Calcium Release at Fertilization in Starfish Eggs Is Mediated by Phospholipase Cγ

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Abstract. Although inositol trisphosphate (IP$_3$) functions in releasing Ca^{2+} in eggs at fertilization, it is not known how fertilization activates the phospholipase C that produces IP$_3$. To distinguish between a role for PLCγ, which is activated when its two src homology-2 (SH2) domains bind to an activated tyrosine kinase, and PLCβ, which is activated by a G protein, we injected starfish eggs with a PLCγ SH2 domain fusion protein that inhibits activation of PLCγ. In these eggs, Ca^{2+} release at fertilization was delayed, or with a high concentration of protein and a low concentration of sperm, completely inhibited. The PLCγSH2 protein is a specific inhibitor of PLCγ in the egg, since it did not inhibit PLCβ activation of Ca^{2+} release initiated by the serotonin 2c receptor, or activation of Ca^{2+} release by IP$_3$ injection. Furthermore, injection of a PLCγ SH2 domain protein mutated at its phosphotyrosine binding site, or the SH2 domains of another protein (the phosphatase SHP2), did not inhibit Ca^{2+} release at fertilization. These results indicate that during fertilization of starfish eggs, activation of phospholipase Cγ by an SH2 domain-mediated process stimulates the production of IP$_3$ that causes intracellular Ca^{2+} release.

At fertilization, the sperm initiates a propagated rise of Ca^{2+} in the egg, which is of central importance in activating the egg to begin development (Jaffe, 1985; Whitaker and Steinhardt, 1985; Kline, 1988). In echinoderm as well as vertebrate eggs, the rise in Ca^{2+} results, at least in large part, from Ca^{2+} release from the endoplasmic reticulum in response to a rise in inositol trisphosphate (IP$_3$[1]; Whitaker and Irvine, 1984; Ciapa and Whitaker, 1986; Miyazaki et al., 1992; Mohri et al., 1995; Jaffe, 1996). However, it has not been established how the IP$_3$ is generated at fertilization.

IP$_3$ is produced from phosphatidylinositol 4,5-bisphosphate (PIP$_2$), by the action of a phospholipase C (PLC; Rhee and Choi, 1992). This family of enzymes includes β, δ, and γ isoforms. PLCβ is activated by G proteins, while PLCγ is activated by tyrosine kinases. The regulation of PLCδ is poorly understood, although the enzymatic activity of all 3 PLC isoforms can be stimulated by an increase in Ca^{2+} (Park et al., 1992; Wahl et al., 1992; Banno et al., 1994). Very likely the generation of IP$_3$ at fertilization results from the activation of one of these isoforms of phospholipase C.

Both PLCβ and PLCγ pathways are present in eggs. Expression in frog, mammalian, and starfish eggs of exogenous receptors known to release Ca^{2+} by a G protein/PLCβ pathway, such as serotonin 2c or muscarinic m1 receptors, allows Ca^{2+} release in eggs when the corresponding agonists are applied (Kline et al., 1988; Williams et al., 1992; Shilling et al., 1994). This indicates that functional PLCβ and corresponding G proteins are present. Likewise, expression in frog and starfish eggs of exogenous receptors known to release Ca^{2+} by a tyrosine kinase/PLCγ pathway, such as receptors for EGF or PDGF, allows Ca^{2+} release in response to these agonists (Shilling et al., 1994; Yim et al., 1994). Point-mutated receptors that do not activate PLCγ do not cause Ca^{2+} release (Shilling et al., 1994; Yim et al., 1994). These findings indicate that a functional PLCγ is present. Such experiments have not been carried out in mammalian eggs, but the presence of PLCγ has been demonstrated by immunoblotting (Dupont et al., 1996).

Several previous studies have examined whether PLCβ or PLCγ pathways cause Ca^{2+} release at fertilization (Miyazaki, 1988; Crossley et al., 1991; Moore et al., 1994;

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1. *Abbreviations used in this paper*: GST, glutathione-S-transferase; IP$_3$, inositol trisphosphate; PLC, phospholipase C; PIP$_2$, phosphatidylinositol 4,5-bisphosphate.

Moore and Kinsey, 1995; Dupont et al., 1996). However, the conclusions from these studies have been uncertain due to questions about the specificity of the pharmacological inhibitors used (see Discussion). To distinguish whether fertilization involves PLCγ- or PLCβ-mediated Ca^{2+} release mechanisms, we injected starfish eggs with a recombinant portion of the PLCγ protein that specifically inhibits PLCγ but not PLCβ activation.

PLCγ is activated when its two tandem src homology-2 (SH2) domains interact with a specific SH2 binding site on an activated protein tyrosine kinase, thus bringing PLCγ in close contact with the kinase and allowing it to be phosphorylated (Kim et al., 1991; Rhee and Choi, 1992). The kinase can be a transmembrane receptor kinase, such as the PDGF receptor, or a cytosolic kinase such as Syk (Sillman and Monroe, 1995). SH2 domain-mediated enzyme activation is widespread and highly specific; for example the PDGF receptor kinase has at least eight sites that specifically bind particular SH2 domain-containing enzymes or adaptor proteins (Claesson-Welsh, 1994). The SH2 domains of each of these proteins are different, and none of these proteins binds to the same site on the PDGF receptor as PLCγ (Claesson-Welsh, 1994; Gish et al., 1995; Pawson, 1995). Similarly for the EGF receptor, the SH2 domains of PLCγ show specificity among the several SH2 domain binding sites present on the receptor, with much higher affinity for one particular site compared to the others (Rotin et al., 1992).

Recombinant proteins containing the two SH2 domains of PLCγ have been used to disrupt the signalling between the tyrosine kinase and PLCγ in fibroblasts (Roche et al., 1996). In an analogous way, we examined the effect of injecting a glutathione-S-transferase (GST) fusion protein composed of the two SH2 domains of PLCγ (NH$_2$- and COOH-terminal), into eggs of the starfish *Asterina miniata*. Our findings indicate that the PLCγ pathway initiates Ca^{2+} release at fertilization in starfish eggs.

