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Flowering Plant Sperm Contains a Cytosolic Soluble Protein Factor Which Can Trigger Calcium Oscillations in Mouse Eggs

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There is evidence showing that the sperm-induced Ca^{2+} oscillations in mammalian eggs at fertilization are triggered by a sperm-derived protein factor. It was established recently that the activity of the putative sperm protein in causing Ca^{2+} oscillations in mammalian eggs is not species-specific in vertebrates (1, 16). Here we report that cytosolic soluble extracts derived from flowering plant sperms in *Brassica campestris* can also induce fertilization-like Ca^{2+} oscillations when microinjected into mouse eggs. The factor responsible for inducing Ca^{2+} oscillations in the plant sperm was sperm-specific and heat- or trypsin-labile. Eight to ten sperm equivalents of the plant sperm extracts had enough activity to trigger Ca^{2+} oscillations in mouse eggs. Our study suggests that, although plant and mammal are evolutionary divergent species, the activity of the putative sperm protein factor in triggering Ca^{2+} signaling in mammalian eggs is not specific to the animal kingdom. © 2001 Academic Press

Key Words: **calcium oscillations; sperm factor; egg; mouse; *Brassica campestris*.**

In all species so far studied, it is a common phenomenon that sperm activates the egg by triggering a transient increase in the intracellular free calcium ion concentration (1–3). This calcium increase may take the form of a single rise in most nonmammalian animals, as in sea urchin (4) and *Xenopus* (5), or a series of repetitive calcium spikes (calcium oscillations) in mammals (6) and some marine invertebrates. In response to this signal, the fertilizing egg completes meiosis and initiates its embryonic development (7).

The mechanism by which sperm causes Ca^{2+} release in the egg is still unknown. There is evidence showing that at fertilization the sperm introduces into egg cytoplasm a cytosolic soluble factor, which serves as the physiological trigger of Ca^{2+} signaling in the fertilizing egg (3). Several laboratories have showed that cytosolic sperm extracts could induce fertilization-like Ca^{2+} transients when microinjected into eggs (8–13). The sperm factor responsible for this activity was shown to be protein-based, and appears to be sperm-specific (8). The sperm factor mobilizes Ca^{2+} release from the intracellular stores through InsP3 receptor-mediated mechanisms, because blocking the function of the InP3 receptor completely inhibited sperm-induced Ca^{2+} release (14, 15). In addition, we have shown recently that the action of the sperm factor is triggering Ca^{2+} oscillations in mammalian eggs is critically dependent upon the presence of a maternal machinery (13).

So far, several sperm factor candidates have been proposed, but no recombinant protein has yet been found to introduce Ca^{2+} oscillations consistent with those seen at fertilization. Microinjection studies have shown that the activity of the sperm factor in triggering Ca^{2+} release is not species specific in mammals (9, 10). The activity can also extend between phyla, since human sperm extracts can produce repetitive Ca^{2+} spikes in ascidian oocytes (16) and the sperm extracts of fish, *Xenopus* and chicken can trigger Ca^{2+} oscillations in mouse eggs (17). These data raise the possibility that fertilization may involve a similar sperm protein factor in wide range of different species. In plants, it is also observed that a transient elevation of free cytosolic Ca^{2+} occurs following fusion of sperm and egg cell (18, 19). However, it is unknown whether the sperm factor is conserved in function between plants and animals. In the present study, we prepared sperm extracts from a model plant, *Brassica campestris*, and examined their Ca^{2+}-releasing ability upon microinjection into mouse eggs. Our results showed, for the first time, that *Brassica* sperm also possess a protein-based cytosolic factor that can induce fertilization-like Ca^{2+} oscillations in mouse eggs.

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MATERIALS AND METHODS

Preparation of mouse eggs. MII eggs were collected from 4- to 6-week-old female ICR mice as described previously (17). Female were superovulated by injection of 7.5 IU of pregnant mare's serum gonadotropin (PMSG) followed 48 h later by 7.5 IU of human chronic gonadotropin (hCG). Ovulated eggs were collected from the oviducts at 14–15 h post-hCG. The cumulus cells were removed by a brief incubation at 37°C in 300 IU/ml hyaluronidase (bovine testis, Type S, Sigma) in M2 medium. Eggs were then washed 3 times and transferred to a microdrop of M2 medium under paraffin oil. All eggs were microinjected within 3.5 h after collection.

