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Injection of sperm extract mimics spatiotemporal dynamics of Ca^{2+} responses and progression of meiosis at fertilization of ascidian oocytes

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SUMMARY

Sperm extract (SE) of the ascidian, *Ciona savignyi*, injected into oocytes induced repetitive intracellular Ca^{2+} increases with kinetics consistent with those at fertilization and caused reinitiation and progression of meiosis as in fertilized oocytes with the formation of polar bodies. The Ca^{2+} response comprised two sets of Ca^{2+} oscillations separated by 5 minutes and correlated with the first and second meiotic metaphase. The effects of SE were dose dependent and the critical dose corresponded roughly to a single spermatozoon. In the first Ca^{2+} transient observed by confocal microscopy, a Ca^{2+} wave started from the SE injection site at the peripheral region of the oocyte and propagated across the ooplasm. The similar wave was produced by injection at the central region, starting from an arbitrary cortical area after 30 seconds, probably after SE had diffused to the cortex. The sensitivity to SE is thought to be preferentially higher in the cortex. The effective component of SE was heat-unstable, and its molecular weight was estimated as in the range between 10×10^4 and 3×10^4 using membrane filters. These results suggest that, in ascidian fertilization, a cytosolic sperm protein factor is introduced to the oocyte cortex and induces Ca^{2+} waves and thereby meiotic resumption, leading to cell-cycle-correlated Ca^{2+} oscillations.

Key words: Fertilization, Ascidian oocyte, Sperm factor, Meiotic division, Intracellular calcium, Calcium wave, Calcium oscillation

INTRODUCTION

A dramatic increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is one of the early events in oocytes at fertilization that is common to all species studied to date. The increase in $[Ca^{2+}]_i$ triggers egg activation and the reinitiation of meiosis, and also affects subsequent embryonic development (Whitaker and Swann, 1993; Swann and Ozil, 1994). In deuterostome oocytes, the $[Ca^{2+}]_i$ rise is caused by release of Ca^{2+} from intracellular stores (Jaffe, 1983), and the Ca^{2+}-release mechanism has been extensively analyzed in various species (Whitaker and Swann, 1993; Miyazaki et al., 1993). However, the mechanism by which the sperm triggers Ca^{2+} release is still unclear. It has been proposed that soluble cytosolic sperm factors, which possess Ca^{2+}-release-inducing activity in oocytes, may be introduced into the ooplasm through the cytoplasmic continuity formed after sperm-egg fusion (Whitaker and Swann, 1993; Swann, 1996). This is supported by the fact that intracytoplasmic injection of sperm extract into various mammalian oocytes induces Ca^{2+} oscillations (Stice and Robl, 1990; Swann, 1990, 1996; Dale et al., 1996; Wu et al., 1997), which are the common Ca^{2+} response pattern in mammalian fertilization (Miyazaki et al., 1993). In sea urchin eggs, injected sperm extract causes breakdown of cortical granules (Dale et al., 1985) and, in the marine worm *Cerebratulus lacteus*, it causes fertilization-like responses including Ca^{2+} oscillations (Stricker, 1997). Interestingly, in mammals, sperm extract is capable of inducing Ca^{2+} oscillations in heterologous oocytes (Swann, 1992; Sousa et al., 1996; Wu et al., 1996). Furthermore, it has been shown that activity can extend between phyla; human sperm extract produces repetitive Ca^{2+} spikes in ascidian oocytes (Wilding et al., 1997), and sea urchin sperm extract causes membrane current changes similar to those observed at fertilization in ascidian oocytes (Dale, 1988). Thus, ascidian oocytes are responsive to heterologous sperm factors. Although some calcium changes have been shown to be induced by injection of ascidian sperm extract (Wilding et al., 1997), ascidian sperm factor-induced Ca^{2+} oscillations have not been analyzed precisely. Therefore, we have investigated the function of sperm extract in egg activation in an ascidian, *Ciona savignyi*.

Ciona oocytes obtained from the oviducts of matured animals are arrested at metaphase I (MI) of meiotic division. After fertilization, the oocytes reinitiate meiosis, protrude the

