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Soluble sperm extract specifically recapitulates the initial phase of the Ca^{2+} response in the fertilized oocyte of *P. occelata* following a G-protein/ PLCβ signaling pathway

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Summary

Matured oocytes of the annelidan worm *Pseudopotamilla occelata* are fertilized at the first metaphase of the meiotic division. During the activation by fertilizing spermatozoa, the mature oocyte shows a two-step intracellular Ca^{2+} increase. Whereas the first Ca^{2+} increase is localized and appears to utilize the inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores, the second Ca^{2+} increase is global and involves Ca^{2+} influx via voltage-gated Ca^{2+} channels on the entire surface of the oocyte. To study how sperm trigger the Ca^{2+} increases during fertilization, we prepared soluble sperm extract (SE) and examined its ability to induce Ca^{2+} increases in the oocyte. The SE could evoke a Ca^{2+} increase in the oocyte when it was added to the medium, but not when it was delivered by microinjection. However, the second-step Ca^{2+} increase leading to the resumption of meiosis did not follow in these eggs. Local application of SE induced a non-propagating Ca^{2+} increase and formed a cytoplasmic protrusion that was similar to that created by the fertilizing sperm at the first stage of the Ca^{2+} response, important for sperm incorporation into the oocyte. Our results suggest that the fertilizing spermatozoon may trigger the first-step Ca^{2+} increase before it fuses with the oocyte in a pathway that involves the G-protein-coupled receptor and phospholipase C. Thus, the first phase of the Ca^{2+} response in the fertilized egg of this species is independent of the second phase of the Ca^{2+} increase for egg activation.

Keywords: Annelida oocyte, Ca^{2+} function, Ca^{2+} increase, Egg activation, Inositol 1, 4, 5-trisphosphate (IP_3), Sperm extract

Introduction

During fertilization, there is an increase in intracellular Ca^{2+} in the oocyte that is triggered by sperm. This increase is necessary and sufficient stimulus for egg activation and early development, but the exact mechanism of the sperm-induced Ca^{2+} increase is not well known and may vary in different animal species (Stricker, 1999; Runft *et al.*, 2002; Miyazaki, 2006; Whitaker, 2006; Dale *et al.*, 2010; Kashir *et al.*, 2013). In the fertilized oocyte of deuterostomes, the Ca^{2+} increase initiates at the spermatozoon-interaction site and propagates to the whole oocyte as a single Ca^{2+} wave. In mammals and ascidians, periodic Ca^{2+} increases (Ca^{2+} oscillations) occur in the oocyte (Kyozuka *et al.*, 1998; Dale *et al.*, 1999; Deguchi *et al.*, 2000; Miyazaki, 2006; Swann & Yu, 2008). The Ca^{2+} oscillations control the meiotic cell cycle after fertilization in ascidian oocytes (Russo *et al.*, 1996). These Ca^{2+} increases are generally provided by inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release (IICR) though the IP_3-receptors on the endoplasmic reticulum (ER) (Terasaki & Sardet, 1991). IP_3 is produced as a result of hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC), which exists in several different isoforms being regulated in different pathways (Rhee, 2001). In addition, a synchronous Ca^{2+} increase in the whole egg surface area due to Ca^{2+} influx (cortical flash) at the moment of fertilization

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is found in echinoderm and lophotrochozoans, which is regulated by voltage-gated Ca^{2+} channels (Stricker, 1999; Churchill *et al.*, 2003; Moccia *et al.*, 2006, Deguchi, 2007). Non-Ca^{2+}-dependent ion currents also work as the activation current during fertilization in ascidian oocytes (Cuomo *et al.*, 2006; Tosti *et al*, 2011). Sea urchin eggs possess another pathway for Ca^{2+} release from the ER, which is due to cyclic ADP-ribose-sensitive Ca^{2+} releasing through the ryanodine receptor (Stricker, 1999; Miyazaki, 2006). The signaling pathway leading to the Ca^{2+} increase in fertilized eggs varies considerably in different animal species. There are two fundamental hypotheses as to how sperm activate the egg and induce Ca^{2+} release (Stricker, 1997; Swann & Parrington, 1999; Runft *et al.*, 2002). The first model requires sperm–egg fusion, and the sperm to trigger Ca^{2+} release by conducting a soluble content directory into the egg cytoplasm. This idea is supported by the fact that intracytoplasmic sperm injection (ICSI) or injection of sperm extract (SE) induces a Ca^{2+} increase that may develop into Ca^{2+} oscillations, an event similar to that in the normally fertilized eggs of mammals, ascidians, and nemertean worm (Stricker, 1997; Kyozuka *et al.*, 1998; Sato *et al.*, 1999; Runft & Jaffe, 2000; Yoon & Fissore, 2007). In mammals, a key sperm factor was identified as a novel sperm-specific PLC, PLCζ (Saunders *et al.*, 2002; Yoon & Fissore, 2007; Swann & Yu, 2008).

The second hypothesis on how sperm activate the egg and increase Ca^{2+} during fertilization is based on signal transduction through the receptors on the egg surface. The binding of sperm to its receptor on the egg surface activates G-proteins and/or protein tyrosine kinase (PTK), then the downstream effector PLCs produce IP_3, which in turn evokes Ca^{2+} release from the intracellular stores. Whereas PLCβ is activated by G-proteins, PLCγ is activated by PTK. In *Xenopus* and echinoderms, where application of sperm protease causes egg activation, PTK inhibitors such as PP1 and genistein either inhibit or cause significant delay of Ca^{2+} increase at fertilization (Carroll & Jaffe, 1995; Mizote *et al.*, 1999; Shen *et al.*, 1999; Abassi *et al.*, 2000; Sato *et al.*, 2000). The SH2 domain proteins, which work as a specific competitive inhibitors of PTK or PLCγ activity, inhibit Ca^{2+} increase and egg activation in echinoderms (Abassi *et al.*, 2000; Runft *et al.*, 2002). The PTK inhibitors and these SH2 domain proteins, however, did not block Ca^{2+} increase in the fertilized eggs of mouse, in which IP_3-induced Ca^{2+} increase is essential for Ca^{2+} release at fertilization (Dupont *et al.*, 1996; Mehlmann & Jaffe, 2005). Mammalian oocytes overexpressing the G-protein/PLCβ interactive receptor showed a Ca^{2+} increase upon the addition of its ligand PDGF (Mehlmann *et al.*, 1998). This result indicates that the mammalian oocyte, which is categorized as a fusion type oocyte for Ca^{2+} increase, also possess the potentiality to respond to the ligand from the outside of the oocyte. In amphibian, urodele newt oocytes can be activated by injection of sperm extract. However, the factor that induced Ca^{2+} increase was citrate synthase, and not PLCs, although the anuran frog oocyte was considered to employ a receptor-based signaling cascade at fertilization (Harada *et al.*, 2007, 2011). Therefore, although fertilization is such a fundamental process for conservation of species, the exact mechanism of egg activation varies widely among animal species, and it is difficult to allocate mechanisms strictly into the two types.

Two different modes of Ca^{2+} increase are observed in echinoderm fertilization, these are the cortical flash and the Ca^{2+} wave (Churchill *et al.*, 2003; Moccia *et al.*, 2003). These two events take place at a short interval apart or almost at the same time during fertilization. The oocyte of a given species may have several different mechanisms of increasing Ca^{2+} during fertilization. When multiple types of Ca^{2+} releasing mechanisms are at work during fertilization, each phase of the Ca^{2+} response may play a distinct physiological role and may provide a unique opportunity to study their relationships with each other.

In this study, we used the annelidan worm *Pseudopotamilla occelata*, which has a time lag between two different Ca^{2+} increases in the fertilized oocyte (Nakano *et al.*, 2008). It is a suitable animal model in which to analyze how sperm induce Ca^{2+} increase and initiate egg activation. In this species, it is evident that two different mechanisms of Ca^{2+} increase are at work during fertilization, but it is still unclear how these Ca^{2+} increases occur and how the fertilizing sperm trigger these Ca^{2+} increases in the oocyte. Here, we have examined how egg activation at fertilization initiates in this species in the light of the aforementioned hypotheses. To this end, we prepared soluble SE from this species. External application of SE to the matured oocyte mimicked the first-step Ca^{2+} increase at fertilization, but it could not induce the second phase of the Ca^{2+} increase in the whole oocyte, suggesting that these two phases of the Ca^{2+} increase are controlled by two different mechanisms.

