Ca\textsuperscript{2+} oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation

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Abstract

Sperm-specific phospholipase C zeta (PLCζ) is known to induce intracellular Ca\textsuperscript{2+} oscillations and egg activation when expressed in mouse eggs by injection of RNA encoding PLCζ. We investigated the expression level and spatial distribution of PLCζ in the egg in real time and in relation to the initiation and termination of Ca\textsuperscript{2+} oscillations by monitoring fluorescence of a yellow fluorescent protein 'Venus' fused with PLCζ. Ca\textsuperscript{2+} oscillations similar to those at fertilization were induced at 40–50 min after RNA injection, when expressed PLCζ reached 10–40 \times 10\textsuperscript{15} g in the egg. PLCζ–Venus increased up to 3 h and attained a steady level at 4–5 h. Interestingly, PLCζ–Venus is accumulated to the pronucleus (PN) formed at 5–6 h and continuously increased there. Ca\textsuperscript{2+} oscillations stopped in most eggs before initiation of the accumulation. A variant of PLCζ that lacks three EF hand domains was much less effective in induction of Ca\textsuperscript{2+} oscillations and little accumulated in the pronucleus, indicating a critical role of those domains. The ability of the accumulation to the pronucleus qualifies PLCζ for a strong candidate of the Ca\textsuperscript{2+} oscillation-inducing sperm factor, which is introduced into the ooplasm upon sperm–egg fusion and concentrated to the pronucleus after inducing egg activation.

Keywords: Phospholipase C zeta; Phospholipase C delta 1; Calcium oscillation; Translocation; Pronucleus; Mouse egg; Sperm factor

Introduction

Repetitive transient increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) occurs at fertilization of mammalian eggs (Jones, 1998; Miyazaki et al., 1993). The first Ca\textsuperscript{2+} transient and some subsequent Ca\textsuperscript{2+} spikes are responsible for egg activation characterized by cortical granule exocytosis leading to blocking of the polyspermy and resumption and completion of the second meiotic division (Jones, 1998; Kline and Kline, 1992). Later, long-lasting Ca\textsuperscript{2+} oscillations facilitate early embryonic development such as pronucleus (PN) formation (Ducibella et al., 2002; Swann and Ozil, 1994). Each [Ca\textsuperscript{2+}]\textsubscript{i} rise is caused by Ca\textsuperscript{2+} release from the endoplasmic reticulum (ER) mainly through inositol 1,4,5-trisphosphate (InsP\textsubscript{3}) receptors (Miyazaki et al., 1993). Similar Ca\textsuperscript{2+} oscillations are induced by extracts from the hamster (Oda et al., 1999; Swann, 1992), bovine (Wu et al., 1997), and human sperm (Palermo et al., 1997; Sousa et al., 1996) when injected into eggs of the mouse as well as the same species. Accumulated evidence suggests that repeated Ca\textsuperscript{2+} release is caused by cytosolic sperm factor to be introduced into the ooplasm upon sperm–egg fusion (Jones, 1998; Swann, 1996), although the sperm factor has not been biochemically identified. After fertilization of mouse eggs, the sperm factor is thought to diffuse over the ooplasm and then becomes associated with the male and female pronuclei (Kono et al., 1995; Ogonuki et al., 2001), as the activity of inducing Ca\textsuperscript{2+} oscillations or egg activation was examined by the transfer of the ooplasm or PN to unfertilized eggs. Ca\textsuperscript{2+} oscillations in fertilized mouse eggs cease at about the time of PN formation (Deguchi et al., 2000; Jones et al., 1995) and resume at the nuclear envelope breakdown of the first mitotic division (Day et al., 2000; Kono et al., 1996). Taken together, it has been suggested that nuclear sequestration and release of the sperm factor may regulate cessation and resumption of Ca\textsuperscript{2+} oscillations (Kono et al., 1996; Marangos et al., 2003).
Recently, Saunders et al. (2002) have found a sperm-specific phospholipase C (PLC, the enzyme that hydrolyzes membrane phosphatidylinositol 4,5-bisphosphate, PtdInsP$_2$, and produces InsP$_3$ and diacylglycerol, DAG). This novel type of PLC, phospholipase C zeta (PLC$_\zeta$), is the smallest PLC identified to date, lacking an N-terminal pleckstrin homology (PH) domain that is found in all isoforms of PLC $\beta$, $\gamma$, and $\delta$. PLC$_\zeta$ induces Ca$^{2+}$ oscillations similar to those at fertilization and causes embryonic development to the blastocyst when PLC$_\zeta$ RNA is injected into mouse eggs at the concentration that leads to expression of PLC$_\zeta$ at a level comparable to the content in a single sperm (Saunders et al., 2002). The Ca$^{2+}$ oscillation-inducing activity of sperm extract is lost when pretreated with an antibody against PLC$_\zeta$ (Saunders et al., 2002). Thus, PLC$_\zeta$ is a strong candidate of the mammalian sperm factor.

