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Review

Intracellular calcium signaling in the fertilized eggs of Annelida

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ABSTRACT

Fertilization is such a universal and indispensable step in sexual reproduction, but a high degree of variability exists in the way it takes place in the animal kingdom. As discussed in other reviews in this issue, recent works on this subject clarified many points. However, important results on the mechanisms of fertilization are obtained mainly from a few restricted model organisms. In this sense, it is utterly important to collect more information from various phyla. In this review, we have re-introduced Annelida as one of the most suitable models for the analysis of fertilization process. We have briefly reviewed the historical works on the fertilization of Annelida. Then, we have described recent findings on the two independent Ca^{2+} increases in the fertilized eggs of Annelida, which arise from two different mechanisms and may have distinct physiological roles toward sperm entry and egg activation. We propose that the Ca^{2+} increase in the fertilized eggs reflect the specific needs of the zygote in a given species.

Contents

1. Introduction

During fertilization, a successful spermatozoon binds to the surface of the oocyte, passes though the egg envelopes, and enters the egg. The essential parts of fertilization that still remain largely a mystery are the fusion and incorporation of the spermatozoon into the egg. The egg is activated during these processes, but the exact changes that take place in the egg at each stage of egg activation is largely unknown. These events proceed in a short period of time, and it is not easy to analyze each step. In sea urchin eggs, interdisciplinary approaches combining electrophysiology, ultrastructural analysis, and fluorescence microscopy with the use of DNA-specific fluorochrome to clarify the sequence of events from sperm binding, gamete membrane fusion and egg activation [1–6].

Similar to the case of sea urchin gametes, Annelida is convenient experimental system because fully grown fertilizable gametes are easily available on schedule. In the case of *Nereis limbata*, mature warms with fully grown gonads swim up to the surface of the sea for reproduction during their breeding season at the precise time, which correspond to the phase of the moon [7]. Swarming worms are ready to release fertilizable gametes [8], and it is extremely easy to obtain matured oocytes and sperm for the fertilization study.

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Similar behavior of the swarming individuals is observed with Japanese Palolo, *Tylorrynchus heterochaetus* [9].

The fine surface structures of the Annelida oocytes have been reported in several species before and after fertilization. The egg surfaces of *Hydroides hexagonus* and *N. limbata* show thick egg envelopes and a myriad of microvilli protruding from the oolemma towards the egg envelope [10,11]. In *Neanthes japonica* and *T. heterochaetus*, the tips of microvilli are exposed to the outer surface of the egg envelope, which makes a specialized structure with which the fertilizing sperm interact [12,13]. At first, the fertilizing spermatozoon, attaches to the tip of the microvilli exposed on the egg envelope in *Chaetopterus pergamentaceus* and *T. heterochaetus* [12–16]. During the sperm penetration into the oocyte, the cortical protrusion from the egg surface grows up in the peri-vitelline space. The spermatozoon penetrates into the oocyte though the egg envelope and the perivitelline space owing to this cortical protrusion [17].

On the surface of the oocytes in many species, there are several layers surrounding them. The fertilizing spermatozoon passes though the layers by its own intrinsic capability or aided by the positive response of the egg surface. The egg envelope lysine in abalone and the specific protease, acrosin, in mammalian sperm are the examples for the former case [18–21]. A remarkable example of the positive response of the egg surface towards the incorporation of spermatozoon is in the elongation of microvilli and formation of cortical protrusion. Sperm engulfing response by microvilli and the development of fertilization cone are observed on the surface of the oocyte in mammalian and echinoderm oocytes, respectively [22–24]. To induce these responses on the surface of the oocyte, actin microfilaments in the microvilli and cortex of the oocyte are coordinately reorganized [24,25]. Indeed, sperm incorporations into sea urchin eggs and starfish oocytes are inhibited when they are inseminated in seawater containing cytochalasin B. Implying a similar role, the actin cytoskeleton is well developed in microvilli of annelid oocytes [26]. During fertilization, complex interactions among the fertilizing spermatozoon, egg envelopes, and egg surface with microvilli take place in a short period of time. Fertilization in Annelida illustrates drastic gamete interactions, as the egg surface with microvilli responds to the fertilizing sperm with a dynamic rearrangement of the actin cytoskeleton and a series of explosive intracellular Ca^{2+} increases.