Preparation of plant sperm extracts. Fresh mature pollens from the anthers of the field grown plants of *Brassica campestris* were collected. The procedures of sperm isolation were adopted as described by Mo *et al.* (20). A two-step osmotic shock method was adopted for isolation of viable sperm cells in large quantities from pollen grains. Pollen grains at the day of anthesis were hydrated in 25% sucrose solution for 30 min. After centrifugation and removal of the supernatant, the pellet was shocked by a medium containing 12.5% sucrose, 0.1 g/L KNO_3, 0.36 g/L $CaCl_2$, 0.6% BSA, and 1.3% polyvinylpyrrolidone (PVP). After removal of pollen wall debris by filtration with 400-mesh sieve and centrifugation, the sperm cell-rich pellets were suspended in extracting buffer (5 mmol/L EDTA, 20 mmol/L Tris (pH 7.2), 5 mmol/L KCl, 1 mmol/L $MgSO4.7H_2O$, 1 mmol/L PMSF). The sperm density of each preparation was determined using Hoechest 33255 under a fluorescent microscope. A sperm suspension of 7–9 $\times$ 10^9 sperm/ml was lysed by sonication at an amplitude of 20%, a duration of 9 s for 3 times (Ultrasonic Homogenizer, Cole Parmer). After sonication, homogenates were spun at 40,000*g* for 30 min at 4°C. The clear supernatant was collected as "sperm extracts." To determine if the activity contained in the sperm extracts are heat-labile, the crude extracts were incubated at 90°C for 10 min, and injected into mouse eggs after being cooled. In proteinase-treatment experiments were performed following the procedures as described by Dong *et al.* (17). The sperm extracts were fractionated using desalting column. The early follow-through high molecular weight fractions were collected for injection.

Microinjection. Microinjection of the eggs was performed following the procedures as described by Dong *et al.* (17). All injections were carried out using a pair of manually operated pressure microinjectors (IM-6, Narishige, Japan) filled with paraffin oil. Microinjection pipettes (Clark Electronmedical Instruments UK) were pulled by a micropipette puller (Sutter Instrument Company, Model P-87, U.S.A.) to give an open tip (1–2 μm). The injection volume was quantified as described by Dong *et al.* (17). With a graticule on the eyepiece of the microscope, we can determine the volume of the sperm extracts injected. Two picoliters is about 1% mouse egg volume which is taken as 180 pl.

Calcium measurement. Before $(Ca^{2+})_i$ measurement, mouse eggs were loaded with 2 μM of Fura-2 AM (Molecular Probes) in M2 medium containing 4 mg/ml BSA at 37°C for 30 min. The procedures and equipment for calcium measurement were the same as described by Dong *et al.* (17). Calibration of $(Ca^{2+})_i$ was conducted according to the method of Poenie *et al.* (21). $(Ca^{2+})_i$ was calculated simultaneously by computer from the ratio equation described by Grynkiewica (22).

RESULTS AND DISCUSSION

Sperm Extracts Derived from Brassica Could Trigger Fertilization-like Ca^{2+} Oscillations When Microinjected into Mouse Eggs

Figure 1 shows typical patterns of Ca^{2+} release in mouse eggs upon stimulation with sperm extracts derived from *Brassica* and bovine. The plant sperm extracts-induced Ca^{2+} oscillations have all the distinctive features of those seen at fertilization. These Ca^{2+} oscillations consist of an initial large Ca^{2+} spike and subsequent repeated Ca^{2+} rises, with the initial transient usually lasting longer and displaying higher amplitude than subsequent rises. Every Ca^{2+} transient displayed rapid online and decline and occurred at fairly regular intervals. The number of Ca^{2+} spikes varied rather than the amplitude. For the same egg, the subsequent repetitive transients were almost identical in amplitude and the frequency of spikes declined with time. Table 1 illustrates that Ca^{2+} oscillations induced by *Brassica* sperm extracts displayed similar characteristics to those induced by bovine sperm extracts during the first half hour of sperm extracts injection. We found that majority of the eggs injected with *Brassica* sperm extracts (17 out of 19 eggs) displayed Ca^{2+} oscillations for approximately 30 min. In Ca^{2+} free medium 9/14 eggs examined underwent only a single Ca^{2+} transients, suggesting that external Ca^{2+} is essential for the maintenance of Ca^{2+} oscillations

