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Involvement of mouse and porcine PLCζ-induced calcium oscillations in preimplantation development of mouse embryos

Akihiro Yoneda [a, b, *], Tomomasa Watanabe [a]

[a] Laboratory of Animal Breeding and Reproduction, Graduate School of Agriculture, Hokkaido University, Japan
[b] Division of Molecular Therapeutics, Center for Food & Medical Innovation, Hokkaido University, Japan

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ABSTRACT

In mammals, phospholipase Cζ (PLCζ) has the ability to trigger calcium (Ca^{2+}) oscillations in oocytes, leading to oocyte activation. Although there is a species-specific difference in the PLCζ-induced Ca^{2+} oscillatory pattern, whether PLCζ-induced Ca^{2+} oscillations affect preimplantation embryonic development remains unclear. Here, we show that Ca^{2+} oscillations in mouse PLCζ cRNA-injected oocytes stopped just before pronuclear formation, while that in porcine PLCζ cRNA-injected oocytes continued for several hours after pronuclei had been formed. This difference of Ca^{2+} oscillations in oocytes after pronuclear formation was dependent on the difference in the nuclear localization signal (NLS) sequence of PLCζ between the mouse and pig. However, mouse and porcine PLCζ cRNA-injected oocytes parthenogenetically developed to blastocysts regardless of the absence or presence of Ca^{2+} oscillations after pronuclear formation. Furthermore, the developmental rate of mouse or porcine PLCζ-activated oocytes injected with round spermatids to the blastocyst stage was not significantly different from that of strontium-activated oocytes injected with round spermatids. These results suggest that the PLCζ-induced Ca^{2+} oscillatory pattern in mouse oocytes is dependent on the NLS sequence of PLCζ and injection of PLCζ may be a useful method for activation of round spermatid-injected and somatic nuclear transferred oocytes.

1. Introduction

During fertilization, repetitive rise of intracellular calcium (Ca^{2+}) concentration, termed Ca^{2+} oscillations, is triggered by the penetration of spermatozoa into oocytes [1,2]. Ca^{2+} oscillations in oocytes induce oocyte activation including cortical granule exocytosis, resumption of the second meiotic division and extrusion of the second polar body, leading to the subsequent embryonic development. Thus, Ca^{2+} oscillations during fertilization are a priming event for the initiation of embryonic development. Ca^{2+} oscillations have been proposed to be triggered by soluble sperm factors that diffuse in oocytes after the fusion of sperm with an oocyte [3–5]. Phospholipase Cζ (PLCζ) was first identified in the mouse as a sperm-specific phospholipase C isoform responsible for the initiation of Ca^{2+} oscillations [6]. Since then, PLCζ has been isolated in various vertebrates [7–11]. PLCζ has the ability to hydrolyze phosphatidyleinositol 4,5-bisphosphate to produce inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. PLCζ drives the production of IP_3 and binding to its receptor, which promotes release of Ca^{2+} from the endoplasmic reticulum and triggers Ca^{2+} oscillations in oocytes [12–15]. Therefore, PLCζ is considered to be a strong candidate for the oocyte-activating factor during fertilization.

There are a species-specific differences in the frequency and duration of PLCζ-induced Ca^{2+} oscillations in oocytes [11,16]. In the mouse, Ca^{2+} oscillations triggered by the injection of mouse PLCζ cRNA into mouse oocytes continue for several hours and terminate just before pronuclear formation [17]. Mouse PLCζ has a nuclear localization signal (NLS) sequence in its molecule and accumulates in the pronucleus in pronuclear-stage embryos [11,17–19]. On the other hand, Ca^{2+} oscillations in oocytes injected with rat, bovine or human PLCζ continue for several hours even after pronuclei have been formed [11,16,20]. These PLCζs have NLS-like sequence in their molecules but localize in the cytoplasm of pronuclear-stage embryos. Site-direct mutagenic experiments have demonstrated that Ca^{2+} oscillations in mouse oocytes injected with mutated-NLS mouse PLCζ that evenly distributes in the cytoplasm persist even

* Corresponding author. Division of Molecular Therapeutics, Center for Food & Medical Innovation, Hokkaido University, Nishi-11, Kita-21, Kita-ku, Sapporo 001-0021, Japan. Fax: +81 11 706 9463.
E-mail address: ayoneda@sci.hokudai.ac.jp (A. Yoneda).

