SH2 Domain-Mediated Activation of Phospholipase Cγ Is Not Required to Initiate Ca\(^{2+}\) Release at Fertilization of Mouse Eggs

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The initiation of Ca\(^{2+}\) release at fertilization of mammalian eggs requires inositol trisphosphate (Miyazaki et al., 1992, Science 257, 251–255), indicating that an enzyme of the phospholipase C family is probably activated. Because Ca\(^{2+}\) release at fertilization in echinoderm eggs is initiated by SH2 domain-mediated activation of phospholipase C\(\gamma\) (Carroll et al., 1997, J. Cell Biol. 138, 1303–1311), we examined the possible role of PLC\(\gamma\) in initiating Ca\(^{2+}\) release at fertilization in mouse eggs. Both PLC\(\gamma\) isoforms, PLC\(\gamma\)1 and PLC\(\gamma\)2, are present in mouse eggs and sperm, and stimulation of these enzymes in the egg by way of an exogenously expressed PDGF receptor causes Ca\(^{2+}\) release. Recombinant SH2 domains of PLC\(\gamma\)1 and PLC\(\gamma\)2 inhibit PLC\(\gamma\)1 and PLC\(\gamma\)2 activation by the PDGF receptor, completely preventing Ca\(^{2+}\) release in response to PDGF when injected at an ∼20- to 40-fold excess over the concentrations of endogenous proteins. However, even at an ∼100- to 400-fold excess over endogenous protein levels, PLC\(\gamma\)1 and PLC\(\gamma\)2 SH2 domains do not inhibit Ca\(^{2+}\) release at fertilization. These findings indicate that Ca\(^{2+}\) release at fertilization of mouse eggs does not require SH2-domain-mediated activation of PLC\(\gamma\). However, activation of PLC\(\gamma\) in the egg by an alternative pathway, or introduction of activated PLC\(\gamma\) from the sperm, may be important. © 1998 Academic Press

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INTRODUCTION

Fertilization of mammalian eggs, like that of other species, initiates release of Ca\(^{2+}\) from the egg's endoplasmic reticulum by way of inositol 1,4,5-trisphosphate (IP\(_3\)) (Miyazaki et al., 1992; Xu et al., 1994; Mehlmann et al., 1996; Jaffe, 1996). In mammals, Ca\(^{2+}\) release causes the exocytosis of cortical granules, which blocks polyspermy, and the reinitiation of meiosis, which marks the beginning of the embryonic cell cycle (Kline and Kline, 1992). Ca\(^{2+}\) release in mouse eggs has been measured to begin ∼3 min after sperm–egg membrane fusion (Lawrence et al., 1997), in contrast to ∼7 s for sea urchin (McCulloh and Chambers, 1992; Mohri et al., 1995). So although IP\(_3\) production and Ca\(^{2+}\) release appear to be common features of fertilization, the earliest signaling events may differ among species.

IP\(_3\) is generated by the phospholipase C (PLC) family of enzymes that hydrolyze the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) (Rhee and Bae, 1997). The PLC family includes 10 known isoforms, which can be divided into 3 types, \(\beta, \gamma,\) and \(\delta\). PLC\(\beta\) is activated by heterotrimeric \(G\)-proteins, and PLC\(\gamma\) is activated by protein tyrosine kinases. All 3 isozymes can be activated by Ca\(^{2+}\) and by factors that favor PLC access to its membrane substrate (see Discussion).