first and second polar bodies, and complete meiosis (Conklin, 1905). Fertilized oocytes exhibit a transient increase in $[Ca^{2+}]_i$ followed by Ca^{2+} spikes (Speksnijder et al., 1989, 1990). The overall Ca^{2+} response comprises two sets of Ca^{2+} oscillations, which correspond to the first and second meiotic division, respectively (Russo et al., 1996). This Ca^{2+} response of ascidian oocytes at fertilization and during meiosis is so characteristic that the sperm-extract-induced $[Ca^{2+}]_i$ rises can be correlated with the Ca^{2+} response after fertilization and distinguished from an artifactual $[Ca^{2+}]_i$ rise due to injection procedure as occurs in sea urchin eggs (Hafner et al., 1988; Shen, 1995). Furthermore, the effect of sperm extract on egg activation and meiosis can be precisely examined, as the first and second meiotic divisions are completed within a period as short as 30 minutes in *Ciona* oocytes. In the present study, we examined the effect of injected sperm extract on the resumption and progression of meiosis as well as on temporal and spatial Ca^{2+} dynamics, by simultaneous observation of morphological changes and $[Ca^{2+}]_i$ rises using video image acquisition with infrared light and Ca^{2+} imaging with confocal laser scanning microscopy. Changes in $[Ca^{2+}]_i$ were also monitored with a photomultiplier.

MATERIALS AND METHODS

Materials

Ciona savignyi were collected near Asamushi Marine Biological Station during May through July 1997. The thick oviduct was dissected from a full-grown animal with small scissors and the oocytes were transferred to a Petri dish filled with sea water using a fine pipette. Oocytes with the intact chorion were used for fertilization or injection of sperm extract after removing the surrounding follicle cells by gentle pipetting. Spermatozoa for fertilization were obtained directly from the ductus and kept as dry sperm, which were diluted in sea water immediately before insemination.

Preparation of sperm extract

All procedures were performed at 4°C. Spermatozoa released from the ductus of 100 animals were suspended in 100 ml Ca^{2+}-free sea water (CaFSW) and resuspended in CaFSW containing 1 mM EGTA after centrifugation (4,000 ***g***, 5 minutes). After washing in CaFSW plus EGTA twice by centrifugation, spermatozoa were suspended in 10 ml of CaFSW. The sperm concentration of this suspension was 4.5×10^7 sperm/ml, counted with a hemocytometer using 10 μl sperm suspension mixed with 90 μl CaFSW containing 1% formalin. Sperm suspension was divided equally into 10 small tubes and centrifuged. Spermatozoa were resuspended in 0.2 M phosphate buffer (pH 7.4) and centrifuged again. After removing the supernatant, packed spermatozoa (about 100 μl of the volume) were obtained in each tube and they were kept frozen at –95°C until use. To obtain sperm extract, frozen spermatozoa in each tube were added with 'extraction buffer' containing 140 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes (pH 7.0) to obtain the final volume of 350 μl, and then homogenized in the same tube using a microhomogenizer. After centrifugation of the homogenate (20,000 ***g***, 10 minutes), supernatant was collected as soluble sperm extract (SE). The protein concentration in SE was about 10 mg/ml. SE was divided into tubes and kept frozen until use.

Bioassay of sperm extract

The relation between the dose of SE and the induction of meiotic resumption was examined by co-injection of a mixture of fluorescent isothiocyanate dextran (Sigma, St Louis, MO; M_r 7×10^4; 10 mg/ml in extraction buffer) and SE (1:19, FITC-SE) under an inverted microscope (IMT-2, Olympus, Tokyo, Japan) equipped with a photomultiplier. The extrusion of polar bodies was examined 45-60 minutes later. Oocytes freed from the chorion with fine needles were used for this experiment since several dechorionated oocytes could be injected with SE using a single micropipette in a short period, without plugging the pipette. Dechorionated oocytes were used to observe the number of polar bodies formed on the egg surface, since test cells and follicle cells that adhere to the chorion would hinder the observation of polar bodies. The fluorescence intensity (F_{FITC}) of individual oocytes was measured by a photomultiplier using 488 nm excitation filter and 530 nm emission filter. The relationship between F and the injected volume of FITC-SE was first obtained by injection of certain amount (8 or 14 pl) of FITC-SE in some oocytes. Other oocytes were injected with various volumes of FITC-SE which were estimated from F_{FITC}. All of these procedures as well as $[Ca^{2+}]_i$ measurement (described below) were performed at 18°C.

Measurement of $[Ca^{2+}]_i$ using photomultiplier

For $[Ca^{2+}]_i$ measurement during fertilization or after injection of SE, oocytes were preinjected with the fluorescent Ca^{2+} indicator dye Calcium Green Dextran (CGD, M_r 1×10^4; Molecular Probes Inc., Eugene, OR; 2 mM CGD in extraction buffer). The amount injected was about 2% of the egg volume. The intracellular CGD concentration was calculated to be about 20 μM. The $[Ca^{2+}]_i$ measuring system has been described previously (Kyozuka et al., 1997). Briefly, 488 nm and 530 nm band-pass filters for passing the excitation and emission light, respectively, were combined with a computer-controlled photomultiplier system (OSP-3, Olympus). F was continuously measured and normalized against the initial value of F (F_0) by the equation $(F - F_0)/F_0$ (Cornell-Bell et al., 1990). As it was necessary to start $[Ca^{2+}]_i$ measurement in the oocyte during injection of SE, bright-field observation with red light was performed using 630 nm long-pass filter during $[Ca^{2+}]_i$ measurement though an eyepiece.