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after pronuclei have been formed [17,21]. These findings suggest that Ca^{2+} oscillations in oocytes are regulated by the localization of mouse PLCζ. Whether the difference of PLCζ-induced Ca^{2+} oscillations in oocytes between the mouse and other mammalian species affects the subsequent preimplantation embryonic development remains elusive.

In the present study, we first investigated Ca^{2+} oscillations triggered by the injection of mouse or porcine PLCζ cRNA into mouse oocytes. To address the effect of Ca^{2+} oscillations on the preimplantation embryonic development, we examined the development of mouse or porcine PLCζ cRNA-injected mouse oocytes to the blastocyst stage. Furthermore, we examined whether mouse or porcine PLCζ-activated mouse oocytes injected with round spermatids develop to the blastocyst stage.

2. Materials and methods

2.1. Animals

Eight-to twelve-week-old C57BL/6 male and female mice were purchased from SLC Japan (Shizuoka, Japan). All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals at Hokkaido University.

2.2. Synthesis of mouse and porcine PLCζ cRNA

Mouse and porcine PLCζ cDNA encoding the open reading frame was synthesized by reverse-transcription-polymerase chain reaction against total RNA extracted from testes of C57BL/6 male mice and Landrace boars obtained at a local abattoir, respectively. The PLCζ cDNA fragment was subcloned into the pT_NT expression vector (Promega, Madison, WI, USA). Then mouse and porcine PLCζ cRNA was synthesized by using a Ribomax RNA synthesis system (Promega) after linearization of PLCζ cDNA inserted into the pT_NT vector by digestion with the EclHKI restriction enzyme. The synthesized mouse and porcine PLCζ cRNA was diluted with an RNase-free injection buffer (120 mM KCl and 20 mM HEPES, pH 7.4).

2.3. Collection of oocytes

Metaphase II-arrested (MetII) oocytes were collected from the oviducts of 8-week-old C57BL/6 female mice that had been superovulated by pregnant mare's serum gonadotropin (Teikokuzoki Pharmaceutical, Tokyo, Japan) followed by human chorionic gonadotropin (Teikokuzoki Pharmaceutical) 48 h later. The MetII oocytes were placed in M2 medium (Sigma–Aldrich, St. Louis, MO, USA) and treated with 0.01% hyaluronidase (Sigma–Aldrich) to remove cumulus cells. The MetII oocytes were incubated in KSOM medium at 37 °C under 5% CO_2 in air until use.

2.4. Microinjection of cRNA

MetII oocytes were injected with mouse and porcine PLCζ cRNA at the amount of about 4 pl and were cultured in KSOM medium at 37 °C in 5% CO_2 in air.

2.5. Preparation of epididymal spermatozoa and round spermatids

The cauda epididymis was removed from 8- to 12-week-old C57BL/6 male mice, and the caudal portion was excised. The dense mass of spermatozoa was collected by compression of the tissue and was transferred into KSOM medium. After centrifugation, the pellets containing spermatozoa were suspended with KSOM medium. To collect spermatogenic cells, seminiferous tubules of the testes from C57BL/6 male mice were cut into small pieces and pipetted gently to disperse the spermatogenic cells. The cell suspension was filtrated through a cell strainer (40 μm in pore size) and washed three times by centrifugation. After centrifugation, the pellets were suspended with HEPES-buffered CZB medium. A 1-μl aliquot of the sperm or spermatogenic cell suspension was mixed with 10 μL of HEPES-CZB medium containing 12% (w/v) polyvinylpyrrolidone (PVP; 360 kDa).