In echinoderm eggs, IP\(_3\) appears to be generated by the activation of PLC\(\gamma\), by a process involving the src homology 2 (SH2) domains of the PLC\(\gamma\) protein (Carroll et al., 1997; D. J. Carroll, D. T. Albay, M. Terasaki, L. A. Jaffe, and K. R. Foltz, unpublished results). Injection of starfish or sea urchin eggs with SH2 domains of PLC\(\gamma\) inhibits Ca\(^{2+}\) release at fertilization. The injected SH2 domains probably compete with the SH2 domains of PLC\(\gamma\) in the egg, preventing an upstream kinase from phosphorylating and activating the enzyme. The inhibitory action of the PLC\(\gamma\) SH2 domains appears to be specific, since SH2 domains from
another protein have no inhibitory effect, and PLCγ SH2 domains have no inhibitory effect on PLCβ activation. Furthermore, a function-blocking point mutation in the PLCγ SH2 domains eliminates their inhibitory activity. In vitro, SH2 domains from different signaling proteins bind to specific tyrosine-containing sequences from particular kinases (Ottinger et al., 1998). PLCγ SH2 domains have also been used as inhibitors of PLCγ activation in other studies in vivo (Chen et al., 1994; Roche et al., 1996; Wang et al., 1998) and in vitro (Bae et al., 1998).

In mammalian eggs, the function of PLC in releasing Ca\(^{2+}\) at fertilization is supported by the findings that an antibody against the IP\(_3\) receptor inhibits Ca\(^{2+}\) release at fertilization in hamster and mouse (Miyazaki et al., 1992; Xu et al., 1994), and that the PLC inhibitor, U73122, partially inhibits Ca\(^{2+}\) release at fertilization in mouse (Dupont et al., 1996). U73122 inhibits both PLCβ (Dupont et al., 1996; Lee and Shen, 1998) and PLCγ (Chen et al., 1994); however, it also has nonspecific actions on molecules other than PLC (Willems et al., 1994; Lee and Shen, 1998). Phosducin, which inhibits the GTP-dependent events of mouse fertilization (IVF) medium containing 3% bovine serum albumin (BSA, Calbiochem, La Jolla, CA) (Kline and Kline, 1992). Sperm were collected from the cauda epididymides and vas deferens and capacitated in in vitro fertilization (IVF) medium containing 3% BSA, under mineral oil. Ten microliters of sperm suspension was added to 90 \(\mu\)l of medium containing eggs, resulting in a final sperm concentration of 2–5 \(\times\) 10\(^5\) sperm/ml. This sperm concentration was used to obtain rapid and synchronous fertilization. Preliminary experiments showed that with a 10-fold lower sperm concentration, the time between insemination and the initiation of Ca\(^{2+}\) release was often increased.

**Immunoblotting**

Aliquots of eggs or sperm were frozen with liquid N\(_2\) in SDS sample buffer, or in a solution containing protease inhibitors (100 mM K glutamate, 10 mM EGTA, 3 mM MgCl\(_2\), 20 mM Hepes, pH 7.2, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin, 100 \(\mu\)g/ml soybean trypsin inhibitor, 1 mg/ml Pefabloc (Boehringer Mannheim, Indianapolis, IN)), and stored at −70°C. Recombinant rat PLCγ1 was purified from baculovirus-infected Trichoplusia ni (High five) insect cells (Horstman et al., 1995), and recombinant rat PLCγ2 was purified from Hela cells (Park et al., 1992; Bae et al., 1998) (Fig. 1). Protein content of the egg samples was based on the previously determined value of 30 ng/fully grown oocyte (Schultz and Wassarman, 1977). Protein content of sperm samples and recombinant protein samples was determined using the BCA assay (Pierce Chemical Co., Rockford, IL) with BSA as standard.

Proteins were separated by SDS-PAGE under reducing conditions, using an 8% gel and high range molecular weight standards (Bio-Rad Laboratories, Hercules, CA). The proteins were transferred to nitrocellulose, and the blots were blocked in a buffer containing 0.5% milk, incubated overnight with primary antibodies, washed, incubated for 1 h with secondary antibody, washed, and developed using ECL reagents (Amersham Life Science, Inc., Arlington Heights, IL). The sources of the antibodies used to probe the blots were as follows: monoclonal antibody B-10-3 against bovine PLCγ1 (S. G. Rhee Laboratory, see Suh et al., 1988), mixed monoclonal antibody against bovine PLCγ1 (Upstate Biotechnology Inc., Lake Placid, N.Y. No. 05-163), polyclonal antibody against a peptide comprised of the 20 amino acids at the C
FIG. 1. Recombinant proteins used in this study. Lane 1, PLCγ1 (0.8 μg); lane 2, PLCγ2 (0.4 μg); lane 3, GST-PLCγ1SH2 (1 μg); lane 4, GST-PLCγ2SH2 (1 μg); lane 5, GST-SHP2SH2 (1 μg). 8% SDS-PAGE gel stained with Coomassie blue. Lower staining intensity of the GST-PLCγ2SH2 sample was seen reproducibly, and could be due to lower staining of this protein by Coomassie blue.