Materials and methods

Biological materials and gametes

Sexually mature *Pseudopotamilla occelata (P. occelata)* were collected from rocky shores around the Asamushi Marine Biological Station or obtained from the fishing-tackles store in Kuji City (Iwate Prefecture) during the breeding season from May to November in 2008, 2009

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and 2010. Animals were kept in running seawater at 5–15°C. Prophase-arrested oocytes (immature oocytes) from the ovary were collected by dissection and were suspended and washed several times in filtered seawater (FSW). To obtain mature oocytes suitable for fertilization, these immature oocytes were stimulated with 400 μM 8-bromo-cAMP (8-Br-cAMP; Sigma) and 10 mM Tris (pH 9.0–9.5) for 20 min at 20°C, as has been descried previously (Nakano *et al.*, 2008). Only the optimal oocytes displaying germinal vesicle breakdown 20 min after the incubation were used in the experiment. Likewise, sperm were obtained from fully matured male worms and kept without dilution at 4°C until use.

Ca^{2+} imaging

Immature oocytes were pressure injected with calcium green-1 dextran, (CGD; Molecular Probes; 3000 MW) at 1 mM in the injection buffer (100 mM potassium aspartate and 10 mM HEPES, pH 7.0) in low Ca^{2+} seawater, which was a mixture of FSW and Ca^{2+}-free sea water (CaFSW) (1:19). The injection volume was typically 4–8% of the total oocyte volume. The dye-loaded oocytes were then induced to mature by use of 8-Br-cAMP, as described above.

For Ca^{2+} measurement, oocytes were put in a chamber, which was a 3-cm plastic dish with 1-cm hole at the bottom. The hole was sealed with a coverslip. A piece of double-sided Scotch® tape and another small piece of coverslip were placed on top to make a 130-μm space between the two coverslips. The oocyte to be used for measurement was placed in the space between the upper and lower coverslips, and soaked in about 400 μl of the medium.

Intracellular Ca^{2+} changes were measured by the fluorescence change in CGD using a cooled charge coupled device (CCD) camera (Coolsnap cf, Nippon Rooper) attached to an inverted epifluorescence microscope (IMT-2, Olympus). The image processing method was essentially the same as those described previously (Deguchi & Morisawa, 2003; Deguchi *et al.*, 2005). The fluorescence intensity after the Ca^{2+} response (F) was normalized to the resting value (F_0) and indicated relative fluorescence was calculated (F/F_0).

Sperm extract

All the procedures for sperm extraction were performed at 4°C. Undiluted 400–600 μl dry sperm were washed twice in CaFSW containing 1 mM ethylene glycol tetra-acetic acid (EGTA) and centrifuged at 1000 *g* for 5 min. After washing in CaFSW without EGTA, the sperm concentration was adjusted to ~8.2×10^{12} sperm/ml using a hemacytometer and divided into 200 μl in each 1.5 ml tube. The sperm suspension was centrifuged at 4000 *g* for 5 min. After removing the supernatant, the packed sperm were frozen at –80°C. Frozen sperm were re-suspended in 400 μl of extraction buffer containing 100 μg/ml soybean trypsin inhibitor (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), and 5 mM HEPES (pH 7.0). The suspended sperm pellet was homogenized with a Teflon microhomogenizer. After centrifugation at 15,000 *g* for 30 min, the supernatant was transferred into a new tube and centrifuged again at 15,000 for 20 min. The supernatant was collected as soluble SE. The concentration of protein and estimated number of sperm in the SE solution were about 3.3 mg/ml and 4.1×10^{12} sperm/ml, respectively. The SE was lyophilized and stored at –20°C. Powdered SE was diluted in the injection buffer (SE, ~330 μg proteins/ml) prior to use. The effect of addition of 10 times concentrated SE (10× SE, ~3.30 mg proteins/ml) was examined in some experiments.

Sperm extract characterization

To test the heat stability of SE, SE was boiled at 100°C for 15 min. The boiled SE was centrifuged at 15,000 *g* for 30 min, and the supernatant was examined as a 'boiled SE'. To examine the protease sensitivity, the boiled SE was incubated with 0.1% protease (actinase E, Kaken Chemical Co., Japan) at 20°C for 3 or 6 h. After the treatment, SE was boiled again for 15 min to inactivate protease activity and centrifuged at 15,000 *g* for 30 min.

Removal of the vitelline envelop

For microinjection into the mature oocyte, the vitelline envelope was removed with a razor blade in a hypertonic solution as described previously in a mixture of 1.5 M NaCl and CaFSW (2:1) (Nakano *et al.*, 2008). The vitelline envelope-free oocytes were washed with FSW briefly and subjected to the quantitative injection in CaFSW.

Reagents

Stock solutions of a PLC inhibitor, U73122 (Biomol Research Labs., Inc) and its inactive analogue U73343 (Biomol Research Labs., Inc.), and Src family PTK inhibitor set (Calbiochem) including genistein, herbimycin A (HA), PP2 were dissolved in dimethylsulfoxide (DMSO) as stock solutions. Caged IP_3 (Calbiochem), a metabolically stable analogue of guanosine diphosphate (GDPβS, Sigma) and a hydrolysis-resistant analogue of guanosine triphosphate (GTPγS Sigma) were dissolved in the injection buffer. These reagents were kept at –20 or –80°C until use. U73122, U73343, genistein and HA were diluted to 1 μM, 10 μM, 100 μM and 5 μg/ml (8.7 μM) in FSW or CaFSW prior to

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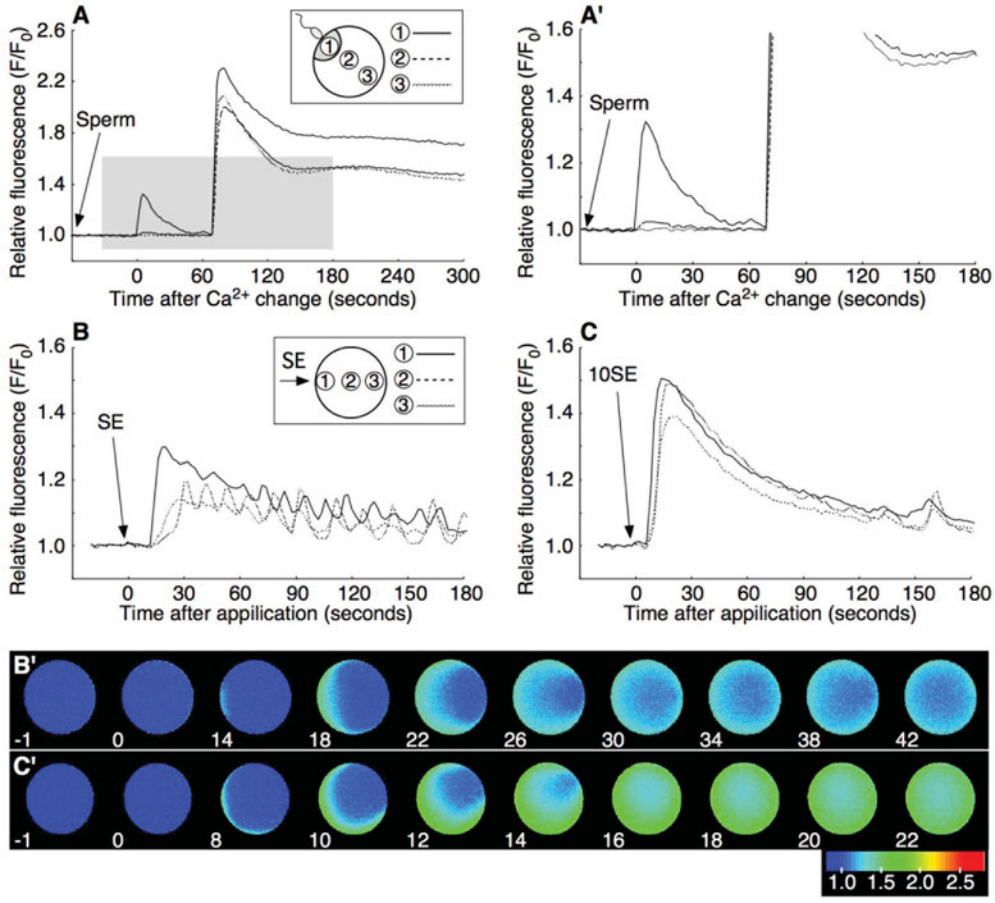

Figure 1 Sperm or sperm extract (SE) induces an intracellular Ca^{2+} increase. An unfertilized oocyte underwent a two-step Ca^{2+} increase following insemination (*A*). (*A′*) is the shaded region of (*A*). The Ca^{2+} increase was induced by external application of 1× SE (*B*, *B′*) or 10× SE (*C*, *C′*) in filtered seawater (FSW). Arrows indicate the time of insemination or SE addition. SE (10–15 μl) was added to oocytes in 400 μl FSW. F/F_0 values were calculated at the initiation site of local Ca^{2+} increase or the SE added side, the center, or the antipode, separately, and are represented by three different lines (shown in insets of *A*, *B*). The profiles of fluorescence changes were also expressed as pseudocolor fluorescence images (*B′*, *C′*). The pseudocolor images were acquired by normalizing the resting image just before the Ca^{2+} increase in a pixel-to-pixel manner at the indicated time (s).

use. In other experiments, genistein, PP2, GDPβS and caged IP_3 were co-injected with CGD into immature oocytes at 4–8% of the oocyte volume. While pipette concentrations of genistein, PP2 and caged IP_3 were 2 mM, 200 μM and 100 μM, GDPβS concentration was 20 or 40 mM. To activate the caged IP_3, the loaded oocytes were irradiated with ultraviolet (UV) light for 3 s.