Materials and Methods

Obtaining Oocytes and Sperm

Starfish (*Asterina miniata*) were obtained from Marinus, Inc. (Long Beach, CA). Ovaries and testes were collected through a hole in the dorsal surface of the animal, made with a 3-mm sample corer (Fine Science Tools, Foster City, CA). Follicle cell-free oocytes were obtained by mincing the ovary in ice-cold, Ca^{2+}-free sea water followed by washing in natural sea water. Oocyte maturation was induced by addition of 1 μM 1-methyladenine (Sigma Chemical Co., St. Louis, MO). Sperm were obtained by mincing the testis, followed by centrifugation for 1 min at 3,000 g to separate the sperm suspension from other testis tissue. Except as indicated, the sperm suspension was diluted 1:5,000 before use. All experiments were performed in natural sea water at 18–20°C.

GST Fusion Proteins and RNA

Plasmid DNAs encoding GST fusion proteins of bovine PLCγSH2(N+C), PLCγSH2(N), and PLCγSH2(C) (Fig. 1) in pGEX2T′6 (Roche et al., 1996) were obtained from S. Courtneidge (Sugen, Inc., Redwood City, CA). DNA for a GST fusion protein of the tandem NH$_2$- and COOH-terminal SH2 domains of the phosphatase SHP2, encoding amino acids 2 to 216 of the murine protein (Feng et al., 1993; Adachi et al., 1996), was obtained from T. Pawson (Mt. Sinai Hospital, Toronto, Canada) and was inserted in frame into the BamHI and EcoRI sites of pGEX2T (Pharmacia Biotech, Piscataway, New Jersey). DNA for SH2(N+C)-wt and SH2 (N+C)-mut GST fusion proteins (Fig. 1) was derived from DNA for wild-
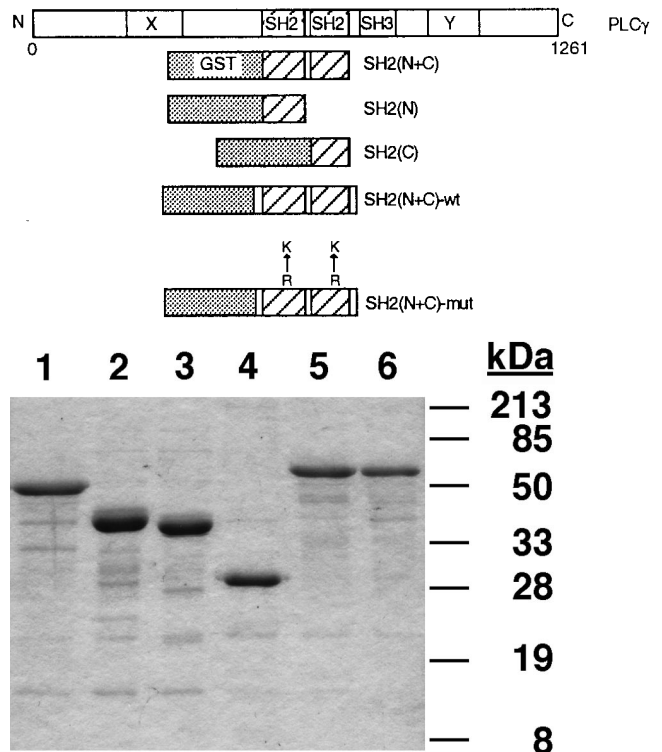

Figure 1. Bacterial fusion proteins of PLCγ SH2 domains. The diagram shows the NH$_2$- and COOH-terminal SH2 domains, the SH3 domain, and the X and Y catalytic domains of the 1261 amino acids of PLCγ, as well as the regions used to make the GST fusion proteins. Proteins contained the following amino acid residues as well as GST: (lane *1*) SH2(N+C), 547-752; (lane *2*) SH2(N), 547-659; (lane *3*) SH2(C), 663-752; (lane *4*) GST; (lane *5*) SH2(N+C)-mut, 518-771, R586/694K; (lane *6*) SH2(N+C)-wt, 518-771. Lanes *1–3* are bovine sequences; lanes *5* and *6* are human sequences. 12% SDS-PAGE gel, 2–3 μg protein per lane, stained with Coomassie blue.

type and mutant constructs containing the SH2 and SH3 domains of human PLCγ (see Huang et al., 1995) obtained from P. Huang (Merck Research Laboratories, West Point, PA). To do this, the SH2(N+C) portions of the SH2(N+C)SH3 constructs were cut out using Bsp 120I, and the overhangs were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I. The DNA was subsequently digested with BglII, and the fragments were inserted in frame into the SmaI and BamHI sites of pGEX2T′6.

GST fusion proteins were produced as described by Gish et al., 1995, purified using glutathione agarose (Sigma Chemical Co.) or glutathione sepharose (Pharmacia Biotech), dialyzed extensively in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$), and concentrated to 3–50 mg/ml, using a 10-kD cutoff Ultrafree-4 centrifugal filter device (Millipore Corp., Bedford, MA). Protein concentrations were determined using a BCA assay (Pierce Chemical Co., Rockford, IL) with BSA as the standard. The correct folding of the PLCγSH2(N+C) and SHP2-SH2(N+C) recombinant GST fusion proteins was confirmed by testing their ability to bind to the activated PDGF receptor (see Roche et al., 1996).

DNA for the serotonin 2c receptor, in the vector Bluescript SK$^-$, was obtained from D. Julius (University of California, San Francisco). Synthetic RNA was made as previously described (Shilling et al., 1990).