2.6. Oocyte activation and microinsemination with spermatozoa and round spermatid injection

To induce oocyte activation, MetII oocytes were placed in Ca^{2+}-free KSOM containing 5 mM $SrCl_2$ for 20 min. After activation of the oocytes, they were used for intracytoplasmic sperm injection and round spermatid injection. Intracytoplasmic sperm injection and round spermatid injection were carried out as described previously [22,23]. Briefly, for intracytoplasmic sperm injection, the head of each spermatozoa was separated from the tail by applying pulses to the head–tail junction by means of a Piezo-driven pipette (PrimeTech, Ibaraki, Japan). The sperm head was injected into a MetII oocyte. For round spermatid injection, a round spermatid nucleus was injected into mouse or porcine PLCζ cRNA-injected oocyes. The treated oocytes were incubated in KSOM medium at 37 °C under 5% CO_2 in air.

2.7. Embryo culture

Treated oocytes were cultured in KSOM medium for 48 h and 144 h at 37 °C under 5% CO_2 in air to examine the development to 2-cell and blastocyst stages, respectively.

2.8. Measurement of intracellular Ca^{2+} concentration

Measurement of intracellular Ca^{2+} concentration in oocytes injected with PLCζ cRNA was performed according to the method described by Yoneda et al. [9] with slight modification. The oocytes were incubated in M2 medium containing 2 μM Fura-2 AM (Dojindo, Kumamoto, Japan) at 37 °C for 30 min. The oocytes loaded with Fura-2 AM were transferred into M2 medium and set on the stage of an inverted microscope (NIKON Tokyo, Japan) heated at 37 °C. Measurement of intracellular Ca^{2+} level in oocytes was carried out at 60 min or 210 min after the injection of mouse or porcine PLCζ cRNA. The Argus 50 software program (Hamamatsu Photonics version 3.5 Shizuoka, Japan) was used for the recording of emission fluorescence at a wavelength of 510 nm to evaluate intracellular Ca^{2+}. When Fura-2 AM couples with Ca^{2+}, the emission fluorescence at 510 nm is intensified by exposure to UV light at 340 nm (F340) but is not changed by exposure to UV light at 380 nm (F380). Intracellular calcium levels were estimated from the F340/F380 ratio (R).

2.9. Statistical analysis

Data for the developmental rate of the oocytes to 2-cell and blastocyst stages are shown as means ± SEM. Differences between groups were tested for statistical significance using Student's t-test or analysis of variance (ANOVA). Statistical significance was determined at $P < 0.05$.

3. Results

3.1. Ca^{2+} oscillations in mouse oocytes injected with mouse or porcine PLCζ cRNA

We first examined the change of intracellular Ca^{2+} in mouse oocytes injected with mouse or porcine PLCζ cRNA. As shown in

Fig. 1A, a repetitive rise of intracellular Ca^{2+} was detected in mouse oocytes injected with mouse or porcine PLCζ cRNA. In mouse oocytes injected with mouse PLCζ cRNA, Ca^{2+} oscillations continued for several hours (n = 15, 189 ± 3.7 min) after the measurement of intracellular Ca^{2+} and terminated just before the time point (n = 15, 191.2 ± 2.7 min) at which pronulei were formed. On the other hand, when porcine PLCζ cRNA was injected into mouse oocytes, Ca^{2+} oscillations persisted during the experimental period (300 min) regardless of the formation of pronulei (n = 15, 195 ± 3.5 min).

3.2. Involvement of NLS of PLCζ in Ca^{2+} oscillations in mouse oocytes injected with mouse or porcine PLCζ cRNA

In the present study, we generated NLS-mutated mouse PLCζ by substitutions of Lys and Arg at positions 377 and 378 to Glu (Fig. 1B). As shown in Fig. 1C, Ca^{2+} oscillations in mouse oocytes injected with mouse PLCζ cRNA stopped just before pronuclear formation (n = 15, 38.6 ± 1.1 min), while those in oocytes injected with NLS-mutated mouse PLCζ cRNA persisted even after pronuclei had been formed (n = 15, 32.1 ± 1.7 min).