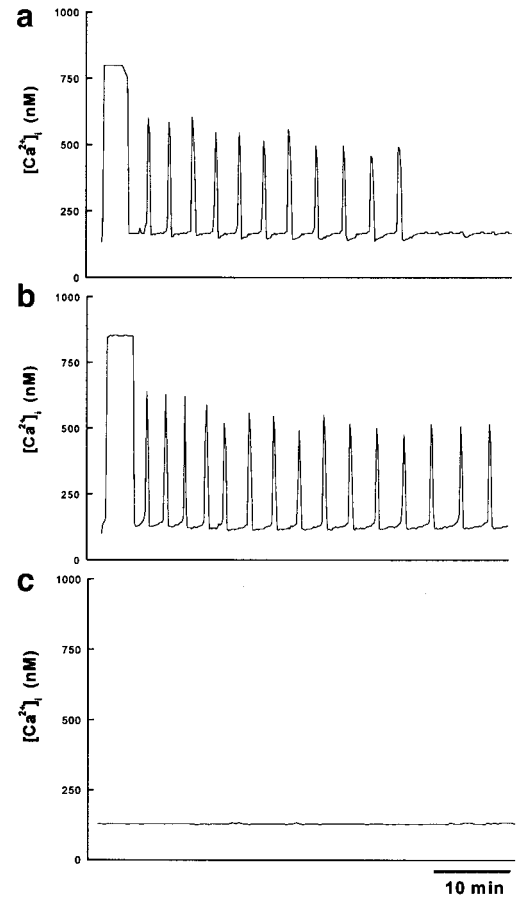

FIG. 1. Typical patterns of Ca^{2+} oscillations observed in mouse eggs activated by injection of sperm extracts derived from *Brassica* (a) and bovine (b). None of eggs displayed any Ca^{2+} spikes when injected with *Brassica* leaf extracts (c).

TABLE 1

Characteristics of Ca^{2+} Oscillations in Mouse Eggs Injected with *Brassica* Sperm Extracts[a]

Sperm extracts	Number of cells injected	Baseline (nM Ca^{2+})	First peak (nM Ca^{2+})	First peak duration (s)[b]	Ca^{2+} rise number in 0.5 h	Ca^{2+} rises interval (s)
Bovine	21	140 ± 20	870 ± 190	253 ± 55	14 ± 8	160 ± 120
Brassica	19	90 ± 20	720 ± 180	322 ± 150	8 ± 6	180 ± 90

[a] Data are presented as mean ± SD.
[b] The duration of the first Ca^{2+} transients is taken from beginning on the rising phase at 100 nM above baseline and ending at the corresponding point on the falling phase.

induced by the plant sperm extracts. In addition, egg injected with active plant sperm extracts were activated to form pronucleus (10 out of 18 eggs examined), while none of the eggs injected with heat-inactivated plant sperm extracts showed pronuclear formation ($n = 15$). In summary, these findings suggest that plant sperm contains a cytosolic soluble factor that can induce both Ca^{2+} oscillations and egg activation in the mouse.

The Plant Sperm Factor Responsible for Inducing Ca^{2+} Oscillations Is Protein-Based, Functional Only When Microinjected into Egg Cytoplasm, and Appears to Be Sperm-Specific

To examine the nature of the plant sperm factor responsible for inducing Ca^{2+} oscillations, the extracts was heated at 90°C for 10 min or treated with proteinase K for 30 min. Upon injection, the heated treated *Brassica* sperm extracts failed to trigger any Ca^{2+} increase in the eggs ($n = 8$). Similarly, all the eggs injected with sperm extracts treated by trypsin showed no response ($n = 7$). Whereas eggs injected with the active plant sperm extracts exhibited Ca^{2+} releasing ($n = 9$), and 7/9 eggs showed Ca^{2+} increase (Table 2). In addition, after applied to desalt column, most of the small molecules in the sperm extracts, such as Ca^{2+} and IP3, were expected to be removed. The flow-through fraction of the *Brassica* sperm extracts remained its activity in triggering Ca^{2+} oscillations (6/7 examined). Our results therefore indicate that the component in the *Brassica* sperm extracts that responsible for inducing Ca^{2+} oscillations is protein-based, heat-liable and of high molecular weight.

To determine whether the activity of plant cytosolic extracts in triggering Ca^{2+} oscillations is seen specifically in the sperm or universally in other tissues, we subsequently injected mouse eggs with cytosolic extracts derived from leaf, root, shoot, and sperm, and compared their ability in causing Ca^{2+} oscillations. Table 3 summarizes the response of eggs to injection of the plant extracts. It was observed that Ca^{2+} increases had occurred in all the eggs injected with the plant sperm extracts ($n = 5$), whereas eggs injected with similar protein concentration of cytosolic extracts derived from leaf ($n = 6$), root ($n = 6$) and shoot ($n = 5$) failed to display any Ca^{2+} increase (Table 3). In addition, we examined whether extracellular application of the sperm extracts to the egg surface could induce Ca^{2+} increase in mouse eggs. No response had been observed in any eggs exposed to the sperm extracts even in a protein concentration 3–5 times higher than the intracellular concentration required for inducing Ca^{2+} release. These findings suggest that the plant protein factor responsible for inducing Ca^{2+} oscillations appears to be sperm specific, and acts only from inside the cell.