Results

External application of SE induces Ca^{2+} increase in the oocyte

Mature oocytes of *P. occelata* have a wide perivitelline space between the plasma membrane and the vitelline envelope of the oocyte. During fertilization, the spermatozoon is attached to the vitelline envelope. Then the first-step local Ca^{2+} increase occurs at the cortex under the fertilizing spermatozoon before it enters the newly formed cytoplasmic protrusion (CP) in the perivitelline space. The second-step global Ca^{2+} increase in the whole cortex of oocyte takes place after the fertilizing spermatozoon has entered the CP (Fig. 1*A*, *A′*) (Nakano *et al.*, 2008). To understand how the initial interaction between sperm and oocyte induces an intracellular Ca^{2+} increase in the oocyte, we prepared soluble SE and examined its capability for Ca^{2+} increase. External application of SE induced a Ca^{2+} increase in most of the oocytes examined (Fig. 1*B*, *B′*). The Ca^{2+} increase started at 4–55 s after SE application from the region where SE was added, and the wave propagated to the antipode though the cortex (31/34; Figs 1*B*, *B′*). The Ca^{2+} peak value around the SE-applied region was similar to that at the initiation site of the first-step Ca^{2+} increase during fertilization

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Table 1 Characteristics of Ca^{2+} change and following cytoplasmic protrusion (CP) formation during fertilization or by external application of sperm extract (SE)

	% of Oocytes with Ca^{2+} rise (n)	Time to Ca^{2+} rise (s)[a]	Peak amplitude (F/F_0)[b]			% of Oocytes with CP (n)[c]
			Initiation or additional site	Center	Antipode	
Sperm						
First-step	100 (7)	–	1.35 ± 0.11	1.09 ± 0.09	1.02 ± 0.03	100 (7)
Second-step	100 (7)	–	2.30 ± 0.13	2.09 ± 0.16	2.28 ± 0.22	–
Application to whole[d]						
SE in FSW	91 (34)	15.9 ± 12.6	1.30 ± 0.13	1.18 ± 0.09	1.22 ± 0.10	35 (31)
SE in CaFSW	93 (28)	20.9 ± 17.4	1.26 ± 0.09	1.15 ± 0.06	1.19 ± 0.07	27 (26)
10× SE in FSW	100 (6)	8.2 ± 0.8	1.47 ± 0.06	1.35 ± 0.08	1.42 ± 0.11	33 (6)
Application to local[d]						
SE in FSW	100 (12)	10.8 ± 5.3	1.38 ± 0.10	1.18 ± 0.10	1.11 ± 0.09	58 (12)
10× SE in FSW	100 (5)	6.8 ± 2.6	1.50 ± 0.09	1.35 ± 0.06	1.30 ± 0.02	80 (5)

[a]Values refer to the time between SE application and the first detection of Ca^{2+} increase (mean ± standard deviation (s.d.)).
[b]Peak values in the oocytes after insemination or SE application are calculated in the initiation site of Ca^{2+} increase (or SE additional site), the center, and the antipode, separately. Values include only those eggs that showed a Ca^{2+} increase, which defined by a >1.06 peak value (mean ± s.d.).
[c]The formation of CP was monitored after oocytes showed Ca^{2+} increases.
[d]SE was applied externally to the oocytes at the exposed whole or local surface.

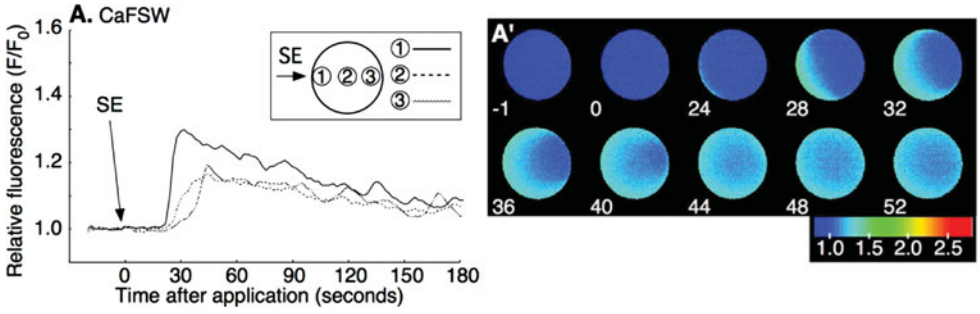

Figure 2 Ca^{2+} increase induced by external application of sperm extract (SE) in Ca^{2+}-free seawater. The Ca^{2+} increase was induced by external application of 1× SE in Ca^{2+}-free filtered seawater (CaFSW) (*A*). Arrows indicate the time of SE addition. The pseudocolor images were acquired by normalizing the resting image just before the Ca^{2+} increase in a pixel-to-pixel manner at the indicated time (s) (*A*′).

(Table 1). The Ca^{2+} increase induced by the external application of 10× SE was larger than that of 1× SE (6/6; Fig. 1*C*, *C*′), but it did not induce the second-step Ca^{2+} increase (Table 1). The CP was observed in some oocytes after SE application (Table 1), but polar body protrusion and further meiotic division did not proceed.

Our previous study indicated that the first-step Ca^{2+} increase at fertilization does not require the presence of external Ca^{2+}. To see whether the SE-induced Ca^{2+} increase also needed no external Ca^{2+}, SE was applied in Ca^{2+}-free seawater (CaFSW). Most oocytes showed a Ca^{2+} increase after SE application (26/28; Fig. 2*A*, *A*′). There was neither a significant decrease of the Ca^{2+} peak amplitude nor a delay in the onset of the Ca^{2+} increase in CaFSW compared with that in FSW ($P > 0.1$; Table 1). Then SE was injected into the peripheral region of denuded oocytes in CaFSW. The injected amount was restricted to 0.4–0.5% (6.3–7.8 pl) of the oocyte volume. No Ca^{2+} response (8/10) or a small Ca^{2+} transient just around the tip of the needle (2/10) was observed during microinjection, the peak amplitude of which was barely above the background level, e.g. 1.13 and 1.16 (F/F_0), in contrast with the high Ca^{2+} increase induced by fertilizing sperm (2.6–3.2 sperm/oocyte). The eggs microinjected with 10× SE (equivalent to 25.5–31.9 sperm/oocyte) made no difference except that the small Ca^{2+} increase observed around the tip of needle lingered for a few seconds (8/10; Fig. 3*A*, *A*′). The Ca^{2+} peak value of 10× SE injection was merely 1.19 ± 0.08 ($n = 8$) at the microinjected site, a value far smaller than that of the first-step Ca^{2+} increase at fertilization ($P < 0.05$, see Table 1). No Ca^{2+} change was observed by injecting

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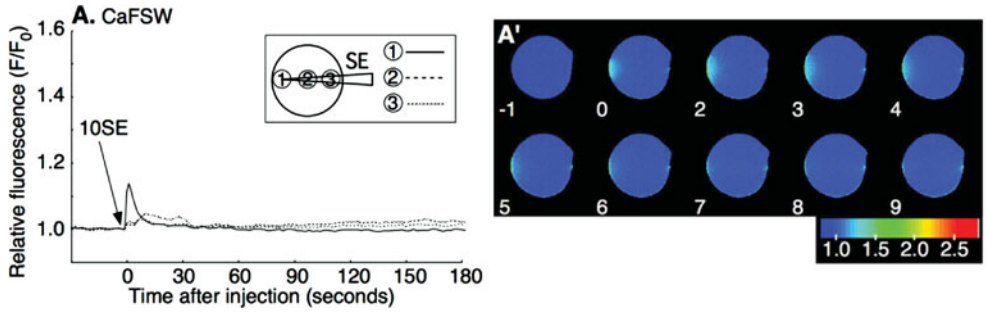

Figure 3 Microinjection of sperm extract (SE) into the oocyte in Ca^{2+}-free seawater. Oocytes were microinjected with 10× SE (10SE) at time zero. Arrow indicates the time of microinjection. 10× SE was microinjected at the peripheral region of the oocyte and F/F_0 values were calculated at the initiation site of local Ca^{2+} increase or the SE-injected side, the center, or the antipode, separately, and are represented by three different lines (*A*). The pseudocolor images were acquired by normalizing the resting image just before the Ca^{2+} increase in a pixel-to-pixel manner at the indicated time (s) (*A*′).

the buffer as a control (0/7; data not shown). These results show that SE has no evident direct role inside the oocyte, and that the Ca^{2+} increase induced by the external application of SE is derived from the internal stores of the oocyte.