In comparison to mouse PLCζ, substitution of two amino acids and deletion of two amino acids were detected in the NLS-like sequence of porcine PLCζ (Fig. 1B), suggesting that the mutations of the NLS-like sequence in porcine PLCζ are responsible for Ca^{2+} oscillations in porcine PLCζ cRNA-injected mouse oocytes after pronuclear formation. Thus, we generated porcine PLCζ with the NLS sequence of mouse PLCζ (mNLS-porcine PLCζ) by substitution of the NLS-like sequence of porcine PLCζ to the NLS sequence of mouse PLCζ (Fig. 1B), and we investigated the change of intracellular Ca^{2+} in mouse oocytes injected with mNLS-porcine PLCζ cRNA. As shown in Fig. 1C, Ca^{2+} oscillations in mouse oocytes injected with porcine PLCζ cRNA continued during the experimental period (50 min) even after pronuclei had been formed (n = 15, 32.9 ± 1.5 min). On the other hand, Ca^{2+} oscillations in mouse oocytes injected with mNLS-porcine PLCζ cRNA terminated just before pronuclear formation (n = 15, 32.2 ± 1.3 min). These results suggest that Ca^{2+} oscillations in porcine PLCζ cRNA-injected mouse oocytes after pronuclear formation are dependent on the mutation of the NLS-like sequence in porcine PLCζ.

3.3. Effect of Ca^{2+} oscillations after pronuclear formation on preimplantation development of mouse oocytes injected with mouse or porcine PLCζ cRNA

We next examined whether the difference in the Ca^{2+} oscillatory pattern affects preimplantation development of mouse oocytes injected with mouse and porcine PLCζ cRNA. As shown in Table 1, there was no significant difference in the developmental rate of mouse oocytes to the 2-cell stage among oocytes injected with mouse PLCζ cRNA (220/249, 89%), NLS-mutated mouse PLCζ