The present study aimed to record Ca$^{2+}$ oscillations continuously in relation to the expression level of PLC$_\zeta$ and to observe any change in the localization of PLC$_\zeta$ by expressing PLC$_\zeta$ fused with a variant of yellow fluorescent protein 'Venus' (Nagai et al., 2002) in mouse eggs. PLC$_\zeta$–Venus was efficiently expressed by injection of its RNA with an added long poly(A) tail (Aida et al., 2001), and fluorescence of Venus allowed us to know the expression level as well as the distribution of PLC$_\zeta$ in the egg in real time. We found that PLC$_\zeta$ is accumulated to the PN and that both Ca$^{2+}$ oscillation-inducing activity and the ability of nuclear accumulation of PLC$_\zeta$ are much higher than a variant of PLC$_\zeta$ that lacks three EP hand domains or PLC$_\delta 1$ that possesses basically similar domain features to those of PLC$_\zeta$ except the PH domain.

Materials and methods

Preparation of gametes and insemination

Mature eggs were collected from the oviducts of superovulated B6D2F1 female mice (for details, see Kumakiri et al., 2003) and were freed from cumulus cells by a brief treatment with 0.05% hyaluronidase (Sigma, St. Louis, MO). M2 medium (Fulton and Whittingham, 1978) was used for egg preparation and experiments. The eggs were loaded with the Ca$^{2+}$-sensitive fluorescent dye fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes Inc., Eugene, OR) by incubation in M2 medium containing 5 $\mu$M fura-2 AM for 8 min at 37°C. Eight to ten eggs were transferred to a 200-$\mu$l drop of M2 medium covered with paraffin oil in a glass-bottomed plastic dish, which was placed on the stage of an inverted fluorescence microscope (TMD, Nikon, Tokyo) and heated at 30–32°C.

For insemination, the zona pellucidae were removed by brief exposure to acidic Tyrode solution and subsequent gentle pipetting (Nakano et al., 1997). Spermatozoa were collected from the cauda epididymides of B6D2F1 male mice and were incubated at 37°C (5% CO$_2$ in air) for several hours for capacitation and acrosome reaction of the spermatozoa (for details, see Kumakiri et al., 2003). A small amount of sperm suspension was added to the drop containing the experimental eggs.

Construction of plasmid

cDNA encoding full-length PLC$_\zeta$ (see Fig. 2A) was cloned from cDNA library originated from mouse testis mRNAs. PLC$_\zeta$ cDNA was amplified by PCR using Pfu polymerase and the following primers: 5'-GCAACGCGGCCGATCATG-3' (forward primer), 5'-GGAATTCATGATGAGTACATGGCCACACTTCTCATGAG-3' (internal forward primer involving an EcoRI site), 5'-CTAAGCTTACATGGCCACACTTCTCATGAG-3' (reverse primer), and 5'-GTCTAGATTACTCTCTGAAGTACAAACATAAACAC-3' (internal reverse primer involving an XbaI site). PLC$_\zeta$ was subcloned into pBluescript II SK (+) (Stratagene, La Jolla, CA) using EcoRI and XbaI sites. The correct nucleotide sequence of PLC$_\zeta$ (Saunders et al., 2002) was confirmed by sequencing the obtained PLC$_\zeta$ cDNA.

cDNA of a PLC$_\zeta$ variant registered as AK006672 in EMBL (see Fig. 2A) was also cloned in the same way. The shorter PLC$_\zeta$ designated as s-PLC$_\zeta$ was amplified using the following primers: 5'-GTCATGGATGGTCTCTACACGACGGAGGAG-3' (forward primer), 5'-GGAATTCATGATGAGTACATGGCCACACTTCTCATGAG-3' (internal forward primer involving an EcoRI site), and the same reverse primers as those for PLC$_\zeta$. cDNA of PLC$_\delta 1$ was a kind gift from Dr. T. Takenawa (Institute of Medical Science, University of Tokyo), cloned from rat liver cells (Kato et al., 1992).

PLC$_\zeta$ cDNA in the plasmid was amplified using the following primers: 5'-TAGGTACGGATCCGACCGCTCAGGAGGAGGAG-3' and 5'-CACACTAGTCTCTCTGAAGATCAAAAAAC-3'. The forward primer was 5'-GGGTTACCCCACTATGGAGATGAGCATTCTCATCTGATT-3' for s-PLC$_\zeta$ and 5'-CCGCTCGAGAAGGAGCTTGCCATGACCTCGGAGG-3' for PLC$_\delta 1$.

Subsequent procedures were basically similar among cDNAs of PLC$_\zeta$, s-PLC$_\zeta$, and PLC$_\delta 1$. Fragments of the PLC$_\zeta$ cDNA were digested with KpnI and SpeI and ligated to the KpnI and SpeI sites of pBluescript II KS (+). cDNA of Venus (Nagai et al., 2002) was a kind gift from Dr. A. Miyawaki (Brain Science Institute, RIKEN). Venus cDNA in a plasmid was amplified using the following primers: 5'-GACACTAGTGGATCCGACCGCTCAGGAGGAGGAG-3' and 5'-GGGTTACCCCACTATGGAGATGAGCATTCTCATCTGATT-3'. Fragments of Venus cDNA were digested with SpeI and NotI and ligated to the SpeI and NotI sites of PLC$_\zeta$-pBluescript II KS (+). cDNA of FLAG in a plasmid was amplified using the following primers: 5'-CTAAGCTTACATGGCCACACTTCTCATGAG-3' and 5'-GGAATTCATGATGAGTACATGGCCACACTTCTCATGAG-3'. Fragments of FLAG cDNA were digested with SpeI and NotI and ligated to the SpeI and NotI sites of PLC$_\zeta$-pBluescript II KS (+).
RNAs and polyadenylation