terminus of human PLCγ2 (Santa Cruz Biotechnology Inc., No. SC-407), horseradish peroxidase-conjugated antibody against mouse IgG (Pierce Chemical Co., No. 31434), horseradish peroxidase-conjugated antibody against rabbit IgG (Santa Cruz Biotechnology, Inc., No. SC-2030). The PLCγ1 and PLCγ2 antibodies were shown by immunoblotting to be specific for the corresponding recombinant protein isoform (data not shown).

PLCγ1 and PLCγ2 content of the eggs was determined by comparing the immunoblot density with a standard curve obtained from recombinant protein samples run in parallel. Immunoblot densities were quantitated as previously described (Gallo et al., 1996).

GST Fusion Proteins and RNA

Plasmid DNAs encoding GST fusion proteins were obtained from the following sources: bovine PLCγ1SH2(N + C) (S. Courtney, Sugen, Inc., Redwood City, CA, see Stahl et al., 1988; Carroll et al., 1997), murine PLCγ2SH2(N + C) (L. Rehrsneider, Fred Hutchinson Cancer Research Center, Seattle, WA, see Bourette et al., 1997), murine SHP2SH2(N + C) (T. Pawson, Mt. Sinai Hospital, Toronto, Canada, see Feng et al., 1993, Carroll et al., 1997), human PLCγ2SH2(N + C)SH3 (P. S. Huang, Merck Research Laboratories, West Point, PA, see Huang et al., 1995; Carroll et al., 1997). GST fusion proteins were produced as described previously (Gish et al., 1995; Carroll et al., 1997) (Fig. 1).

DNA for the human platelet-derived growth factor (PDGF) β receptor in the vector Bluescript was obtained from A. Kazlauskas (Harvard Medical School, Boston, MA, see Gronwald et al., 1988). Synthetic RNA was made as previously described (Shilling et al., 1990), using XbaI to linearize the DNA, and T7 polymerase.

Microinjection and Ca2+ Measurements

Quantitative microinjection was performed using mercury-filled micropipets (see Mehlmann and Kline, 1994). This method allows injection of precisely defined picoliter volumes. To minimize damage, the temperature during injection of mature eggs was reduced to 25–28°C. Concentrations of injected substances were calculated based on a cytoplasmic volume of 200 pl.

Ca2+ measurements were performed using Ca green 10-kDa dextran (Molecular Probes, Eugene, OR), at a concentration of 10 μM in the egg. The temperature on the recording stage was maintained at 34–37°C, using a Peltier heating stage (Model TC-202, Medical Systems Corp., Greenvale, NY) with a laminar flow of 5% CO2 in air over the recording chamber. Ca green was excited by 470 nm light from a 100-W halogen lamp operated at 40–65 W and applied to the egg through a 40×, 0.75 NA objective on an inverted microscope (Diaphot-TMD, Nikon Inc., Melville, NY). Emitted 540-nm light was measured using a photodiode with a built in amplifier (Model 71882, Oriel Instruments, Stratford, CT), mounted on the microscope. The gain of the photodiode amplifier was set at 109 V/amp, and the filter of the amplifier was set at either “medium” or “minimum.” The output of the photodiode amplifier was recorded on a chart recorder (Model 220, Gould Instrument Systems, Valley View, OH), with an RC filter at the chart recorder input (τ = 20 ms), and with the gain of the chart recorder preamplifier boosted 5×. The baseline photodiode signal from the egg before stimulation was typically 2–5 mV (2–5 pA).

