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Events during Fertilization of Sea Urchin Eggs

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At fertilization, sea urchin eggs undergo a series of activation events, including a Ca^{2+} action potential, Ca^{2+} release from the endoplasmic reticulum, an increase in intracellular pH, sperm pronuclear formation, MAP kinase dephosphorylation, and DNA synthesis. To examine which of these events might be initiated by activation of phospholipase Cγ (PLCγ), which produces the second messengers inositol trisphosphate (IP_3) and diacylglycerol, we used recombinant SH2 domains of PLCγ as specific inhibitors. Sea urchin eggs were co-injected with a GST fusion protein composed of the two tandem SH2 domains of bovine PLCγ and (1) Ca^{2+} green dextran to monitor intracellular free Ca^{2+}, (2) BCECF dextran to monitor intracellular pH, (3) Oregon Green dUTP to monitor DNA synthesis, or (4) fluorescein 70-kDa dextran to monitor nuclear envelope formation. Microinjection of the tandem SH2 domains of PLCγ produced a concentration-dependent inhibition of Ca^{2+} release and also inhibited cortical granule exocytosis, cytoplasmic alkalinization, MAP kinase dephosphorylation, DNA synthesis, and cleavage after fertilization. However, the Ca^{2+} action potential, sperm entry, and sperm pronuclear formation were not prevented by injection of the PLCγSH2 domain protein. Microinjection of a control protein, the tandem SH2 domains of the phosphatase SHP2, had no effect on Ca^{2+} release, cortical granule exocytosis, DNA synthesis, or cleavage. Specificity of the inhibitory action of the PLCγSH2 domains was further indicated by the finding that microinjection of PLCγSH2 domains that had been point mutated at a critical arginine did not inhibit Ca release at fertilization. Additionally, Ca^{2+} release in response to microinjection of IP_3, cholera toxin, cADP ribose, or cGMP was not inhibited by the PLCγSH2 fusion protein. These results indicate that PLCγ plays a key role in several fertilization events in sea urchin eggs, including Ca^{2+} release and DNA synthesis, but that the action potential, sperm entry, and male pronuclear formation can occur in the absence of PLCγ activation or Ca^{2+} increase.

Key Words: **sea urchin; signal transduction; calcium; fertilization; DNA synthesis; pH; MAP kinase; activation.**

INTRODUCTION

An increase in intracellular free calcium (Ca^{2+}) is a universal feature of egg activation during fertilization (Jaffe, 1983). In sea urchins, this Ca^{2+} increase takes the form of a wave that is initiated at the sperm entry site and passes through the cytoplasm to the opposite pole of the egg (Steinhardt *et al.*, 1977; Eisen *et al.*, 1984); however, the molecular mechanism that links sperm–egg interaction to the initiation of the Ca^{2+} wave is unclear. Recent evidence suggests that, as in mammals (Miyazaki *et al.*, 1992; Xu *et al.*, 1994), *Xenopus* (Nuccitelli *et al.*, 1993), and starfish eggs (Carroll *et al.*, 1997), inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release is responsible for the Ca^{2+} increase during fertilization of sea urchin eggs (Mohri *et al.*, 1995; Lee and Shen, 1998). However, since the experiments in sea urchin eggs have relied on the use of relatively nonspecific inhibitors, and since agents unrelated to the IP_3 pathway, such as cADP ribose (cADPR), cGMP, and ryanodine, can also stimulate calcium release in sea urchin eggs (Whalley *et al.*, 1992; Shen and Buck, 1993), this question deserves further study.

IP_3 and 1,2-diacylglycerol (DAG) are produced from phosphatidylinositol 4,5-bisphosphate (PIP_2) by members of the