The constructed plasmids were digested with NotI, and the resulting fragments were used as templates for in vitro transcription. RNAs were synthesized with T3 polymerase using T3 Message Machine Kit (Ambion, Austin, TX). Synthesized RNAs were treated with phenol–chloroform before precipitation with ethanol, and dried RNAs were resolved in H2O. RNA was polyadenylated by incubation for 30 min at 37°C in the presence of 100 μM ATP and 60 U/ml poly(A) polymerase (GIBCO BRL, Rockville, MD) in a buffer containing (mM) 250 NaCl, 50 Tris–Cl (pH 8.1), 10 MgCl2, 2 DTT, supplemented with 900 U/ml RNasin (Promega, Madison, WI), and 100 μg/ml BSA. Dried RNA was resolved in 150 mM KCl solution (final concentration, approximately 3 μg/μl RNA). RNA was diluted to the range between 1 and 200 ng/ml before injection into eggs. To avoid folding of RNA, the diluted RNA was heated at 85°C for 3 min and then cooled on ice. RNA was injected into approximately 10 eggs in a dish within 5 min using a glass micropipette (injected amount, approximately 5 pl/egg). The estimated concentration of RNA in the egg ranged between 0.025 and 5 ng/μl, assuming the egg volume is approximately 200 pl.

Synthesis and purification of recombinant Venus

Venus cDNA was constructed into pGEX-4T-1 (Amersham Bioscience Corp., Piscataway, NJ), and glutathione S-transferase (GST) Venus fusion protein was expressed in XL1-Blue Escherichia coli. After 12-h culture in the presence of isopropyl-1-thio-β-D-galactopyranoside, the bacteria were collected and disrupted by a French Press (5051, Ohtamke Works, Tokyo). GST–Venus fusion protein was purified using glutathione–Sepharose chromatography (Amersham) and digested with thrombin to remove GST. Venus protein was further purified with a glutathione–Sepharose and Resource Q columns (Amersham) and dialyzed against intracellular solution (120 mM KCl, 1 mM EDTA, 20 mM HEPES/KOH pH 7.5).

\[ \text{Ca}^{2+} \]i measurement

\[ \text{Ca}^{2+} \], was measured by a conventional \text{Ca}^{2+} imaging method using an image processor (Argus 50; Hamamatsu Photonics, Hamamatsu, Japan; for details, see Aida et al., 2001). The fluorescence intensity (F) of fura-2 was measured without interference with that of Venus by applying 340- and 380-nm UV lights alternatively and by leading emission light through a 400-nm dichroic mirror (DM400; Nikon) and a 500- to 520-nm bandpass filter. Fluorescence was detected by a silicon intensifier target camera (C2400-08; Hamamatsu Photonics). \text{Ca}^{2+} images were acquired at intervals of 20 s and processed to calculate the ratio R = F340/F380 later using NIH Image (a public domain image processing software for the Macintosh computer).

To induce \text{Ca}^{2+} oscillations in some experiments, InsP3 (potassium salt; Dojindo, Kumamoto, Japan) was injected by pressure with the solution containing 120 mM KCl, 0.1 mM EDTA, 10 mM HEPES (pH 7.3), and 100 μM InsP3. In some experiments, \text{Ca}^{2+} oscillations were inhibited by injection of function-blocking monoclonal antibody (mAb) 18A10 against the InsP3 receptor type 1 (Miyazaki et al., 1992). 18A10 was a kind gift from Dr. K. Mikoshiba (Institute of Medical Science, University of Tokyo).

Measurement of Venus fluorescence

Fluorescence of Venus ligated to PLCζ and expressed in eggs was investigated using the equipment for [\text{Ca}^{2+}], measurement (for details, see Aida et al., 2001). Excitation light was passed through a 470- to 490-nm bandpass filter and a ×20 objective lens (Fluor 20, Nikon). Emitted fluorescence passed through the objective lens, a 510-nm dichroic mirror (DM510; Nikon), and a 520- to 560-nm bandpass filter. Three fluorescence images were acquired at a defined time after RNA injection. The fluorescence intensity (F) was averaged in an egg and presented as an arbitrary unit of values. The relationship between F and the arbitrary unit was calibrated by measuring F of fluorescein (Sigma). The optical system and detector were set at the same conditions throughout the present study. The localization of PLCζ–Venus in the egg was observed using a confocal laser scanning microscope (LSM310, Carl Zeiss, Oberkochen, Germany) with an argon laser (488 nm) (for details, see Aida et al., 2001; Shiraishi et al., 1995). When [\text{Ca}^{2+}], and Venus fluorescence were simultaneously measured, fura dextran (Molecular Probes) was used by injection together with PLCζ–Venus RNA. Fura dextran was excited by a 340-nm light, and emitted fluorescence was passed through a 510-nm dichroic mirror, separated from Venus fluorescence. [\text{Ca}^{2+}], was presented as F340.