2. Plural Ca^{2+} changes proceed in an oocyte during fertilization

The relationship between the intracellular Ca^{2+} increase and egg activation was first shown by Mazia [27]. The pattern of the Ca^{2+} changes in the oocyte fertilized by sperm is different in each species. However, the studies of the intracellular Ca^{2+} change in many species have shown that it signals re-initiation of meiosis or embryonic development [28–38]. Artificial induction of Ca^{2+} increase in the oocyte induced parthenogenetic activation of the oocytes without sperm [39,40]. These facts indicate that Ca^{2+} increase in the oocyte may be the necessary and sufficient condition for the egg activation at fertilization.

At the fertilization of deuterostome oocytes, Ca^{2+} increase starts at the sperm-interaction site and propagates to the entire oocyte as a Ca^{2+} wave [28,41]. The Ca^{2+} increase mainly derives from the endoplasmic reticulum (ER), which is the major Ca^{2+} store in the cytoplasm of oocyte, though the inositol 1,4,5-trisphosphate (IP_3) dependent Ca^{2+} releasing pathway [42]. IP_3 is produced in the oocyte from phosphatidylinositol 4,5-bisphosphate (PIP_2), which is one of the components of the plasma membrane, by the catalytic reaction of phospholipase C (PLC) in the oocyte [43,44].

Concerning the intracellular of Ca^{2+} stores contributing to the Ca^{2+} increase at fertilization, not only IP_3-dependent Ca^{2+} release but also other Ca^{2+}-releasing mechanisms such as cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate (NAADP)-dependent Ca^{2+} release are at work in the oocytes of sea urchin, starfish, and ascidian [45–52].

In the case of protostomes, Ca^{2+} influx from the outside of the mollusk oocyte has a major contribution to the increase of intracellular Ca^{2+} in the fertilized eggs [53–56]. On the other hand, the Ca^{2+} increase in the deuterostome eggs at fertilization has two components: (i) the Ca^{2+} release from the internal stores and (ii) the Ca^{2+} influx at the entire cortex as 'cortical flash' shortly after the arrival of the fertilizing spermatozoon on the surface. The voltage dependent Ca^{2+} channels on the plasma membrane of the oocyte are involved in the cortical flash in the cortex of the oocyte [46,54,56,57]. In mammals, store-operated Ca^{2+} influx is involved in the Ca^{2+} oscillations during fertilization in pig oocyte [58]. In protostomes, IP_3-dependent Ca^{2+} release from the inside of the oocyte take place in the nemertean worm and the Japanese clam [36,59].

Therefore, several different Ca^{2+} releasing mechanisms function in an oocyte within a short period during fertilization. It is necessary to clarify the distinct roles that each Ca^{2+} increase work independently for the different cell function or it covers each other and cooperate together.

3. A unique Ca^{2+} change in the fertilized eggs of Annelida

The Ca^{2+} increase at fertilization in Annelida has been studied in *C. pergamentaceus*, *Urechis caupo*, and *Pseudopotamilla occelata* [34,53,60–62]. In *C. pergamentaceus* and *P. occelata*, fertilization takes place at metaphase I of the meiotic division. The first Ca^{2+} response in these eggs is restricted to a localized Ca^{2+} increase at the cortex of the oocyte that doesn't propagate to the entire oocyte [34,62]. Then, the global Ca^{2+} increases follow with the repetitive Ca^{2+} oscillations as was reported in *C. pergamentaceus* [34] .

As the type 1 IP_3 receptor was detected in *C. pergamentaceus*, the Ca^{2+} release through the type-1 IP_3-gated Ca^{2+} channels are thought to be involved at fertilization [61]. At the same time, the membrane potential change by hyperdepolarization takes place during fertilization in *C. pergamentaceus* oocyte. The Ca^{2+} influx through voltage dependent Ca^{2+} channels are involved in the increase of Ca^{2+} in these oocytes [63]. Artificial induction of hyper-depolarization in the plasma membrane by adding excess potassium in medium induced the Ca^{2+} increase in the oocyte and re-initiation of meiosis [34,64,65]. These works indicate that plural Ca^{2+} releasing mechanisms increase the intracellular Ca^{2+} by use of the Ca^{2+} stores both inside and outside of the fertilized oocytes of Annelida.