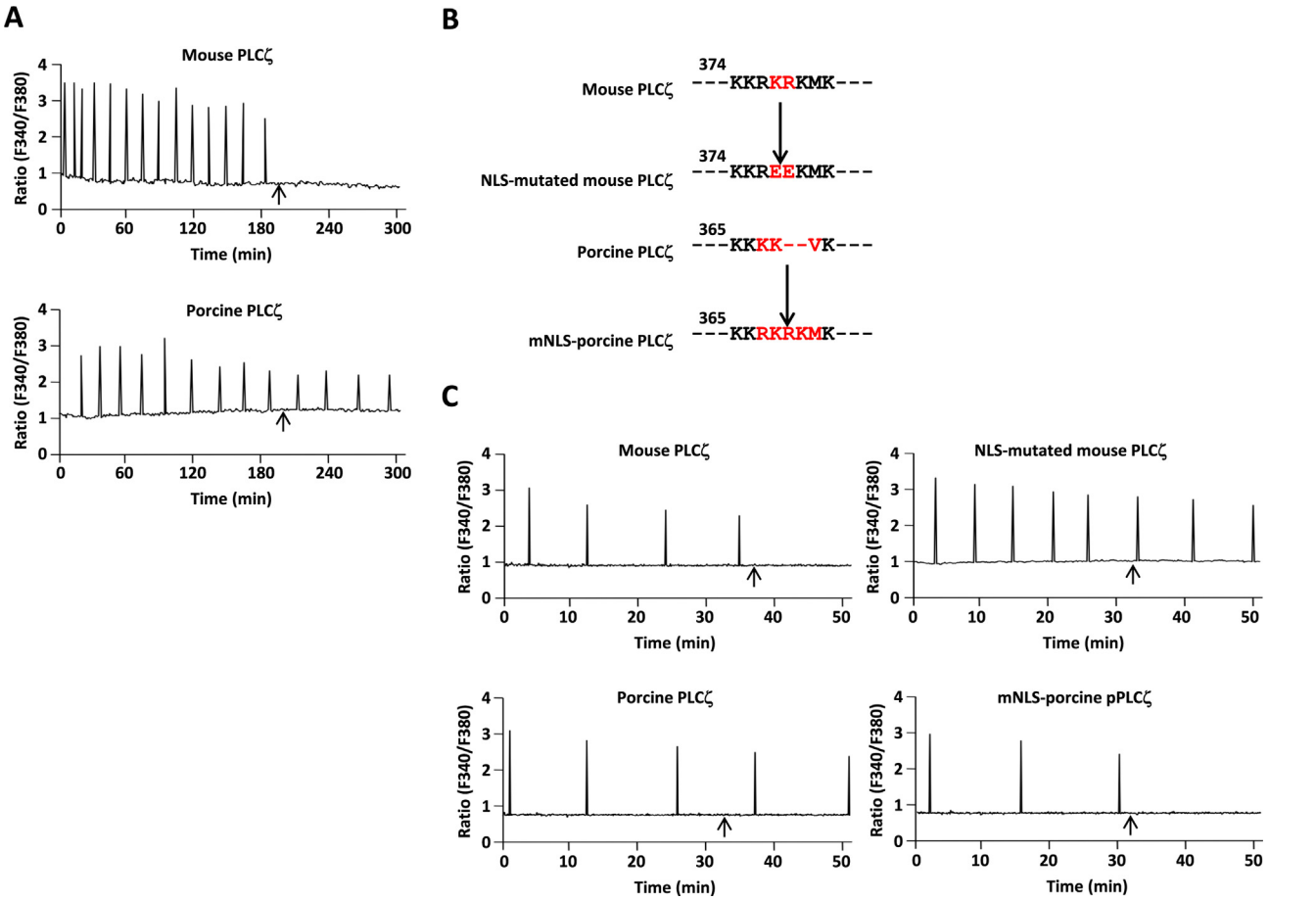

Fig. 1. Ca^{2+} oscillations in mouse oocytes injected with mouse and porcine PLCζ cRNA. (A) Measurement of change in the intracellular Ca^{2+} concentration in mouse oocytes injected with mouse PLCζ cRNA or porcine PLCζ cRNA was carried out for 300 min. An arrow indicates the time point at which pronuclei were formed. (B) Amino acid sequences of the nuclear localization signal (NLS) in mouse and porcine PLCζ proteins. The number indicates the number of the amino acid sequence in PLCζ protein. (C) Measurements of changes in intracellular Ca^{2+} concentrations in mouse oocytes injected with mouse PLCζ, NLS-mutated mouse PLCζ cRNA, porcine PLCζ cRNA and mNLS-porcine PLCζ cRNA were carried out for 50 min. An arrow indicates the time point at which pronuclei were formed.

cRNA (226/265, 85%), porcine PLCζ cRNA (267/297, 90%) and mNLS-poricne PLCζ cRNA (260/301, 86%). Furthermore, there was no significant difference in the developmental rate of oocytes to the blastocyst stage among oocytes injected with mouse PLCζ cRNA (120/249, 49%), NLS-mutated mouse PLCζ cRNA (115/265, 47%), porcine PLCζ cRNA (125/297, 42%) and mNLS-poricne PLCζ cRNA (125/301, 41%). These results imply that the preimplantation development of PLCζ-activated mouse oocytes is not affected by the Ca^{2+} oscillatory pattern in mouse oocytes after pronuclear formation.

3.4. Activation of mouse or porcine PLCζ cRNA-injected mouse oocytes followed by injection of round spermatids

We investigated the activation of mouse oocytes injected with mouse or porcine PLCζ cRNA followed by injection of round spermatid. As shown in Fig. 2A, Ca^{2+} oscillations in mouse oocytes injected with spermatozoa were detected until the time point ($n = 18$, 28.3 ± 1.9 min) at which pronuclei were formed. On the other hand, a Ca^{2+} transient was not detected in mouse oocytes only injected with round spermatids. In strontium-treated oocytes injected with round spermatids, a Ca^{2+} transient was not detected, but pronuclei were formed ($n = 18$, 26.3 ± 1.9 min). Ca^{2+} oscillations were observed in mouse PLCζ cRNA-injected mouse oocytes injected with round spermatids and they terminated just before pronuclear formation ($n = 18$, 27.8 ± 2.5 min). On the other hand, Ca^{2+} oscillations in porcine PLCζ cRNA-injected mouse oocytes injected with round spermatids continued even after pronuclei had been formed ($n = 18$, 30.7 ± 2.1 min). As shown in Fig. 1B, there was no significant difference in activation rates of mouse PLCζ cRNA-injected (126/160, 78.7%) or porcine PLCζ cRNA-injected mouse oocytes (128/170, 77.0%) compared to those of oocytes injected with spermatozoa (132/155, 85.3%) and strontium-treated oocytes injected with round spermatids (129/162, 79.9%).