Immunohistochemistry

In some experiments, the localization of expressed PLCζ–FLAG instead of PLCζ–Venus was investigated using a monoclonal antibody against FLAG. Eggs were fixed for 20 min by 2% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.025% polyvinyl alcohol (P-PBS). The fixed eggs were permeabilized by exposure to 0.2% Triton X-100 in P-PBS for 15 min and blocked by bathing them in 1% BSA-containing P-PBS overnight at 4°C. For immunostaining, eggs were incubated successively with a primary and a secondary antibody (Ab) for 1 h, each at room temperature followed by careful washing. The primary Ab used was an mAb against FLAG (M2, 1:150 dilution; Sigma). The secondary Ab was a goat BODIPY-labeled anti-mouse IgG Ab (b-2752, 1:200; Molecular Probes). The stained eggs were observed by confocal microscopy with a 488-nm argon laser.
Results

Features of expression of PLCζ–Venus in the egg

Fig. 1A shows the relationship between the calculated intracellular concentration of recombinant Venus injected into the egg (in pmol/µl) and the fluorescence intensity (F; arbitrary unit) of Venus in the egg under a conventional fluorescence microscope. Autofluorescence of the egg was subtracted from total fluorescence. A calibration line was obtained by a linear regression through the origin. PLCζ (molecular weight, 75 kDa) fused with Venus (27 kDa) at the N-terminus was expressed in mouse eggs by RNA injection. In Fig. 1C, five eggs were injected with 100 ng/µl RNA of PLCζ–Venus at the zero time, and averaged F of expressed PLCζ–Venus in the egg was sampled every 5 min. An elevation of F level became detectable 40–50 min after RNA injection, and then F increased during at least 150 min. F of PLCζ–Venus at 180 min after RNA injection had a linear relationship to the concentration of RNA in the injection solution up to 200 ng/µl (Fig. 1B). Thus, expressed PLCζ–Venus was proportional to the injected RNA concentration in this range when measured at 180 min.

Ca²⁺ oscillations induced by expressed PLCζ–Venus

Expressed PLCζ–Venus induced Ca²⁺ oscillations in a dose-dependent manner (Figs. 2B–D), as expressed PLCζ does (Saunders et al., 2002). The first Ca²⁺ transient lasted longer than succeeding Ca²⁺ spikes, as always seen at fertilization (Deguchi et al., 2000; Jones et al., 1995). The critical RNA concentration to induce Ca²⁺ oscillations was approximately 0.5 ng/µl, although F of PLCζ–Venus in this case was indistinguishable from the egg autofluorescence. The time lag from RNA injection (the zero time) to the occurrence of the first Ca²⁺ transient was approximately 90 min for 1 ng/µl RNA (Fig. 2B) and it was shortened with the higher RNA concentration (Figs. 2C–D). Quantitative analysis showed that the time lag was remarkably affected by the RNA concentration up to 20 ng/µl, while the relation at higher RNA concentrations exhibited an asymptote reaching to a minimal value of 25 min (Fig. 3). The relation between the time lag and RNA concentration was basically similar between PLCζ–Venus and Venus-free PLCζ.

Precise comparison of Ca²⁺ spike frequencies was basically hard because the frequency increased progressively as Ca²⁺ oscillations advanced (Figs. 2C–D), probably due to

![Fig. 1. Fluorescence intensity (F) as the function of various factors. (A) F versus calculated intracellular concentration of recombinant Venus that was injected into mouse eggs. (B) F at 180 min after RNA injection versus concentration of PLCζ–Venus RNA in the injection solution. (C) Increase in F after injection of 100 ng/µl PLCζ–Venus RNA. Autofluorescence of the egg was subtracted from the total fluorescence. About 5 pl of solution containing protein or RNA was injected into an egg of which volume is approximately 200 pl. The mean ± SD is presented in each point in A (n = 4) and B (n = 7).]
continuous increase of expressed PLC\~. There was a tendency of the higher Ca\textsuperscript{2+} spike frequency for the higher RNA concentration, while the amplitude of Ca\textsuperscript{2+} spikes was unaffected. The spike frequency is usually 3 or 4 per 60 min in the early stage of IVF in monospermic mouse eggs (Deguchi et al., 2000). Comparable Ca\textsuperscript{2+} oscillations were obtained during a certain period after injection of 10–50 ng/\mu l s-PLC\~–Venus RNA (Fig. 2C). Expressed PLC\~ during 180 min after RNA injection was estimated from Figs. 1A to B as 100–500 fg/egg. It was estimated as 10–40 fg/egg from F values at the time when the first Ca\textsuperscript{2+} transient appeared.

With 100–200 ng/\mu l PLC\~–Venus RNA, a burst of Ca\textsuperscript{2+} spikes occurred 120–180 min after RNA injection (Fig. 2D). These features of the Ca\textsuperscript{2+} oscillation-inducing activity were comparable to those produced by Venus-free PLC\~ (data not shown).

