Published in final edited form as:
Dev Biol. 2007 December 1; 312(1): 321–330.

Alterations of PLCβ1 in mouse eggs change calcium oscillatory behavior following fertilization

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Abstract

Inositol 1, 4, 5-trisphosphate generated by the action of a phospholipase C (PLC) mediates release of intracellular Ca^{2+} that is essential for sperm-induced activation of mammalian eggs. Much attention currently focuses on the role of sperm-derived PLCζ in generating changes in egg intracellular Ca^{2+} despite the fact that PLCζconstitutes a very small fraction of the total amount of PLC in a fertilized egg. Eggs express several isoforms of PLC, but a role for an egg-derived PLC in sperm-induced Ca^{2+} oscillations has not been examined. Reducing egg PLCβ1 by a transgenic RNAi approach resulted in a significant decrease in Ca^{2+} transient amplitude, but not duration or frequency, following insemination. Furthermore, over-expressing PLCβ1 by microinjecting a *Plcb1* cRNA significantly perturbed the duration and frequency of Ca^{2+} transients and disrupted the characteristic shape of the first transient. These results provide the first evidence for a role of an egg-derived PLC acting in conjunction with a sperm-derived PLCζin egg activation.

Introduction

A universal feature of fertilization in eggs of all species studied to date is a transient elevation in intracellular Ca^{2+} concentration. In mammals, the sperm evokes a series of repetitive Ca^{2+} oscillations that persist for several hours and terminate with pronucleus (PN) formation (Jones et al., 1995; Marangos et al., 2003). This pattern of repetitive Ca^{2+} oscillations in mice is essential for both early events of egg activation (e.g., resumption of meiosis, CG exocytosis, recruitment of maternal mRNAs) and the developmental program (Ducibella et al., 2002; Ozil and Huneau, 2001; Ozil et al., 2006).

Release of Ca^{2+} from the inositol 1, 4, 5-trisphosphate (IP_3)-sensitive Ca^{2+} pool is essential for egg activation, because inhibiting its release with a monoclonal antibody that blocks the IP_3 receptor (Miyazaki et al., 1992) inhibits egg activation (Xu et al., 1994). Calmodulin-modulated protein kinase II (CaMKII) is likely a major target of the released Ca^{2+} because expressing a constitutively active form of CaMKII results in egg activation in the absence of any increase in the concentration of intracellular free Ca^{2+} (Madgwick et al., 2005). CaMKII, whose activity changes in parallel with the changes in intracellular Ca^{2+} (Markoulaki et al.,

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2003; Markoulaki et al., 2004), may do this by "summing" the amount of Ca^{2+} released during the course of egg activation (Ducibella et al., 2002; Ozil et al., 2005).

Fertilization likely triggers the increase in intracellular Ca^{2+} via PLC-catalyzed production of IP_3 in the egg (Runft et al., 2002). The IP_3 then acts on type 1 IP_3 receptors to initiate Ca^{2+} release. How the fertilizing sperm activates PLC has been intensively studied for the past 15 years. During this time a paradigm shift occurred from a sperm ligand-egg receptor model using either G protein-coupled or tyrosine kinase receptors to a model proposing that a soluble, sperm-derived protein factor is responsible for activating PLC and thereby evoking Ca^{2+} oscillations in mammalian eggs (Swann and Lai, 1997). The seminal observations leading to the new model are that mouse sperm and egg fusion precedes the onset of oscillations by ~1-5 min (Lawrence et al., 1997) and that injecting mouse sperm in the egg, which causes fertilization-like Ca^{2+} oscillations, supports full-term development (Kimura and Yanagimachi, 1995).

A novel, sperm-specific PLC, termed PLCζ, is now thought to be the sperm factor. Expression of PLCζ cRNA in mouse eggs that results in synthesis of PLCζ estimated to be equivalent to the amount in a single sperm triggers Ca^{2+} oscillations that closely resemble the pattern observed following fertilization (Saunders et al., 2002; Yoda et al., 2004). Furthermore, immunodepleting PLCζ from sperm extracts abolishes the Ca^{2+} releasing activity, suggesting that PLCζ is the sole Ca^{2+} releasing component (Saunders et al., 2002). Last, recent biochemical studies using recombinant PLCζ protein demonstrated that its activity is ~70% maximal at 100 nM Ca^{2+}, the resting level of Ca^{2+} in the egg (Kouchi et al., 2004). This finding supports the model in which the sperm delivers a PLC that is readily activated on exposure to the egg's cytoplasm. Consistent with this proposal is our observation that reducing sperm PLCζ protein by a transgenic RNAi approach significantly perturbs the number of Ca^{2+}oscillations (but not their amplitude) when eggs are inseminated with these sperm (Knott et al., 2005). Furthermore, no transgenic offspring were derived from founder males mosaic for the transgene suggesting that sperm-derived PLCζ is essential for development to term (Knott et al., 2005).

Several observations suggest a role for an endogenous egg PLC in generating the calcium oscillatory pattern during fertilization. First, it is estimated that only about 50 fg of PLCζ enters the egg, which contains ~25,000,000 fg of protein (Saunders et al., 2002). Second, the egg likely contains significantly greater amounts of numerous PLC isoforms, including β,γ, and δ. Third, these egg PLCs, which are regulated by Ca^{2+}, are likely activated by increases in intracellular Ca^{2+} that now reach stimulatory concentrations. Taken together, these results suggest a model in which each Ca^{2+} transient is triggered by a sperm-derived PLCζ resulting in IP_3 production and an initial increase in intracellular Ca^{2+}. In turn, this initial increase in Ca^{2+} activates an egg PLC that results in the further production of IP_3 and an increase in intracellular Ca^{2+} that can last for minutes. Consistent with this proposal is the observation that modulating the amount of PLCζ in eggs affects the frequency but not the amplitude of the Ca^{2+} oscillations (Saunders et al., 2002).

Except for PLCζ, members of each of the PLC families are expressed in eggs (Table 1) and in theory any of these could be involved in modulating Ca^{2+} oscillations. Based on the finding that the litter size of null females is normal, we excluded as candidates isoforms β4, δ1, δ4, and ε. We excluded isoforms β2, δ2, and δ3 because their transcripts are not expressed in the oocyte. Microinjecting recombinant PLCγ1 protein into mouse eggs can induce persistent Ca^{2+} oscillations similar to oscillations induced by sperm (Mehlmann et al., 2001). It is unlikely, however, that PLCγ1 or γ2 collaborate with sperm PLCζ to generate Ca^{2+} oscillations because a dominant-negative approach employing recombinant SH2 domain to inhibit PLCγ1 and γ2 did not inhibit the Ca^{2+} oscillatory pattern during fertilization (Mehlmann et al.,

1998); these experiments had an appropriate positive control to demonstrate efficacy of PLCγ inhibition.