Eight to Ten Plant Sperm Contain Enough Active Factor to Trigger Ca^{2+} Oscillations in Mouse Eggs

The frequency of Ca^{2+} spikes increases with increasing amounts of sperm extracts injected and it is of interest determine the minimal amount of *Brassica*

TABLE 2

The Effect of Heat and Proteinase K Treatment on the Ability of *Brassica* Sperm Extracts to Induce Ca^{2+} Oscillation in Mouse Eggs

Sperm extracts	Treatment	Eggs injected	Eggs with a single Ca^{2+} spike	Eggs with Ca^{2+} oscillations
	Heat treatment	8	0	0
Brassica	Proteinase K	5	0	0
	Control	9	2	7

TABLE 3

The Ability of Different *Brassica* Tissue Extracts to Induce Ca^{2+} Oscillation in Mouse Eggs

Tissue extracts	Eggs injected	Eggs with a single Ca^{2+} spike	Eggs with Ca^{2+} oscillation
Sperm	5	1	4
Leaf	6	0	0
Root	6	0	0
Shoot	5	0	0

sperm extracts required to cause Ca^{2+} oscillations upon injection into mouse eggs. Based on an average of 4.3×10^9 sperm/ml for *Brassica* sperm extracts, theoretically 1 pl of the extracts is 4.3 sperm equivalents. Injecting 2 pl of the sperm extracts to deliver 8.6 sperm equivalents, 6/8 eggs showed a single Ca^{2+} rise and 2/8 generated fertilization-like Ca^{2+} oscillations. It was therefore evident that 8–10 plant sperms contain enough active factor to trigger Ca^{2+} oscillations in the mouse eggs.

In summary, our findings in this study show for the first time that intracellular injection of plant sperm extracts can cause mouse eggs to undergo calcium oscillations resembling those seen at fertilization. This study also describes for the first time that calcium oscillations can be generated in a heterologous combination of gametes obtained from plant and animal. Such cross-reactivity between distantly related taxa suggests that the activity of the putative sperm factor in triggering Ca2+ signaling in mammalian eggs is not specific to the animal kingdom.

It has been reported that human sperm extracts produces repetitive Ca^{2+} spikes in ascidian oocytes (16), and porcine sperm extracts caused Ca^{2+} release in sea urchin or *Xenopus* egg homogenates (23). Recently, we reported that sperm extracts of fish, *Xenopus* and chicken can trigger Ca^{2+} oscillations in mouse eggs respectively (17). These data and the findings of the present study raise the possibility that fertilization associated transient Ca^{2+} rises may involve a similar sperm protein factor in wide range of different species.

In higher flowering plants, eggs exhibit only a single Ca^{2+} transient at fertilization (18, 19), however, its sperm extracts could induce fertilization-like Ca^{2+} oscillation in mouse eggs. The plant sperm factor may cause Ca^{2+} oscillations through InsP3 receptor-mediated mechanism, because 600 μM low-molecular weight heparin (MW = 3000) could completely inhibit Ca^{2+} oscillations induced by *Brassica* sperm extracts (Li and Sun, unpublished observations). However, the mechanism as to how the plant sperm factor induces Ca^{2+} oscillations in mammalian eggs is still mysterious.

The plant sperm extracts induced Ca^{2+} oscillations in mouse eggs may be attributed to some characteristic cytoplasmic components developed in mammalian eggs. Recently, we have shown that the ability of the sperm factor in triggering Ca^{2+} oscillations in mouse eggs depends on the presence of a maternal machinery (13). It was found that this maternal machinery functions only once in mammalian oocytes and eggs and is inactivated by the sperm extracts in a non-regenerative manner. Based on this finding, we speculate that the difference between mouse and plant eggs in response to plant sperm factor stimulation in generating Ca^{2+} transients may reflect the function of this putative machinery. It is possible that although the sperm factor is capable of causing the first Ca^{2+} transient, the absence of this putative maternal machinery may result in the absence of Ca^{2+} oscillations in plant eggs during fertilization. Further studies on the nature and identity of the maternal machinery are under investigation in our laboratory.

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