—Mini Review—

Mammalian Sperm Factor and Phospholipase C Zeta

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Abstract: *In mammals, eggs exhibit a series of transient increases in intracellular calcium ion (Ca^{2+}) concentrations (Ca^{2+} oscillations) at the time of fertilization, which are prerequisite and sufficient for egg activation. Recent studies have been revealing the molecular mechanism of how sperm induce Ca^{2+} oscillations in fertilized eggs, that has remained unclear for a longtime. Especially in mammals, the sperm factor theory is being confirmed, which postulates that a cytoplasmic factor in the spermatozoon (the "sperm factor") is introduced into the egg through the sperm-egg cytoplasmic connection and activates the egg. In 2002, a new isoform of phospholipase C (PLC), PLC zeta, was identified as a strong candidate for the mammalian sperm factor. In this mini-review, the history of the mammalian sperm factor theory and the search for the mammalian sperm factor is reviewed. In addition, whether PLC zeta is truly the mammalian sperm factor or not is discussed.*

Key words: *Fertilization, Calcium, Oscillations, Sperm, Phospholipase*

Introduction

In almost all animals, including mammals, an egg exhibits a rise of intracellular calcium ion (Ca^{2+}) concentrations at the time of fertilization [34, 46, 52]. Immediately after the sperm attaches to the egg, increases in intracellular Ca^{2+} in the egg are first seen near the site of sperm-egg fusion, then spread throughout the egg in a wave (Ca^{2+} wave) in echinoderms, ascidians, and vertebrates. The velocity of the Ca^{2+} wave, the first Ca^{2+} rise in fertilized mouse eggs, is approximately 20 μm/sec [3]. Among mammals, mouse fertilized eggs exhibit a series of Ca^{2+} rises (Ca^{2+} oscillations), which are sustained for 3–4 hours [3], while the rise in bovine eggs lasts longer than 20 hours [36]. Increases in Ca^{2+} activate the egg machinery inducing the cortical reaction, the release from Metaphase II arrest; this signal is critical for the normal development of a fertilized egg into an embryo [16, 41, 62]. Numerous granules (cortical granules) containing proteases, peroxidases, and other enzymes reside beneath the plasma membrane of the unfertilized egg [1, 9]. Ca^{2+} induces the exocytosis of these cortical granules, releasing the enzymes into the perivitelline space. These enzymes modify the *zona pellucida*, transforming it into the fertilized form. This modification forms the molecular basis for the rejection of additional sperm access (polyspermy block) [10, 57, 61]. In both mammals and other vertebrates, the mature oocyte cell cycle is stopped at the metaphase of mitosis II (MII arrest), due to the high activity of maturation promoting factor (MPF) that persists until the egg is fertilized. In the fertilized egg, Ca^{2+} activates the ubiquitin/proteosome pathway to induce the degradation of cyclin B1, a component of the active MPF complex, reducing MPF activity [11, 38]. The cell cycle of the fertilized egg then restarts, and the egg protrudes the second polar body, completing mitosis. Increases in intracellular Ca^{2+} in the fertilized egg also activate calmodulin, the most abundant modulator of Ca^{2+} signaling in the cell, and the activated calmodulin triggers Ca^{2+} calmodulin-dependent protein kinase II (CaMKII), inducing several but as-yet unidentified enzymes that are critical for embryonic development [11, 30].

How Does the Sperm Activate the Egg?

One of the most important questions remaining in biology is, "How does the sperm activate the egg?" In essence, this question really asks, "How does the sperm induce a rise in intracellular calcium

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concentration in the fertilized egg?" This question has remained unanswered for many decades; the molecular mechanisms governing sperm-induced activation of the egg, however, are slowly being revealed by recent studies [46].

Studies by Miyazaki and his colleagues examining the molecular mechanisms by which sperm induce a rise in Ca^{2+} in fertilized hamster eggs indicated that Ca^{2+} is released from the endoplasmic reticulum (ER) through the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) [35]. Using a monoclonal antibody against IP_3R type 1 that specifically inhibits the function of this receptor, they demonstrated that the hamster eggs injected with this antibody did not exhibit any Ca^{2+} oscillations after insemination. Together with data demonstrating that injection of IP_3 can parthenogenetically activate mammalian eggs of multiple species, including humans [8], mice [17, 39], hamsters [33], bovines [36], rabbits [4], and horses [28], it has become widely accepted that sperm induce Ca^{2+} release from ER in mammalian eggs through activation of the IP_3 receptor. It is now necessary to address the mechanisms by which the sperm induce Ca^{2+} release through IP_3R.