Because there are conflicting data regarding the fertility of PLCβ3-deficient female mice (Table 1), we tested the ability of egg PLCβ1 to modulate sperm-induced Ca^{2+} oscillations. We report here that reducing egg PLCβ1 using a transgenic RNAi approach results in a decrease in amplitude of the Ca^{2+} oscillations but no change in their duration or frequency. Over-expressing PLCβ1 in eggs prior to fertilization does not result in spontaneous Ca^{2+} oscillations but following fertilization these eggs exhibit changes in the oscillatory pattern including an altered shape of the characteristic first oscillation, shorter duration, and decreased frequency of oscillations.

Materials and Methods

Generation of transgenic mice

A RNAi transgene was designed such that the *Zp3* promoter (Millar et al., 1991) directs oocyte-specific expression of Plcb1 double-stranded RNA (dsRNA). To prepare the inverted repeat for generating the dsRNA hairpin, a portion of the *Plcb1* coding sequence (nucleotides 301 to 854, Genbank accession no. NM_019677) was amplified by PCR and then ligated 3' to 3'. The inverted repeat was transferred to the pRNAi-ZP3 cassette (Stein et al., 2003). Transgenic animals were generated by PN injection at the University of Pennsylvania Transgenic and Chimeric Mouse Facility. The zygotes for microinjection were produced by mating of $B6SJLF_1$ mice. Transgenic founders and subsequent generations of transgenic mice were mated to wild-type C57Bl6/J mice. Genotyping was performed by PCR of enhanced green fluorescent protein from tail DNA as previously described (Ma et al., 2006). Experimental oocytes and eggs were obtained from transgenic female offspring, and controls were obtained from their non-transgenic littermates; these will be referred to as "TG" and "NTG" oocytes and eggs, respectively. All animal experiments were approved by the University of Pennsylvania Institutional Animal Use and Care Committee and were consistent with National Institutes of Health guidelines.

Complementary RNA synthesis

The pTRIamp19 vector (Ambion, Austin, TX) was modified for the generation of *in vitro* transcribed, stable, and polyadenylated mRNAs for microinjection. The 5' and 3' *Xenopus* β-globin untranslated regions were amplified from pXT7 (a generous gift from Dr. Sergei Sokol, Harvard Medical School) and cloned into unique *HindIII* and *EcoRI* sites of pTRIamp19, respectively. The 3' globin untranslated region was engineered to end with a polyA(33) and polyC(12) tail. This expression vector was renamed pIVT (plasmid *In Vitro* Transcription) and was used to generate the overexpression constructs.

The complete 3651 bp coding region of mouse PLCβ1 (GenBank accession no. NM_019677) was engineered using PCR to contain an N-terminal abbreviated Kozak sequence (Kozak, 1987) and a C-terminal T7 epitope tag (MASMTGGQQMG). A catalytically inactive C-terminal deletion mutant of PLCβ1 (Δ830-1041; GenBank accession no. NP_062651) (Litosch, 2000; Ross et al., 2006) containing a C-terminal T7 epitope tag was generated in similar fashion. The coding region of DsRed-Monomer was amplified by PCR from pDsRed-Monomer-C1 (Clontech, Mountain View, CA). The resulting cDNAs were subcloned into pIVT using the unique *SphI* and *XbaI* sites. Complementary RNA (cRNA) was synthesized from linearized plasmid DNA using T3 or T7 RNA polymerase and the mMESSAGE mMACHINE kit (Ambion, Austin, TX) according to the manufacturer's instructions. The cRNA was cleared and purified by using MEGAclear (Ambion) and MicroPoly(A)Purist

(Ambion), respectively. The final cRNA concentration was determined by spectrophotometry, and cRNA integrity was confirmed by analyzing a sample on a formaldehyde gel.

Gamete collection and culture

Female CF-1 mice (6-8 wk old) were obtained from Harlan Sprague-Dawley (Indianopolis, IN). Fully-grown, GV-intact oocytes and metaphase II (MII)-arrested eggs were collected from gonadotropin-treated females as previously described (Manejwala et al., 1986). The collection medium for oocytes was bicarbonate-free minimal essential medium (Earle's salts) containing 0.01% polyvinyl alcohol (PVA), 25 mM Hepes, pH 7.3, and 2.5 μM milrinone to prevent germinal vesicle (GV) breakdown. After collection, oocytes were cultured in Whitten's medium (Whitten, 1971) containing 0.01% PVA (Whittens/PVA) and 2.5 μM milrinone. Metaphase II (MII)-eggs were collected 13-14 h post-hCG administration into modified Whitten's medium containing 15 mM Hepes, pH 7.2, 7 mM Na_2HCO_3, 10 μg/ml gentamicin, and 0.01% polyvinyl alcohol (modWhittens/PVA). The cumulus cells were removed by brief hyaluronidase treatment. When necessary, z*ona pellucida* (ZP)-free oocytes and eggs were obtained by removing the ZP with acidic Tyrodes solution, pH 1.6 (Bornslaeger and Schultz, 1985). The eggs were cultured until use in Whittens/PVA under light mineral oil. All gametes and embryos were cultured at 37° C in a humidified atmosphere of 5% CO_2 in air.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated from groups of 20-30 GV-intact oocytes using the Absolutely RNA Microprep kit (Strategene) according to the manufacturer's protocol. The RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamer primers. Real-time PCR analysis was carried out using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). TaqMan probes corresponding to PLCβ1 (ABI assay ID Mm00479987_m1) and histone H2A (ABI assay ID Mm00501974_s1) were used. A single oocyte equivalent of cDNA was used in each reaction and performed in triplicate. The amount of PLCβ1 cDNA was normalized by the comparative C_t method (http://www.ambion.com) using amplification of endogenous histone H2A in the same samples.

Microinjection and in vitro maturation of oocytes

Injections were done in 10 μl drops of modWhittens/PVA containing 2.5 μM milrinone. Approximately 10 pl of cRNA or diethyl pyrocarbonate (DEPC)-treated water was injected into the cytoplasm of GV-intact oocytes using a Harvard Apparatus PLI-100 Pico-Injector on the stage of a Nikon Eclipse TE300 microscope equipped with Hoffman optics and Narishige micromanipulators. After microinjection, oocytes were cultured in the presence of milrinone for 1-6 hr to allow for exogenous protein production. The oocytes were washed free of milrinone and then matured for 16 hr in CZB medium (Chatot et al., 1989).