Under these conditions, the full response time of the measurement system was <1 s with the medium setting, or <0.1 s with the minimum setting. Since the Ca2+ transients in the egg were relatively slow, these response times were sufficient to obtain accurate measurements. In particular, the peak response usually occurred during the oscillations superimposed on the first Ca2+ transient, and these oscillations had a period of >10 s.

Figures were made by scanning the chart records into a computer. Because recording conditions varied somewhat between experiments, the baseline signal from the egg before stimulation was used as a standard, and the peak amplitude of the response to fertilization or PDGF stimulation was expressed as the ratio of the peak signal from the photodiode (total of the baseline current + the increase in current after stimulation), divided by the baseline signal. This baseline signal is indicated by a scale bar in each figure.

For experiments involving PDGF, immature oocytes in MEM containing 100 μg/ml dbcAMP were injected with 6–12 pg of RNA encoding the PDGF β receptor. After 2–8 h, oocyte maturation was initiated by washing the oocytes into MEM without dbcAMP. At 17.5–20 h after removing dbcAMP, the mature eggs were injected with SH2 domain proteins and 10 μM Ca green dextran, or with Ca green dextran alone. One to 6.5 h later, human recombinant PDGF B/B (Boehringer Mannheim) was added while recording Ca green fluorescence. The PDGF was present throughout the recording period.

For experiments involving fertilization, mature eggs were co-injected with SH2 domain proteins and 10 μM Ca green dextran. The zona pellucidae were then removed with a brief treatment (<1 min) in acid Tyrode’s solution (Mehlmann et al., 1996), and eggs were adhered to a glass coverslip coated with Cell-Tak (Collaborative Research, Bedford, MA). The eggs were exposed to sperm while recording Ca green fluorescence.

RESULTS

Mouse Eggs and Sperm Contain Both PLCγ1 and PLCγ2 Proteins

As previously reported, PLCγ1 protein is present in mouse eggs (Dupont et al., 1996). To estimate the amount of PLCγ1 in the eggs, we compared the immunoblot signal from the eggs with that from known amounts of recombi-
As a mixed monoclonal antibody against bovine PLC-γ (Fig. 2A). These results indicated that each egg contains 1.5 ± 0.2 pg of PLC-γ protein (mean ± SD of 2 similar experiments). To examine whether and how much PLC-γ2 is present in mouse eggs, we compared the immunoblot signal from eggs with that from known amounts of recombinant rat PLC-γ2 protein (Fig. 2B). These results indicated that each egg contains 2.4 ± 1.4 pg of PLC-γ2 protein (mean ± SD of 2 similar experiments). Thus both PLC-γ isoforms are present in the egg, at ~52 and ~85 nM for PLC-γ1 and PLC-γ2 respectively.

We also examined PLC-γ in mouse sperm and found that both γ1 and γ2 isoforms are present (Fig. 2C). PLC-γ in mouse sperm has also been reported by others (Dupont et al., 1996; Tomes et al., 1996).

**Activation of the PDGF Receptor Causes Ca2+ Release and Pronuclear Formation**

Since PLC-γ1 and PLC-γ2 are present in mouse eggs and could potentially function at fertilization, we tested if activation of these enzymes would result in Ca2+ release like that occurring at fertilization. To do this, we used the PDGF receptor, which activates both PLC-γ1 and PLC-γ2 isoforms (Sultzman et al., 1991; Homma et al., 1993). The PDGF receptor was introduced into the mouse eggs by injecting RNA encoding the human PDGF β receptor into GV stage oocytes. After in vitro maturation, the eggs were injected with Ca green dextran to monitor Ca2+ release. Application of PDGF-BB (0.5–9 nM) caused oscillating Ca2+ rises, resembling those at fertilization (Fig. 3, Table 1). The response consisted of an initial Ca2+ rise lasting for a few minutes, followed by additional Ca2+ rises of shorter duration. The peak amplitudes of the Ca2+ release in...
response to PDGF and sperm were similar, but the frequency of the repetitive Ca^{2+} transients in response to PDGF was somewhat greater than at fertilization, having a period of about 3 min, compared to about 7 min for fertilization (Table 1). When PDGF was applied to eggs that had not been injected with PDGF receptor RNA, no Ca^{2+} oscillations were seen; in some cases a single sustained Ca^{2+} rise occurred, but this was not seen consistently.