Local application of SE mimics the first phase of the Ca^{2+} response to the fertilizing sperm by inducing a non-propagating intracellular Ca^{2+} increase

The external application of SE induced a Ca^{2+} increase in the cortex. During fertilization, the first-step Ca^{2+} increase was localized underneath a fertilization sperm. Thus, we tested if local application of SE would bring about such an effect. To this end, the oocyte was sucked into a glass capillary (inside diameter of the tip; 100–130 μm) and only the local surface of the oocyte was exposed to SE, which was added to the FSW in the chamber. The external application of SE in the chamber induced a Ca^{2+} increase around the open region of the surface but the Ca^{2+} increase did not propagate well to the antipode of the oocyte deep in the capillary (12/12; Fig. 4*A*, *A*′). The external application of 10× SE induced a larger local Ca^{2+} increase, but again the Ca^{2+} increase did not propagate to the whole cortex (5/5; Fig. 4*B*). An oocyte sucked into narrower glass capillary showed clearly that the Ca^{2+} increase at the cortex was larger than that at center (Fig. 4*C*, *C*′).

During fertilization, the CP is formed following the first-step Ca^{2+} increase. Here, 58% (7/12) of SE-exposed oocytes formed the CP around the SE-applied region (Fig. 4*A*′ arrowhead, 120 s), and 80% (4/5) of oocytes formed the CP with the application of 10× SE. The Ca^{2+} peak value around the CP formed by SE application was more than 1.35 (F/F0) (Fig. 4*D*), which was similar to that of the first-step Ca^{2+} increase during fertilization (Table.1). Thus, local application of SE induced a non-propagated Ca^{2+} release with the formation of the CP in the cortex, a result comparable with the first-step Ca^{2+} response at fertilization.

PLC inhibitor U73122 suppresses the SE-induced intracellular Ca^{2+} increase

The first-step Ca^{2+} increase during fertilization depends upon IICR, which is inhibited by PLC inhibitor, U73122 (Nakano *et al.*, 2008). To see if the SE-induced Ca^{2+} release is also due to IICR, we examined the effect of U73122 on SE application. The mature oocytes were preincubated for 5 min in CaFSW containing 10 μM U73122, and SE was applied in the same medium. The Ca^{2+} increase was blocked in 69% (11/16) of the cases (Fig. 5*A*). The rest of the U73122-treated oocytes (5/16) showed a much reduced Ca^{2+} peak amplitude and a significant delay of Ca^{2+} increase (Table. 2). Addition of U73343, the inactive analogue of U73122, had no such effect, displaying virtually the same Ca^{2+} increase as in non-treated control eggs (15/15; Fig. 5*B*). These results indicated that the SE induced the Ca^{2+} response through the IICR, just as the first-step Ca^{2+} response to the fertilizing sperm depends on it.

The SE-induced intracellular Ca^{2+} increase is inhibited by GDPβS.

As SE induced the Ca^{2+} increase in the oocyte only when it was applied externally, we speculated that a cell surface receptor-mediated signaling pathway must be involved in the process. Upon ligand–receptor interaction, G-protein or PTK is thought to activate PLC to produce IP_3 and Ca^{2+}. To test if the SE-induced Ca^{2+} increase is due to G-protein, or a PTK related pathway, we examined the effect of a stable deactivator of G-protein (GDPβS) and the PTK inhibitors (genistein, HA, and PP2).

The microinjection into immature oocytes of 20 mM or 40 mM of GDPβS (pipette concentration) did not affect the progress of the maturation process,

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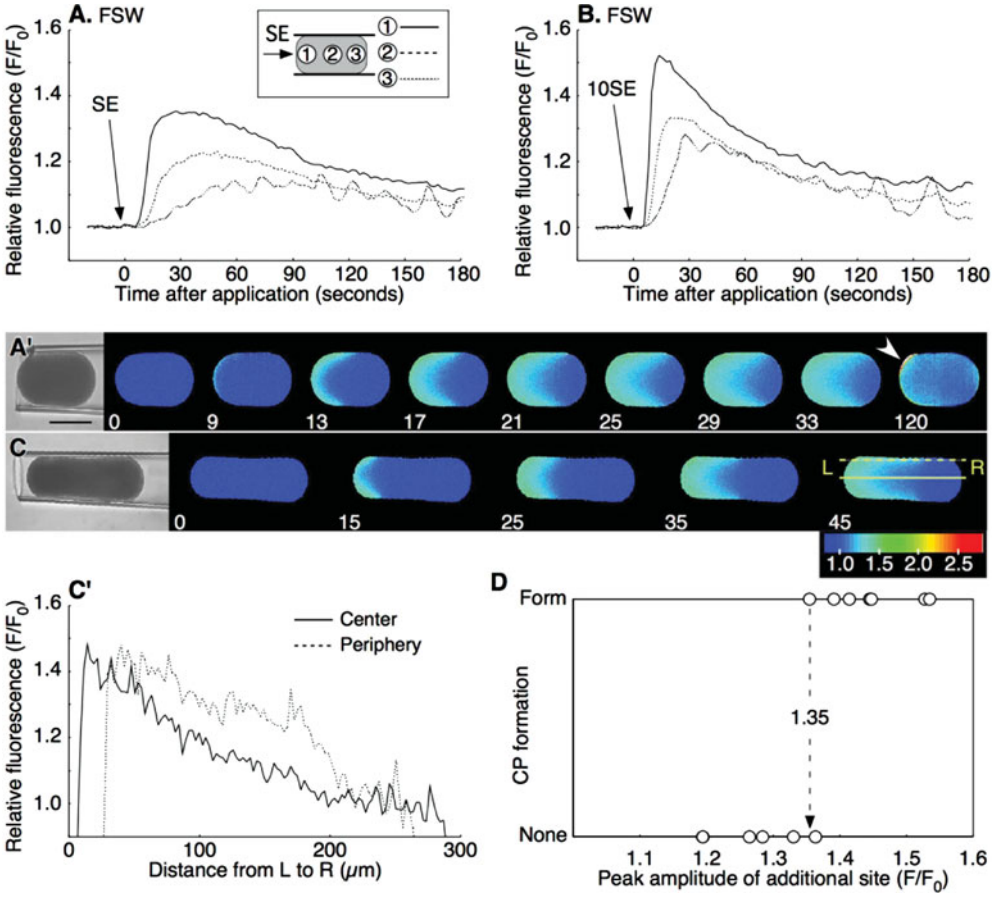

Figure 4 External application of sperm extract (SE) to the partial cortex induces an intracellular Ca^{2+} increase and the formation of a cytoplasmic protrusion (CP). 1× SE (SE) (*A*, *A′*); or 10× SE (10SE) (*B*); was applied to the oocytes placed within the glass capillary (diameter of ~100 μm) in filtered seawater (FSW). The oocyte in (*A*) is the same as in (*A′*). Arrowheads indicate the formation of the CP (*A′*, 120 s). The difference in relative fluorescence (F/F_0) in the oocyte in the glass capillary at the peak of calcium level from the SE-applied side (L) to the opposite side (R) (*C*, *C′*). The periphery region (dotted line shown in (*C*) 45 s) and the center region of the oocyte (continuous line shown in (*C*) 45 s) were measured separately (*C′*). The peak calcium level in each oocyte at the SE-applied region. When the relative fluorescence (F/F0) was higher than 1.35, the CP was formed (*D*).

which was induced by cAMP (data not shown). However, the same treatment significantly inhibited the SE-induced Ca^{2+} increase in a dose-dependent manner. No Ca^{2+} increase was observed in 30% of eggs (3/10) microinjected with 20 mM GDPβS (final concentration: 0.8–1.6 mM), and at 40 mM GDPβS (final concentration: 1.6–3.2 mM), over 64% (9/14) of oocytes failed to display a Ca^{2+} increase after the external application of SE (Fig. 6*A*, *E*, *F*). In comparison with the control eggs, the amplitude of the Ca^{2+} peak was also reduced in the eggs injected with 20 mM and 40 mM GDPβS-injected oocytes (Table 2).