Calcium Measurements

Ca^{2+} measurements were performed using eggs at first meiotic metaphase, the stage at which fertilization normally occurs. The eggs were injected with 10 μM Ca-green 10-kD dextran (Molecular Probes, Eugene, OR) and were held between two coverslips, with ∼1 egg diam (∼180 μm) be-

tween the coverslip edge and the egg surface (Kiehart, 1982). Sperm were added to the front of the chamber. Ca-green fluorescence was measured with a photomultiplier (Shilling et al., 1994) or imaged with a confocal microscope (MRC600; Bio Rad Laboratories, Hercules, CA) with a 20×, 0.5 N.A. neofluar objective (Zeiss, Inc., Thornwood, NY). The video output from the confocal microscope was stored on an optical memory disk recorder (OMDR; 3038F; Panasonic). Each scan was automatically recorded by using the confocal sync to trigger the OMDR (details available at http://www.uchc.edu/~terasaki/trigger.html). To make the figure, data from the OMDR was digitized and the images were assembled using NIH Image (Wayne Rasband, Research Services Branch, National Institutes of Health, Bethesda, MD) and Photoshop (Adobe Systems, Inc., Mountain View, CA).

Microinjection

Quantitative microinjection was performed using mercury-filled micropipets (Kiehart, 1982) or micropipets with an oil-filled constriction (Kishimoto, 1986). These methods allow injection of precisely defined picoliter volumes. Injected volumes were 1–5% of the egg volume (3,100 pL), except for the PLCγSH2(N+C)-wt and PLCγSH2(N+C)-mut proteins, which were injected at 5–7% of the egg volume. In general, protein concentrations in the stock solutions were adjusted so that the volume injected was the same for control and test injections in the same series of experiments. Injections of control proteins at these volumes had no inhibitory effect on Ca^{2+} release. Protein concentrations given in the text indicate the final values in the egg cytoplasm.

Results

Delay of Ca^{2+} Release at Fertilization by PLCγSH2(N+C)

The Ca^{2+} rise seen in starfish eggs at fertilization consists of two phases. The initial response is a Ca action potential, resulting from Ca^{2+} entry through voltage-gated channels in the plasma membrane (Miyazaki et al., 1975; Miyazaki and Hirai, 1979); this is followed by a much larger Ca^{2+} rise, resulting from the release of Ca^{2+} from intracellular

stores in a wave across the egg (Stricker et al., 1994). The Ca action potential functions to establish a fast electrical block to polyspermy (Jaffe, 1976) and probably occurs simultaneously with sperm–egg fusion (McCulloh and Chambers, 1992). In photomultiplier records of eggs injected with Ca-green dextran, the action potential appeared as a transient deflection preceding the main Ca^{2+} rise (Fig. 2 A). In confocal microscope images, it appeared as a transient ring of brightness at the egg surface preceding the Ca^{2+} wave (see Fig. 3). Previous studies in sea urchin eggs have demonstrated that these Ca^{2+} signals are due to the action potential (McDougall et al., 1993; Shen and Buck, 1993). In starfish eggs from different animals, the amplitude and duration of the Ca^{2+} signal due to the action potential were variable, with a duration of ~5–10 s (compare Figs. 2 A and 4 A). In the present study, we used the action potential as a time marker, with respect to which we measured the timing of Ca^{2+} release from intracellular stores.

In eggs injected with a GST fusion protein including the NH_2- and COOH-terminal SH2 domains of PLCγ (90–220 μg/ml, final intracellular concentration), intracellular Ca^{2+} release, as detected with photomultiplier records of Ca-green dextran fluorescence, did not begin until an average of 75 s after the rise of the action potential (Fig. 2 B; Table I), compared with 6 s in control eggs (Fig. 2 A; Table I). The magnitude of the delay was concentration dependent; the lowest concentration at which the delay was significantly different from the control was ~90 μg/ml (1.8 μM; Table I). In eggs injected with 90–220 μg/ml PLCγSH2(N+C), the rise to the peak Ca-green fluorescence often included several smaller amplitude increases (see Fig. 5 B), and the peak amplitude was slightly less than in control eggs (Table I). When the concentration of the PLCγSH2(N+C) protein was increased to 900 to 1,000 μg/ml in the egg cytoplasm, the delay between the action potential and the initiation of Ca^{2+} release was increased further, and the
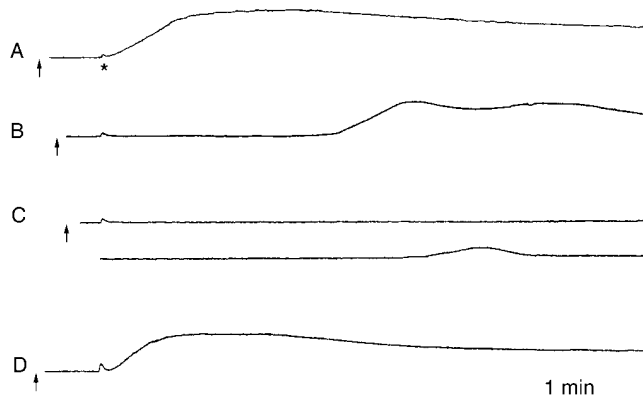

Figure 2. Delay of Ca^{2+} release during fertilization of starfish eggs injected with PLCγSH2(N+C) protein. Eggs were co-injected with 10 μM Ca-green dextran; photomultiplier current indicating Ca-green fluorescence is shown as a function of time. Each fluorescence trace was normalized to the fluorescence of the unfertilized egg. Arrows indicate the time of sperm addition (1:5,000 dilution of the suspension from the testis). (*A*) An egg that was injected with 1,100 μg/ml of the GST control protein. The asterisk indicates the action potential. (*B* and *C*) Eggs that were injected with 105 (*B*) or 900 (*C*) μg/ml of the PLCγSH2(N+C) protein. In *C*, the lower line shows the continuation of the same record. (*D*) An egg that was injected with 1,000 μg/ml of the SHP2-SH2(N+C) control protein.