Confocal microscopy

The spatiotemporal distribution of $[Ca^{2+}]_i$ rises was observed by confocal laser scanning microscopy (CLSM; RCM 8000, Nikon, Tokyo) attached to an inverted microscope (TMD-300, Nikon). Ratio images with two dyes were obtained by injecting 0.4 mM CGD and 4 mM Texas Red Dextran (TRD; Ca^{2+}-insensitive dye; M_r 1×10^4; Molecular Probes) into an oocyte. For excitation of the dyes, light from a 488 nm argon laser was led to the oocyte through a ×20 objective lens (Fluor 20; NA 0.75; Nikon), after being reflected by the first dichroic mirror. Emission fluorescence (green and red) passed through the objective lens and the same dichroic mirror. Interference between excitation and emission lights was avoided, as the dichroic mirror had two band-pass ranges only for emission light: between 510 and 535 nm and between 570 and 680 nm. The emitted light was then separated by the second dichroic mirror at 565 nm after passing through the pinhole. Each signal passed through a band-pass filter (520±15 nm) for green channel and a long-pass filter (>610 nm) for red channel, and was detected by separate photomultiplier tubes. The focal plane of CLSM was adjusted at center the oocyte.

Recording and image processing

Two-channel fluorescence images were acquired every 1.5 to 3 seconds until the completion of egg contraction (about 2 minutes after fertilization), and subsequently every 5 to 10 seconds to avoid cell damages caused by continuous irradiation with the laser during meiosis. Each image was constructed by accumulating 32 frames to improve signal-to-noise ratio. Images were led to an image processor (MAX VIDEO) and stored on an optical memory disc recorder (Model TQ-3800F, Panasonic Co., Tokyo). Processing was performed with built-in image analyzing software for confocal laser unit

controlling system (Nikon). Fluorescence ratio CGD/TRD was calculated in a pixel-to-pixel manner.

Bright-field images with infrared light (IR images)

Video-rate Bright-field images were simultaneously acquired using transmitted infrared (IR) light at video-rate. IR images did not interfere with fluorescence images of CLSM, since a dichroic beam splitter at 650 nm was inserted between the objective lens and the first dichroic mirror described above. Images were led to an IR camera (VC 820L, Tokyo Electronic Industry Co., Tokyo) through a band-pass filter (735-770 nm) and were recorded on an SVHS video cassette recorder (HR-VX11, Victor, Yokohama, Japan). Other details have been described previously (Mohri et al., 1998).

Characterization of sperm extract

To examine the heat stability, 10 μl of SE in a 1.5 ml tube was treated in boiling water for 10 minutes and then the 'boiled SE' was injected. To determine the approximate molecular size of the presumptive active factor, membrane filters of M_r 3×10^4 and 1×10^5 (Artkiss, Artchem Co., Tokyo) were used. Samples were centrifuged at 4,000 ***g*** for 5 minutes. Both passed and non-passed fractions were collected and examined for ability to induce $[Ca^{2+}]_i$ rises in unfertilized oocytes.

RESULTS

Ca^{2+} oscillations and meiotic division following fertilization or injection of sperm extract

Fig. 1A shows the typical Ca^{2+} response in the *Ciona savignyi* oocyte at fertilization and after reinitiation of meiosis. The response comprises two sets of Ca^{2+} oscillations correlated with the first and second meiotic division, respectively, similar to the response in the *Ciona intestinalis* oocyte (Speksnijder et al., 1989, 1990; Russo et al., 1996; Albrieux et al., 1997). The Ca^{2+} response showed a relatively large Ca^{2+} transient with a rapid rising phase, a slower declining phase and the total duration of 3-4 minutes. Egg contraction associated with ooplasmic segregation, which is one of the earliest morphological indicators of egg activation in *Ciona* (Sawada and Osanai, 1981), was recognized about 2 minutes after the onset of the $[Ca^{2+}]_i$ rise, during the declining phase of the first Ca^{2+} transient. The first Ca^{2+} transient was then immediately followed by several Ca^{2+} spikes of the much shorter duration (Fig. 1A). There was a gap of about 6 minutes after the first set of Ca^{2+} oscillations before the second set of Ca^{2+} oscillations which then continued for an additional 15 minutes. The first and second polar bodies were formed at the end of the first and second set of Ca^{2+} oscillations, respectively.