Two theories have been proposed to explain the induction of Ca^{2+} release in fertilized eggs following fertilization; the sperm receptor theory and the sperm factor theory. The sperm receptor theory is a by-product of scientific progress examining the hormone receptor. Despite efforts to identify the sperm receptor, no molecule has been yet been identified on the egg plasma membrane. Foltz *et al.* reported the identification of a sperm receptor on a sea urchin egg [5]. This molecule, however, does not appear to be capable of transducing the fertilization signal to the egg [14]. In addition to sea urchins, *Xenopus* sperm appear to have the ability to activate the egg through a sperm receptor on the egg surface [47].

In contrast, the sperm factor theory postulates that a cytoplasmic factor in the spermatozoon (the "sperm factor"), which is introduced into the egg through the sperm-egg cytoplasmic connection, activates the egg. This theory was not seriously considered, until Swann implicated an egg-activating protein in the cytoplasm of hamster sperm in 1990 [53]. Injection of sperm extract, a soluble fraction prepared from hamster sperm, into unfertilized eggs demonstrated that the injected eggs exhibited the Ca^{2+} oscillations characteristic of fertilized mammalian eggs. Egg activation by sperm factor has also been seen in newts [60], ascidians [24], and nematodes [51], in addition to mammals.

Sperm Factor Theory in Mammals

In the 1990's, evidence accumulated for the sperm factor theory, especially in mammals. Mouse eggs subjected to intra-cytoplasmic sperm injection (ICSI) exhibit Ca^{2+} oscillations, which resemble those induced by sperm at the time of fertilization [22, 37, 48]. These oscillations occur in the absence of the interaction of the sperm with the egg plasma membrane. This result contradicts the sperm receptor theory, in which sperm-egg interactions at the plasma membrane are required for sperm-induced Ca^{2+} oscillations.

The study of knockout mice has also provided strong evidence for the sperm factor theory. CD9 is a surface molecule required for cell-cell fusion of osteoclasts [56] and myoblasts [54], whereas it prevents fusion of monocytes and alveolar macrophages [55]. In 2000, three laboratories independently generated CD9-deficient mice. CD9-deficient female mice are nearly infertile. Although sperm from CD9-deficient male are fertile, the CD9-deficient eggs cannot fuse with sperm [15, 27, 32]. Kaji *et al.* showed that only a small subset of CD9-deficient eggs (less than 5%) was competent to fuse with sperm, and only those eggs exhibited Ca^{2+} oscillations when inseminated [15]. Their result indicates that sperm-egg fusion is a prerequisite for Ca^{2+} oscillation. Using fluorescent probe transfer, Lawrence *et al.* demonstrated that sperm-egg fusion precedes Ca^{2+} oscillations during mouse fertilization [26]. Together, these results strongly suggest that sperm-egg fusion precedes Ca^{2+} oscillations, supporting the sperm factor theory. Kumakiri *et al.* demonstrated that *Clostridium difficile* toxin B inhibits sperm-egg fusion and that this toxin also severely disturbed the Ca^{2+} oscillations induced by insemination. In the presence of this toxin, approximately half of the sperm "kissed" the egg, fusing once, but lost the cytoplasmic connection. As the sperm was not incorporated into the egg, this toxin severely disturbed the generation of Ca^{2+} oscillations [21].

These findings strongly support the sperm factor theory and researchers have tried to identify the sperm factor originally described by Swann in 1990 [53]. To identify sperm factor in hamster sperm, sperm were first isolated from the epididymis of male hamsters and carefully washed with saline to remove seminal plasma. Then, the sperm were disrupted by ultra-sonication and fractionated by ultracentrifugation. The prepared soluble cytosolic fraction was designated sperm extract.

In 1996, a mammalian homologue of *E. Coli* glucosamine-6-phosphate isomerase, dubbed oscillin,

was reported to be the sperm factor in hamster sperm extract [42]. Oscillin was biochemically purified from hamster sperm extract. As oscillin localizes in the equatorial regions of hamster, human, and boar sperm, the region at which mammalian sperm are believed to fuse first with the egg [63], oscillin was a strong candidate for mammalian sperm factor. Recombinant oscillin, however, failed to induce Ca^{2+} oscillations in unfertilized mouse eggs, which eliminated oscillin as a candidate for mammalian sperm factor [50, 58, 59].