Immunocytochemical analysis

For PLCβ1 detection, ZP-free MII-arrested eggs were fixed in 3.7% paraformaldehyde in PBS for 1 h at room temperature, permeabilized in PBS containing 0.3% BSA and 0.1% Triton X-100 for 15 min, and then blocked in PBS containing 0.3% BSA and 0.01% Tween-20 (blocking solution). The eggs were incubated in a monocolonal anti- PLCβ1 antibody (50 μg/ml in blocking solution; cat# 05-164, Upstate Biotechnology) for 1 h at room temperature and then washed in blocking solution. The primary antibody was detected using a donkey anti-mouse Cy5-conjugated antibody (final concentration 7 μg/ml; Jackson Immunoresearch) for 1 h at room temperature. For detection of the T7 tag, the same procedure was used except the eggs were fixed in 2.5% paraformaldehyde and the primary antibody was a monoclonal anti-T7 tag antibody (0.2 μg/ml in blocking solution; Novagen). DNA was detected by incubation in 0.02% SITOX® Green (Invitrogen) for 15 min at room temperature. Eggs were mounted in

Vectashield (Vector Laboratories) and observed with a laser-scanning confocal microscope (Leica DMRE, Leica Microsystems, Inc.). The images were acquired with Leica confocal software, and processed and quantified using Photoshop (Adobe).

The PLCβ1 immunofluorescence signal intensity was quantified by drawing an elliptical region over the entire egg and determining the mean pixel intensity for that egg. Background pixel intensity was determined by measuring the mean pixel intensity of eggs stained with secondary antibody alone in the same experiment. At least 10 TG and 10 NTG eggs were examined in each of two independent experiments for a total of more than 20 eggs examined in these groups.

In vitro fertilization, embryo culture, and Ca^{2+} imaging

Sperm were collected from the caudae epididymides of $B6SJLF_1$ male mice (8-12 wks old; Jackson Laboratories, Bar Harbor, ME). The tissues were incised and the sperm allowed to swim out into TYH medium (Toyoda et al., 1971). The sperm were incubated in TYH medium for 90 min to allow capacitation. ZP-intact and ZP-free MII eggs were inseminated with 2×10^5/ml and 2.5×10^4/ml sperm in TYH medium, respectively. Three h after insemination, the eggs were washed free of unbound sperm and transferred into KSOM medium with amino acids (Specialty Media) for culture.

To create an artificial grid for egg placement and to prevent movement artifacts during fertilization and Ca^{2+} imaging, we used a 71 μm pore nylon mesh (Nippon Rikagaku Kikai Co., Ltd) coated with Cell-Tak (Becton Dickinson Labware) (Takahashi et al., 2003). The coating solution was a fresh mixture of 50 μl Cell-Tak, 1445 μl 0.1 M $NaHCO_3$, pH 8.0, and 5 μl 5N NaOH. Pieces of nylon mesh (6 × 6 mm with 71 μm^2 pores) were incubated in the coating solution for 20 min, and then washed with sterile water to remove the bicarbonate. The nylon mesh pieces were air-dried and stored at 4° C until use.

For Ca^{2+} imaging, ZP-free eggs were incubated for 20 min in Whittens/PVA containing 10 μM fura-2-acetoxymethylester (fura-2; Molecular Probes Inc., Eugene, OR), and 0.025% Pluronic F-127 (Poenie et al., 1986). A piece of Cell-Tak-coated nylon mesh was placed on a cover slip in a Leiden chamber and covered with 500 μl Whittens medium not containing BSA. The fura-2 loaded eggs were transferred into individual spaces on the mesh. The chamber was placed on a temperature-controlled microscope stage at 37° C under laminar flow of 5% CO^2 in air. After a 10-min incubation to allow the eggs to stick to the nylon mesh and coverslip, 500 μl of Whittens medium containing 30 mg/ml of BSA was added to achieve a final BSA concentration of 15 mg/ml. For insemination, sperm were added such that the final concentration was 2.5×10^4 sperm/ml. Measurements of intracellular Ca^{2+} were carried out as described previously (Xu et al., 2003). By using the grid created by the Cell-Tak-coated nylon mesh, different experimental groups of eggs were easily distinguished, and Ca^{2+} oscillations were measured in eggs simultaneously under exactly the same conditions.

Measurements and statistics

The percentages of eggs to form PNs at various times following *in vitro* fertilization were compared using the chi-square test. Differences between means were judged by the one-way analysis of variance (ANOVA) followed by Sheffe's F-test using StatView software (Abacus Concepts). Significant difference was defined as $p < 0.05$.

Measurements of various parameters of the Ca^{2+} oscillations (amplitude, duration, frequency, persistence) were expressed as a percentage of the mean for control (NTG or RFP) eggs for the same experimental replicate. Each parameter was compared between the two experimental groups using a T test (Prism 4.0; GraphPad Software, San Diego, CA).

Results

Knockdown of PLCβ1 in eggs from transgenic females

A transgenic RNA interference approach using a long inverted repeat RNA under control of the *Zp3* promoter (Fig. 2A) was used to reduce production of PLCβ1 during oocyte growth (Stein et al., 2003). Success of the knockdown approach was determined by performing real time quantitative PCR for *Plcb1* mRNA using GV-intact oocytes from each transgenic founder line; oocytes of non-transgenic littermates were used as controls. Oocytes derived from females of one transgenic founder line had ~25% of the amount of *Plcb1* mRNA as control oocytes, whereas oocytes from several other founder lines had comparatively more *Plcb1* mRNA (data not shown). Targeting of *Plcb1* appeared specific because there was no decrease in the amount of *Plcb4*, a PLC isoform highly related to *Plcb1* (data not shown). The percentage PLCβ1 protein knockdown in this founder line was ascertained by obtaining MII-arrested eggs from transgenic females and non-transgenic littermates and immunostaining them for PLCβ1 (Fig. 2, B-D). Quantification of the immunofluorescence signal indicated that after subtracting the background fluorescence present in control eggs, TG eggs from this line contained ~66% of the amount of PLCβ1 when compared to NTG control eggs (Fig. 2E).

Sperm-induced calcium oscillations in transgenic PLCβ1 knockdown eggs

To determine if a reduction in egg PLCβ1 protein levels affected sperm-induced Ca^{2+} oscillations, ZP-free NTG and TG eggs were inseminated simultaneously with capacitated sperm and alterations in intracellular Ca^{2+} were recorded. Representative examples of the Ca^{2+} oscillation patterns observed for NTG and TG eggs are shown (Fig. 3, A and B). When the Ca^{2+} oscillation patterns in each group were analyzed across all eggs, there was no significant difference between TG and NTG eggs in the baseline 340/380 fluorescence ratio, persistence of Ca^{2+} oscillations, amount of time to the first Ca^{2+} transient, or length of the first Ca^{2+} transient. The only parameter that we observed to be modified was peak amplitude, which was decreased ~20% (Fig. 3C). Such changes in peak amplitude can arise artefactually if acquisition settings for fura-2 are changed between experiments (e.g., increasing exposure time at 340 nm while maintaining the 380 nm exposure time). However this could not have been the case here because we kept the exposure times the same across all experiments, and the data were acquired simultaneously from both TG and NTG eggs. The modest, but significant, ~20% reduction in amplitude is consistent with the ~34% reduction in the amount of PLCβ1 protein. These findings support the hypothesis that egg PLCβ1 is involved in modulating the overall Ca^{2+} oscillatory pattern induced by the sperm at fertilization.