When PDGF receptor RNA-injected eggs were observed at 17–20 hours after application of PDGF, a pronucleus was seen in 58% of the eggs examined (n = 26). Control eggs, not injected with PDGF receptor RNA, did not form pronuclei in response to PDGF (n = 20). These results indicated that activation of the PDGF receptor causes oscillating Ca^{2+} rises and resumption of meiosis, like events occurring at fertilization.

**Ca^{2+} Release by the PDGF Receptor Is Inhibited by the SH2 Domains of PLC-γ**

Isolated SH2 domains act to inhibit PLC-γ activation, by competing with the SH2 domains of full-length PLC-γ for the SH2 binding site on kinases such as the PDGF receptor (see Introduction). To establish that PLC-γ SH2 domains were effective inhibitors of PLC-γ in mouse eggs, we injected eggs expressing the PDGF receptor with GST fusion proteins of the SH2 domains of either PLC-γ1 or PLC-γ2. In eggs injected with $100 \mu$g/ml (2 $\mu$M, final concentration in the egg cytoplasm) of either PLC-γ1 or PLC-γ2 SH2 domains, Ca^{2+} release in response to PDGF was completely inhibited (Figs. 4 and 5). At 10 $\mu$g/ml (200 nM) it was not inhibitory. At 1000 $\mu$g/ml (20 $\mu$M), the SH2 domains of a control protein, the phosphatase SHP2, had no inhibitory effect (Figs. 4 and 5). The amino acid sequences of the SH2 domains of PLC-γ1 or PLC-γ2 SH2 domains, Ca^{2+} release in response to PDGF was completely inhibited (Figs. 4 and 5). At 10 $\mu$g/ml (200 nM) it was not inhibitory. At 1000 $\mu$g/ml (20 $\mu$M), the SH2 domains of a control protein, the phosphatase SHP2, had no inhibitory effect (Figs. 4 and 5). The amino acid sequences of the SH2 domains of PLC-γ1 (Stahl et al., 1988) and PLC-γ2 (Emori et al., 1989) are 65% identical. The amino acid sequence of the SH2 domains of SHP2 (Feng et al., 1993) is ≈50% identical to either PLC-γ1 or PLC-γ2.

Although the site on the PDGF receptor for binding the SH2 domains of PLC-γ1 is well characterized (Valius and Kazlauskas, 1993; Ottinger et al., 1998), the PDGF receptor binding site for the SH2 domains of PLC-γ2 has not been determined. Our results indicate that PLC-γ1 SH2 domains can inhibit PLC-γ2 activation by the PDGF receptor, and
vice versa, suggesting that both PLC\(\gamma\) isoforms bind to the same or physically close sites on the PDGF receptor.

**Ca\(^{2+}\) Release at Fertilization Is Not Inhibited by the SH2 Domains of PLC\(\gamma1\) and PLC\(\gamma2\)**

The results described above indicate that the SH2 domains of PLC\(\gamma1\) and PLC\(\gamma2\) are effective inhibitors of SH2 domain-mediated activation of these enzymes in mouse eggs. To test the effect on Ca\(^{2+}\) release at fertilization of inhibiting SH2 domain-mediated activation of PLC\(\gamma\), mouse eggs were injected with SH2 domain fusion proteins and Ca green dextran. After removal of the zonae, the eggs were exposed to sperm while Ca green fluorescence was recorded. Neither PLC\(\gamma1\) nor PLC\(\gamma2\) SH2 domains had any inhibitory effect on Ca\(^{2+}\) release at fertilization, even at a concentration of 500–1000 \(\mu\text{g/ml}\) (5–10 times higher than that used to block the PDGF response) (Fig. 6, Table 2). The delay between insemination and the initial Ca\(^{2+}\) rise was not significantly different from that in control eggs, and the amplitude and duration of the initial Ca\(^{2+}\) rise and the frequency of the subsequent Ca\(^{2+}\) transients were also similar.