3.5. Preimplantation embryonic development of mouse or porcine PLCζ cRNA-injected mouse oocytes followed by injection of round spermatids

We further examined the preimplantation embryonic development of mouse and porcine PLCζ-activated mouse oocytes injected with round spermatids. As shown in Table 2, there was no significant difference in the developmental rate to the 2-cell stage among spermatozoa-injected oocytes (81/84, 96%), strontium-activated oocytes (83/86, 96%) and mouse PLCζ-activated oocytes injected with round spermatids (81/85, 94%). On the other hand, the developmental rate of PLCζ-activated oocytes (50/85, 59%) to the blastocyst stage was significantly lower than that of spermatozoa-injected oocytes (63/84, 75%) but was not significantly different from that of strontium-activated oocytes injected with round spermatids (57/86, 66%).

In porcine PLCζ-activated mouse oocytes injected with round spermatids, the developmental rate of the oocytes (80/87, 91%) to the 2-cell stage was not significantly different from those of spermatozoa-injected, strontium-activated, or mouse PLCζ-activated oocytes injected with round spermatids. On the other hand, the developmental rate (47/87, 54%) of porcine PLCζ-activated oocytes to the blastocyst stage was significantly lower than those of spermatozoa-injected and strontium-activated oocytes but was not significantly different from that of mouse PLCζ-activated oocytes injected with round spermatids.

4. Discussion

The present study demonstrated that injection of porcine PLCζ cRNA into mouse oocytes triggered Ca^{2+} oscillations that continued for several hours even after pronuclei had been formed, while injection of mouse PLCζ cRNA induced Ca^{2+} oscillations that stopped just before the time point at which pronuclear formation was detected. Substitution and deletion of two amino acids were detected in the NLS-like sequence of porcine PLCζ in comparison to that of mouse PLCζ. When mutated porcine PLCζ cRNA, which was generated by substitution of the NLS-like sequence of porcine PLCζ to the NLS sequence of mouse PLCζ, was injected into mouse oocytes, Ca^{2+} oscillations were triggered and they terminated before pronuclear formation. Previous studies revealed that the substitution, insertion and deletion of amino acids in the NLS-like sequence are present in PLCζ of the other mammals including rats, humans, cattle and horses in comparison to mouse PLCζ [11,20]. These mammalian PLCζs are evenly distributed in the cytoplasm of pronuclear-stage embryos, but mouse PLCζ localizes in the pronucleus. Ca^{2+} oscillations in mouse oocytes persist for several hours even after pronuclei have been formed when human, bovine or equine PLCζ cRNA are injected [11,16,20]. Furthermore, site-directed mutagenesis has demonstrated that Ca^{2+} oscillations in mouse oocytes persist even after pronuclear formation when NLS-mutated mouse PLCζ, which localizes in the cytoplasm, is injected [17,18,21]. These findings and results of the present study imply that Ca^{2+} oscillations in mouse oocytes are dependent on the intracellular localization of PLCζ.

During fertilization, the Ca^{2+} oscillatory pattern in mouse oocytes has been reported to affect oocyte activation events (cortical granule exocytosis, the completion of the second meiosis and pronuclear formation), gene expression profile and development to term [24–26]. Cortical granule exocytosis requires 4-8 Ca^{2+} oscillations, and completion of the second meiosis and recruitment of maternal mRNA require 16–24 Ca^{2+} oscillations. On the other hand, only 20 Ca^{2+} oscillations in mouse oocytes have been reported to reduce postimplantation embryonic development. These results imply that the Ca^{2+} oscillatory pattern before pronuclear formation is an important parameter for the development to term. However, whether Ca^{2+} transients in oocytes after pronuclear formation affect preimplantation development to the blastocyst stage had been unclear. The present study showed that the developmental rates of mutated-NLS mouse PLCζ cRNA-injected and porcine PLCζ cRNA-injected mouse oocytes, in which Ca^{2+} oscillations were induced even after pronuclear formation to 2-cell and blastocyst

Table 1
In vitro development of mouse PLCζ- or porcine PLCζ-injected mouse oocytes to the 2-cell and blastocyst stages.