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phosphatidylinositol phospholipase C (PLC) enzyme family. There are currently three known members of the PLC family: PLC*β*, *δ*, and *γ*. PLC*β* is activated by the α_q or the *βγ* subunits of heterotrimeric G proteins (Rhee and Bae, 1997). The mechanism by which PLC*δ* is activated is unclear, although there is some evidence that the GTP-binding protein G_h (Feng *et al.*, 1996), or the GTPase-activating protein RhoGAP, may be involved (Homma and Emori, 1995). PLC*γ* activity is regulated by the association of its tandem SH2 domains with a specific phosphorylated tyrosine on a receptor or cytoplasmic tyrosine kinase, resulting in phosphorylation of PLC*γ* (Rhee and Bae, 1997). The SH2 domains of PLC*γ* have been used as specific *in vivo* inhibitors of PLC*γ* function in fibroblasts (Roche *et al.*, 1996; Wang *et al.*, 1998), epithelial cells (Wang *et al.*, 1998), and starfish eggs (Carroll *et al.*, 1997). In starfish eggs, microinjection of the tandem SH2 domains of PLC*γ* completely blocks Ca^{2+} release during fertilization without affecting Ca^{2+} release via PLC*β* (Carroll *et al.*, 1997). Interestingly, the injection of PLC*γ*SH2 or SH3 domains into mouse eggs does not affect sperm-induced calcium release, although the tandem SH2 domains do specifically block calcium release induced by activation of a heterologously expressed PDGF receptor tyrosine kinase, a well-established signaling pathway that includes PLC*γ* (Mehlmann *et al.*, 1998).

A number of egg activation events in addition to Ca^{2+} release may depend upon the activation of a PLC during fertilization. The cytoplasmic pH of sea urchin eggs increases (from ~6.8 to ~7.2) as a result of fertilization (Johnson *et al.*, 1976; Shen and Steinhardt, 1978). As with the Ca^{2+} increase, the exact components of the signaling pathway(s) leading to the pH increase during fertilization are unknown. The direct mediator of the pH increase is thought to be an activated Na^+/H^+ antiporter in the egg membrane (see Shen and Steinhardt, 1979; Payan *et al.*, 1983; Epel, 1990). The Na^+/H^+ antiporter can be stimulated in the absence of fertilization by the treatment of sea urchin eggs with synthetic diacylglycerol or phorbol esters (Swann and Whitaker, 1985; Shen and Burgart, 1986; Lau *et al.*, 1986), leading to the hypothesis that the DAG produced by the action of a PLC during fertilization activates protein kinase C (PKC) which, in turn, regulates the activity of the Na^+/H^+ antiporter. However, Shen and Buck (1990) demonstrated that a 17-amino-acid peptide from the pseudosubstrate domain of PKC completely blocks the pH increase in response to phorbol ester but only partially inhibits the pH increase during fertilization, suggesting that Na^+/H^+ antiporter activation may occur by both PKC-dependent and -independent mechanisms. The PKC-independent mechanism may involve regulation of the Na^+/H^+ antiporter by Ca^{2+}-calmodulin kinase (Shen, 1989). This issue remains to be resolved.

Other later egg activation events, perhaps mediated in concert by Ca^{2+} and pH changes, include protein synthesis and DNA synthesis (Shen and Steinhardt, 1978; Winkler *et al.*, 1980) and the formation of the male pronucleus (Poccia and Collas, 1997). The transformation of the sperm nucleus involves removal of the poreless nuclear envelope, decondensation of chromatin, and reformation of a nuclear envelope with pores, followed by swelling, all of which can occur within 30 min (Poccia and Collas, 1997). Using cell-free sea urchin egg extracts, pH (among other parameters) has been suggested to be important in mediating this transformation (Cameron and Poccia, 1994; Raskin *et al.*, 1997).

The initiation of DNA synthesis after fertilization is another activation event that may be linked to early signaling events. In sea urchins, DNA replication can be stimulated by increasing intracellular free Ca^{2+} in the egg by applying a Ca^{2+} ionophore (Steinhardt and Epel, 1974). However, whether Ca^{2+} release is necessary for the initiation of DNA synthesis is unknown. In particular, the relationships between Ca^{2+}, the MAP kinase signaling pathway, and DNA synthesis need to be examined. In the starfish *Asterina pectinifera* and *A. miniata*, the dephosphorylation of MAP kinase and the down regulation of its kinase activity are necessary and sufficient for the initiation of DNA synthesis following fertilization (Tachibana *et al.*, 1997; Sadler and Ruderman, 1998; see also Picard *et al.*, 1996). Importantly, inactivation of MAP kinase, even in the absence of fertilization, results in the initiation of DNA synthesis (Tachibana *et al.*, 1997; Sadler and Ruderman, 1998). It could be that factors other than, or in addition to, the fertilization Ca^{2+} increase regulate MAP kinase activity. Recent work indicates that fertilization stimulates the dephosphorylation of MAP kinase and a down regulation of MAP kinase activity in sea urchin eggs as well (D. T. Albay and K. R. Foltz, unpublished observations). However, the signaling pathway(s) that leads to inhibition of MAP kinase and the pathway(s) that links MAP kinase to the regulation of DNA synthesis remain to be described.