**Injection of s-PLC\~ and PLC\~I**

mRNA encoding a protein similar to PLC\~ has been found to exist in the mouse testis (AK006672 in EMBL). This protein (designated as s-PLC\~) lacks 110 amino acid residues from the N-terminus corresponding to EF1, EF2, and EF3 domains of PLC\~ but is identical to PLC\~ in EF4 and succeeding region (Fig. 2A). Since s-PLC\~ gene has not been found in the mouse genome, s-PLC\~ is probably a splicing variant of PLC\~. Ca\textsuperscript{2+} oscillations were generated by s-PLC\~ (Fig. 2E), although at least 500 ng/\mu l RNA was necessary (six eggs). F in the eggs at 180 min after injection...
of 500 ng/μl s-PLCζ–Venus RNA was approximately 100, which corresponded to the expression of PLCζ–Venus by 100 ng/μl RNA according to Fig. 1B. The efficiency of expression of s-PLCζ–Venus appeared to be fivefold lower than that for PLCζ–Venus. The pattern of Ca2+ oscillations was different from that caused by PLCζ–Venus. Small Ca2+ oscillations first appeared 120 min after injection of s-PLCζ–Venus RNA, and they were followed by discrete Ca2+ spikes with the small but incremental amplitude, associated with the interspike intervals of approximately 30 min (Fig. 2E). Such series of Ca2+ spikes occurring after a long time lag could be produced by PLCζ–Venus RNA in the range of 1 ng/μl (Fig. 2B). Thus, the Ca2+ oscillation-inducing activity of s-PLCζ is estimated to be roughly two orders of magnitude lower than that of PLCζ.

Some eggs were injected with 1.5 μg/μl PLCγ1–Venus RNA. The efficiency of expression was so low that F of PLCγ1–Venus at 180 min corresponded to that of PLCζ–Venus expressed by 80 ng/μl RNA. Ca2+ oscillations could not be induced by PLCγ1–Venus (data not shown). The situation is consistent with that in the previous work (Saunders et al., 2002). Any PLC isoform at high-enough concentration is predicted to induce Ca2+ oscillations, as shown by injection of recombinant PLCγ1 into mouse eggs (Mehlmann et al., 2001). The expression level of PLCγ1 in the present experimental conditions is thought to be not high enough to induce Ca2+ oscillations.

Accumulation of expressed PLCζ in the pronuclei

The distribution of expressed PLCζ in the egg was investigated by fluorescence of Venus that was fused with PLCζ using a confocal microscope 6–8 h after injection of PLCζ–Venus RNA (50 ng/μl). Eggs had been activated on account of repetitive [Ca2+]i rises caused by PLCζ, and the female PN had been formed. Interestingly, the PN that was identified in a bright field image (Fig. 4A, right) exhibited a remarkably higher level of fluorescence compared with the cytoplasm (Fig. 4A, left). Fig. 4D is a control image showing faint autofluorescence of the egg.

Fig. 3. The time lag to the first Ca2+ transient after injection of PLCζ RNA or PLCζ–Venus RNA at various concentrations. The mean ± SD is presented in each point (n = 6). The PLCζ–Venus RNA concentration (ng/μl) was converted to the calculated value as PLCζ RNA.

Fig. 4. Distribution of expressed PLCζ. Confocal fluorescence images (left) and bright field images (right) of the egg. (A) An egg 6 h after injection of PLCζ–Venus RNA (50 ng/μl). (B) An egg injected with PLCζ–FLAG RNA (100 ng/μl) and stained with anti-FLAG mAb 7.5 h later. Bright tiny spots in the perivitelline space are unwashed secondary antibody (goat BODIPY-labeled anti-mouse IgG antibody). (C) An egg injected with PLCζ–Venus RNA (200 ng/μl) and inseminated 10 min later. Faint fluorescent patches are autofluorescence, probably derived from oxidized flavins of mitochondria. (D) A control image showing faint autofluorescence in an egg injected with Venus-free PLCζ RNA. (E) A control image to B (an egg injected with Venus-free, FLAG-free PLCζ RNA and treated with anti-FLAG mAb) showing that bright spots in the perivitelline space are artifact due to unwashed secondary antibody. Images of C, D, and E were taken at 7 h after RNA injection. Scale bar is 20 μm.
injected with Venus-free PLCζ RNA. The predominant localization of F in the PN was observed in all 60 eggs examined. With precise inspection, F in the nucleolus, which was identified as a round structure with a clear circumference in the bright field image, was substantially lower than that in the nucleoplasm 6 h after RNA injection. (Fig. 4A, left). F was not significantly accumulated in the second polar body.

The special localization in the PN took place not only for PLCζ–Venus, but also for PLCζ–FLAG (addition of only 10 amino acids in the N-terminus of PLCζ), as observed in the eggs (n = 7) that were injected with PLCζ–FLAG RNA (100 ng/μl) and subjected to immunohistochemical staining with anti-FLAG mAb 7.5 h later (Fig. 4B). Fig. 4E is a control image of an egg injected with FLAG-free PLCζ RNA. Fig. 4B shows that the nucleoplasm was clearly stained, although the antibody appeared not to access into the nucleolus. The result indicates that PLCζ but not Venus is responsible for predominant localization of PLCζ–Venus in the PN. Thus, expressed PLCζ is accumulated in the PN during early embryogenesis. In some experiments, eggs (n = 6) were injected with PLCζ–Venus RNA (200 ng/μl) and inseminated about 10 min later. PLCζ–Venus was accumulated in the male PN, as well as the female PN, when examined 7 h after RNA injection (Fig. 4C).