Embryo development of fertilized transgenic PLCβ1 knockdown eggs

Because the total Ca^{2+} signal experienced by the egg regulates completion of fertilization-associated events (Ducibella et al., 2002; Ozil et al., 2005), we hypothesized that the decrease in amplitude of the Ca^{2+} transients in TG eggs would result in a delay of egg activation as indicated by later PN formation. To test this idea, we performed *in vitro* fertilization of ZP-free TG and NTG eggs and monitored the timing of PN formation. “Early” and “late” PN formation were determined at 5.5 to 6 h and 7 to 8 h after insemination, respectively. There were no significant differences in the percentage of TG and NTG eggs that had early or late PN formation, or cleavage to the 2-cell stage (Table 2). When these embryos were cultured to the blastocyst stage, no differences in the rate of development or percentage of embryos to develop to fully expanded blastocysts were observed. These results suggested that the observed alterations in peak amplitude in the TG eggs did not affect the total Ca^{2+} signal enough to alter egg activation events. These findings were confirmed by a six-month mating trial in which no differences in fertility (number of litters and number of pups delivered per litter) were observed when comparing TG females and NTG littermates (data not shown).

Effect of over-expressing PLCβ1 on sperm-induced calcium oscillations

To explore further our hypothesis that an egg PLC is involved in generating Ca^{2+} oscillations at fertilization, we examined the effects of over-expressing exogenous PLCβ1 in wild type eggs on the characteristics of the Ca^{2+} oscillatory pattern induced by sperm. The full-length (~3.6 kb) coding region of *Plcb1* with a C-terminal T7 epitope tag (*Plcb1-T7*) was inserted into the pIVT vector, which allows efficient *in vitro* transcription of stable polyadenylated cRNA (Fig. 4A). The coding region of a red fluorescent protein (RFP) was inserted into pIVT and cRNA encoding RFP was generated for use as a control. As an additional control, cRNA encoding a C-terminal deletion mutant of PLCβ1 (Δ830-1041) was generated. GV-intact oocytes were microinjected with *Plcb1*-T7 or RFP cRNA and subsequently matured *in vitro*. PLCβ1 expression in these MII-arrested eggs was increased over the endogenous protein level as indicated by immunostaining (Fig. 4, B and C). At least some of the exogenous PLCβ1 protein was full length, because the T7 tag was detected in the *Plcb1-T7* cRNA-injected eggs (Fig. 4, D and E). RFP was clearly observed by epifluorescence microscopy in the control RFP cRNA injected eggs (data not shown).

After maturation *in vitro*, eggs from each experimental group were simultaneously inseminated and the Ca^{2+} oscillatory patterns recorded; it should be noted that eggs injected with *Plcb1-T7* cRNA but not inseminated displayed no changes in intracellular Ca^{2+}. The majority of eggs over-expressing PLCβ1 (26/28) displayed a distinctly altered Ca^{2+} oscillatory pattern when compared to RFP-injected eggs (Fig. 5, A and B). We analyzed five parameters of individual Ca^{2+} oscillations in these eggs: amplitude, duration, frequency, persistence, and shape. There was no difference in either the amplitude or persistence in eggs over-expressing PLCβ1 (data not shown). These eggs, however, had a shorter duration of the first Ca^{2+} transient and a symmetric shape of each Ca^{2+} transient (Fig. 5, B and C). In addition, the interval between Ca^{2+} transients was longer in these eggs (Fig. 5D). We also analyzed the "onset" and "restore" times of the 1^{st} and 2^{nd} Ca^{2+} transients in these eggs. Onset time refers to the time from an initial increase in Ca^{2+} from the baseline to when the first sharp change in positive slope occurs. Reciprocally, restore time refers the time at the end of the transient when the sharp reduction in negative slope occurs to the time of return to baseline; these time points were determined by visual examination of each Ca^{2+} transient (Fig. 6A). Results of these analyses revealed a significant increase in both the onset and restore times in eggs over-expressing PLCβ1 with the restore time exhibiting more than a 10-fold increase (Fig. 6B). These perturbations in Ca^{2+} oscillatory behavior were not observed when eggs expressing the inactive form of PLCβ1 were inseminated (data not shown). It was also possible that the observed differences were due to reduced calcium stores in eggs over-expressing PLCβ1. This was unlikely because virtually identical amounts of Ca^{2+} were released in response to the calcium ionophore A23187 when eggs were injected with either *Plcb1* or RFP cRNA (data not shown).

A more detailed analysis of the first Ca^{2+} transient was undertaken because of the pronounced effects on its shape (Fig. 7). The signature pattern of a normal sperm-induced first transient is characterized by a shoulder that is often followed by a series of initial spikes (Fig. 7, A and B). These features were largely absent in the eggs over-expressing PLCβ1, e.g., only 1 of 28 eggs displayed a transient that had normal spikes but even this one had no shoulder (Fig. 7C). A large fraction (43%) displayed no shoulder or spikes (Fig. 7D), 43% displayed an abnormally large single initial spike followed by a plateau (Fig. 7E), and 11% displayed a single very large spike with no plateau (Fig. 7F).

The results shown above clearly indicated that decreased amounts of total Ca^{2+} are released in eggs over-expressing PLCβ1. Because events of early egg activation show a graded response to the amount of Ca^{2+} released (Ducibella et al., 2000; Ozil et al., 2005), we anticipated that the timing of the PN formation would be delayed in these eggs. ZP-free eggs over-expressing either PLCβ1 or RFP were inseminated and the number of eggs that formed PN at "early" or

"late" times were counted. As anticipated, the timing of PN formation was slower in eggs over-expressing PLCβ1 than controls as indicated by the smaller number of fertilized eggs that displayed a PN at the early time point (Table 3).

Discussion

The results presented here strongly suggest that the Ca^{2+} oscillatory pattern observed in a fertilized egg is not solely due to a sperm-derived PLCζ but rather represents a response to both sperm and egg-derived PLCs. Such a model provides a mechanism for amplifying the initial sperm-derived signal, amplification being a common feature of signal transduction pathways. In addition, this mechanism would foster development and implantation of "healthy" eggs, i.e., eggs that have accumulated appropriate amounts of PLC and other signaling components required to generate Ca^{2+} oscillations sufficient for development to term (Ozil et al., 2006). Although we focused on egg-derived PLCβ1 for the reasons discussed in the Introduction, our results neither address nor exclude a function for other egg PLCs in modulating Ca^{2+} oscillatory behavior in fertilized eggs.