Phospholipase C ζ

In mammals, sperm induce Ca^{2+} release from the ER via activation of IP_3R [34]. Thus, the most likely action of sperm factor in the egg cytoplasm is the production of IP_3, and a likely candidate, therefore, for sperm factor would be phospholipase C (PLC), as PLC is the only enzyme known to produce IP_3 in living cells. Recombinants of all known PLC isozymes failed to induce Ca^{2+} oscillations in unfertilized mouse eggs [12]. These results suggested that none of the known PLCs were the mammalian sperm factor, though it remained possible that a new type of PLC might be the sperm factor in mammals, if this factor were indeed a PLC isoform.

Based on the assumption that mammalian sperm factor is a PLC, Swann and his colleagues searched the EST database of the mouse testis, seeking to find a new PLC isoform. In 2002, they identified a new PLC expressed only in the testis, designated PLC ζ (PLCZ) [49].

PLCZ possesses almost all of the domains of PLC delta (PLCD), including EF hand-like domains, catalytic X and Y domains, a linker sequence, and a C2 domain at the C-terminus, though it lacks the pleckstrin homology (PH) domain. The PH domain is thought to be essential for PLCD targeting the plasma membrane in response to extracellular signals. A fusion of PLCD and a fluorescent protein, Venus (PLCD-Venus), targets the plasma membrane when expressed by RNA injection in unfertilized eggs [64]. As PLCZ lacks the PH domain, a fusion of PLCZ and Venus (PLC-Venus) distributed evenly throughout the cytoplasm following RNA injection into unfertilized mouse eggs [64]. The EF hand-like domain is critical for calmodulin detection of Ca^{2+} rises and resulting activation. The X and Y domains, which are highly conserved among species, are catalytic domains essential for PLC activity. These domains are followed by a linker sequence, which contains a nuclear localization sequence (NLS). The amino acid sequence of the linker sequence varies greatly among mammalian species [2]. The structure of PLCZ is very similar to that of PLCD1, with the exception that PLCZ lacks an N-terminal PH domain [44].

Following injection of polyadenylated PLCZ cRNA into unfertilized mouse eggs, PLCZ was efficiently expressed [64]. Exogenous PLCZ expression induced Ca^{2+} oscillations in mouse eggs. The delay between the time of injection and the start of the induced Ca^{2+} oscillations decreased and the frequency of Ca^{2+} spikes increased with increasing concentrations of PLCZ cRNA injected. In contrast, injection of PLCD cRNA was much less effective, requiring greater than 50-fold more PLCD cRNA than PLCZ cRNA to induce Ca^{2+} oscillations (unpublished data). Kouchi *et al.* produced a recombinant PLCZ protein using a baculovirus system. The recombinant PLCZ induced Ca^{2+} oscillations in unfertilized mouse eggs, in a similar manner to injected hamster sperm extract [20]. These results indicate that PLCZ can induce Ca^{2+} oscillations in unfertilized mouse eggs, supporting the candidature of PLCZ as the mammalian sperm factor.

If PLCZ is the mammalian sperm factor, it should exhibit high PLC activity at resting Ca^{2+} concentrations. After sperm-egg fusion, sperm factor would be introduced into the egg, which is not yet activated. In the unactivated cells, the Ca^{2+} concentration is approximately 100 nM. The activities of all known PLC isoforms are low at Ca^{2+} concentrations of 100 nM. Therefore, Kouchi *et al.* produced recombinant PLCZ and PLCD proteins and investigated the Ca^{2+} dependence of their PLC activity *in vitro*. The PLC activity of PLCZ was highly Ca^{2+} sensitive, it could be activated at very low Ca^{2+} concentrations (below 100 nM), while the PLC activity of PLCD was minimal at such low Ca^{2+} concentrations [20]. The high Ca^{2+} sensitivity of PLCZ is an important feature of this protein suggesting that it might be the mammalian sperm factor.

When the cytoplasm of a fertilized egg in anaphase or telophase is transferred into an unfertilized egg, the recipient egg exhibits increases in Ca^{2+} and the production of the pronucleus, classic indications of egg activation. The cytoplasm of a fertilized egg that retains a pronucleus (an egg at interphase), however, does not possess this ability to induce Ca^{2+} increases. Kono *et al.* were the first group to report that the pronucleus of a fertilized mouse egg possesses egg-activating activity. Following transplantation of the pronucleus of a fertilized egg into an unfertilized egg, the transplanted egg was activated, exhibiting increases in intracellular

Ca^{2+}. These Ca^{2+} increases occurred only when the transplanted pronucleus was disrupted during the manipulation [19]. Ogunuki *et al.* confirmed these results, confirming that the egg-activating activity of the mouse fertilized egg accumulates in the pronucleus [40]. These results suggest that mammalian sperm factor accumulates in the pronucleus. The proposed model suggests that mammalian sperm factor would be introduced into the egg cytoplasm through sperm-egg cytoplasmic connections, initially distributing evenly throughout the cytoplasm. When the egg enters the interphase, resulting in the formation of pronuclei, the sperm factor would then accumulate in the pronuclei.