A Ca^{2+} response that was quite similar to that following fertilization was induced by injection of 14 pico-liter (pl) of *Ciona* sperm extract (SE) into the oocyte (Fig. 1B), a dose roughly comparable to the extract from 1.8 spermatozoa (see Discussion). The first large Ca^{2+} transient was generated soon after injection of SE and lasted for ~4 minutes, associated with egg contraction in the declining phase. The first Ca^{2+} transient was immediately followed by several Ca^{2+} spikes, before $[Ca^{2+}]_i$ returned to the basal level. The protrusion of the first polar body was observed at the end of the first set of Ca^{2+} oscillations, about 10 minutes after injection of SE. After a resting period of 6 minutes, the second set of Ca^{2+} oscillations consisting of Ca^{2+} spikes was generated for another 10 minutes. The second polar body was formed at the end of the second Ca^{2+} oscillations. Thus, the injected SE caused the reinitiation and progression of meiotic division as well as the induction of Ca^{2+} oscillations. Neither cleavage nor further development proceeded after the completion of meiosis following injection of SE, although they proceeded normally after fertilization. The measurement of $[Ca^{2+}]_i$ using photomultiplier with 488 nm excitation wave did not block embryonic development.

The characteristics of Ca^{2+} oscillations induced by injection of SE were precisely compared with those after insemination (Table 1). There was no significant difference in the number of Ca^{2+} spikes in the first and second set of Ca^{2+} oscillations. The duration of the first set of Ca^{2+} oscillations including the first Ca^{2+} transient, the interval between the two sets of Ca^{2+} oscillations, and the duration of the second set of Ca^{2+} oscillations were quite similar between sperm- and SE-induced Ca^{2+} responses. Thus, injection of *Ciona* SE precisely mimicked fertilization by the sperm in terms of the induction of Ca^{2+} oscillations and the completion of meiosis.

The dose effect of SE on egg activation was examined by injecting different volumes of FITC-labeled SE and identifying the formation of polar bodies (Fig. 2) in dechorionated oocytes. Injected volumes were estimated by the fluorescence intensity of FITC (F_{FITC}) measured after homogeneous distribution of FITC throughout the oocyte was attained. F_{FITC} for injection of 14 pl SE was about 400 (arbitrary unit). Injection of SE less than 14 pl failed to induce the formation of polar bodies or caused the protrusion of only the first polar body. With volumes more than 14 pl, two polar bodies were formed in most oocytes

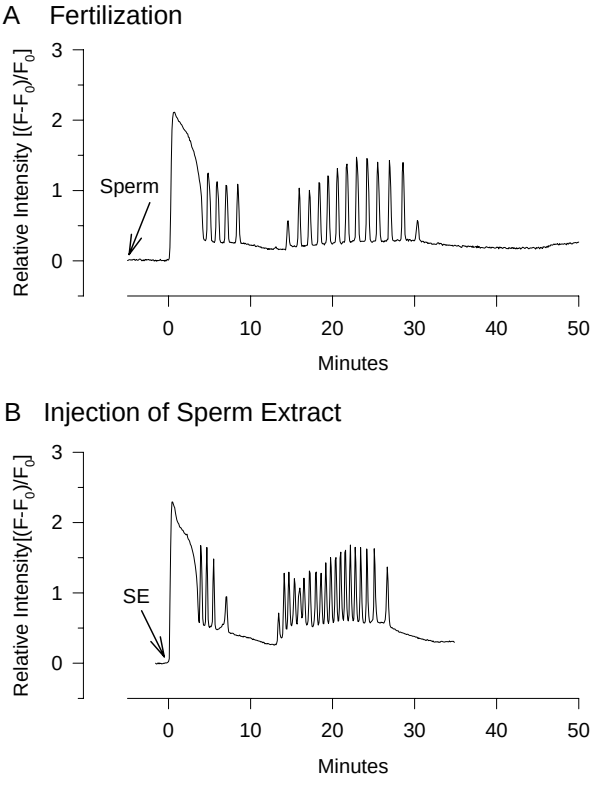

Fig. 1. Changes in $[Ca^{2+}]_i$ following fertilization of the *Ciona savignyi* oocyte (A) or injection of SE into an oocyte (B). Arrows indicate the time when sperm suspension was added or SE was injected. The onset of the first Ca^{2+} transient was taken as the zero time.

Table 1. Characteristics of Ca^{2+} oscillations induced by fertilization and injection of sperm extract

	Number of Ca^{2+} spikes		Duration (minutes)		
	1st set*	2nd set*	1st set*	Intermission*	2nd set*
Fertilization (*n*=8)	4.5±1.3†	14.5±4.2	8.1±1.0	6.4±2.3	14.8±2.4
Injection of sperm extract (*n*=8)	3.8±1.2	15.0±4.1	7.4±1.2	5.7±2.0	13.8±1.3

*1st set and 2nd set of Ca^{2+} oscillations
†mean ± s.d.