4. Involvement of Ca^{2+} increase in sperm incorporation and egg activation

Resent principal topics on the Ca^{2+} response in the fertilized eggs include: (i) how several different types of Ca^{2+} increases are regulated in a oocyte, (ii) how these Ca^{2+} increases are triggered by the fertilizing spermatozoon, and (iii) what is the role of each Ca^{2+} increase towards successful fertilization.

Two major theories proposed for how sperm induces the release of intracellular Ca^{2+} in the oocyte are the "sperm factor theory" and "sperm receptor theory" [38,66–68]. In the former case, a molecule involved in the Ca^{2+} increase in an oocyte is introduced directory from the fertilizing spermatozoon by gametes membrane fusion. In the latter case, molecule(s) from the spermatozoon may bind to the specific receptor(s) on the oocyte surface as the "ligand" that triggers a signaling cascade through the plasma membrane. It is extremely difficult to distinguish the gametes membrane fusion

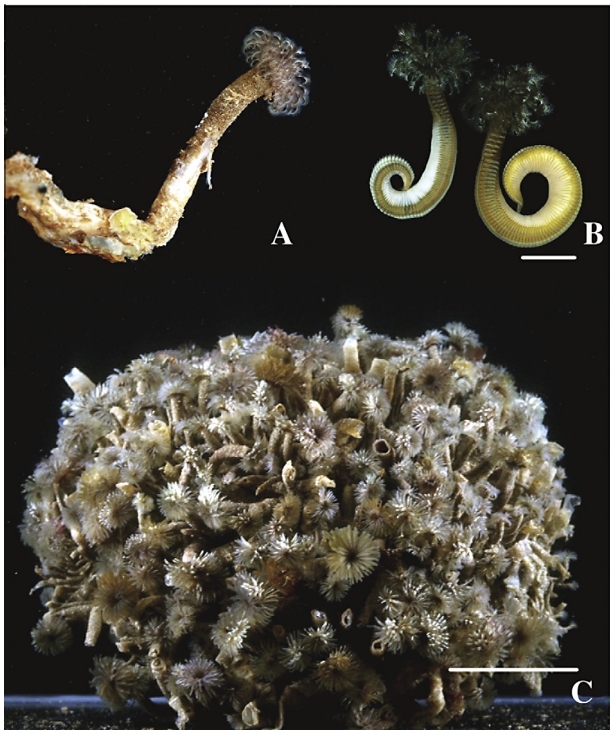

Fig. 1. Individual and colony of Annelida *Pseudopotamilla occelata*. (A) An isolated individual of *P. occelata* from the colony with an intact tube. (B) Denuded individuals from the tube. White color body (left) and cream color body (right) of the individuals show the testis of a male and the ovary of a female, respectively. (C) A colony of *P. occelata*. Scale bars in (B) and (C) are 1 cm and 5 cm, respectively.

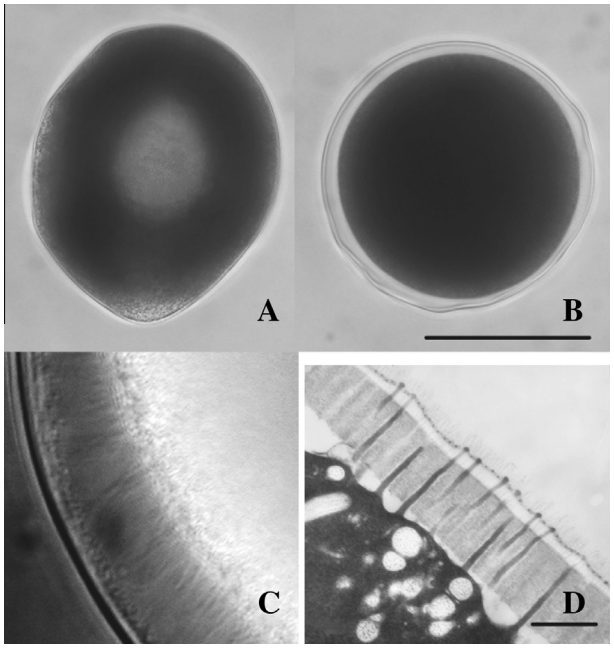

Fig. 2. Isolated oocyte and the enlarged surface of the matured oocyte. (A) An immature oocyte of *P. occelata* with a germinal vesicle at the center of the oocyte. (B) A matured oocyte after the breakdown of germinal vesicle. Egg envelope was detached from the surface and the peri-vitelline space was formed. Scale bar is 100 μm. (C) Peri-vitelline space of mature oocyte (enlarged). Microvilli elongated in the peri-vitelline space. (D) Transmission electron microscopy on surface of matured oocyte in *T. heterochaetus*. The tips of the microvilli are exposed to the surface of the egg envelope. Scale bar is 1 μm.