Table I. Effect of PLCγSH2(N+C) on the Delay between the Action Potential and the First Detectable Ca^{2+} Release at Fertilization

Injected protein (μg/ml)	Delay (s)	Peak amplitude	n
No injection	6 ± 0.2	1.25 ± 0.05	14
PLCγ(N+C) (10–80)	10 ± 3	1.26 ± 0.06	10
PLCγ(N+C) (90–130)	46 ± 12*	1.00 ± 0.09*	10
PLCγ(N+C) (160–220)	93 ± 12*	1.01 ± 0.05*	16
PLCγ(N+C) (900–1000)	410 ± 70*	0.33 ± 0.14*	7
GST (200–1100)	6 ± 0.6	1.21 ± 0.03	15
SHP2(N+C) (240–1000)	6 ± 0.4	1.16 ± 0.03	8
PLCγ(N) (180)	6 ± 0.4	1.22 ± 0.05	6
PLCγ(C) (140)	5 ± 0.2	1.22 ± 0.04	11
PLCγ(N+C)-wt (180–250)	44 ± 12*	1.04 ± 0.06*	13
PLCγ(N+C)-mut (180–260)	5 ± 0.3	1.30 ± 0.05	10

Eggs were inseminated with a 1:5,000 dilution of the suspension from the testis. Measurements were made from photomultiplier records of Ca-green fluorescence. Values for delay indicate the time between the rise of the action potential and the time at which the fluorescence versus time trace started its second rise. Values for peak amplitude are expressed as the change in fluorescence after fertilization divided by the fluorescence of the unfertilized egg. Data are expressed as the mean ± SEM. Each data point was obtained with eggs from 2 to 5 animals. Points marked with asterisks are significantly different from controls, comparing PLCγSH2(N+C) with GST, and PLCγSH2(N+C)-wt with PLCγSH2(N+C)-mut. Data were analyzed using the nonparametric Mann-Whitney test (Instat software; GraphPad, San Diego, CA) to calculate *P* values. *P* values <0.01 were considered to be statistically significant. *n* = number of eggs.
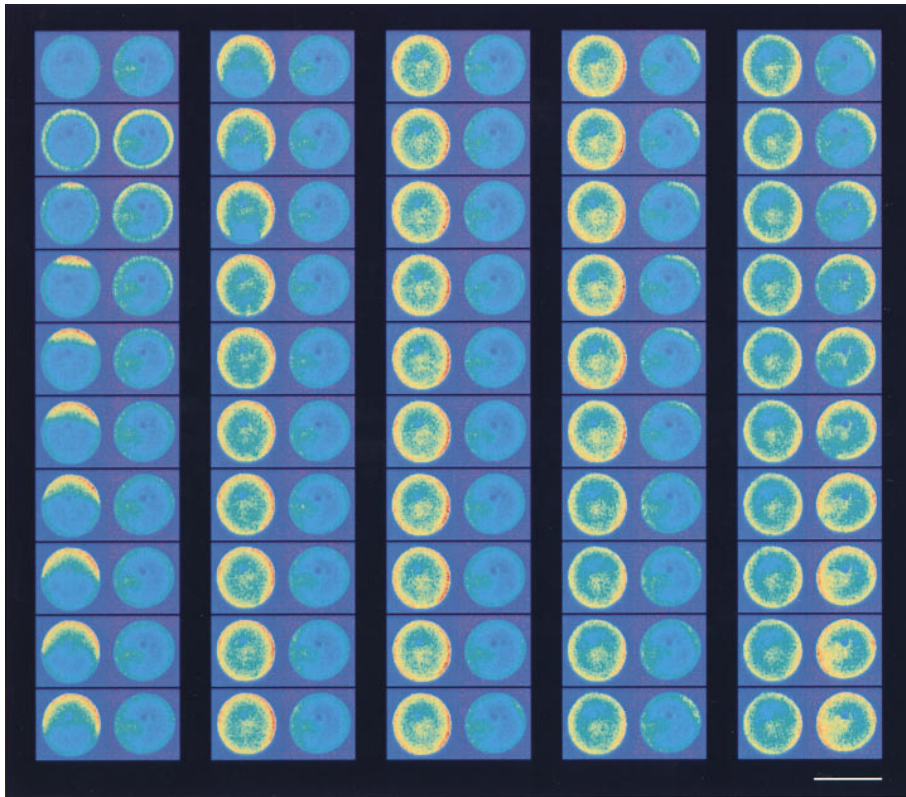
Figure 3. Ca^{2+} imaging during fertilization of starfish eggs injected with PLCγSH2(N+C) or GST proteins. The control egg on the left was injected with 190 μg/ml GST and 10 μM Ca-green dextran. The egg on the right was injected with 130 μg/ml PLCγSH2(N+C) and 10 μM Ca-green dextran. Fluorescence intensity (0–255) is displayed using the 32-color look-up table of NIH Image (increasing values correspond to purple, blue, green, yellow, orange, red). Images are shown at 3-s intervals; the sequence is shown reading down each column, starting at the upper left. Sperm (1:5,000 dilution of the suspension from the testis) were added to the recording chamber 14 s before the first image. In the second image in the first column, an action potential occurred in both eggs (increased fluorescence at the egg surface). In the third image in the first column, the Ca^{2+} wave is starting in the control egg. In the first image in the fourth column, a local Ca^{2+} rise occurs in the PLCγSH2(N+C)-injected egg but does not propagate across the egg. In the last image in the fourth column, another Ca^{2+} rise occurs in the PLCγSH2(N+C)-injected egg, and a Ca^{2+} wave propagates across the egg in the fifth column. The difference in fluorescence intensity between the egg cortex and interior is due at least in part to greater absorbance of light in the center of the egg where the specimen is thicker; it is also possible that there is a real difference in Ca^{2+} activity between the cortex and interior. A Quicktime movie sequence from this experiment is available at: http://www.uchc.edu/~terasaki/data/sh2.html. Bar, 200 μm.

amplitude of the Ca^{2+} release was also reduced (Fig. 2 *C*; Table I). The PLCγSH2(N+C) protein did not change the Ca^{2+} level in the unfertilized eggs, the amplitude or duration of the action potential, or the time between sperm addition and the occurrence of the action potential. The cytoplasm of the PLCγSH2(N+C)-injected eggs appeared normal as observed with transmitted light microscopy.