(Fig. 2). Where no polar bodies were observed, they might have detached from the oocyte before observation, or formed on the opposite surface of the oocyte. Fig. 2 demonstrates that the reinitiation and progression of meiotic division are induced dose-dependently by the injected SE, and that 14 pl SE was the critical dose for egg activation in the present experimental condition.

Ca^{2+} waves at fertilization and upon injection of SE

Spatiotemporal dynamics of the $[Ca^{2+}]_i$ rise in the first Ca^{2+} transient were investigated by Ca^{2+} imaging every 1.5 to 3 seconds using confocal microscopy. At fertilization, a Ca^{2+} wave started from the sperm binding site and propagated to the antipode across the deep cytoplasm in ~20 seconds (Fig. 3A). A similar Ca^{2+} wave was induced by injection of 14 pl SE at the peripheral region of the oocyte (Fig. 3B). SE was injected at the 9 o'clock position of the peripheral region opposite the micropipette penetration site, to avoid any effect of a slight elevation of $[Ca^{2+}]_i$ at the penetration site (probably due to Ca^{2+} leakage from outside the oocyte) on the initiation of a Ca^{2+} wave. The time when injection of SE was finished was defined as the zero time. A small $[Ca^{2+}]_i$ rise occurred during injection at the injection site near the tip of the micropipette (see the image marked by −1.5 seconds in Fig. 3B) and ceased after several seconds. This localized transient $[Ca^{2+}]_i$ rise was considered to be an artefact, since a similar $[Ca^{2+}]_i$ rise was produced even by injection of buffer without SE (not shown). A small amount of Ca^{2+} might be introduced to the cytoplasm from the tip of the micropipette into which sea water had diffused before insertion, or localized Ca^{2+} release might occur from the ER subjected to mechanical stimulation by the stream of injected solution. In Fig. 3B, the genuine $[Ca^{2+}]_i$ rise began at the injection site 7.5 seconds after the completion of injection and led to a Ca^{2+} wave. The wave propagated not only in the cortex but also in the central region of the cytoplasm, and arrived at the opposite pole in 15 seconds.

When the same amount of SE was injected into the central region of the oocyte, no $[Ca^{2+}]_i$ rise was observed for a while except the small artifactual $[Ca^{2+}]_i$ rise at the injection site (see the image marked by −12.0 seconds in Fig. 3C). In Fig. 3C, a clear $[Ca^{2+}]_i$ rise began at the 2 o'clock position of the egg's cortex at 22.5 seconds after the completion of injection. The $[Ca^{2+}]_i$ rise led to a Ca^{2+} wave that propagated toward the opposite pole in about 20 seconds. Thus, the Ca^{2+} wave was generated from a cortical area instead of the injection site in the deep cytoplasm, and was preceded by a long lag time. The trigger region of the Ca^{2+} wave was often near the micropipette penetration site as in Fig. 3C, at which $[Ca^{2+}]_i$ was slightly elevated probably due to leakage of Ca^{2+}. However, the trigger region was sometimes a site of the cortex other than the pipette penetration site (at the 3 o'clock position of the egg's cortex) in 6 of 14 oocytes.

The characteristics of Ca^{2+} waves induced by injection of SE were precisely compared in terms of the injection sites and with those after insemination (Table 2). The lag time was defined as the time difference between the completion of SE injection and the start of the Ca^{2+} wave. The mean value was 6.6 seconds for injection at the peripheral region, as opposed to 33.8 seconds for injection in the central region. The propagation time of the SE-induced Ca^{2+} wave to traverse the oocyte was about 12 seconds in average, irrespective of the

Table 2. Characteristics of Ca^{2+} waves induced by fertilization and injection of sperm extract (SE)

	Lag time[1] (seconds)	Propagation time[2] (seconds)
Fertilization (*n*=5)	–	14.0+3.2
Injection of SE		
At peripheral region (*n*=5)	6.6±3.9*,†	12.0±3.2
At central region (*n*=5)	33.8±17.8†	11.7±3.6

*mean ± s.d.
†Difference significant $P<0.02$ by Student's *t*-test.
[1]The time required from the completion of SE injection to 10% increase of fluorescence (the difference between the basal level and the maximal level was taken as 100%). Average fluorescence intensity in the optical section of the oocytes (observed by confocal microscopy) was measured in a concentric circle of about 2/3 of the oocyte diameter, in order to eliminate the change of the peripheral area due to egg contraction.
[2]The time between 10% and 90% increase of fluorescence intensity which was measured as described above.