As described above, PLC\(\gamma1\) and PLC\(\gamma2\) SH2 domains appear to cross-react in inhibiting Ca\(^{2+}\) release in response to the PDGF receptor. However, this would not necessarily be true for unknown kinases that might activate PLC\(\gamma1\) and PLC\(\gamma2\) at fertilization. To be sure that both PLC\(\gamma1\) and PLC\(\gamma2\) activation would be inhibited, we injected mouse eggs with a mixture of SH2 domains from PLC\(\gamma1\) and PLC\(\gamma2\) (500 \(\mu\text{g/ml\ of each}). No inhibition of Ca\(^{2+}\) release at fertilization was seen (Fig. 6, Table 2).

We also tested the effect of injecting a fusion protein comprised of the SH3 as well as the SH2 domains of PLC\(\gamma1\), since this construct is sometimes, but not always, a more effective inhibitor than the SH2 domains alone (Bae et al., 1998; Wang et al., 1998). This construct was also of interest because it could potentially inhibit an SH3-mediated activation of PLC\(\gamma\). However, no inhibition or delay of Ca\(^{2+}\) release at fertilization was seen in 3 eggs injected with 500 \(\mu\text{g/ml of PLC\(\gamma\)SH2(N + C)SH3}\) (data not shown).

**DISCUSSION**

Both isoforms of PLC\(\gamma\), \(\gamma1\) and \(\gamma2\), are present in mouse eggs and sperm (this paper as well as Dupont et al., 1996; Tomes et al., 1996). PLC\(\beta\) isoforms have also been inferred to be present in mouse eggs, since stimulation of G\(_\alpha\) family proteins causes Ca\(^{2+}\) release (Williams et al., 1998). PLC\(\delta\) isoforms in mouse eggs have not been investigated.

Although PLC\(\gamma1\) and PLC\(\gamma2\) are present in mouse eggs, SH2 domain-mediated activation of these enzymes is not required to initiate Ca\(^{2+}\) release at fertilization. This was concluded based on injections of eggs with recombinant SH2 domains of PLC\(\gamma1\) and PLC\(\gamma2\). At an \(\approx\)20- to 40-fold excess over the concentrations of the endogenous proteins, PLC\(\gamma1\) or PLC\(\gamma2\) domains completely inhibited Ca\(^{2+}\) release in response to PDGF. However, even at an \(\approx\)100- to
400-fold excess, PLCγ1 and PLCγ2 SH2 domains did not inhibit Ca\(^{2+}\) release at fertilization. These findings, together with the evidence against a role for G-protein activation of PLCβ (see Introduction), argue against the two best established PLC activation mechanisms in initiating Ca\(^{2+}\) release at fertilization of mouse eggs.

It is possible that SH2 domain-mediated PLCγ activation and G-protein-mediated PLCβ activation provide redundant pathways for producing IP\(_3\) at fertilization, such that inhibition of either alone does not inhibit Ca\(^{2+}\) release at fertilization. However, it would be surprising in this case that inhibition of either pathway alone has no significant inhibitory effect at all, such as an increased delay between insemination and Ca\(^{2+}\) release (see also Williams et al., 1998).