In contrast, the general inhibitors of PTK make no difference to the SE-induced Ca^{2+} increase in the oocytes. When mature oocytes were preincubated for 30–60 min in the presence of 100 μM genistein or 5 μg/ml HA in FSW, all the oocytes showed a normal Ca^{2+} increase after the application of SE (4/4, 5/5 respectively; Fig. 6*A*–*C*). Likewise, microinjection of 200 μM PP2 (final concentration: 8–16 μM) showed the same Ca^{2+} increase as the non-injected control after SE addition (5/5; Fig. 6*A*, *D*). These results indicated that the G-protein-linked receptor on the oocyte surface, but not the PTK-linked receptor, is involved in the Ca^{2+} increase in response to SE.

GDPβS inhibited the first-step Ca^{2+} increase during fertilization

Based on the above results, we examined whether the first-step Ca^{2+} increase during fertilization is also regulated by the G-protein-coupled receptor. We found that all the oocytes injected with 20 mM GDPβS produced only a small and short-lived first-step Ca^{2+} increase at fertilization (7/7; Fig 7*A*, *A′*). The Ca^{2+} peak (F/F_0) was 1.23 ± 0.03 at the initiation site of the Ca^{2+}

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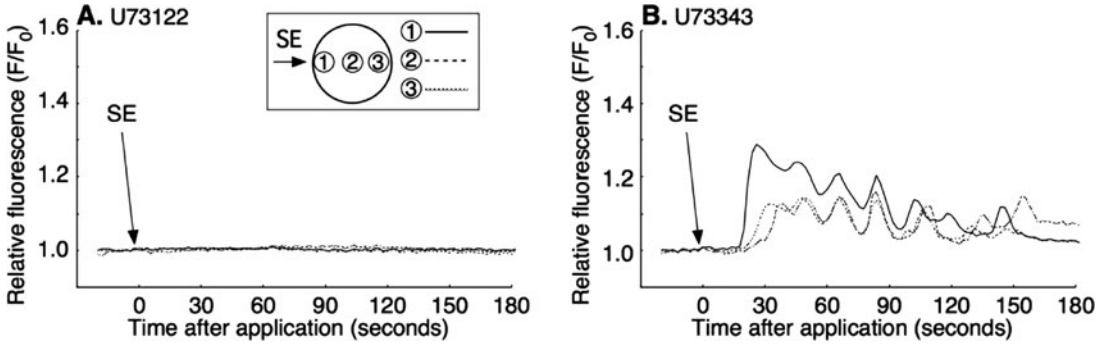

Figure 5 Inhibition of sperm extract (SE)-induced intracellular Ca^{2+} increase by U73122. Oocytes were incubated for 5 min in Ca^{2+}-free filtered seawater (CaFSW) containing 10 μM U73122 (*A*); or U73343 (*B*); and 1× SE was added in same solution.

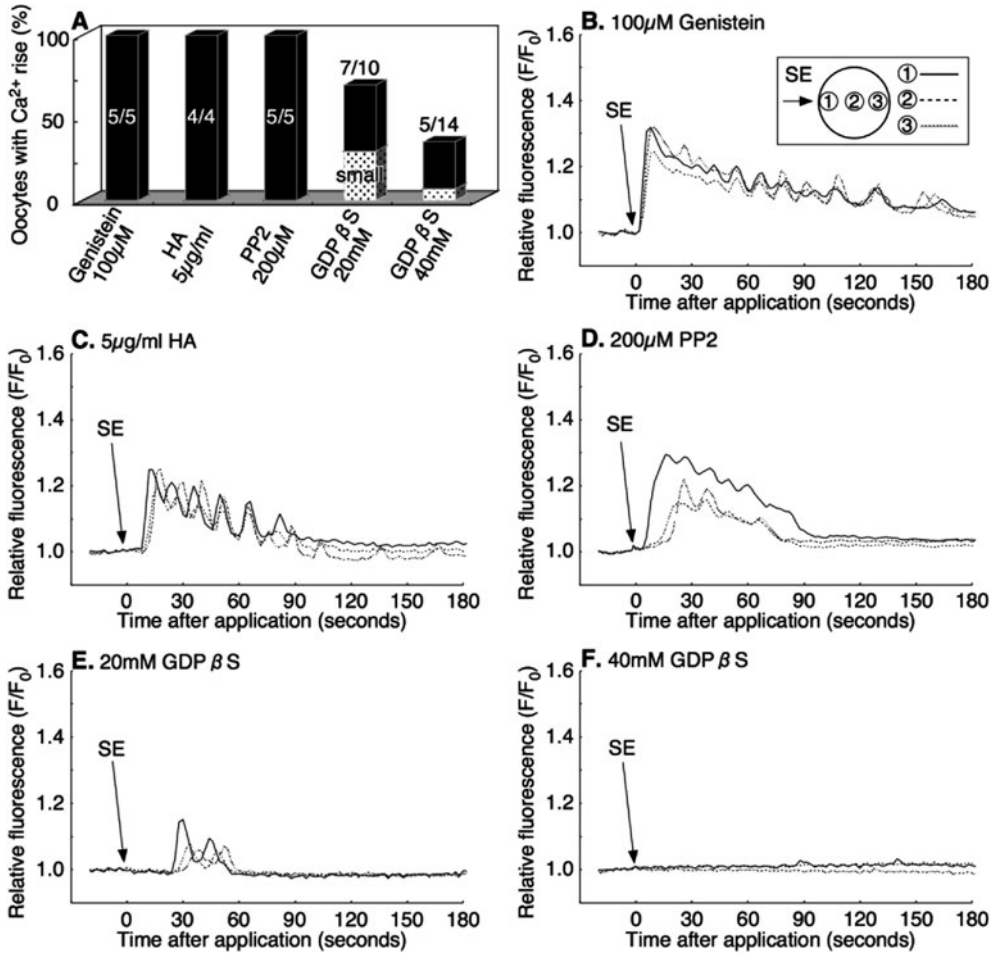

Figure 6 Inhibition of sperm extract (SE)-induced intracellular Ca^{2+} increase by GDPβS but not by tyrosine kinase inhibitors. The rate of Ca^{2+} increase after SE application in various inhibitors (*A*). Oocytes were incubated for 30–60 min in filtered seawater (FSW) containing 100 μM Genistein (*B*); or 5 μg/ml herbimycin A (HA) (*C*); then 1× SE was added in the same solution. The other oocytes were co-injected with calcium green dextran and 200 μM PP2 (final concentration: 8–16 μM) (*D*); 20 mM GDPβS (final concentration: 0.8–1.6 mM) (*E*); or 40 mM GDPβS (final concentration: 1.6–3.2 mM) (*F*); then 1× SE was added into the same solution.

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Table 2 Inhibitory effects on Ca^{2+} increase induced by external application of sperm extract

Reagents	% of Oocytes with Ca^{2+} rise (n)	Time to Ca^{2+} rise (s)[a]	Peak amplitude (F/F_0)[b]		
			Additional site	Center	Antipode
10 μM U73122	31 (16)	97.2 ± 18.4[c]	1.16 ± 0.09[d]	1.15 ± 0.04	1.15 ± 0.06
10 μM U73343	100 (15)	18.6 ± 8.9	1.26 ± 0.08	1.15 ± 0.06	1.20 ± 0.10
20 mM GDPβS	70 (10)	25.7 ± 13.6	1.14 ± 0.06[e]	1.08 ± 0.04	1.10 ± 0.06
40 mM GDPβS	36 (14)	23.2 ± 8.4	1.22 ± 0.06[e]	1.07 ± 0.04	1.03 ± 0.02

[a]Values refer to the time between SE application and the first detection of Ca^{2+} increase (mean ± standard deviation (s.d.)).
[b]Peak values in the oocytes after SE application are calculated in the SE additional site, the center, and the antipode, separately. Values include only those eggs that showed Ca^{2+} increase, which was defined by a >1.06 peak value (mean ± s.d.).
[c]Significant longer than U73343-treated oocytes (Student's t-test, $P < 0.001$).
[d]Significant less than U73343-treated oocytes (Student's t-test, $P < 0.05$).
[e]Significant less than non-injected control oocytes (Table 1) (Student's t-test, $P < 0.05$).