We observed a modest reduction in the amount of egg PLCβ1 using the transgenic RNAi approach even though PLCβ1 mRNA was substantially reduced. A likely explanation for this finding is that PLCβ1 is accumulated early during oocyte growth and the protein is relatively stable. Nevertheless, even this modest reduction in egg PLCβ1 results in a consistent and statistically significant reduction in amplitude of the Ca^{2+} oscillations in fertilized eggs. This result suggests that other egg PLCs do not compensate for loss of PLCβ1 function, implying a pivotal role for PLCβ1 in egg activation. In addition, previous work demonstrates that events of early development depend on the total amount of Ca^{2+} released during the oscillatory period. In our transgenic eggs, there is a modest reduction in amplitude, with no observable change in duration or frequency Ca^{2+} oscillations. Because neither preimplantation development nor development to term is compromised in embryos derived from transgenic eggs, sufficient amounts of Ca^{2+} are likely released following fertilization.

An inherent caveat of any over-expression study is that non-physiological amounts of protein are present and this may result in non-physiological responses. Nevertheless, if sperm-derived PLCζ were solely responsible for the signature Ca^{2+} oscillatory pattern, over-expressing an egg PLC should not significantly change the pattern. Instead, we noted that over-expressing PLCβ1 in mouse eggs has a pronounced effect on sperm-induced Ca^{2+} oscillations. This result complements the results obtained from the knockdown experiments and provides additional support for involvement of an egg-derived PLC. Particularly noteworthy, is the dramatic effect of over-expressing PLCβ1 on the characteristic shape of the first Ca^{2+} oscillation (Jones et al., 1998; Miyazaki et al., 1986). Remarkably, little is known regarding the molecular basis why the first Ca^{2+} transient is of longer duration and exhibits initial spikes, but these properties must reflect, at least in part, Ca^{2+} release and re-uptake mechanisms active in the fertilized egg. Perhaps the shorter duration and effects on the initial spikes in fertilized eggs over-expressing PLCβ1 reflects faster release of Ca^{2+} from intracellular stores that in turn stimulates even faster Ca^{2+} re-uptake than normally occurs. An enhanced re-uptake mechanism may also explain failure to observe an increase in amplitude in these fertilized eggs.

Over-expressing PLCβ1 in eggs results in a substantial reduction in the total amount of Ca^{2+} released following fertilization. This change is due to the shorter duration, particularly that of the first transient, and decreased frequency of Ca^{2+} oscillations. Consistent with the finding that experimentally decreasing the total amount of Ca^{2+} released delays the time course for PN formation (Tóth et al., 2005) is that a similar delay occurs in eggs over-expressing PLCβ1. This finding also minimizes concerns about using electroporation to manipulate intracellular

Ca^{2+} concentrations to study egg activation (Ducibella et al., 2002; Ozil et al., 2005, 2006) because similar results are observed with both methods.

In summary, the results presented here provide the first evidence for a role of an egg-derived PLC in sperm-induced egg activation in mammals. Although these studies focused on PLCβ1, a similar approach could unmask functions for other egg-derived PLCs in egg activation.

Acknowledgements

The authors thank Dr. Shin Murai for conducting the calcium ionophore experiments reported as data not shown. This research was supported by a grant from the NIH (HD22732 to RMS and CJW). HI was supported in part by a grant from Yamagata University, School of Medicine. JGK was supported by training grant T32 HD007305.

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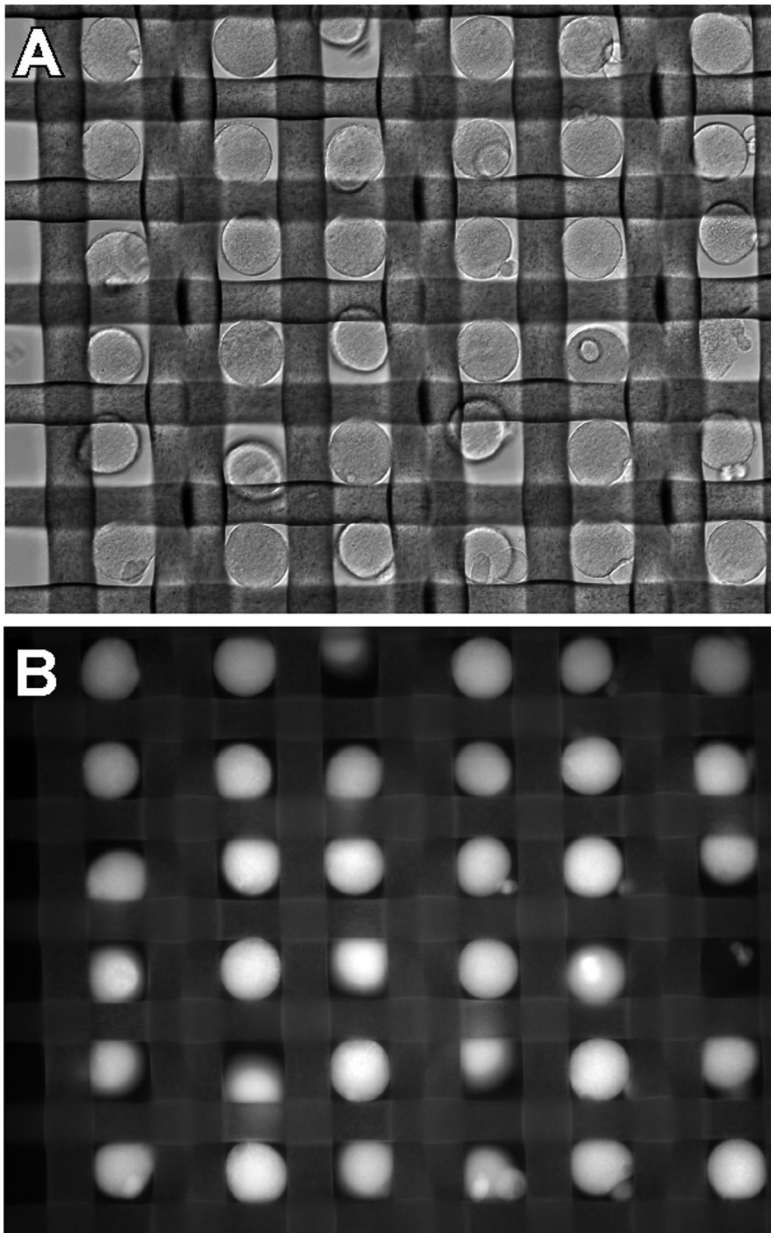

Figure 1.
Photograph of nylon mesh creating grid for fura-2-loaded eggs. A. Brightfield image. B. Fluorescence image.