Accumulation into the pronucleus is a unique feature of mammalian sperm factor. If PLCZ is the mammalian sperm factor, it is expected that this protein would accumulate in the pronucleus. Yoda *et al.* synthesized cRNA encoding a fusion of PLCZ and Venus, injected this cRNA into unfertilized mouse eggs, and examined the real-time localization of PLCZ in activated eggs. Injection of PLCZ-Venus cRNA activated the injected eggs, inducing the formation of pronuclei. Continuous observation of those eggs by fluorescence microscopy clearly demonstrated that the exogenously expressed PLCZ-Venus distributed evenly throughout the cytoplasm before the formation of pronuclei, then accumulated in the pronuclei after their formation [64]. The accumulation of PLCZ in the pronucleus was also reported by Larman *et al.* using a myc-tagged PLCZ [25].

Sperm-induced Ca^{2+} oscillations cease at the time of pronuclei formation and the cessation of Ca^{2+} oscillation with appropriate timing is required for the normal development of the mammalian embryo [7]. Marangos *et al.* reported that pronucleus formation is required for the cessation of Ca^{2+} oscillations [29]. Larman *et al.* constructed a PLCZ mutant which did not localize in the pronucleus, and this mutant induced Ca^{2+} oscillations that did not terminate with pronucleus formation [25]. These results strongly suggest that the sequestration of PLCZ into the pronucleus results in cessation of Ca^{2+} oscillations in the fertilized eggs. However, Yoda *et al.* reported that the time at which PLCZ-induced Ca^{2+} oscillations stoped did not correspond exactly with the time of pronucleus formation. Ca^{2+} oscillations terminate significantly before pronucleus formation in some eggs, while in others they do not stop until after pronucleus formation [64]. This observation suggests that there may be significance for the localization of PLCZ in the pronucleus other than the termination of Ca^{2+} oscillations.

In addition to results supporting the candidature of PLCZ as the mammalian sperm factor, antiserum against PLCZ was reported to neutralize the sperm factor activity of hamster sperm extracts [49]. This experimental result, which has been confirmed by Fujimoto *et al.*, is the most convincing evidence that PLCZ is the mammalian sperm factor [6].

Is PLCZ Truly the Mammalian Sperm Factor?

A number of research studies have shed doubt on the identity of PLCZ as mammalian sperm factor. Mehlmann *et al.* measured the amount of PLC activity in a single mouse spermatozoon, reporting that a single mouse spermatozoon contained too little PLC activity to induce Ca^{2+} oscillations in unfertilized mouse egg [31]. Rice *et al.* reported contradictory results indicating that a single mouse spermatozoon contained sufficient PLC activity [45]. The measured activity published by Rice *et al.* was more than 100 times that determined by Mehlmann *et al.* As both experimental methods employed recombinant PLCγ as a control in the measurements, both measurements appear to be reliable, so it is unclear whether sufficient PLC activity is found in mouse sperm. Further studies are necessary to determine if additional factors, which modify the PLC activity in these sperm samples, are present.

Kurokawa *et al.* reported that the distribution of the sperm factor activity corresponds roughly, but not exactly, with that of PLCZ after fractionation of porcine sperm extracts by chromatography [23]. Although it remains possible that PLCZ might undergo some modification, such as phosphorylation, influencing the activation state of the molecule, these studies shed some doubt on the identity of PLCZ as the mammalian sperm factor.

Perry *et al.* fractionated mouse sperm, hypothesizing that egg-activating activity corresponding to sperm factor, requires two distinct factors, designated sperm-borne oocyte activating factors I and II, SOAF-I and SOAF-II. Each SOAF was necessary, but not sufficient, to induce egg activation [43]. The author and collaborators obtained results supporting the synergistic action of two distinct components of mammalian sperm factor. The sperm factor activity in hamster sperm extracts was separated into distinct two fractions, which exhibited no activity in isolation, but demonstrated a synergistic effect when injected together (unpublished data). It is likely that a complex of unknown composition is the active factor in sperm extracts and it will be necessary in the future to determine the

relationships between SOAF I, SOAF II, PLCZ, and the two components of the hamster sperm factor fractionized by our group and their relative representation in the sperm factor complex.