**Cytoplasmic distribution of expressed s-PLCζ and PLCζ1**

Since expressed s-PLCζ–Venus induced small Ca2+ oscillations associated with a long time lag and long interspike intervals (Fig. 2E), PN formation was much delayed; more than 10 h after RNA injection during which F tended to decrease. To form the PN earlier, RNA of s-PLCζ–Venus RNA (200 ng/μl) and subjected to immunohistochemical staining with anti-FLAG mAb 7.5 h later (Fig. 4B). Fig. 4E is a control image of an egg injected with FLAG-free PLCζ RNA. Fig. 4B shows that the nucleoplasm was clearly stained, although the antibody appeared not to access into the nucleolus. The result indicates that PLCζ but not Venus is responsible for predominant localization of PLCζ–Venus in the PN. Thus, expressed PLCζ is accumulated in the PN during early embryogenesis. In some experiments, eggs (n = 6) were injected with PLCζ–Venus RNA (200 ng/μl) and inseminated about 10 min later. PLCζ–Venus was accumulated in the male PN, as well as the female PN, when examined 7 h after RNA injection (Fig. 4C).

**Time course of accumulation of expressed PLCζ in the pronucleus**

The accumulation of PLCζ–Venus in the PN was followed up during egg activation and formation of the PN. In Fig. 6A, F in six eggs under a conventional fluorescence microscope was sampled every 5 min for 480 min after injection of RNA (50 ng/μl). F was homogeneous in the entire egg and increased up to 180 min (Fig. 6A, dotted lines), as shown in Fig. 1C. The rate of rise of F during this period is called R0. The rate of rise of F gradually decreased later than 180 min. The formation of the PN was identified by its clear boundary in the ooplasm in the bright field image between 300 and 360 min after injection of PLCζ–Venus RNA and between 240 and 300 min after the onset of Ca2+ oscillations. Localization of F in the PN area was detectable in about 30 min before the identification of the PN (Fig. 6A, inset). F in the PN increased at a higher rate than R0 continuously from 300 min up to at least 480 min (solid lines). On the other hand, F in the cytoplasm remained at a steady level or, in some eggs, slightly declined (Fig. 6A, dotted lines). Consequently, F in the PN area became more pronounced with time, contrasted to the cytoplasm. These results suggest that expressed PLCζ translocates into the PN formed after egg activation.

Fig. 6B presents F of two eggs in which a mixture of s-PLCζ–Venus RNA (500 ng/μl) and PLCζ RNA (50 ng/μl) was injected and the PN was formed. F in the cytoplasm increased up to 180 min but reached a steady level later than 240 min. The temporal pattern was similar to that for expression of PLCζ–Venus in the cytoplasm (Fig. 6A, dotted lines). F of s-PLCζ–Venus in the PN area never
exceeded but was comparable to that in the cytoplasm under a conventional fluorescence microscope (Fig. 6B, solid lines; inset) in which F of the cytoplasm was superimposed on F of the PN area. These results indicate that the rate of accumulation of s-PLCζ to the PN is thought to be much smaller than that of PLCζ.

Cessation of Ca2+ oscillations

PLCζ-induced Ca2+ oscillations ceased in the majority of eggs later than 100 min after RNA injection. For example, of 17 eggs injected with 40 ng/μl PLCζ–Venus RNA, Ca2+ oscillations ceased within 6 h in 14 eggs, while they continued in 3 eggs during recording for 7 h. To describe this phenomenon, the time between the first Ca2+ transient and the last Ca2+ spike is defined as the ‘duration’ of Ca2+ oscillations. The values of the duration were scattered in a wide range but separated into two groups; relatively longer duration ranged between 210 and 340 min in eight eggs and shorter duration between 90 and 140 min in six eggs. Representative records of the two groups are shown in Figs. 7A–B, together with simultaneously recorded F in the cytoplasm and the PN. Ca2+ oscillations shown in Fig. 7A were accelerated in the Ca2+ spike frequency from 90 min after the onset of the first Ca2+ transient but stopped 30 min before the appearance of PLCζ–Venus in the PN (Fig. 7A). In all of the eight eggs, Ca2+ oscillations ceased before the accumulation of PLCζ–Venus in the PN became detectable. The accumulation occurred in the absence of Ca2+ oscillations in these eggs. In another group of eggs, Ca2+ oscillations

Fig. 6. Changes in F in the cytoplasm and the PN after injection of PLCζ–Venus RNA (50 ng/μl) (A) or s-PLCζ–Venus RNA (500 ng/μl) (B). Autofluorescence of the egg was subtracted from the total fluorescence. Insets are images of an egg under a conventional fluorescence microscope at the periods indicated by arrows.

Fig. 7. Simultaneous recording of F of PLCζ–Venus in the cytoplasm and the PN (A and B) and Ca2+ oscillations (A’ and B’) after injection of 40 ng/μl RNA shown in two examples in which the duration of Ca2+ oscillations was different. (C) An example of Ca2+ oscillations after injection of 200 ng/μl RNA. The ordinate is F340 of fura dextran in the egg (arbitrary unit). Total fluorescence including autofluorescence of the egg is presented. In A and B, an oscillatory change of F was seen in association with each Ca2+ transient in the early phase of Ca2+ oscillations. This is probably due to a Ca2+-dependent change in cell autofluorescence derived from oxidized flavins (Mironov and Richter, 2001).
oscillations shown in Fig. 7B’ stopped 90 min after the onset of the first Ca\textsuperscript{2+} transient, without transition to accelerated Ca\textsuperscript{2+} spikes. As shown in Fig. 7B, Ca\textsuperscript{2+} oscillations ceased far before the PN was formed in the six eggs. In another three eggs Ca\textsuperscript{2+} oscillations did not stop during recording for 420 min, although the PN was formed and nuclear accumulation of PLC\textsuperscript{~}–Venus was observed.