**Other Factors That Regulate PLC**

Phospholipase C isoforms can also be activated by a number of other factors, which could be modulated during fertilization. PLCβ, γ, and δ can all be stimulated to some degree by 1–10 μM Ca\(^{2+}\) (Wahl et al., 1992; Jhon et al., 1993; Hwang et al., 1996), suggesting that Ca\(^{2+}\) introduced as a consequence of sperm–egg fusion could be the activating factor. Ca\(^{2+}\) is elevated in acrosome-reacted mammalian sperm (Florman, 1994; Suarez and Dai, 1995), and this elevation is due at least in part to the opening of Ca channels in the sperm plasma membrane (Florman et al., 1992). These observations support the possibilities that Ca\(^{2+}\) entering the egg from the sperm cytoplasm or via Ca channels in the sperm plasma membrane might initiate Ca\(^{2+}\) release (Jaffe, 1980; Créton and Jaffe, 1995). The idea that Ca\(^{2+}\) influx initiates Ca\(^{2+}\) release in the egg was tested by injection of Ca\(^{2+}\) into hamster and mouse eggs (Igusa and Miyazaki, 1983; Kline and Kline, 1994). This produced a regenerative Ca\(^{2+}\) rise, but did not produce the normal series of repetitive Ca\(^{2+}\) rises, even with continuous injection of Ca\(^{2+}\) by iontophoresis, or with use of a Ca\(^{2+}\) buffer to maintain Ca\(^{2+}\) at an elevated level. These results argue against the idea that Ca\(^{2+}\) influx from the sperm accounts for the activation of PLC that initiates Ca\(^{2+}\) release at fertilization in these eggs, although this mechanism is still a possibility.

Phospholipase C isoforms can also be regulated by their access to their lipid substrate, phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). Both lipid composition of the membrane containing the PIP\(_2\) and the degree of stretch on the membrane, affect the access of the enzyme to its substrate (Irvine et al., 1984; Hurley and Grobler, 1997; James et al., 1997). Both of these factors could be changed when the...
sperm membrane becomes continuous with the egg membrane during membrane fusion. In particular, the phospholipids in the plasma membrane of ram sperm have a large proportion of highly unsaturated acyl chains, a condition that results in a more loosely packed membrane (Nolan and Hammerstedt, 1997). A looser packing of membrane lipids favors PLC insertion in the lipid bilayer, thus increasing enzyme activity (Irvine et al., 1984; Hurley and Grobler, 1997; James et al., 1997).

Another possibility is suggested by the finding that in eggs of sea urchin (Turner et al., 1984; Kamel et al., 1985; Ciapa and Whitaker, 1992) and frog (Snow et al., 1996), PIP2 synthesis increases at fertilization. In the frog study, the method used was specific for the 4,5-isomer of PIP2. If PI-4,5-P2 was also synthesized at fertilization of mouse eggs, the amount of IP3 produced could be increased due to the increase in the PLC substrate, even if PLC activity remained constant. This effect could be particularly significant because in monolayer studies, a small increase in PIP2 (1.6×) can produce a large increase (7×) in PIP2 hydrolysis (James et al., 1997). Thus stimulation of IP3 production could be accomplished by stimulation of phosphatidylinositol-4-phosphate-5-kinase (PIPkin) (James et al., 1997; Hinchcliffe et al., 1998).

**Proteins That Enter the Egg from the Sperm**

Injection of a mouse egg with a mouse sperm initiates a normal sequence of Ca2+ oscillations (Nakano et al., 1997). However, injection of a spermatid has no such effect, indicating that something unique to mature sperm cells is responsible for this response (Kimura and Yanagimachi, 1995; Sato et al., 1998). Much recent work has been directed at identifying a protein in sperm that is responsible for this activity, but this question has not yet been conclusively answered. Candidates include a cytosolic protein (Swann, 1990), a nuclear protein (Uehara and Yanagimachi, 1976; Kuretake et al., 1996), glucose-6-phosphate deaminase or “oscillin” (Parrington et al., 1996; but see Wolosker et al., 1998), and a truncated form of the receptor tyrosine kinase c-kit (Sette et al., 1997). Such a protein from sperm might...
activate PLC (or PIPkin) in the egg, either directly or indirectly. Activation of PLC by protein interactions that do not appear to involve tyrosine phosphorylation or G-proteins have been previously described for the EGF receptor under particular conditions (Hernandez-Sotomayor and Carpenter, 1993), and for the microtubule-associated protein tau (Hwang et al., 1996).