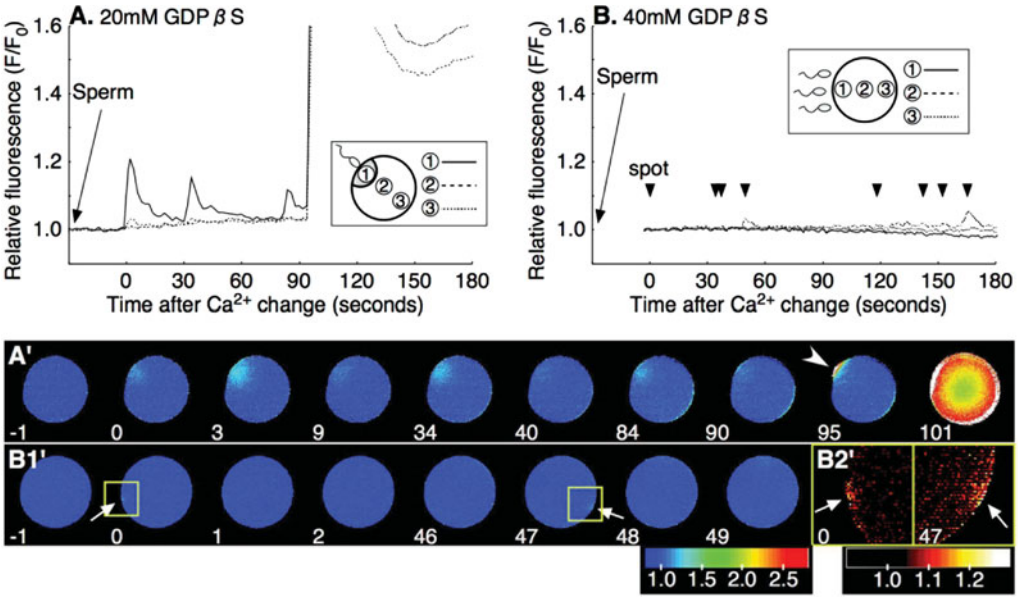

Figure 7 Inhibition of first-step Ca^{2+} increase by GDPβS but not by tyrosine kinase inhibitors. Oocytes were co-injected with calcium green dextran and 20 mM GDPβS (final concentration: 0.8–1.6 mM) (*A*, *A′*); or 40 mM GDPβS (final concentration: 1.6–3.2 mM) (*B*, *B1′*); and inseminated. Arrowheads indicate moments of frequent Ca^{2+} rise before the first-step Ca^{2+} increase, which involved formation of the cytoplasmic protrusion. (*B2′*) 0 and 47 s are magnified images of (*B1′*) 0 and 47 s (yellow squares) by contrasting pseudocolor, respectively.

increase ($n = 7$), which was significantly reduced from that of the non-injected control oocytes ($P < 0.05$, see Table. 1). The CP was not formed immediately after a small Ca^{2+} increase. These small Ca^{2+} increases were repeated several times at the same region, then the CP formation was accomplished (Fig. 7*A′* arrowhead, 95 s). Nonetheless, the second-step Ca^{2+} increase followed in an apparently usual way, suggesting that G-protein receptor signaling pathway is not involved in the second stage of the Ca^{2+} response at fertilization. Likewise, the Ca^{2+} response of the oocytes after injection of 40 mM GDPβS showed only sporadic miniature Ca^{2+} spots that lasted few seconds (7/10, Fig. 7*B*, 7*B1′*, *B2′* arrows, 0 and 47 s). Conversely, PTK inhibitors, such as genistein, HA, and PP2, had no effect on the first-step Ca^{2+} increase during fertilization (data not shown). These results clearly indicated that the first-step Ca^{2+} increase during fertilization was regulated by a G-protein mediated pathway.

GDPβS does not affect the exogenous IP_3 induced Ca^{2+} increase

To ensure that IICR acts downstream of G-protein, a mixture of 100 μM caged-IP_3 (final concentration: 4–8 μM) and GDPβS were co-injected into immature

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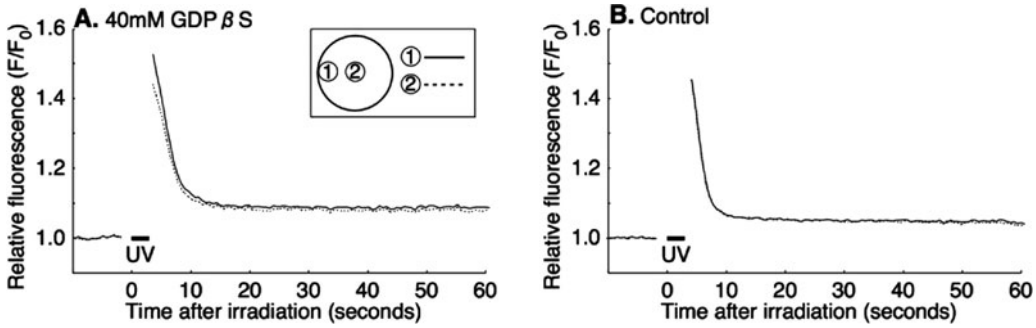

Figure 8 Exogenous IP_3-induced intracellular Ca^{2+} increase after GDPβS injection. Oocytes were co-injected with 100 μM caged IP_3 (final concentration: 4–8 μM) and 40 mM GDPβS (final concentration: 1.6–3.2 mM) (*A*); or injected with 100 μM caged IP_3 as control (*B*); and then maturation was induced. Caged IP_3-loaded mature oocytes were uncaged by 3 s UV irradiation.

oocytes before inducing maturation. The oocytes injected with caged-IP_3 but without GDPβS were used as a control. The UV irradiation-induced Ca^{2+} increase in these oocytes (7/7; Fig. 8*A*) was virtually the same as in the control eggs that had been injected only with caged-IP_3 without GDPβS (11/11; Fig. 8*B*). Thus, GDPβS did not inhibit the Ca^{2+} increase when IP_3 was supplied from an exogenous source. These result suggested that GDPβS does not affect the IICR itself, but its upstream events such as IP_3 production by PLC.

GTPγS injection induces the intracellular Ca^{2+} increase from the cortical region

The results that GDPβS inhibited the first-step Ca^{2+} increase during fertilization and the SE-induced Ca^{2+} release in the oocyte of *P. occelata* indicated that a G-protein-mediated pathway is involved in the process. Using a stable activator G-protein, GTPγS, we tested whether G-protein directly induced Ca^{2+} release in the oocyte. As expected, microinjection of 80 mM GTPγS (final concentration: 3.2–4.0 mM in the oocyte) at the peripheral region of the oocytes started to caused a Ca^{2+} increase around the tip of the needle immediately after injection (8/8; Fig. 9*A*, *A*′ 0 s), which grew further within seconds. The CP was formed around the injected region within 10 s (6/8; Fig. 9*A*′ 20 s arrowhead). The Ca^{2+} increase did not propagate well in the cortical region, and was terminated by 3–5 min. When 80 mM GTPγS was injected at central region of oocyte, a smaller Ca^{2+} increase compared with the injection at the peripheral region was detected in the whole cortex 24–36 s after injection (6/6; Fig. 9*B*′). The CP was not formed (0/6). In contrast, a large Ca^{2+} increase was observed upon GTPγS injection at the peripheral region (Fig. 9*C*, *D*). These results further supported the idea that the fertilized oocyte of *P. occelata* induces the initial Ca^{2+} signals through a G-protein-coupled receptor and the functional PLCβ near the plasma membrane.

Characteristics of SE activity

To characterize the putative active factor in SE that induces the Ca^{2+} increase, we examined its heat stability, protease sensitivity, and molecular weight. The SE was boiled for 15 min and centrifuged. The supernatant was obtained as a 'boiled SE'. The external application of boiled SE induced a Ca^{2+} increase, which was similar to the non-boiled control SE (10/10; Fig. 10). The amount of total protein after boiling dropped down to about 4.2% (from 330 to 14 μg proteins/ml), but the Ca^{2+} peak value of boiled SE, which was 1.35 ± 0.03 (F/F_0) ($n = 10$) at applied site, was larger than that of the crude SE (see Table 1).

To elucidate whether the SE activity is derived from protein, we tested the protease sensitivity of SE. The boiled SE was incubated with 0.1% protease at 20°C for 3 or 6 h, and then boiled for 15 min to deactivate the protease activity. The Ca^{2+}-increasing activity of the protease-treated SE was decreased after 3 h digestion, and was lost fully by 6 h (Fig. 11).

These results suggested that SE contained the putative active factor to induce the first-step Ca^{2+} increase during fertilization, which was the heat-stable small peptide.