Microinjection of GST alone, at 200 to 1,100 μg/ml, or a GST fusion protein composed of the tandem NH$_2$- and COOH-terminal SH2 domains of another protein, the phosphatase SHP2 (240–1,000 μg/ml), had no effect on the kinetics or amplitude of Ca^{2+} release (Fig. 2, *A* and *D*; Table I). Fusion proteins composed of the individual NH$_2$- or COOH-terminal SH2 domains of PLCγ were also without effect at the concentrations tested (140–180 μg/ml; Table I). Although these individual SH2 domains of PLCγ can also bind to kinases, the affinity of the interaction is lower (Anderson et al., 1990; Rotin et al., 1992; Larose et al., 1995).

Injections of the SH2 proteins were performed 28–130 min before insemination. Within this range, the time of injection had no effect on the kinetics or amplitude of the fertilization-induced Ca^{2+} rise. When injections were performed 13–19 min before insemination, PLCγSH2(N+C) delayed the Ca^{2+} release, but the delay was smaller (42 ± 9 s for 7 injections of 220 μg/ml versus 100 ± 13 s for 14 injections of the same amount of protein made >28 min before insemination). The time required to see the full inhibitory

effect may be related to the time for the protein to spread to the site of sperm interaction at the egg plasma membrane. This time is comparable to the times seen for some other proteins to spread in the cytoplasm of eggs (Hamaguchi et al., 1985; Mabuchi et al., 1985).

At the time of injection of the PLCγSH2(N+C) protein, oocytes were at either the prophase or first metaphase stage. When the PLCγSH2(N+C) protein was injected before applying 1-methyladenine to cause the transition from prophase to first metaphase, there was no effect on the time of germinal vesicle breakdown, and the delay of the fertilization-induced Ca^{2+} release was the same as in oocytes injected at the metaphase stage.

Imaging of Ca^{2+} in PLCγSH2(N+C)-injected Eggs

Imaging of the Ca^{2+} rise during fertilization of eggs injected with the PLCγSH2(N+C) protein (100–130 μg/ml) also showed an increased delay between the occurrence of the action potential, indicated by a Ca^{2+} rise at the egg surface, and the initiation of Ca^{2+} release, seen as a wave that spread across the egg (Fig. 3; Table II). Multiple local Ca^{2+} rises occurred before the initiation of a Ca^{2+} wave that crossed the entire egg. In favorable optical sections, the local Ca^{2+} rises could be seen to occur at sites of sperm interaction, as indicated by the subsequent appearance of cytoplasmic protrusions where sperm had entered the egg ("fertilization cones"). Sometimes more than one local Ca^{2+}

Table II. Effect of PLCγSH2(N+C) on Kinetics of Ca^{2+} Release in Eggs Imaged by Confocal Microscopy

Injected protein (μg/ml)	Delay to Ca^{2+} rise (s)	Delay to Ca^{2+} wave (s)	Wave propagation time (s)
GST (190) or no injection	7 ± 2 (8)	7 ± 2 (8)	28 ± 4 (5)
PLCγ(N+C) (100-130)	69 ± 10 (9)*	147 ± 16 (8)*	23 ± 3 (5)

Values for "delay to Ca^{2+} rise" indicate the time between the start of the action potential and the first detection of a local Ca^{2+} rise. Values for "delay to Ca^{2+} wave" indicate the time between the start of the action potential and the initiation of a Ca^{2+} wave that propagated across the egg. Values for "wave propagation time" indicate the time between the initiation of the Ca^{2+} wave and the time at which the wave reached the opposite pole of the egg. Examples in which the wave was initiated at multiple sites or far outside of the focal plane were not analyzed. Data are expressed as the mean ± SEM. Points marked with asterisks are significantly different from controls (P < 0.001, Mann-Whitney test). n = number of eggs.

rise occurred at the same time, and a wave was initiated from multiple sites. When the wave was initiated from a single site, such that it was possible to measure the time to propagate to the opposite pole of the egg, the propagation time was the same as in control eggs (Fig. 3; Table II).

Polyspermy in PLCγSH2(N+C)-injected Eggs

Eggs injected with 90–220 μg/ml PLCγSH2(N+C) underwent normal, but delayed, cortical granule exocytosis, as indicated by the elevation of a normal fertilization envelope. In a series of experiments in which these eggs were observed at the time of first cleavage, 24/24 eggs injected with 100–220 μg/ml PLCγSH2(N+C) were seen to be polyspermic (first cleavage to multiple cells), while 22/25 control eggs that were injected with GST alone or SHP2-SH2(N+C) (200–1,100 μg/ml) were monospermic (normal early cleavage). The eggs injected with PLCγSH2(N+C) presumably became polyspermic because the delay in Ca^{2+} release delayed the elevation of the fertilization envelope. Also, although the action potential provides a fast electrical block to polyspermy, the positive membrane potential that establishes the electrical block is probably not sustained in the absence of Ca^{2+} release (Kline et al., 1986). Eggs injected with 900–1,000 μg/ml PLCγSH2(N+C) showed little or no fertilization envelope elevation and were highly polyspermic; >10 sperm pronuclei were observed in the cytoplasm of these eggs, using transmitted light microscopy.

Complete Inhibition of Ca^{2+} Release in PLCγSH2(N+C)-injected Eggs Inseminated with a Low Concentration of Sperm

The occurrence of polyspermy in the eggs injected with the PLCγSH2(N+C) protein suggested the possibility that the residual Ca^{2+} release seen in these eggs might be eliminated if we further reduced the probability of PLCγ activation by reducing the number of sperm interacting with the egg. For the experiments described above, we used a relatively high concentration of sperm (1:5,000 dilution of the suspension obtained from the testis). With this sperm concentration, ~100% of eggs in the recording chambers were fertilized. When the sperm concentration was reduced 10-fold (1:50,000 dilution), ~80% of the control uninjected eggs in the recording chambers were fertilized. Of
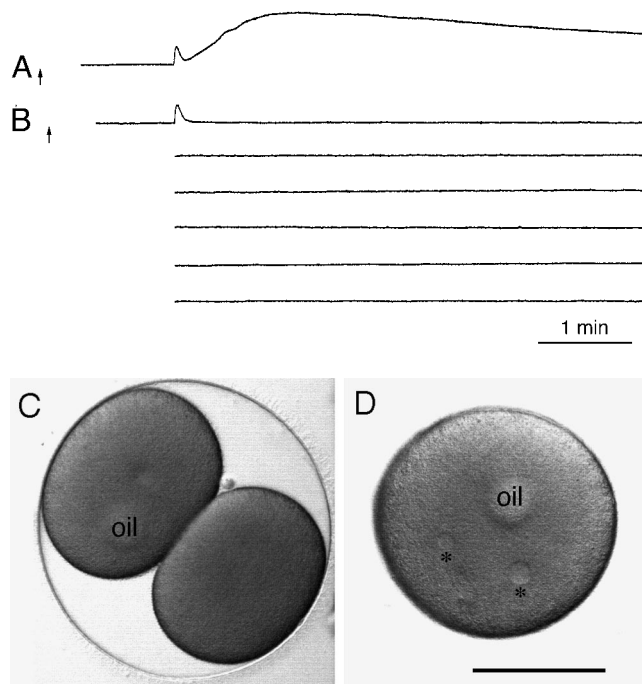