cRNA injected	No. of oocytes injected	No. of oocytes survived	No. (%) of oocytes developed to	
			2-Cell	Blastocyst
Mouse PLCζ cRNA	250	249	220 (89)	120 (49)
Mutated-NLS mouse PLCζ cRNA	270	265	226 (85)	115 (47)
Porcine PLCζ cRNA	300	297	267 (90)	125 (42)
mNLS-porcine PLCζ cRNA	305	301	260 (86)	125 (41)

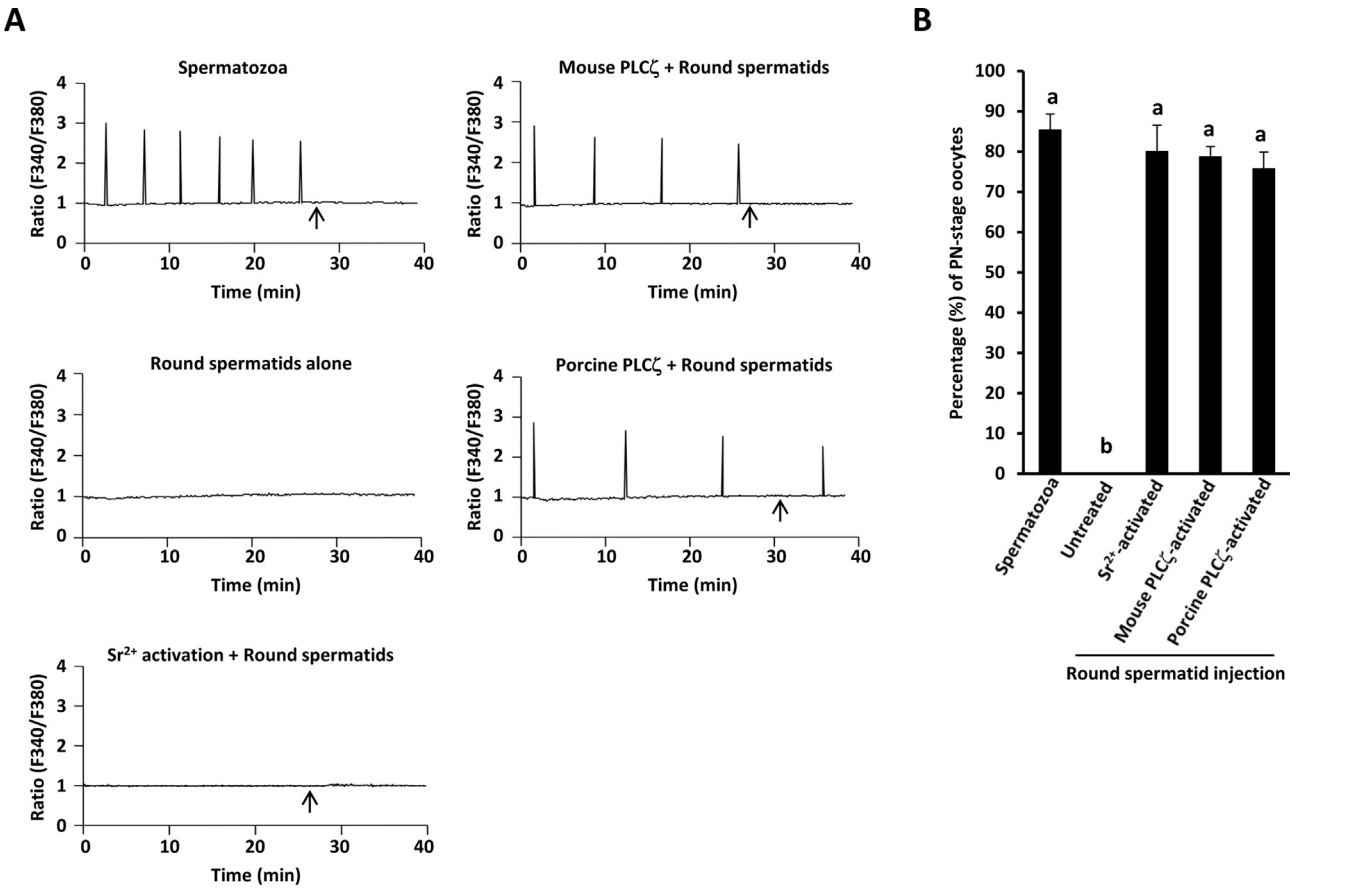

Fig. 2. Activation of mouse and porcine PLCζ cRNA-injected mouse oocytes after injection of round spermatids. (A) Measurement of changes in intracellular Ca^{2+} concentrations in spermatozoa-injected oocytes, round spermatid-injected oocytes, strontium-activated oocytes injected with round spermatids, and mouse PLCζ cRNA-injected and porcine PLCζ cRNA-injected oocytes injected with round spermatids were performed for 40 min. An arrow indicates the time point at which pronuclei were formed. (B) The activation rate of oocytes was examined at 6 h after injection of cRNAs. An oocyte in which two pronuclei were observed was determined as an activated oocyte. Different letters (a and b) represent a significant difference ($P < 0.05$).

stages were not significantly different from those of mouse PLCζ cRNA-injected and mNLS-porcine PLCζ cRNA-injected oocytes, in which Ca^{2+} oscillations terminated before pronuclear formation. These results suggest that Ca^{2+} oscillations before, but not after, pronuclear formation affect oocyte activation and preimplantation embryonic development.

Intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI) have been accepted as reliable means of micro-insemination in several mammalian species [27–29]. There is a difference between the methods used for ICSI and ROSI. In a protocol for ICSI, a single sperm is simply injected into an oocyte, leading to subsequent development to term [22]. On the other hand, in a method for ROSI, the oocyte injected with a round spermatid must be artificially activated before or after injection of the round spermatid because round spermatids have no capability to activate oocytes [23,30,31]. Treatment with an electrical pulse or strontium is typically used to induce oocyte activation for ROSI. The present study revealed that activation and development to the blastocyst stage were normally induced in mouse PLCζ cRNA-injected mouse oocytes treated with injection of round spermatids similar to that for strontium-activated