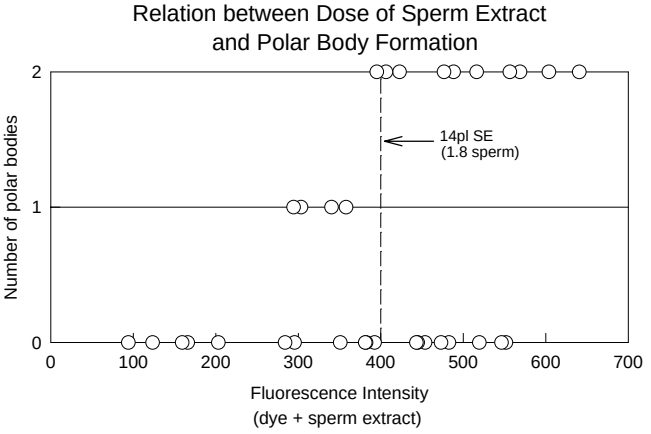

Fig. 2. The relationship between the amount of injected SE and the number of polar bodies formed in the oocyte. The amount of SE was estimated from the fluorescence intensity of FITC dextran which was co-injected together.

injection site (Table 2). The propagation time tended to be slightly shorter than that of the Ca^{2+} wave at fertilization, but not significantly different.

Characterization of *Ciona* sperm extract

Injection of 22 pl of boiled SE caused neither increase in $[Ca^{2+}]_i$ nor reinitiation of meiosis, as shown in Fig. 4 (examined in n=5 oocytes). The responsiveness of these oocytes to SE was confirmed by injection of heat-untreated SE 20 minutes later (Fig. 4, right half). No Ca^{2+} response was produced even by injection of 65 pl of boiled SE (n=3). Thus, the active *Ciona* sperm factor is heat-unstable.

To examine the approximate molecular weight of the active factor of *Ciona* SE, the sample was filtered using membrane filters of M_r 3×10^4 and 10×10^4. The filtered solution through M_r 3×10^4 filter had no activity (Fig. 5A, left; n=6), whereas the non-filtered solution possessed the complete Ca^{2+} oscillation-inducing activity (Fig. 5A, right half) as well as the ability to reinitiate and complete meiosis (n=4). The filtered solution through M_r 10×10^4 filter had complete activity (n=7). It is likely that the molecular size of the active factor is between 3×10^4 and 10×10^4.

DISCUSSION

Effects of injected *Ciona* sperm extract

It is known that fertilized *Ciona* oocytes exhibit well-regulated $[Ca^{2+}]_i$ changes, comprising two sets of Ca^{2+} oscillations that are correlated to meiosis I and II, respectively (McDougal and Sardet, 1995; Russo et al., 1996). The present study demonstrated that *Ciona* SE injected into an unfertilized oocyte induces in a dose-dependent manner the reinitiation and progression of meiosis as well as two sets of Ca^{2+} oscillations that have kinetics almost identical with those at fertilization. Thus, the Ca^{2+} oscillation-inducing factor exists in the *Ciona* sperm and injection of SE faithfully mimics fertilization.

The critical dose of SE for the induction of complete meiosis was 14 pl. As 350 μl SE was obtained from 4.5×10^7 spermatozoa, 14 pl SE corresponds to extract from 1.8 spermatozoa. The estimated value will be closer to 1 spermatozoon, if some fraction of activity of the effective sperm factor was lost during preparation of SE. It was, therefore, reasonable to consider that the sperm factor contained in a single spermatozoon is capable of inducing the fertilization responses. Injection of smaller amounts of SE failed to cause egg activation and no polar body or only the first polar body was induced. It has been shown that a single injection of inositol 1,4,5-triphosphate ($InsP_3$) into ascidian oocytes or photolysis of injected caged $InsP_3$ produces a series of Ca^{2+} oscillations, egg contraction and first polar body formation (Tosti and Dale, 1994; Roegiers et al., 1995; McDougal and Sardet, 1995; Wilding et al., 1997). The second set of Ca^{2+} oscillations and second polar body formation are not detected (Wilding et al., 1997), but they are induced by continuous application of $InsP_3$ through a micropipette (Albrieux et al., 1997). These results suggest that the function of the sperm factor is not simply to trigger the first Ca^{2+} transient which is followed by the first set of Ca^{2+} oscillations but to act continuously to induce the second set of Ca^{2+} oscillations and that the primary action of the sperm factor is to induce $[Ca^{2+}]_i$ rises, which cause the reinitiation of meiotic division.