from the sperm binding and determine the exact timing of egg activation [6]. Even when the injection of sperm or sperm extract succeeded to induce Ca^{2+} increase in the oocyte, it doesn't disprove the pathway involving sperm binding.

The Ca^{2+} increase in the fertilized *Xenopus* eggs may depend on receptor-based signaling pathway, but injection of the sperm extract prepared from *Xenopus* sperm into a mouse oocyte generated Ca^{2+} oscillations as with the mouse sperm extract [69]. Thus, Xenopus sperm have the potential to function not only from on egg surface but also directory from inside of the oocyte. Thus, it seems difficult to classify the each species to either of the two categories.

During the fertilization process of Annelida, dynamic changes of the egg surface proceed step by step, and the sperm-egg interaction and the following egg reaction are separated from the Ca^{2+} change in the oocyte. Thus, it is a suitable model system in which to analyze the sperm-egg interaction and Ca^{2+} response during fertilization.

5. *P. occelata* as a model system for studying fertilization

P. occelata is a species of Annelida, and individuals make a colony in the shadow of a rock at sea shores in northern part of Japan (Fig. 1). An individual animal, which lives in each tube, is attached with each other at the bottom (Fig. 1A and C). The animals can be kept for several months in running seawater at 5–10 degrees. After the removal of the individuals form the tube, maturity of the gonad is judged easily by their body colors. Mature females have dark gray ovary and the male has cream color testis (Fig. 1B). The oocytes from mature ovary are at the first prophase of meiosis (Fig. 2A).

The naturally spawned oocytes are at metaphase I, which is the right period for natural fertilization of *P. occelata*. The maturation inducing hormone for *P. occelata* is still unknown, but the oocyte maturation can be induced within 20 min by the treatment with 8 bromo cyclic AMP (100 microM) in high pH sea water (pH 9.0) [62]. After that, germinal vesicle breakdown can take place in natural seawater within 1 h nearly in a synchronous manner. Then, the oocytes are arrested at metaphase I until the sperm unblock the meiotic arrest.

During oocyte maturation, the egg envelope is detached from the egg plasma membrane to form peri-vitelline space (Fig. 2B). In this space, a myriad of microvilli are projecting (Fig. 2C). Microvilli penetrate the egg envelope and exposed at the outer layer of the envelope in *T. heterochaetus* (Fig. 2D).

When added to the metaphase I oocyte, sperm attached to the egg envelope. Then, the protrusion from the egg surface developed toward the spermatozoon though the peri-vitelline space (Fig. 3A) [62]. When the tip of the cortical protrusion reached the spermatozoon on the egg envelope, the spermatozoon was incorporated into the protrusion complex. The cortical protrusion was then absorbed quickly to the egg cortex and spermatozoa in the cortical protrusion entered the oocyte. The sperm incorporation into the oocyte was completed within 3 min after insemination. Ten minutes after fertilization, the first polar body was extruded, and the second polar body by 40 min.

6. Two modes of the Ca^{2+} increase in the fertilized eggs of *P. occelata* may have different roles

We previously described sperm incorporation in *P. occelata* in relation to the intracellular Ca^{2+} response (Fig. 3) [62]. Briefly, two distinct Ca^{2+} increases with different characteristics took place independently of each other in the fertilized eggs of *P. occelata*. The first one was a local Ca^{2+} increase just beneath the fertilizing spermatozoon and the following one was a global Ca^{2+} increase