With higher RNA concentration (200 ng/\mu l), Ca\textsuperscript{2+} oscillations once ceased within 90 min and reappeared approximately 180 min after the onset of the first Ca\textsuperscript{2+} transient in 8 of 12 eggs (Fig. 7C). The second Ca\textsuperscript{2+} oscillations consisted of a relatively long-lasting Ca\textsuperscript{2+} transient and subsequent high-frequency Ca\textsuperscript{2+} spikes. They ceased 360–420 min after RNA injection when PLC\textsuperscript{~}–Venus was accumulating in the PN.

To examine whether Ca\textsuperscript{2+} oscillations are necessary for PLC\textsuperscript{~} to be modified before its nuclear translocation, PLC\textsuperscript{~}–induced Ca\textsuperscript{2+} oscillations were inhibited by preinjection of a function-blocking mAb, 18A10, against the InsP\textsubscript{3} receptor type 1 (Miyazaki et al., 1992). In Fig. 8, transient Ca\textsuperscript{2+} oscillations were first induced by injection of InsP\textsubscript{3} to induce egg activation and PN formation. The egg was injected with 18A10 200 min later and with PLC\textsuperscript{~}–Venus RNA (50 ng/\mu l) after a 40-min interval. No Ca\textsuperscript{2+} spike was generated because of the inhibitory effect of 18A10. PLC\textsuperscript{~}–Venus was accumulated in the PN even under this condition (inset of Fig. 8). Thus, expressed PLC\textsuperscript{~} does not require repetitive [Ca\textsuperscript{2+}]\textsubscript{i} rises for conditioning before its nuclear translocation.

**Discussion**

The present study demonstrated that PLC\textsuperscript{~} labeled with a fluorescent protein had the Ca\textsuperscript{2+} oscillation-inducing activity in mouse eggs and exhibited accumulation to the PN during early embryogenesis. The improved probe, Venus, undergoes accelerated oxidation of the chromophore after it is expressed in the cell at 37°C (Nagai et al., 2002). This advantage allowed us to visualize PLC\textsuperscript{~}–Venus in the egg cytoplasm as early as 40–50 min after RNA injection and continuously follow-up its nuclear accumulation. The expression level of PLC\textsubscript{ζ}, s-PLC\textsubscript{ζ}, and PLC\textsubscript{δ1} at 180 min could be compared using F of Venus. Thus, the present study provided a quite efficient model system for characterization of functional proteins expressed in mammalian eggs by injection of RNA with an added long poly(A) tail.

Ca\textsuperscript{2+} oscillation-inducing activity of PLC

Activated PLC is predicted to cause Ca\textsuperscript{2+} oscillations via continuous production of InsP\textsubscript{3} since artificial supply of InsP\textsubscript{3} at a sustained low level in the ooplasm produces low-frequency discrete Ca\textsuperscript{2+} spikes similar to those at fertilization (Jones and Nixon, 2000). Application of recombinant PLC\textsubscript{β1}, γ1, γ2, and δ1, which are present in the mammalian sperm, is incapable of inducing Ca\textsuperscript{2+} release in the ooplasm (Jones et al., 2000), or recombinant PLC\textsubscript{γ1} at extremely high dosage produces Ca\textsuperscript{2+} oscillations (Mehlmann et al., 2001). The present study confirmed the previous result by Saunders et al. (2002) that expression of PLC\textsubscript{ζ} by injection of its RNA is effective to induce Ca\textsuperscript{2+} oscillations, although the method has a disadvantage in controlling the amount of the target protein, which is continuously synthesized in the cell. Ca\textsuperscript{2+} oscillations comparable to those at fertilization were produced 40–50 min after injection of PLC\textsubscript{ζ}–Venus RNA at 10–50 ng/\mu l. The expressed PLC\textsubscript{ζ} level to initiate Ca\textsuperscript{2+} oscillations was estimated as 10–40 fg/egg, comparable to the estimated content of PLC\textsubscript{ζ} in a single mouse spermatozoon (20–50 fg; Saunders et al., 2002).

The activation mechanism of PLC\textsubscript{ζ} as well as PLC\textsubscript{δ} is unknown since they lack a regulatory domain such as the G protein binding site of PLC\textsubscript{β} or the SH domain of PLC\textsubscript{γ} for...
phosphorylation by tyrosine kinase. Furthermore, PLCζ lacks the PH domain that is the site for interaction with membrane phospholipids (Katan, 1998). s-PLCζ, which lacks three EF hand domains, was much less effective (roughly two orders of magnitude) in inducing Ca^{2+} oscillations compared with full-length PLCζ. PLCδ1 was also much less effective than PLCζ. It is important to examine the Ca^{2+} sensitivity of recombinant PLCζ, considering the N-terminal EF hand domains as Ca^{2+}-binding site(s). Rice et al. (2000) have shown that boar sperm extract possesses the PLC activity of hydrolyzing PtdInsP_2 at [Ca^{2+}] as low as the resting [Ca^{2+}] of unfertilized eggs (approximately 100 nM; Nakano et al., 1997). This is an appropriate character of the PLC isoform as a candidate of the sperm factor because the factor is introduced into the ooplasm at fertilization and first triggers Ca^{2+} release from the ER. The Ca^{2+} sensitivity of the PLC activity of sperm extract described above is higher than that of PLCδ1 reported (Cheng et al., 1995). PLCζ may be highly sensitive to Ca^{2+} and active in the cell even at the resting state. The activation mechanism of PLCζ is one of the key subjects to be solved for identification of the sperm factor.