Interestingly, sperm-induced Ca^{2+} spikes are cell-cycle dependent. Long-lasting Ca^{2+} oscillations in fertilized mouse oocytes cease at about the time of pronucleus formation, whereas Ca^{2+} oscillations continue for longer than 18 hours when fertilized oocytes are arrested at the metaphase II by application of colcemid (Jones et al., 1995). In the pronuclear

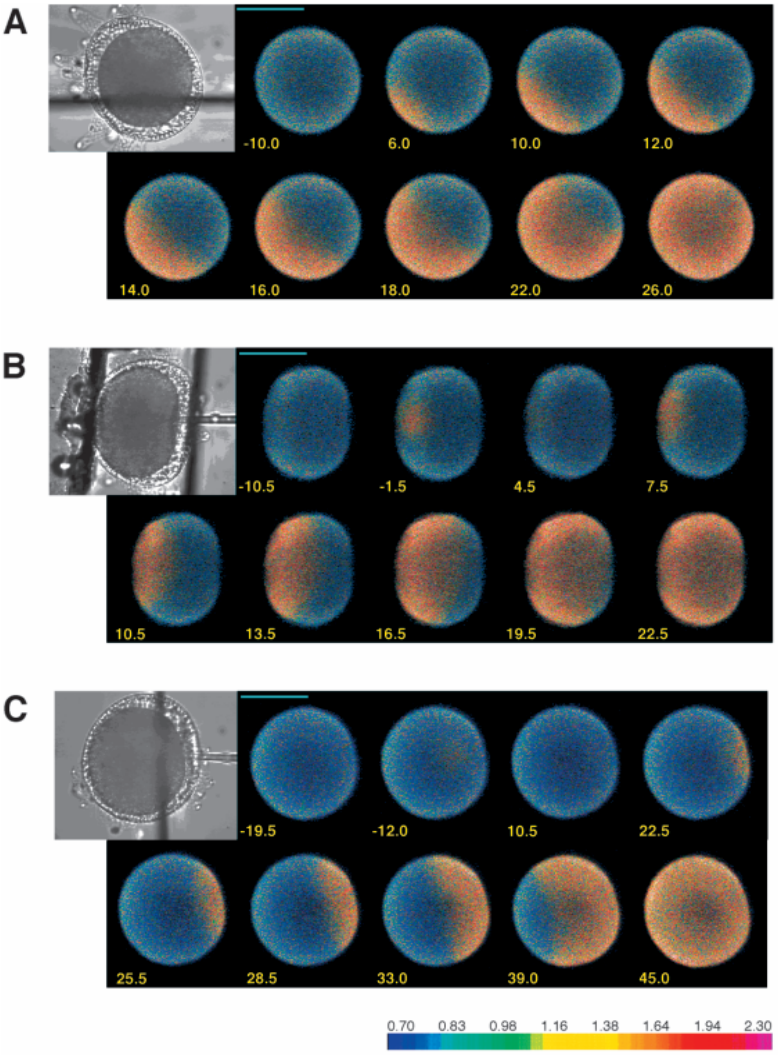

Fig. 3. The Ca^{2+} wave in the first Ca^{2+} transient induced by fertilization (A), injection of 14 pl SE at the peripheral region of the oocyte (B), or injection at the central region (C), observed using confocal microscopy. A bright-field image of the oocyte before insemination or SE injection is shown top left in each figure. The injection pipette is seen in B,C. The distribution of the $[Ca^{2+}]_i$ rise is presented by ratio imaging (fluorescence of CGD divided by that of TRD). The number presented in each image is the time (in seconds) of acquisition after the initiation of the $[Ca^{2+}]_i$ rise (A) or after the end of SE injection (B,C). The color bar indicates ranges of fluorescence ratio. The blue bar (top left) is 100 μm.

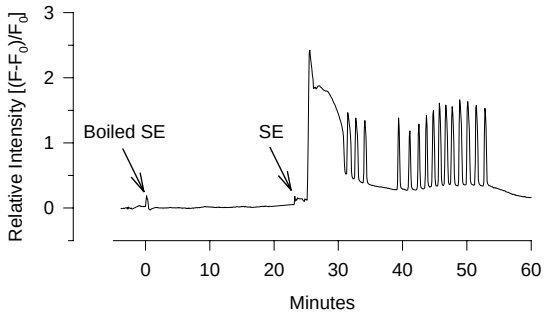

Fig. 4. Changes in $[Ca^{2+}]_i$ following injection of boiled SE and subsequent injection of non-treated SE in the same oocyte.

stage mouse embryo after cessation of Ca^{2+} oscillations, injection of $InsP_3$ hardly induces Ca^{2+} oscillations (Jones et al., 1995). The correlation between the occurrence of Ca^{2+} oscillations and the stage of cell division is demonstrated more clearly in *Ciona* oocytes in which meiosis progresses in a short period. Ca^{2+} oscillations can be easily generated at the metaphase, but are prevented after exit from the metaphase. The ability of oocytes to release Ca^{2+} from the endoplasmic reticulum may be reduced after the metaphase and the sperm factor may be unable to overcome this perturbation.