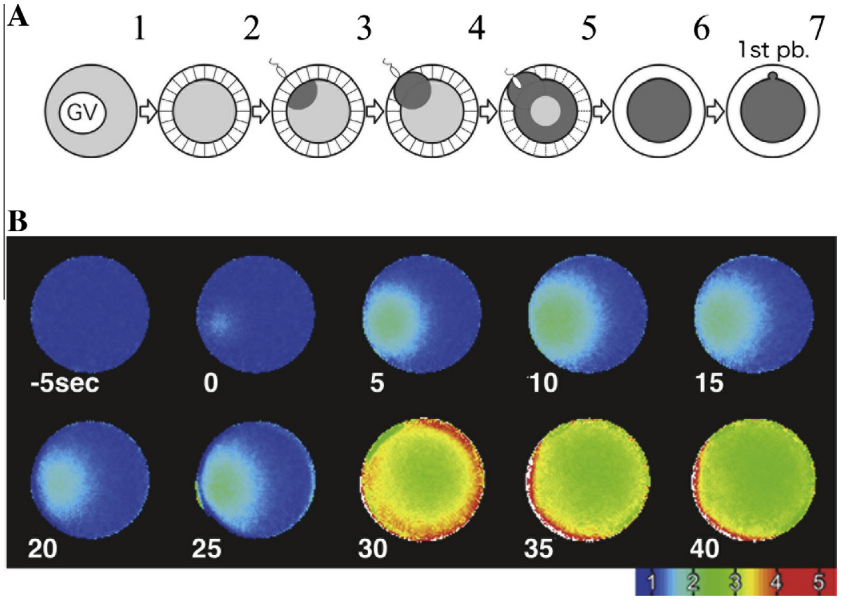

Fig. 3. Sperm egg interaction and Ca^{2+} change in the oocyte during fertilization in *P. occelata*. (A) After the induction of oocyte maturation, peri-vitelline space was formed between egg surface and egg envelop. Microvilli were observed in the peri-vitelline space. When spermatozoa attached on the egg envelope, a local Ca^{2+} increase took place (the dark gray part in the oocyte). Then, cortical protrusion was formed and spermatozoon was contacted with the tip of the cortical protrusion. The second step global Ca^{2+} increase took place in the entire cortex of the oocyte. The cortical Ca^{2+} increase propagated into the entire oocyte. Microvilli in the peri-vitelline space disappeared. Level of Ca^{2+} in the oocyte was reduced to the resting level at 10 min after fertilization, and then the first polar body was protruded. (B) Ca^{2+} changes from step 2 to 6 of (A). Each number represents the time in seconds after fertilization. Panels of time 0 to 20 show the first step local Ca^{2+} increase. Cortical protrusion was formed at 25 s. then the second step global Ca^{2+} started at 30 s.

in entire cortex of the oocyte. When the fertilizing spermatozoon attached to the egg envelope, a local Ca^{2+} increase in the cortex took place just beneath the fertilizing spermatozoon (Fig. 3A2 and A3). At this moment, the spermatozoon located on the egg envelope, which was 10 μm far from the surface of the oocyte above peri-vitelline space. During fertilization in *T. heterochaetus*, a spermatozoon attaches to the tip of microvilli expose on the egg envelope. Then microvilli in the peri-vitelline space develop towards the penetration cone, which help the fertilizing sperm to penetrate the oocyte [13].

After the local Ca^{2+} increase, the cortical protrusion developed in the peri-vitelline space beneath the spermatozoon. The source for the first step of Ca^{2+} increase was from the inside of the oocyte though IP_3 dependent Ca^{2+} releasing pathway. Local injection of IP_3 at the cortex of the oocyte induced the cortical protrusion without insemination. Addition of sperm in sea water containing U73122 blocked the local Ca^{2+} increase and the formation of cortical protrusion.

A specific structure involved in sperm incorporation ('fertilization cone') is formed on the surface of sea urchin and starfish eggs at fertilization, which is similar to the cortical protrusion in *P. occelata*, [23,24,70]. In the fertilization cone, polymerized actin becomes the framework of the structure [24,25]. The precise analysis of the Ca^{2+} changes during sperm incorporation in starfish oocytes shows that a local Ca^{2+} increase takes place at the place where the fertilization cone is formed before the Ca^{2+} wave run in whole oocyte [71]. In *P. occelata* oocytes, pre-injection of heparin also blocked the formation of cortical protrusion with sperm incorporation into it [62]. Heparin or U73122 blocked not only Ca^{2+} release though IP_3 dependent pathway in the oocyte, but also affected actin microfilaments in the cortex of the oocyte [72]. Actin microfilaments in microvilli themselves may control the Ca^{2+} change [73,74]. Thus, the first step Ca^{2+} increase and the cortical actin changes in the fertilized eggs of *P. occelata* might be interdependent and be involved in sperm incorporation.

