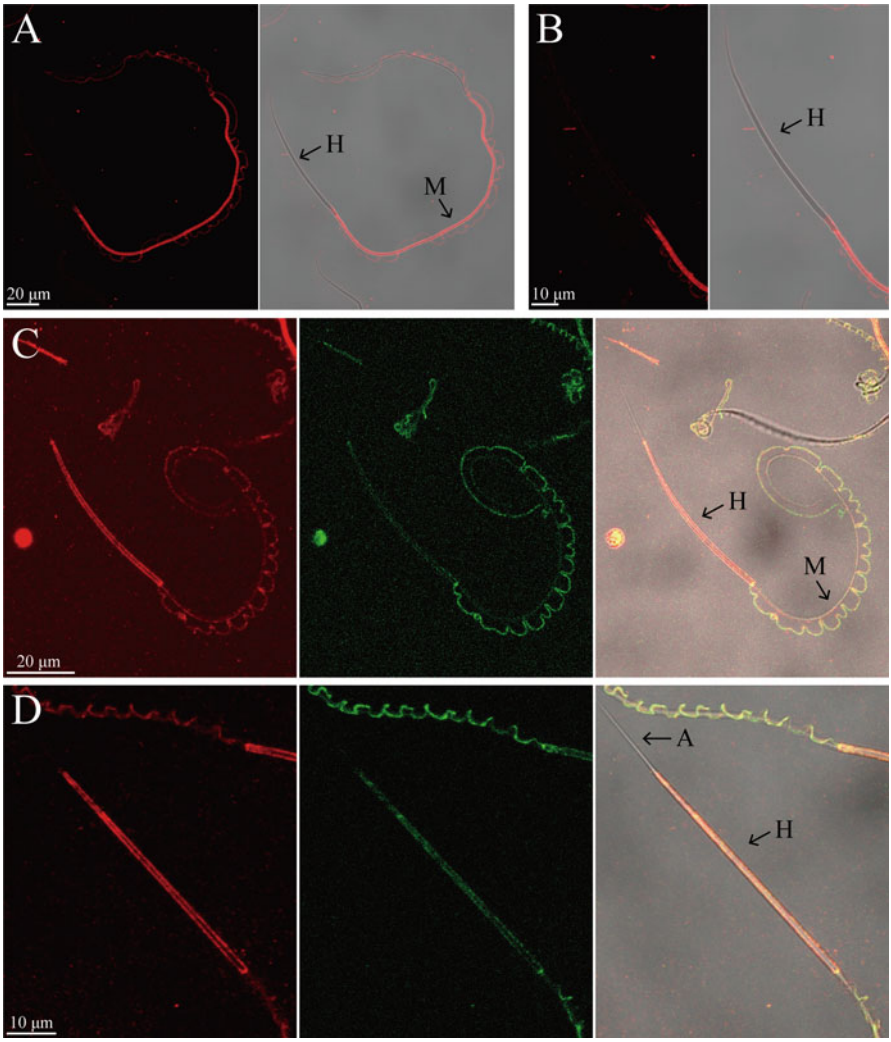

Fig. 15.2 (**A**), (**B**) Newt *Cynops pyrrhogaster* sperm show localization of citrate synthase (*red*) on *left* and merge with the differential interference contrast (DIC) image on *right*. (**C**), (**D**) Salamander *Hynobius nebulosus* sperm show citrate synthase (*red*) on *left*, α-tubulin (*green*) in *middle*, and merge with DIC image on *right*. *A* acrosome, *H* head region, *M* midpiece

15.4 Evolution of a Sperm Factor in Vertebrate Fertilization

It is worth discussing the species specificity of sperm factors to understand the evolution of egg activation in vertebrates. Although it is reported that extract of the monospermic *Xenopus* sperm induces Ca^{2+} oscillation when injected into mouse eggs (Dong et al. 2000), and the injection of several sperm into a *Xenopus* egg causes egg activation (Aarabi et al. 2010), no activity to activate *Xenopus* eggs is

detected in homologous sperm extract (Harada et al. 2011). *Xenopus* eggs do not respond to the newt sperm factor, and no citrate synthase is detected in *Xenopus* sperm (Table 15.1). Not only polyspermic newt sperm, but also monospermic *Hynobius* sperm, contain a large amount of citrate synthase under the plasma membrane in the head region, except for the acrosomal region (Fig. 15.2C). Citrate synthase is distributed in close association with microtubules (Fig. 15.2D). A large amount of sperm citrate synthase is observed in mammalian mouse sperm, but not in fish carp sperm (Iwao and Harada, unpublished observations, 2011). Thus, the extramitochondrial localization of citrate synthase in the sperm appears to be acquired in the transition between monospermy and physiological polyspermy in urodele amphibians.

Taken together, the large and single Ca^{2+} wave induced by the first sperm entry is necessary for ensuring monospermy to elicit the positive fertilization potential mediated by Ca^{2+}-activated Cl^- channels in monospermic vertebrates, such as lampreys (Kobayashi et al. 1994), frogs, and *Hynobius* salamanders (Table 15.1). In the bony fishes, a single Ca^{2+} wave is induced by a fertilizing sperm (Gilkey et al. 1978; Webb and Miller 2013), but monospermy is ensured by a micropyle (canal) on the hard chorion, through which only one sperm approaches the egg (Iwamatsu 2000). Thus, the single Ca^{2+} wave at egg activation is characteristic of monospermic vertebrates (Iwao 2012). In this connection, it is interesting to know the Ca^{2+} increase at the physiological polyspermy of large eggs in sharks and chimera (Hart 1990). In contrast, multiple Ca^{2+} waves are necessary for egg activation in physiological polyspermy because a single newt sperm does not have a sufficient amount of sperm factor to induce egg activation and multiple Ca^{2+} increases are necessary for complete activation of the large eggs (Iwao 2012). Some transitional characteristics are, however, observed in occasionally polyspermic eggs of the frog *Discoglossus picutus* with multiple Ca^{2+} increases (Talevi 1989), or in external and polyspermic fertilization in the Japanese giant salamander *Andrias japonicus* (Table 15.1) (Iwao 2000a). Physiological polyspermy probably appeared in species whose egg size was more than about 2 mm in diameter (Table 15.1). Reptiles and birds lay larger and yolky eggs, but their Ca^{2+} increase at polyspermy remains to be investigated. In primitive mammals, the monotrematous platypus laying big eggs exhibits physiological polyspermy (Gatenby and Hill 1924). Although a small and yolkless egg of the higher eutherian mouse exhibits monospermy, it elicits multiple Ca^{2+} increase to ensure sufficient egg activation (Ozil 1990; Ducibella et al. 2002). Sperm-specific PLCζ is known as a potent sperm factor for egg activation in mammals (Saunders et al. 2002; Kouchi et al. 2004) and birds (Mizushima et al. 2009). In mammalian egg activation, the role of sperm citrate synthase remains unknown.

15.5 Perspective

Thus, comparative studies in fertilization among vertebrates provide better understanding of the role of egg activation in establishment of polyspermy during evolution. Because egg activation by sperm citrate synthase is tightly linked to slow egg

activation in physiological polyspermy, investigations in polyspermic birds and reptiles are important to clarify the evolution of egg activation in vertebrates. It is also interesting to know the mechanisms of egg activation in bony fishes that exhibit monospermy but lack the fast electrical block to polyspermy. In addition, investigations in invertebrates, such as ascidians and sea urchins, may provide us with the ancestral and universal mechanisms of egg activation during the evolution of animal reproduction.

Acknowledgments We thank Tomoyo Ueno for her help on preparing the manuscript. 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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