oocytes injected with round spermatids. Porcine PLCζ cRNA-injected mouse oocytes were also normally activated and they developed to the blastocyst stage after injection of round spermatids. Kim et al. [32] reported that the electrically-stimulated porcine oocytes injected with round spermatids or round spermatid nuclei normally developed to the blastocyst stage. However, the percentage (30–35%) of porcine oocytes normally activated by an electrical stimulation has been

Table 2
In vitro development of mouse PLCζ- or porcine PLCζ-injected mouse oocytes treated with the injection of round spermatids.

Activation	Cells injected	No. of oocytes injected	No. of oocytes survived	No. (%) of oocytes developed to	
				2-Cell	Blastocyst
–	Spermatozoa	86	84	81 (96)	63 (75)[a]
–	Round spermatids	91	91	0 (0)	–
Sr^{2+}	Round spermatids	88	86	83 (96)	57 (66)[b]
Mouse PLCζ	Round spermatids	90	85	81 (94)	50 (59)[bc]
Porcine PLCζ	Round spermatids	93	87	80 (91)	47 (54)[c]

[a,b,c] Numbers with different superscripts indicate significant differences ($P < 0.05$).

reported to be very low. We previously demonstrated that the developmental rate of porcine PLCζ-activated oocytes to the blastocyst stage was not significantly different to that of in vitro fertilized porcine embryos [12]. In the future, whether porcine PLCζ-activated porcine oocytes normally develop to the blastocyst stage after the injection of round spermatids or round spermatid nuclei must be investigated.

In conclusion, the present study demonstrated that mouse PLCζ and porcine PLCζ have the capability to trigger Ca^{2+} oscillations in mouse oocytes and that the Ca^{2+} oscillatory pattern in mouse oocytes after pronuclear formation is species-specific different. The preimplantation development of PLCζ-activated mouse oocytes was unaffected by the difference between the Ca^{2+} oscillatory patterns in mouse oocytes injected with mouse PLCζ cRNA and those injected with porcine PLCζ cRNA. PLCζ-activated mouse oocytes injected with round spermatids progressed to the blastocyst stage, suggesting that injection of PLCζ cRNA may be a useful method for activation of oocytes before injection of round spermatids.

Conflict of interest

The authors have no conflict of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.058.

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