Alternatively, the PLC that initiates egg activation at fertilization might originate in the sperm. Both PLCγ1 and PLCγ2 are present in mouse sperm (our results as well as Dupont et al., 1996; Tomes et al., 1996), and PLC is activated when human and mouse sperm are stimulated to undergo the acrosome reaction (Roldan and Harrison, 1989; Thomas and Meizel, 1989; Roldan et al., 1994; Tomes et al., 1996). Zona-induced PLC activation in mouse sperm is inhibited by tyrophostin, indicating that the PLC isoform is probably PLCγ (Tomes et al., 1996). If this PLC was still activated at the time of sperm-egg fusion, it could produce IP3 and release Ca2+ in the egg cytoplasm. PLCγ SH2 domains in the egg cytoplasm would not be expected to inhibit PLCγ that was already in the active form when it entered the egg, so our present results are consistent with this hypothesis.

In summary, our experiments indicate that the initiation of IP3 production and Ca2+ release at fertilization in mouse eggs differs from that in echinoderm eggs, where an SH2 domain-mediated signaling pathway in the egg activates PLCγ. However, PLCγ could still be responsible for generating IP3 in mouse eggs at fertilization, either by an alternate pathway for activating PLCγ in the egg, or by introduction of activated enzyme from the sperm.

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### REFERENCES


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**TABLE 2**

Ca2+ Responses from Eggs Injected with PLCγ1 and/or PLCγ2 SH2 Domains and Stimulated with Sperm

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PLCγ1SH2 (1000 μg/ml)</th>
<th>Control</th>
<th>PLCγ2SH2 (500 μg/ml)</th>
<th>Control</th>
<th>PLCγ1SH2 + PLCγ2SH2 (500 μg/ml each)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of eggs with Ca2+ transients (n tested)</td>
<td>75 (12)</td>
<td>86 (14)</td>
<td>100 (6)</td>
<td>100 (4)</td>
<td>100 (4)</td>
<td>100 (4)</td>
</tr>
<tr>
<td>Time from insemination to 1st transient (min)</td>
<td>8.3 ± 7.5</td>
<td>9.8 ± 4.4</td>
<td>5.6 ± 3.8</td>
<td>5.8 ± 0.8</td>
<td>11.1 ± 3.2</td>
<td>9.8 ± 2.3</td>
</tr>
<tr>
<td>Peak amplitude</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>1.9 ± 0.4</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Duration of 1st transient (min)</td>
<td>3.0 ± 0.7</td>
<td>3.4 ± 0.9</td>
<td>3.2 ± 0.3</td>
<td>2.6 ± 0.1</td>
<td>3.3 ± 0.8</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>Oscillation period (min)</td>
<td>8.2 ± 5.0</td>
<td>9.3 ± 5.0</td>
<td>6.8 ± 3.5</td>
<td>4.2 ± 0.6</td>
<td>6.5 ± 2.7</td>
<td>4.8 ± 0.8</td>
</tr>
</tbody>
</table>

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*a* ± values indicate standard deviations. Each experiment was repeated on 2-6 separate days. Control values showed some variability from day to day, probably due to experimental conditions such as temperature or sperm concentration, or to variation among different individual animals. For each series of experiments, the set of recordings from eggs injected with protein and Ca green dextran was compared with the set of recordings made on the same days from control eggs injected with Ca green dextran alone. An alternative analysis of the PLCγ1 SH2 data, in which experimental values were compared with control values obtained on the same individual day, also showed no consistent effects of the injected protein.

*b* PLCγ2SH2 was used at 500 rather than 1000 μg/ml because the 33 mg/ml stock solution needed to obtain 1000 μg/ml in the cytoplasm was too viscous to inject.

c The peak amplitude is expressed as the ratio of the peak current from the photodiode divided by the baseline current before stimulation.

d The oscillation period was defined as the average time between the rises of successive Ca2+ transients.


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