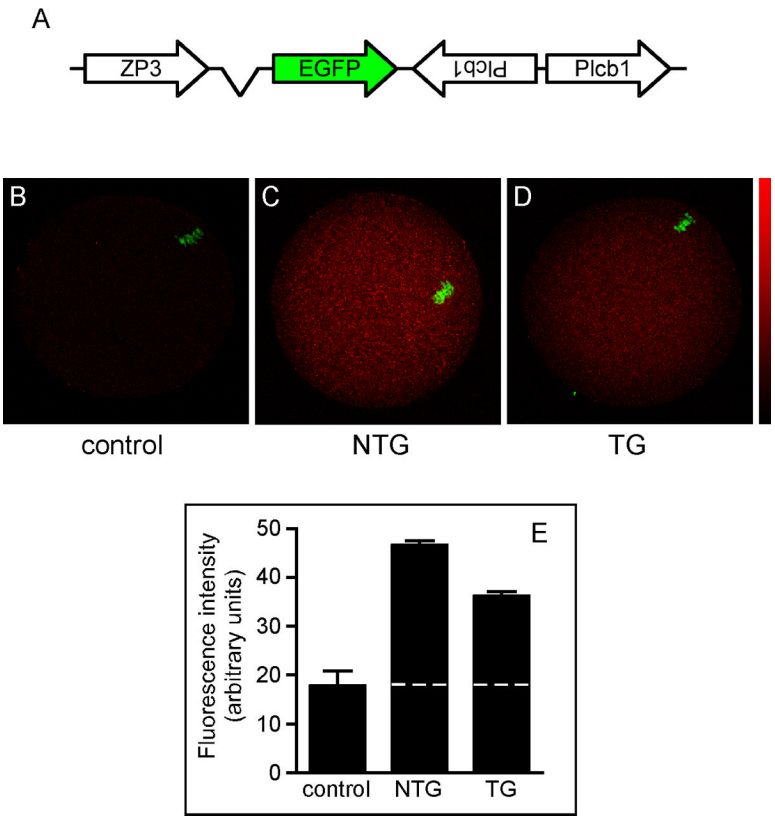

Figure 2.

PLCβ1 expression in non-transgenic and transgenic mouse eggs. A. Schematic of construct for generating transgenic mice expressing dsRNA under control of the oocyte-specific *Zp3* promoter. B-D. Immunofluorescence analysis of PLCβ1 protein in MII-arrested eggs from non-transgenic (NTG) and transgenic (TG) mice. Controls were MII eggs from NTG mice immunostained with secondary antibody alone. Representative images are shown. Color bar shows pixel intensity range from 0 (black) to 255 (red). B, Control; C, NTG; D, TG. E. Graph representing mean pixel intensity of PLCβ1 immunofluorescence signal ± S.E.M. in 5 control, 21 NTG, and 22 TG eggs in two independent experiments. Dashed line indicates mean background fluorescence level in control eggs.

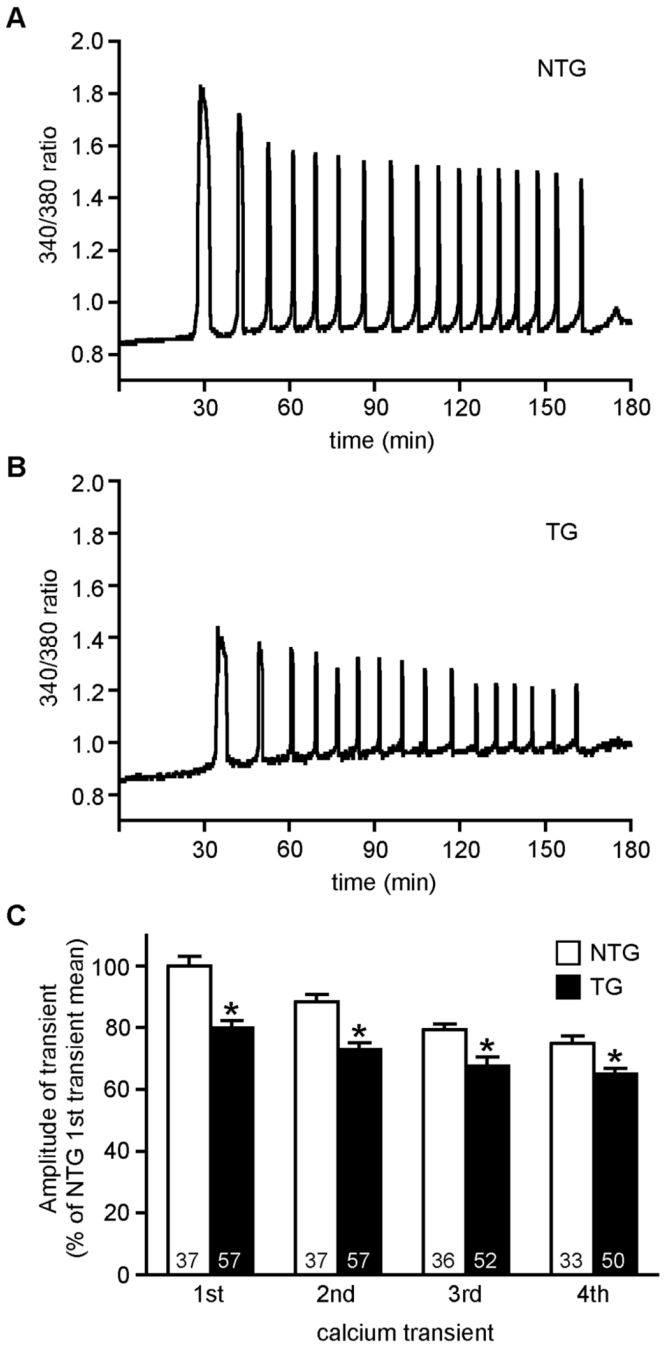

Figure 3.

Sperm-induced Ca^{2+} oscillations in non-transgenic and transgenic mouse eggs. A and B. Representative Ca^{2+} oscillatory patterns for NTG (A) and TG (B) eggs. C. Graph of amplitude of 1st four Ca^{2+} oscillations in NTG and TG eggs. Amplitude is expressed as the percentage of the mean amplitude of the 1st Ca^{2+} transient for NTG eggs for each experimental replicate (mean ± SEM). Asterisks indicate that the mean amplitude of each Ca^{2+} transient was significantly different in TG as compared to NTG eggs (T test; $p < 0.01$). Numbers at the base of each column indicate number of transients measured in 3 independent experiments. Time zero is the time of insemination.