Discussion

When the first-step Ca^{2+} increase occurs during fertilization in *P. occelata*, a fertilizing sperm head still remains on the vitelline envelop, which is ~15 μm distance from the egg surface. The CP is formed after the first-step Ca^{2+} increase and fertilizing spermatozoon is incorporated into it (Nakano *et al.*, 2008). In another annelid species, *Sabellaria vulgaris*, the oocyte has a similarly wide perivitelline space and thus forms a large CP during fertilization (Novikoff, 1939). In contrast, *Chaetopterus* oocyte has an inconspicuous perivitelline space, and its fertilizing sperm does not need to protrude the acrosome process to the egg

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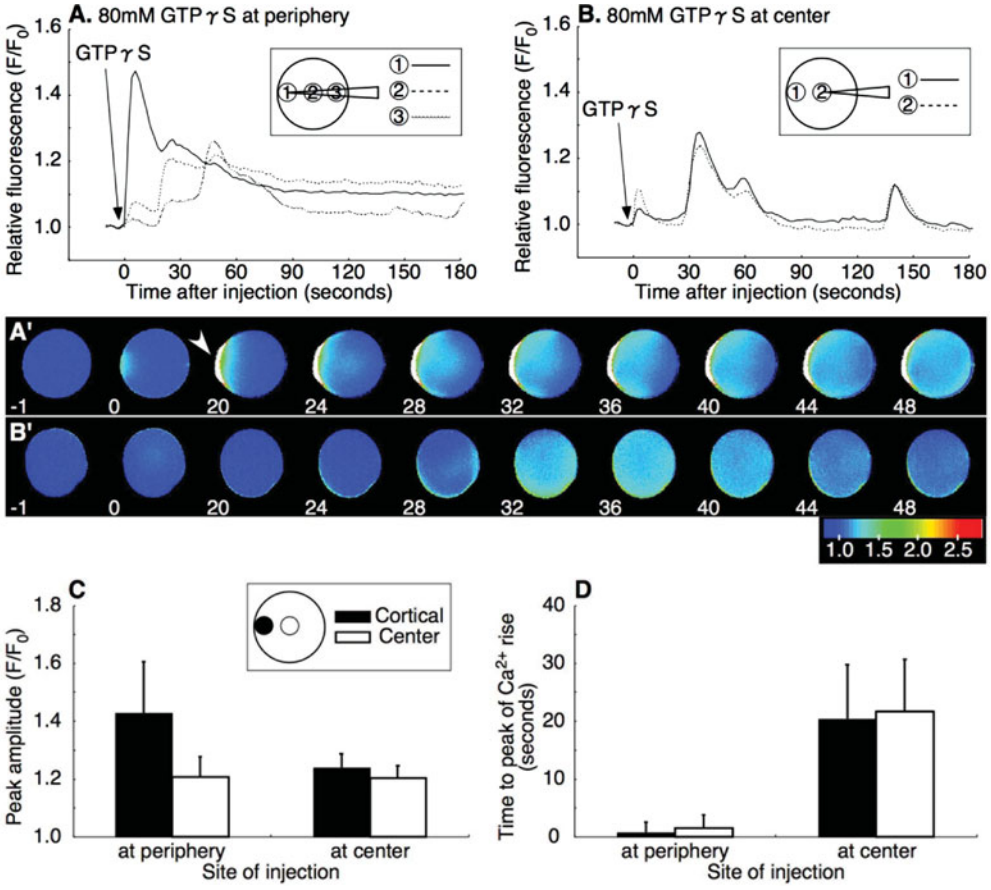

Figure 9 Injection of GTPγS induces an intracellular Ca^{2+} increase. Oocytes were injected with 80 mM (final concentration: 3.2–4.0 mM) GTPγS in at time zero. GTPγS was injected at peripheral (*A*, *A*′); or central (*B*, *B*′); regions of the oocytes. The graphs represented the peak amplitude (*C*) and the time span between the time of GTPγS injection and the time of initiation of the Ca^{2+} increase from the cortex (*D*).

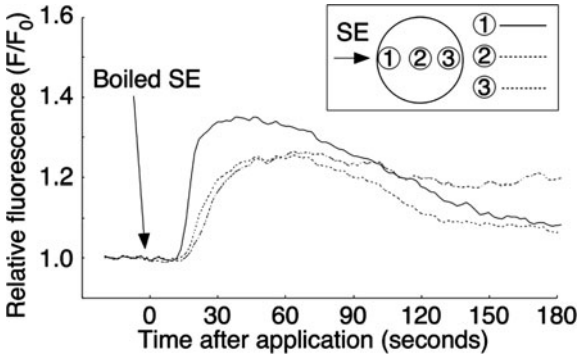

Figure 10 Resistance properties of sperm extract (SE) activity following heat treatment. SE was boiled for 15 min and then centrifuged. The supernatant from the boiled SE was applied externally to the oocyte in filtered seawater (FSW).

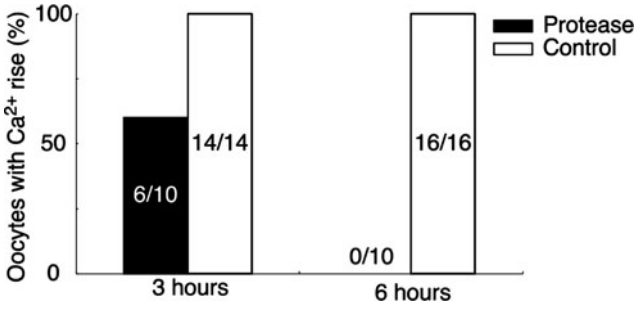

Figure 11 Inactivation of the sperm extract (SE) activity by protease. Boiled SE was incubated with 0.1% protease or deionized water (control) for 3 or 6 h at 20°C and then re-boiled for 15 min. Protease-treated SE or deionized water-treated SE was applied externally to the oocyte and the peak level of Ca^{2+} increase was compared.

surface. It is incorporated in a CP (Anderson & Eckberg, 1983). Therefore, the CP or fertilization cone is required for sperm incorporation, the size of which seems to correspond to the depth of the perivitelline space.

It remains unclear how sperm–oocyte interaction leads to the formation of the CP or fertilization cone. During fertilization in *P. occelata*, the sperm nucleus has been detected only after the retraction of the CP in a dye transfer experiment with Hoechst 33342-loaded

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oocytes. The first-step Ca^{2+} increase was inhibited by U73122 and the dye transfer was not observed in spite of the attachment of sperm on the vitelline envelope (Nakano *et al.*, 2008). When the oocyte was treated with excess K^+ to induce the second-step Ca^{2+} increase immediately after the first-step Ca^{2+} increase took place, the formation of a fully grown CP was blocked, and no sperm was observed in the activated oocyte (Nakano *et al.*, 2008). In the present study, we have demonstrated that the external application of SE can induce a local Ca^{2+} increase that leads to the formation of the CP (Fig. 4*A′* arrow). As SE is a soluble fraction prepared from the sperm, our results indicated that gamete membrane fusion between the fertilizing sperm and oocyte is not necessary for the induction of the first-step Ca^{2+} increase and the following CP formation.

Induction of the Ca^{2+} increase in the *P. occelata* oocytes by the application of SE was observed not only in FSW but also in CaFSW, but was blocked in the FSW that contained U73122. The repressive effect of the PLC inhibitor U73122 was also observed in the case of the first-step Ca^{2+} increase during fertilization by sperm, in which the IICR is involved. As GDPβS blocked the first-step Ca^{2+} increases by both SE and sperm, G-protein is involved in this pathway. We have corroborated this by microinjecting the mature oocytes with GTPγS that induced Ca^{2+} increase as expected. The Ca^{2+} increase was larger when GTPγS was injected at the peripheral region than at the center of the oocyte. The increase in Ca^{2+} always started from the cortex after a certain time lag even when the GTPγS was injected in the central region of the oocyte. Thus, the functional receptors coupled with G-protein and its downstream effectors for inducing the Ca^{2+} increase must exist in the cortex of *P. occelata. Chaetopterus* oocyte has PLCβ, which is very similar to PLCβ4 in mammalian oocyte and PLCβ in sea urchin oocyte (Yin & Eckberg, 2009), and it is thus likely that a similar G-protein-PLCβ pathway is at work in the cortex of the *P. occelata* oocyte. Recent studies have shown that U73122 and GDPβS have an effect on the kinetics of actin microfilaments and modulate the Ca^{2+} signaling in the oocyte (Kyozuka *et al.*, 2008, 2009). The regulation of Ca^{2+} changes with actin microfilaments are closely related to the progression of oocyte maturation and sperm entry in starfish oocytes (Puppo *et al.*, 2008). Actin microfilaments are rich in microvilli and in the cortex of annelid oocytes (Shimizu, 1999). Further studies are necessary to clarify the involvement of the actin microfilaments in the regulation of the local Ca^{2+} increase and CP formation during sperm incorporation in the *P. occelata* oocyte.