Accumulation of PLCζ to the pronucleus

PLCζ was found to be accumulated in the female and male pronuclei. Localization of PLCζ–Venus was recognized in the PN area before the boundary of the PN became clearly visible in the bright field image under a microscope. Subsequently, PLCζ–Venus was accumulated in the PN at a higher rate than the initial expression rate in the cytoplasm. Thus, PLCζ is accumulated to the PN from the early stage of PN formation. The accumulation continued even after cessation of Ca^{2+} oscillations (Figs. 7A–B), implying that [Ca^{2+}]_i rise is not a prerequisite to the accumulation.

The nuclear accumulation of s-PLCζ or PLCδ1 was remarkably lower than that of PLCζ, even in the presence of Ca^{2+} oscillations produced by coexpressed PLCζ, at least at the expression level of s-PLCζ or PLCδ1 threefold higher than that of PLCζ. The difference between PLCζ and s-PLCζ suggests that EF hand domains of PLCζ may be responsible for the ability of nuclear accumulation. Since translocation of PLCζ occurred without previous [Ca^{2+}]_i rises (Fig. 8), it might not rely on Ca^{2+} binding. It has been shown that PLCδ1 is predominantly localized at the plasma membrane and the cytoplasm (Fig. 5B) (Watt et al., 2002), but little is present in the nucleus (Fujii et al., 1999). PLCδ1 has a functional nuclear export signal sequence in the EF hand domain, and thereby not accumulated in the nucleus (Yamaga et al., 1999). Further analysis of the property is required, based on the molecular structure.

Biological significance of nuclear accumulation of PLC

The present finding that PLCζ is accumulated into the PN is of important implication in that PLCζ fulfills a necessary condition as a candidate of the sperm factor since the Ca^{2+} oscillation-inducing activity derived from the sperm is localized in the PN in fertilized eggs (Knott et al., 2003; Kono et al., 1995). Transfer of the male or female PN to an unfertilized mouse egg causes repeated Ca^{2+} spikes at long intervals (Knott et al., 2003; Kono et al., 1995). It has been proposed that the accumulation of the sperm factor into the PN is responsible for the cessation of Ca^{2+} oscillations (Kono et al., 1996; Marangos et al., 2003), which occurs at about the time when the PN is formed in fertilized eggs (Deguchi et al., 2000; Jones et al., 1995). Ca^{2+} oscillations in fertilized eggs persist for approximately 10 h when PN formation was prevented by injection of a lectin, WGA (Marangos et al., 2003). In the present experiments, Ca^{2+} oscillations induced by expressed PLCζ ceased in the majority of eggs before the nuclear accumulation of PLCζ was initiated and when PLCζ distributed throughout the cytoplasm. High-frequency Ca^{2+} oscillations induced by a high level of expressed PLCζ stopped at the period when its nuclear accumulation was going on (Fig. 7C). However, clear evidence for the causal effect of nuclear sequestration of PLCζ on the cessation of Ca^{2+} oscillations was not provided by the present experimental system where PLCζ is accumulatively produced. Ca^{2+} oscillations stopped within several hours, even when PN formation was inhibited by WGA (unpublished result). Other inhibitory effects on Ca^{2+} oscillations might be rather enhanced by overexpression of PLCζ. Ca^{2+} oscillations tended to stop at two periods, between 1.5 and 2 h and 3.5 and 4.5 h after the onset of the first Ca^{2+} transient. A negative feedback via production of DAG and subsequent activation of protein kinase C (PKC) might operate on PLC activity (Ali et al., 1999) when considerable amount of PLCζ was expressed in the egg. Down-regulation of the InsP_3 receptor type 1, which is essential for Ca^{2+} release from the ER and consequent Ca^{2+} oscillations in mouse eggs (Miyazaki et al., 1993; Oda et al., 1999), is known to occur as a result of Ca^{2+} oscillations in mouse eggs, notably approximately 4 h after fertilization or parthenogenetic activation (Brind et al., 2000; Julleter et al., 2000). This down-regulation could be enhanced by overexpressed PLCζ.

A phosphoinositide signaling pathway is known to exist in the nucleus, and PLC plays biological roles in the nucleus (Irvine, 2003). For example, PLCβ1 translocates into the nucleus during G2/M transition in immature mouse oocytes and participates in germinal vesicle breakdown (Avazeri et al., 2000). It is predicted that DAG produced by PLCβ1 attracts PKCβII from the cytoplasm and PKCβII causes lamin phosphorylation leading to nuclear envelope breakdown (Goss et al., 1994; Irvine, 2003). PLCδ4 is predominantly present in the nucleus and increases dramatically at G1/S transition in response to mitogenic stimulation (Liu et al., 1996). It is tempting to address whether PLCζ that accumulated in the PN affects cell proliferation and differentiation in early embryonic development; that is, PLCζ...
may have functions not only via InsP₃ and Ca²⁺ in the cytoplasm, but also via DAG and PKC in the nucleus.

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