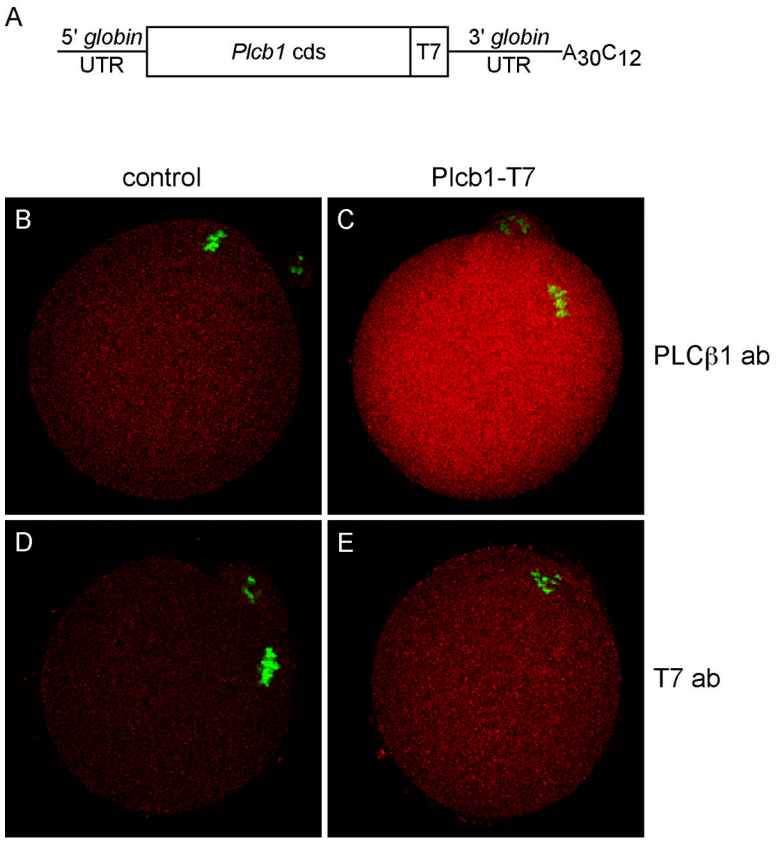

Figure 4.

Over-expression of PLCβ1 in wild type MII eggs. A. Schematic of capped cRNA microinjected into GV-intact oocytes. B-E. Immunofluorescent staining of MII eggs for PLCβ1 or T7 epitope tag, as indicated (both shown in red) and DNA (green). B and D, control non-injected eggs; C and E, eggs injected with *Plcb1*-T7 cRNA. Representative images from two independent experiments are shown.

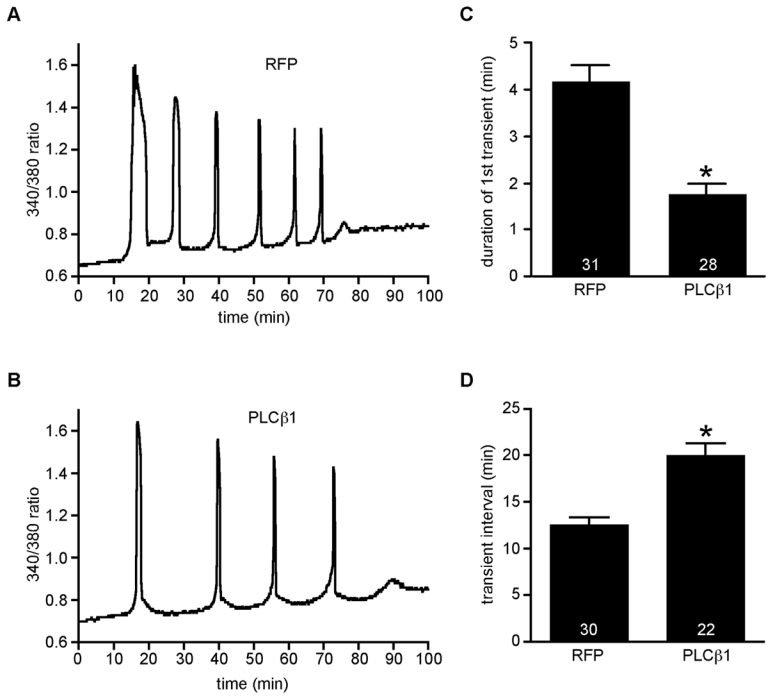

Figure 5.

Sperm-induced Ca^{2+} oscillations in eggs overexpressing red fluorescent protein (RFP) or PLCβ1. A and B. Representative calcium oscillation pattern for RFP (A) and PLCβ1 (B). C and D. Graphs of duration of 1^{st} Ca^{2+} transient (C) and interval between transients (D), expressed as mean ± SEM. Asterisks indicate significant differences (T test; $p < 0.0001$). Numbers at the base of each bar indicate total number of eggs analyzed in 3 independent experiments. Time zero is the time of insemination.

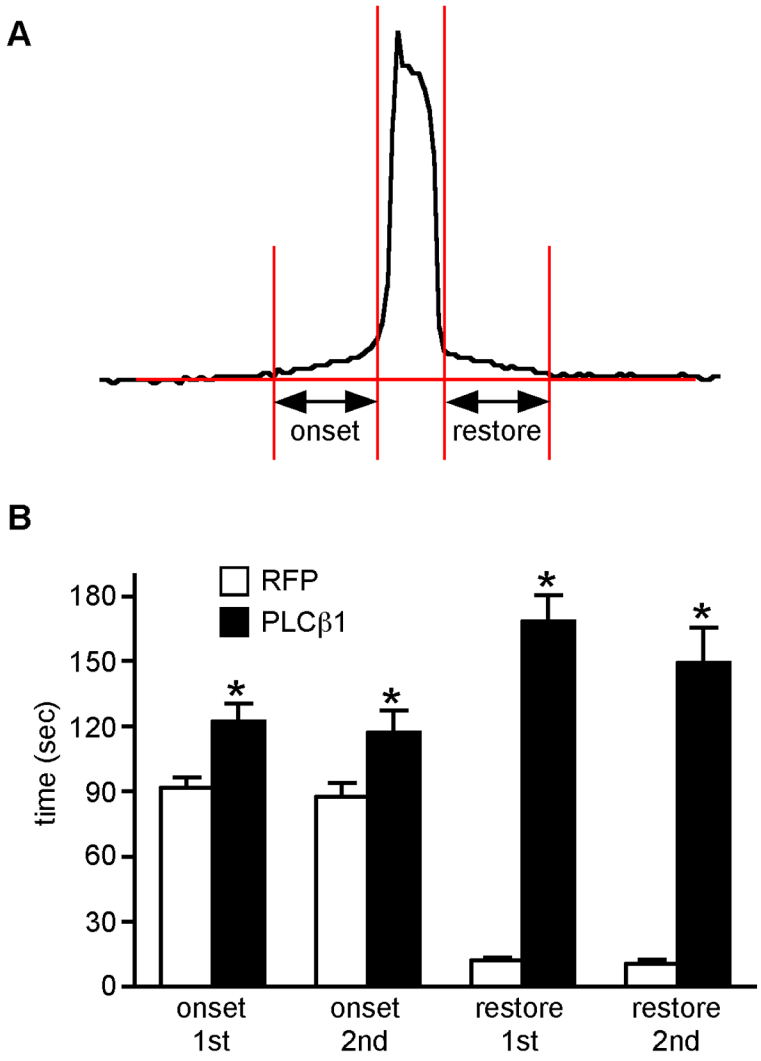

Figure 6.

Analysis of onset and restore times of sperm-induced Ca^{2+} transients in eggs over-expressing RFP or PLCβ1. A. Schematic showing how onset and restore times were measured. B. Graph comparing onset and restore times of 1st and 2nd Ca^{2+} transients in eggs expressing RFP or PLCβ1, as indicated. Asterisks indicate significant differences (T test; *, $p < 0.02$; **, $p<0.0001$). At least 28 1st and 2nd transients were analyzed in 3 independent experiments.