Preferential action of SE on the cortex

A Ca^{2+} wave was initiated from the injection site, when 14 pl SE was injected at the peripheral region of the oocyte. In contrast, when SE was injected at the central region, a Ca^{2+} wave was initiated from an arbitrary cortical area, associated with a delay of 25-35 seconds after injection without any preceding $[Ca^{2+}]_i$ rise except the artifactual transient $[Ca^{2+}]_i$ rise upon injection. Once a Ca^{2+} wave was initiated, the wave per se appeared to be identical as observed in the propagation velocity. It is likely that SE injected in the central region diffused to the peripheral region and then caused the $[Ca^{2+}]_i$ rise at the cortical region. These results indicate that the sensitivity to the sperm factor is preferentially higher in the cortical area than in the interior, although the exact site of action of the sperm factor is still unknown. $InsP_3$ receptors are rich in the cortical area, as shown in *Xenopus* (Kume et al., 1993) or mouse oocyte at the metaphase II (Mehlmann et al., 1996). In *Ciona* oocytes, heparin, a competitive antagonist of $InsP_3$ receptors, blocks Ca^{2+} spikes at fertilization (Russo et al., 1996; Albrieux et al., 1997). If $InsP_3$ receptors distribute abundantly in the cortex of *Ciona* oocytes, this could be one of the causes of preferential initiation of a Ca^{2+} wave at the cortex following injection of SE. This higher sensitivity to the sperm factor in the cortex would result in a Ca^{2+} wave generated as a result of the sperm-egg cytoplasmic continuity at fertilization and supports the sperm factor hypothesis.

There was a lag time of ~7 seconds between injection and the first $[Ca^{2+}]_i$ rise, even when SE was injected at the peripheral region. Beside a slight delay due to diffusion of SE, there may be an inherent delay in the action of the sperm factor leading to Ca^{2+} release. Galione et al. (1997) have demonstrated that the application of hamster sperm extract to homogenates of sea urchin oocytes induces Ca^{2+} release from

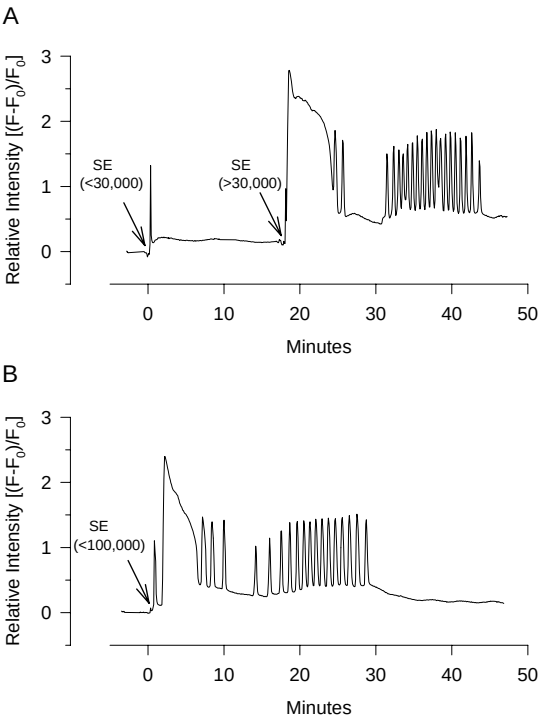

Fig. 5. Changes in $[Ca^{2+}]_i$ following injection of SE solution filtered through M_r 3×10^4 filter (<30,000 in A), non-filtered solution (>30,000 in A), and solution filtered through M_r 10×10^4 filter (<100,000 in B).

both $InsP_3$-sensitive and ryanodine-sensitive Ca^{2+} stores after a delay of at least 100 seconds. They suggested the involvement of a cytosolic egg factor that is activated by the sperm factor and leads to Ca^{2+} release, since SE pretreated with the sea urchin egg cytosol remarkably reduces the lag time (Galione et al., 1997). This presumptive process may take place more readily in the cortical area than the deeper cytoplasm.

Characterization of the sperm factor

The present study suggests that the presumptive *Ciona* sperm factor is heat-unstable and has M_r between 3×10^4 and 1×10^5. The factor is probably a protein, as suggested in the hamster (Swann, 1996) and marine worm sperm (Stricker, 1996). The active component of the hamster sperm has been identified as a 33 kDa protein designated as oscillin (Parrington et al., 1996). Further studies are expected for purification and characterization of the *Ciona* sperm factor. As injection of human SE into ascidian oocytes induces Ca^{2+} oscillations (Wilding et al., 1997), comparison of sperm factors among species is one of the central subjects for the study on the mechanism of fertilization. Furthermore, the fertilized ascidian oocyte could be a useful model system for elucidation of the relationship between Ca^{2+} oscillations and cell cycle.

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