Immediately after sperm incorporation into the fully developed cortical protrusion, the second Ca^{2+} increase takes place in the entire egg cortex (Fig. 3A5 and A6). It propagated toward the center of the oocytes, and the level of Ca^{2+} returns to the resting level by10 min after fertilization (Fig. 3). The cortical protrusion was absorbed to the oocyte during this period.

The second step of Ca^{2+} increase in the whole oocyte was due to the influx of Ca^{2+} though L type Ca^{2+} channel on the oocyte [62]. The global Ca^{2+} increase with egg activation did not take place when oocytes were treated with potassium chloride, in Ca^{2+} free sea water, or in sea water containing L type Ca^{2+} channel blocker, D600. In the oocyte of several limpets, Mollusk, and Annelida, main Ca^{2+} source during fertilization derived from the outside of the oocyte though voltage dependent Ca^{2+} channels on the oocyte. In those oocytes, potassium chloride opened the Ca^{2+} channels on the plasma membrane artificially and parthenogenetic activation proceeded [56,75–77]. Treatment of *P. occelata* oocytes with high potassium chloride induced the global Ca^{2+} increase without adding sperm. Then the first and second polar body formation followed on the same time schedule as fertilization.

The two modes of Ca^{2+} increase may have distinct physiological significance. As aforementioned, D600 inhibited specifically the Ca^{2+} influx at the second phase of the Ca^{2+} response in the fertilized *P. occelata* eggs without affecting the earlier localized Ca^{2+} increase. In these eggs, the cortical protrusion was still formed, but was not absorbed, and additional cortical protrusions were formed in the different places of the oocyte. A spermatozoon was incorporated in each protrusion and as the result, multiple spermatozoa entered in one oocyte. On the other hand, pretreatment of the oocyte with sea water containing excess potassium before insemination inhibited the local Ca^{2+} increase and the formation of the cortical protrusion. As the result, no sperm entered into the oocyte.

During fertilization in general, the egg envelope is converted to the fertilization envelope to prevent the additional spermatozoa from entering and thus guides monospermic fertilization. During fertilization in Annelida, the egg envelope does not change morphologically after sperm penetration [78]. Treatment of unfertilized oocyte with excess potassium blocked sperm incorporation in *P. occelata*. Microvilli in the perivitelline space were disrupted

as with the second global Ca^{2+} change in fertilized oocyte. Monospermic fertilization in Annelida appears to be obtained not by the reinforced egg envelope but by the signaling system to induce a local Ca^{2+} increase and the formation of cortical protrusion.

7. Soluble sperm extract of *P. occelata* and egg activating factor

During fertilization of *P. occelata*, the global Ca^{2+} increase in the cortex takes place after the local Ca^{2+} increase beneath the fertilizing spermatozoon, which was necessary for the re-initiation of meiosis. Interestingly, sperm extract from *P. occelata* induced the local Ca^{2+} increase when it was added to the surface of the oocyte. However, the second step Ca^{2+} increase for egg activation did not follow [79].

Sperm extracts and purified sperm factors from mammalian, ascidian, and polychaeta sperm, all induced intracellular Ca^{2+} increase and egg activation when they were injected into the oocyte [66,80,81]. On the other hand, sperm peptide, derived from the acrosomal vesicle of *U. caupo,* changed of the membrane potential and induced intracellular Ca^{2+} increase when it was applied to the outside of the oocyte [82,83]. As the role of sperm factor has been focused mostly on egg activation, the local interaction between the sperm factor and the egg surface was somehow neglected. Sperm extract from *P. occelata* contained the factors that induce Ca^{2+} increase in the oocyte, but it was not extended to the egg activation. Induction of a local Ca^{2+} increase by sperm extract was indispensable for the sperm incorporation into the oocyte as the first step for the successful fertilization. Our result of sperm extract indicates that sperm incorporation and egg activation are linked with two different Ca^{2+}-related mechanisms. Incorporation of spermatozoon though a deep peri-vitelline space in Annelida takes longer time, and may thus help to study the relationship between the two procedures.