Generally, signal transduction through the receptor-coupled PLC is mediated by G-protein-PLCβ and/or PTK-PLCγ pathways (Berridge, 1993). There are many reports that the PTK-PLCγ pathway participates during the activation of the oocyte by sperm (Runft *et al.*, 2002). In *Chaetopterus* oocytes, PTK-activated PLCγ has a main role in signal transduction by sperm, but the involvement or localization of G-protein and PLCβ was unclear (Yin & Eckberg, 2009). PTK inhibitors effected blockage or delay of the Ca^{2+} increase during fertilization in annelid *Chaetopterus* (Yin & Eckberg, 2009), sea urchin (Shen *et al.*, 1999), ascidian (Ueki & Yokosawa, 1997), and *Xenopus* (Sato *et al.*, 1998; Sato *et al.*, 2000). From our results, PTK inhibitors, such as PP2, genistein, or herbimycin A, did not block the Ca^{2+} increase. In our previous work on the sensitivity of the *P. occelata* oocytes to IP_3, the localized Ca^{2+} increase induced by injection of high concentration IP_3 did not develop into a propagating Ca^{2+} wave (Nakano *et al.*, 2008). A similar Ca^{2+} response was observed in the fertilized oocytes of limpet, which showed a single Ca^{2+} increase that fully depended on a Ca^{2+} influx but not on the Ca^{2+} release by IICR (Deguchi, 2007). The bivalve *Mactra* also showed a Ca^{2+} influx during fertilization. (Deguchi & Morisawa, 2003). The neurotransmitter serotonin triggers Ca^{2+} release in these and bivalve *Spisula* oocytes (Colas & Dube, 1998; Deguchi & Morisawa, 2003). Serotonin mainly induces Ca^{2+} release in the cell through serotonin receptors coupled with G-protein (Noda *et al.*, 2004). In *Xenopus* oocytes, expression of the exogenous serotonin receptor induces IP_3-mediated Ca^{2+} increase though G-protein (Kline *et al.*, 1988). In all these cases, lack of development of Ca^{2+} wave propagation despite the IICR may be related to the intrinsic nature of the Ca^{2+} release mediated by the G-protein-activated PLCβ in the oocytes.

There is no information about the localization of the sperm receptors for activation on the egg surface. Studies on the ultrastructure of annelids *Chaetopterus* (Anderson & Eckberg, 1983), *Tylorrhynchus* (Sato & Osanai, 1983) and *Neanthes* (Sato & Osanai, 1986) oocytes have shown that microvilli extend from the cortex to the outer surface beyond the vitelline envelope, acrosome reacted-sperm fuses with the tips of microvillus where the vesicles are seated (Sato & Osanai, 1983; Anderson & Eckberg, 1983; Sato & Osanai, 1986). These morphological studies suggested that the tips of the microvillus vesicle might be a site of sperm reception. In the unfertilized *P. occelata* oocyte, the perivitelline space is bridged by numerous microvilli, which diminish in the fertilized oocyte significantly within 10 min (Nakano *et al.*, 2008). The microvilli may contain the receptors for the first-step Ca^{2+}increase during fertilization. In *Neanthes* oocytes, the tips of microvilli exposed on the vitelline envelope are about 2% of total surface area (Sato & Osanai, 1986).

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The present study showed that the SE-induced Ca^{2+} increase was similar to the first-step Ca^{2+} increase in the eggs fertilized by sperm. The SE was prepared by extraction from 4.1×10^{11} sperm/ml, and the volume of applied SE was 10 μl, which was equivalent to 4.1×10^{9} sperm. However, it is reasonable to assume that the SE is received only at the tips of microvilli. Therefore, *in vivo*, a small amount of SE might be sufficient to induce the first-step Ca^{2+} increase as one sperm may act on a single microvillus tip, and the effective concentration of SE in the microdomain is sufficient.

External application of protease permits egg activation in starfish (Carroll & Jaffe, 1995) and *Xenopus* (Mizote *et al.*, 1999), and existence of protease inhibitor disturbs *Xenopus* fertilization. However, it is unlikely that the first-step Ca^{2+} increase during fertilization of *P. occelata* involves protease activity as the first- and second-step Ca^{2+} increase and SE-induced Ca^{2+} increase were not affected by the existence of protease inhibitors, such as soybean trypsin and phenylmethylsulfonyl fluoride (data not shown). Meiotic maturation in starfish is reinitiated by a maturation-inducing hormone, 1-methyladenine. Its putative receptor is coupled with a heterotrimeric G-protein (Kishimoto, 1998). 1-Methyladenine also induced Ca^{2+} release by starfish immature oocytes (Santella *et al.*, 2003), although any connections between Ca^{2+} release and G-protein are still unknown. Our findings in the present study of a putative active factor to induce the first-step Ca^{2+} increase during fertilization was of a heat-stable peptide. A preliminary study to estimate the molecular size using a molecular sieve membrane with several cutoff sizes indicated that the active factor was smaller than 3 kDa (data not shown). The major factor in SE may act functionally like a hormone.

During fertilization, the second-step Ca^{2+} increase by Ca^{2+} influx follows the first-step Ca^{2+} increase. Our previous reports have indicated that the second-step Ca^{2+} increase is a necessary and sufficient stimulus for the extrusion of the polar body, resulting in egg activation. In this study, SE induced the first-step Ca^{2+} increase and CP formation; however, SE did not induce the second-step Ca^{2+} increase even in the presence of external Ca^{2+}. Accordingly, this activity differed functionally from the factor previously known as an egg activating sperm factor. It is, however, conceivable that the present factor has an essential role to play in *P. occelata* fertilization as it contributes to sperm entry by forming the CP. It is possible that this function is not seen in other animals as they do not have the wide perivitelline space such as that found in *P. occelata* and *S. vulgaris.* Alternatively, it may be characterized as part of the egg activation process.

Our previous study found that the second-step Ca^{2+} increase was caused by Ca^{2+} influx though voltage-gated Ca^{2+} channels. Subsequently, microvilli in the perivitelline space disappeared and additional CPs could not be formed for sperm incorporation. For the success of monospermic fertilization, the spermatozoon enters the egg at a preferential site or area in most animals (Dale & DeFelice, 2011; Dale, 2014). The binding of spermatozoon at the tip of microvillus on the vitelline envelope and the development of the CP beneath it must both contribute to monospermic fertilization in *P. occelata.* For the second-step Ca^{2+} increase in *P. occelata* oocytes, another active sperm factor is needed. The second-step Ca^{2+} increase occurred around the time that the fertilizing spermatozoon was engulfed by CP. These results indicated that the putative sperm factor works when the sperm contact or fusion with the CP.

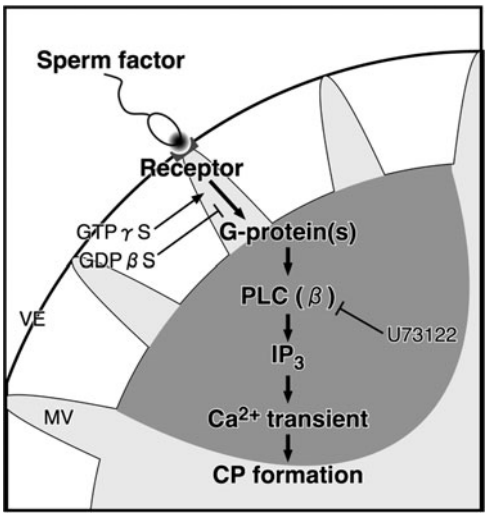

Figure 12 A model for the first-step Ca^{2+} increase during fertilization. External sperm factor is discharged from a spermatozoon on the vitelline envelope (VE). The sperm factor interacts with a receptor and mediates the activation of a G-protein/PLCβ pathway. Then, the IP_3-dependent Ca^{2+} increase induces the formation of the cytoplasmic protrusion for the incorporation of the spermatozoon into the oocyte. CP, cytoplasmic protrusion; GDP, guanosine diphosphate; GTP, guanosine triphosphate ; MV, microvillus; PLC, phospholipase C.

These results in this study are summarized schematically in Fig. 12. When the sperm bind to the vitelline envelope, a sperm–oocyte interaction that is mediated by an external sperm factor and an oocyte receptor, which is located at the microvillus tip, leads to activation of receptor-coupled G-proteins. Thereafter, directly or indirectly activated PLCβ produces IP_3 and causes IICR. The local Ca^{2+} increase in cortex, which is the first-step Ca^{2+} increase, introduces the formation of CP for sperm incorporation. The second-step Ca^{2+} increase, leading to the activation of the oocyte, is

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triggered due to contact/fusion of sperm with the CP surface membrane.

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