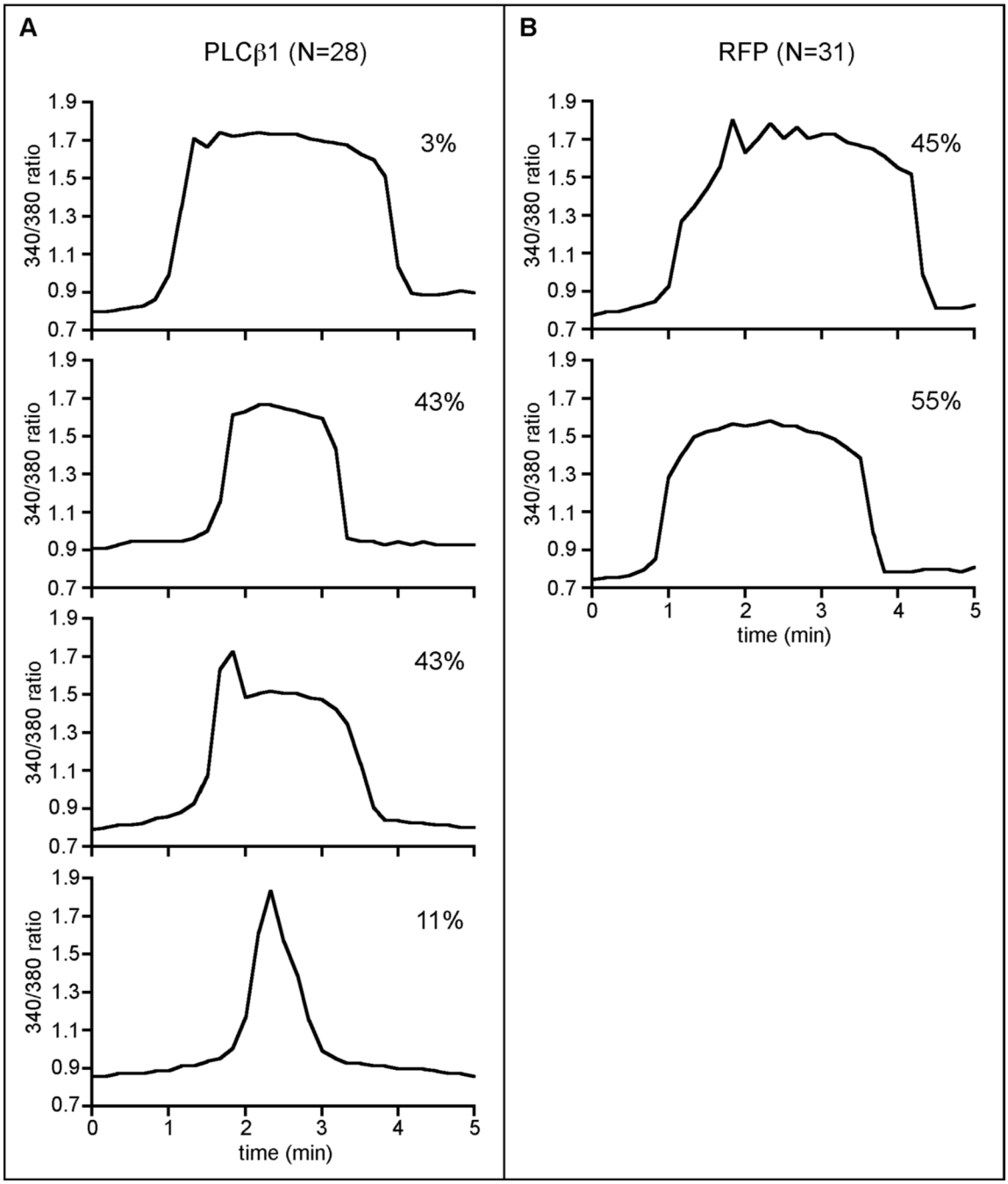

Figure 7.

Analysis of the first Ca^{2+} transient in eggs overexpressing RFP or PLCβ1. Graphs representing observed shapes of the first calcium transient for RFP (A, B) and PLCβ1 (C-F) are shown. The percentage of eggs exhibiting each shape and the total number of eggs analyzed are indicated. The arrows point to the shoulder region of the transient.

Table 1

PLC isoforms expressed in oocytes and knockout phenotype

PLC isoform	Oocyte Expression	Fertility in knockout (KO) females
PLCβ1	Yes (Avazeri et al., 2000; Dupont et al., 1996)	Two KO lines generated: (1) Homozygous females produced smaller litters (L. Upton, personal communication); (2) homozygous females do not reproduce due to behavioral problems (Kim et al., 1997)
PLCβ2	No (Dupont et al., 1996)	
PLCβ3	Yes (Avazeri et al., 2000; Dupont et al., 1996)	Conflicting data; normal litter size (D. Wua, personal communication) but preimplantation lethal for a different knockout line (Wang et al., 1998)
PLCβ4	Yes (our laboratory, unpublished)	Normal litter size (M. Kano, personal communication)
PLCγ1	Yes (Dupont et al., 1996; Mehlmann et al., 1998)	Embryonic lethal E9 (Nakamura et al., 2003)
PLCγ2	Yes (Dupont et al., 1996; Mehlmann et al., 1998)	Conditional KO (Hashimoto et al., 2000)
PLCδ1	Yes (our laboratory, unpublished)	Normal litter size (Nakamura et al., 2003)
PLCδ2	No (our laboratory, unpublished)	
PLCδ3	No (Unigene expression analysis)	
PLCδ4	Yes (our laboratory, unpublished)	Normal litter size (Fukami et al., 2001)
PLCε	Yes (our laboratory, unpublished)	Normal litter size (Bai et al., 2004)
PLCζ	No (Saunders et al., 2002)	

Table 2

Timing of embryo development after fertilization of nontransgenic and transgenic eggs

Group	# MII eggs	Early PN formation* (%)	Late PN formation** (%)	Cleavage to 2-cell (%)	Fully expanded blastocyst (%)
NTG	189	35/189 (18.5)	137/189 (72.5)	163/189 (86.2)	116/163 (71.2)
TG	124	21/124 (16.9)	96/124 (77.4)	102/124 (82.3)	73/102 (71.6)

* Early PN formation was observed at 5.5-6 hrs after insemination

** Late PN formation was observed at 7-8 hrs after insemination

Table 3

Timing of pronucleus formation after fertilization of eggs overexpressing PLCβ1

Overexpressed protein	No. MII eggs	Early PN formation* (%)	Late PN formation** (%)
RFP	78	35/94 (37.2)	65/94 (69.1)
PLCb1	94	14/78 (17.9)†	32/78 (41.0)†

* Early PN formation was observed at 5.5-6 hrs after insemination

** Late PN formation was observed at 7-8 hrs after insemination

† Significantly different from RFP group, chi-square, $p<0.01$