In the case of *U. caupo*, application of insoluble sperm extract induced the calcium influx though voltage dependent Ca^{2+} channels and subsequent egg activation. The electron dense substance exists around the acrosomal process after the induction of acrosome reaction, which stands between the sperm and egg surface [84]. The global Ca^{2+} increase of *P. occelata* oocyte took place when sperm entered into the cortical protrusion. Another factor(s) from the sperm induce the global Ca^{2+} increase at the cortex though voltage gated Ca^{2+} channels. In sea urchin oocytes, NAADP induced a Ca^{2+} increase in the egg though voltage gated Ca^{2+} influx [85,86]. Quantitative analysis of NAADP in sperm showed that certain amount of NAADP is detected in sperm [87]. Another sperm factor which induces the global Ca^{2+} increase and egg activation must be identified in the sperm.

From the fertilization process of Annelida, sperm incorporation and egg activation by the fertilizing sperm could be analyzed separately (Fig. 4). Increases of Ca^{2+} with different Ca^{2+} releasing mechanisms have important roles in both processes. The dynamic response of the fertilization process in Annelida, *P. occelata*, is helpful for the further analysis of how Ca^{2+} response is involved in the relationship between sperm incorporation and egg activation by sperm.

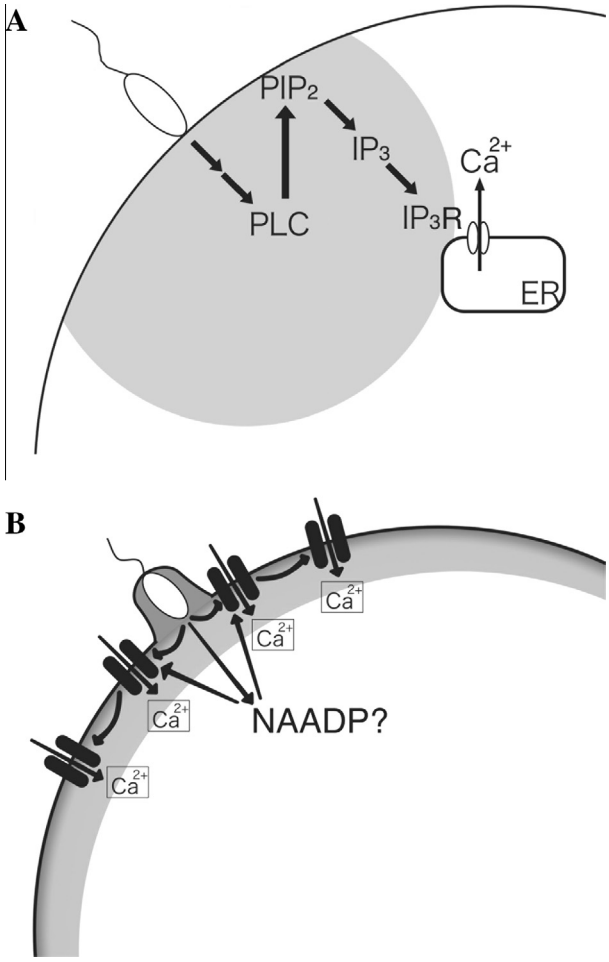

Fig. 4. Model for the first and second step Ca^{2+} release in the oocyte. (A) First step local Ca^{2+} increase was induced by IP_3 dependent pathway. (B) Then the second step global Ca^{2+} increase was induced by the influx of Ca^{2+} though Ca^{2+} channels on the oocyte.

8. Conclusion

During fertilization, sperm incorporation and egg activation are the important steps. Different aspects of the Ca^{2+} increase triggered by fertilizing spermatozoa might have distinct roles towards sperm entry and egg activation. However, most studies on the Ca^{2+} increase by sperm were focused on the egg activation. In Annelida, the remarkable sperm incorporation step precede egg activation, and the two procedures may be linked to the distinct aspects of the Ca^{2+} increases that take place by different mechanisms. The idea that the sperm-induced Ca^{2+} increase is intertwined with several functions during fertilization will help understand the role of Ca^{2+} release in different species.

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