All PLCs, including PLCZ, hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce diacylglycerol (DAG) and IP_3, which binds to IP_3R (type 1) to induce Ca^{2+} release from ER. Therefore, PIP_2 is necessary to mediate the release of calcium from the ER induced by PLC. All known PLCβ and PLCγ isoforms are positioned beneath the plasma membrane, whereas PLCD translocates to the plasma membrane in response to a cell activation signal [44]. At the plasma membrane, PLC hydrolyzes PIP_2 in the plasma membrane to produce IP_3. If PLCZ produces IP_3 in the same manner, PIP_2 must be present at the site at which PLCZ acts within the egg. In hamster and mouse eggs, the Ca^{2+} waves induced by sperm, ICSI, or sperm extract injection propagate not over the surface of the egg, but also through the deep cytoplasm of the egg [39, 48]. When hamster sperm extract is injected into the central part of the egg, the Ca^{2+} wave is initiated from that site, spreading to the egg surface [39]. These results suggest that PIP_2 may be included in membrane structures present within the egg cytoplasm, such as the ER or nuclear membranes, although only the nuclear membrane is known to contain PIP_2 in most cells. In addition, unfertilized mouse eggs do not have a nuclear membrane, because they are arrested in metaphase II. Although it is suggested that the yolk membrane of sea urchin eggs may serve as a source of PIP_2 [45], it remains possible that an unknown intracellular membrane structure exists in the egg, which serves as the source of PIP_2 for PLCZ action.

Knott *et al*. produced transgenic mice expressing short hairpin RNAs specific for PLCZ mRNA. Sperm from these transgenic animals induced a small number of Ca^{2+} rises, but not persistent Ca^{2+} oscillations. In a fraction (20%) of eggs inseminated with sperm from the transgenic mice, sperm and egg fusion proceeded normally, but egg activation did not occur; the process of fertilization was thus arrested with a very large fertilization cone [18]. To confirm that PLCZ is at least part of the mammalian sperm factor complex, it will be necessary to show that a spermatozoon lacking PLCZ fails to induce Ca^{2+} oscillations, are necessary. It is expected that sperm from mice lacking PLCZ will not induce Ca^{2+} oscillations after fusion with an egg. It will also be necessary to demonstrate that sperm extracts prepared from PLCZ-deficient sperm lack the ability to induce Ca^{2+} oscillations after injection into unfertilized mouse eggs.

As reported by Kurokawa *et al*., the distribution of sperm factor activity and PLCZ did not correspond exactly in chromatography, suggesting the presence of additional components of mammalian sperm factor. Before PLCZ can be utilized as a physiological activator for fertilized eggs in clinical medicine, it will be necessary to clarify the mechanisms and the molecular characteristics of mammalian sperm factor. In addition, all of the effects of PLCZ in eggs must be clarified completely, to avoid any unexpected results in the utilization of sperm factor.

Outlook

Currently, several important questions concerning mammalian sperm factor remain to be addressed and the molecular mechanisms governing PLCZ-induced Ca^{2+} release and Ca^{2+} oscillations remain to be identified. Jones and Nixon indicated that continuous production of low levels of IP_3 can mimic sperm-induced Ca^{2+} oscillations in mouse eggs [13], suggesting that continuous PLCZ activity in the egg cytoplasm would result in persistent Ca^{2+} oscillations. The mechanism by which PLCZ can produce IP_3 in the deep cytoplasm of the egg, however, is still unclear. In addition, the localization of PLCZ within a spermatozoon requires additional investigation. Although Fujimoto *et al.* reported the localization of PLCZ in the post-acrosomal region of mouse sperm [6], a precise investigation by immuno-electron microscopy is required, which will also hopefully reveal why PLCZ is quiescent in sperm prior to fusion.

For normal activation of the fertilized egg and normal development into an embryo, Ca^{2+} oscillations must begin after sperm-egg fusion and continue until the pronuclei formation, at which point the oscillations should terminate. When the molecular characteristics of PLCZ and the machinery governing PLCZ activation and inactivation are revealed, allowing PLCZ activity to be completely controlled, PLCZ will likely be an effective and safe physiological activator for eggs in the fields of fertility treatment and animal husbandry.

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