Figure 4. Inhibition of Ca^{2+} release during fertilization of starfish eggs injected with PLCγSH2(N+C) protein and exposed to a low concentration of sperm (1:50,000 dilution of the suspension from the testis). Photomultiplier current as a function of time. (*A*) An egg that was injected with 1,000 μg/ml of the SHP2-SH2(N+C) control protein. (*B*) An egg that was injected with 1,000 μg/ml of the PLCγSH2(N+C) protein. Arrows indicate the time of sperm addition. The lower lines show the continuation of the same record. (*C* and *D*) Images of eggs injected with 1,000 μg/ml SHP2-SH2(N+C) and 1,000 μg/ml PLCγ-SH2(N+C) and inseminated with a 1:50,000 sperm dilution; images were taken at the time of first cleavage in uninjected eggs, using the scanning transmission mode of the BioRad confocal microscope. (*C*) The SHP2-SH2(N+C)-injected egg showed a Ca-green record like that in *A*, elevated a normal fertilization envelope, and cleaved normally. Photographed at 3.0 h after insemination. (*D*) The PLCγSH2(N+C)-injected egg showed a Ca-green record like that in *B* and did not elevate a fertilization envelope or cleave; however, the presence of two pronuclei in the cytoplasm indicated that it was fertilized. Photographed at 2.6 h after insemination. These pronuclei were similar in appearance to those described by Sluder et al. (1989) for normal starfish fertilization, although the time course of their development was not compared with that in control eggs. Asterisks indicate pronuclei; "oil" indicates the oil drop introduced during microinjection. Bar, 100 μm.

10 eggs injected with 1,000 μg/ml PLCγSH2(N+C) and inseminated with sperm diluted 1:50,000, 9 were fertilized, as judged by the occurrence of an action potential and the subsequent observation of at least one sperm pronucleus in the egg cytoplasm (Fig. 4 *D*). However, during the 30-min recording period, 7/9 of these eggs showed no detectable Ca^{2+} release after the action potential (Fig. 4 *B*) and no fertilization envelope elevation. 2/9 of these eggs showed an action potential followed by a small and delayed Ca^{2+} release. In 8/9 control eggs injected with 1,000 μg/ml SHP2-SH2(N+C) and inseminated with a 1:50,000 dilution of sperm, the action potential was followed by normal Ca^{2+} release (Fig. 4 *A*). These results indicate that
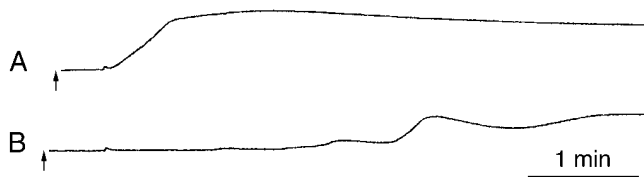
Figure 5. Normal Ca^{2+} release during fertilization of starfish eggs injected with a point-mutated PLCγ SH2(N+C) protein. Photomultiplier current as a function of time. Arrows indicate the time of sperm addition (1:5,000 dilution of the suspension from the testis). (*A*) An egg that was injected with 260 μg/ml of the PLCγ SH2(N+C)-mut protein. (*B*) An egg that was injected with 220 μg/ml of the PLCγ SH2(N+C)-wt protein.

Figure 6. Normal Ca^{2+} release in starfish eggs injected with the PLCγSH2(N+C) protein and treated with IP$_3$ or serotonin. (*A* and *B*) Photomultiplier records from oocytes that were injected with Ca-green dextran and for *B*, 1,000 μg/ml PLCγSH2(N+C), and then matured and injected with 1% of the cell volume of 1 μM IP$_3$ (*arrows*). (*C* and *D*) Prophase-stage oocytes were injected with serotonin 2c receptor RNA (30 pg/oocyte). 24–27 h later the oocytes were injected with 10 μM Ca-green dextran and for *D*, 1,000 μg/ml PLCγSH2(N+C), and then treated with 1-methyladenine to cause maturation to the first meiotic metaphase stage. Serotonin (1 μM) was applied (*arrows*), while recording Ca-green fluorescence. Photomultiplier current indicating Ca-green fluorescence is shown as a function of time.

under low sperm concentration conditions, inhibition of PLCγ activation can completely inhibit Ca^{2+} release at fertilization.

Specificity of the Inhibition of Ca^{2+} Release by PLCγSH2(N+C)

SH2 domains of various enzymes have specific binding sites on their target proteins such as the PDGF receptor (Claesson-Welsh, 1994), and therefore inhibition of enzyme activation by isolated SH2 domains should show specificity for the enzyme from which the domains were derived. However, it was critical to examine whether PLCγSH2(N+C) inhibited other cellular processes besides the activation of PLCγ. As noted above, the PLCγSH2(N+C)-injected eggs appeared normal in all respects except for the release of intracellular Ca^{2+}, and sperm–egg fusion occurred normally as indicated by the entry of sperm nuclei into the egg cytoplasm. Also as described above, two control proteins, GST and a GST fusion protein including the SH2 domains of a different protein (the SHP2 phosphatase), had no inhibitory effect on Ca^{2+} release at fertilization.

As a general control against the possibility that PLCγSH2(N+C) interfered with some cellular process that was unrelated to SH2-domain signaling, we injected a PLCγSH2(N+C) fusion protein that had been point mutated at a particular arginine (Fig. 1) that is conserved in all SH2 domain proteins (the "FLVR" sequence; Koch et al., 1991). For several SH2 domain proteins (Mayer et al., 1992; Iwashima et al., 1994), including PLCγ (Huang et al., 1995), substitution of lysine for this arginine eliminates binding to the target kinase. At the concentrations tested (180–260 μg/ml), injection of the point-mutated protein had no effect on the kinetics or amplitude of Ca^{2+} release at fertilization (Fig. 5 *A*; Table I; Fig. 5 *B* shows the wild-type protein for comparison). Due to precipitation of protein in the concentrator tube when we attempted to prepare a more concentrated protein solution, we were unable to test the mutant protein at higher concentrations. Nevertheless, our results indicated that the inhibitory effect of PLCγSH2 was related to its specific SH2 domain properties. If the inhibitory effect of PLCγSH2 was due to nonspecific binding to a cytoplasmic protein, this point mutation would not be expected to alter its biological effect (Itoh et al., 1996).

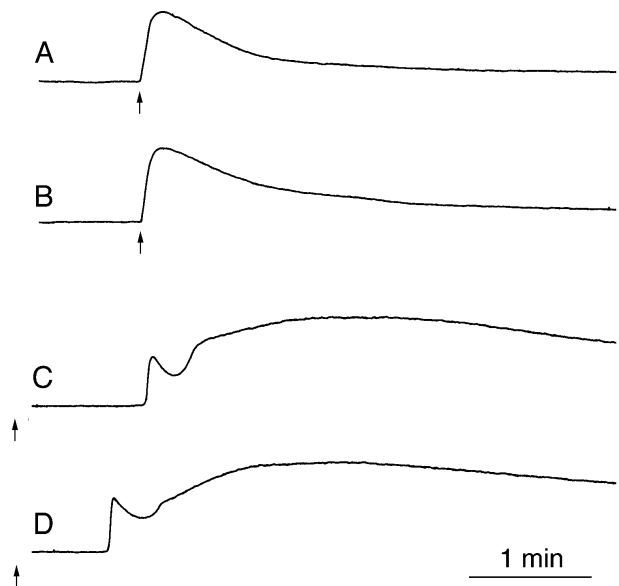To rule out the possibility that PLCγSH2(N+C) directly interfered with IP$_3$-induced Ca^{2+} release, we injected IP$_3$ into eggs that had been injected with the PLCγSH2(N+C) protein. Using an amount of IP$_3$ (1% injection of 1 μM) that was close to the minimum needed to cause Ca^{2+} release (Chiba et al., 1990), we found that 1,000 μg/ml PLCγSH2(N+C) did not delay or attenuate the response (Fig. 6, *A* and *B*). The time between injection of IP$_3$ and the initial increase in Ca-green dextran fluorescence was <2 s for both controls without protein injection (*n* = 17) and eggs injected with PLCγSH2(N+C) (*n* = 11). For both groups, the peak amplitude of the Ca^{2+} response was the same (.94 ± .03, mean ± SEM, peak fluorescence/unstimulated egg fluorescence).

To obtain direct evidence that the PLCγSH2(N+C) protein did not interfere with activation of PLCβ, a PLC isoform that lacks SH2 domains, we tested the effect of PLCγSH2(N+C) on the PLCβ pathway stimulated by the serotonin 2c receptor (Julius et al., 1988; Baxter et al., 1995). In starfish eggs expressing this exogenously introduced receptor, application of serotonin causes a Ca^{2+} rise (Shilling et al., 1990, 1994). When such eggs were injected with PLCγSH2(N+C) at concentrations that delayed or completely inhibited Ca^{2+} release at fertilization, the kinetics and amplitude of the Ca^{2+} rise in response to serotonin were unaffected (Fig. 6, *C* and *D*; Table III). These control experiments further indicated that the PLCγSH2(N+C) protein is a specific inhibitor of the activation of PLCγ.

Table III. No Effect of PLCγSH2(N+C) on the Ca^{2+} Rise in Response to PLCβ Stimulation by the Serotonin 2c Receptor

Injected protein (μg/ml)	Delay (s)	Peak amplitude	n
Series one			
No injection	41 ± 16	0.98 ± 0.06	3
PLCγSH2(N+C) (220)	42 ± 15	1.10 ± 0.08	6
Series two			
No injection	73 ± 10	1.08 ± 0.06	4
PLCγSH2(N+C) (1000)	69 ± 14	1.21 ± 0.04	4

Experiments were performed as described in Fig. 6, *legend,* except that for series one, the RNA was injected 46–50 h before testing with 50 nM serotonin, while for series two, the RNA was injected 23–29 h before testing with 1 μM serotonin. Less serotonin is needed to cause Ca^{2+} release if oocytes are incubated for a longer time after RNA injection (Shilling et al., 1990). Measurements were made from photomultiplier records of Ca-green fluorescence. Values for delay indicated the time to the initial increase in Ca-green fluorescence, after adding serotonin. Values for peak amplitude are expressed as the change in fluorescence after serotonin application divided by the fluorescence of the unstimulated egg. Data are expressed as the mean ± SEM.
n = number of eggs.

Discussion

Our main finding from these studies is that injection of starfish eggs with recombinant SH2 domains of PLCγ can completely and specifically inhibit Ca^{2+} release at fertilization. This confirms that IP$_3$-mediated signaling accounts for the initiation of Ca^{2+} release at fertilization, and furthermore, indicates that activation of PLCγ initiates the IP$_3$-mediated Ca^{2+} release. This finding implicates a tyrosine kinase in the upstream signaling pathway at fertilization.

IP$_3$-mediated Signaling Accounts for the Initiation of Ca^{2+} Release at Fertilization

In hamster eggs, complete inhibition of Ca^{2+} release at fertilization by an antibody against the IP$_3$ receptor has indicated that the IP$_3$ pathway accounts for the initiation of Ca^{2+} release at fertilization (Miyazaki et al., 1992). In frog eggs, heparin can completely block egg activation at fertilization (Nuccitelli et al., 1993), supporting the conclusion that IP$_3$ initiates Ca^{2+} release at fertilization, although heparin is not a highly specific inhibitor (Bezprozvanny et al., 1993). In sea urchin eggs, initial studies indicated that the IP$_3$-mediated pathway was only part of the mechanism by which Ca^{2+} release is initiated at fertilization (Galione et al., 1993; Lee et al., 1993), but subsequent work has shown that inhibition of the IP$_3$ receptor by pentosan polysulfate can completely inhibit Ca^{2+} release at fertilization (Mohri et al., 1995). However, like heparin, pentosan polysulfate is not a specific inhibitor of the IP$_3$ receptor (Bezprozvanny et al., 1993). Our results extend this previous work, since the SH2 domains of PLCγ, a highly specific inhibitor of IP$_3$ production, can completely inhibit Ca^{2+} release at fertilization in starfish eggs.

Activation of PLCγ Initiates IP$_3$-mediated Ca^{2+} Release at Fertilization

Since inhibition of PLCγ activation by injection of SH2 domains can completely block Ca^{2+} release at fertilization, it appears that activation of PLCγ initiates the IP$_3$-mediated Ca^{2+} release. The related question of whether tyrosine kinase activation is necessary for Ca^{2+} release at fertilization has been examined in sea urchin eggs, using pharmacological tyrosine kinase inhibitors. These substances do not inhibit fertilization envelope elevation (Moore and Kinsey, 1995), but it is not certain that activation of PLCγ was completely inhibited under the conditions of these experiments. This study also noted that one of the inhibitors, genistein, caused polyspermy, suggesting a delay in Ca^{2+} release. In mammalian eggs, studies with pharmacological inhibitors of protein tyrosine kinases and phospholipase C suggest a role for PLCγ in initiating Ca^{2+} release at fertilization (Dupont et al., 1996), but other evidence that GDP-β-S injection completely inhibits Ca^{2+} release at fertilization of mammalian eggs indicates that G proteins mediate this signaling (Miyazaki, 1988; Moore et al., 1994). Uncertainty about the specificity of these inhibitory substances complicates the interpretation of these findings (see also Jaffe, 1990; Crossley et al., 1991). Injection of PLCγSH2(N+C) into mammalian eggs should help to resolve the relative roles of G proteins and tyrosine phosphorylation in initiating Ca^{2+} release at fertilization in mammals.

Our findings also indicate that the Ca action potential preceding the large release of intracellular Ca^{2+} is not dependent on SH2 domain-mediated activation of PLCγ. The opening of the voltage-dependent Ca channels that produce the action potential presumably results from a small depolarization of the egg plasma membrane. This depolarization could be produced by the introduction of ion channels from the sperm membrane or by opening of ion channels in the egg membrane (Hagiwara and Jaffe, 1979; McCulloh and Chambers, 1992).

Upstream Components in the Signaling Pathway at Fertilization

The involvement of SH2 domains in the activation of PLCγ at fertilization indicates that the activator is a tyrosine kinase, either a receptor tyrosine kinase or a cytosolic tyrosine kinase (Rhee and Choi, 1992). This kinase could come from either the sperm or the egg; possibilities include activation of a receptor kinase in the egg membrane by contact with a sperm ligand, activation of a receptor in the egg membrane leading to activation of a cytosolic kinase, introduction of a receptor kinase from the sperm membrane into the egg membrane as a consequence of sperm–egg fusion, or introduction of a cytosolic kinase or kinase-activating protein from the sperm into the egg cytoplasm. At present, there is insufficient information to distinguish between these possibilities. Sperm–egg fusion appears to precede Ca^{2+} release in both echinoderms (McCulloh and Chambers, 1992) and mammals (Lawrence et al., 1997), although some uncertainty remains (Longo et al., 1994). However, timing alone does not identify whether Ca^{2+} release is initiated by contact of the sperm with a receptor or by sperm–egg fusion. A hamster sperm-derived protein, oscillin, causes Ca^{2+} release when injected into mouse eggs (Parrington et al., 1996), but there is no evidence how this protein could lead to IP$_3$ production or that the amount of protein in a single sperm is sufficient to cause Ca^{2+} release. A completely different protein derived from mouse sperm, a truncated form of

c-kit, also causes activation when introduced into mouse eggs (Sette et al., 1997). This latter finding is particularly relevant to our findings, since c-kit is a receptor tyrosine kinase. The truncated form of c-kit used in these experiments lacks part of the tyrosine kinase domain, but as discussed by these authors, it might still participate in activating PLCγ. In particular, although activation of PLCγ by receptor tyrosine kinases is known to be caused by phosphorylation of PLCγ, there is also evidence that a noncatalytic interaction with a receptor tyrosine kinase can activate PLCγ in vitro (Hernandez-Sotomayor and Carpenter, 1993). Whether a protein that activates eggs upon injection can be purified from echinoderm sperm is unknown, although activation of sea urchin and nemertean eggs by injection of sperm extracts has been reported (Dale et al., 1985; Stricker, 1997).

Tyrosine kinase activity in sea urchin eggs increases before Ca^{2+} release (Ciapa and Epel, 1991; Abassi and Foltz, 1994), and one of the proteins that is phosphorylated has a molecular mass of ~138 kD, consistent with PLCγ, although its identity is unknown (Ciapa and Epel, 1991). Several cytoplasmic protein tyrosine kinases that could possibly activate PLCγ have been found in sea urchin eggs (Moore and Kinsey, 1994; Kinsey, 1995, 1996), but so far none of these has been found to show increased kinase activity before Ca^{2+} release. It may be possible to use the GST fusion proteins of the SH2 domains of PLCγ as a means to look for such a fertilization-activated kinase in either sperm or egg extracts (Gish et al., 1995; Sillman and Monroe, 1995), and thus to search for upstream components in the signaling pathway at fertilization.

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