Part of the reason it has been difficult to identify the components of these pathways has been the lack of specific activators or inhibitors of molecules that may operate during fertilization. Much of the previous work has relied upon the use of pharmacological agents, such as the ionophore A23187 to elevate intracellular free Ca^{2+} or phorbol esters to stimulate PKC activity, but they probably do not faithfully mimic the mechanism by which sperm causes these same events. We have used recombinant proteins as specific inhibitors of PLC*γ* to begin to define the role of this molecule in the activation of sea urchin eggs during fertilization. We find that the increase of intracellular free Ca^{2+} and cytoplasmic pH, dephosphorylation of MAP kinase, initiation of DNA synthesis, and cleavage depend upon the SH2 domain-mediated activation of PLC*γ*. However, the Ca^{2+} action potential resulting from an influx of Ca^{2+} from the surrounding seawater, the entry of the sperm nucleus, and the formation of the sperm pronucleus can occur independent of PLC*γ* activation.

MATERIALS AND METHODS

Obtaining Gametes

Lytechinus pictus were collected from the Santa Barbara channel near Santa Barbara, California, and were maintained at 10°C in tanks with a constant supply of fresh seawater. *Lytechinus variegatus* were obtained from Sue Decker (Miami, FL) and were maintained in a closed seawater aquarium at room temperature. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Spawned eggs were collected into 0.2 μm filtered natural seawater and rinsed three times in 50 ml of filtered natural seawater before use. Spawned sperm were collected "dry" with a Pasteur pipet from the aboral surface of the sea urchin and kept at 10°C. Except where indicated, the dry sperm suspension was diluted 1:2500–1:5000 in filtered natural seawater just before use. Sperm and eggs were used only on the day they were collected.

Microinjection

Quantitative microinjection was performed using mercury-filled micropipets (Hiramoto, 1962; Kiehart, 1982), allowing injection of precisely calculated picoliter volumes into the eggs. Further details of this method are available at http://www2.uchc.edu/~terasaki/injection. Injection volumes were 3% of the total egg volume, except for experiments in which the eggs were injected with IP_3, cGMP, or cADPR (see below). The volumes of the *L. pictus* and *L. variegatus* eggs were calculated to be 700 pl based upon a diameter of 110 μm. Protein concentrations in the stock solutions were adjusted so that eggs were injected with the same volume under control and experimental conditions. Protein injections were made 10–40 min before insemination or 25–125 min before injection of IP_3, cholera toxin, cGMP, or cADPR. The time between protein injection and stimulation by sperm, IP_3, cholera toxin, cGMP, or cADPR did not influence the Ca^{2+} release observed. The concentrations of the injected proteins (listed as final concentrations in the egg cytoplasm) were converted to molarity for more direct comparison; the molarity values given in the text indicate the calculated final molarity of the protein in the egg cytoplasm.

IP_3 was obtained from Calbiochem (San Diego, CA) and dissolved in 100 mM potassium aspartate, 10 mM Hepes, pH 7.0, at a concentration of 1 μM. Eggs were injected with 1 μM IP_3 to either 1 or 2% of the total egg volume, resulting in a final concentration of 10–20 nM IP_3 in the egg cytoplasm. Cholera toxin (complete molecule) was obtained from List Biological Laboratories (Campbell, CA) in a buffer containing 50 mM Tris, 0.2 M NaCl, and 1 mM EDTA, pH 7.5. A 1 mg/ml stock solution of cholera toxin was activated by incubation for 30 min at 35°C with 25 mM DTT before use in the microinjection experiments. A buffer containing 50 mM Tris, 0.2 M NaCl, 1 mM EDTA, pH 7.5, and 25 mM DTT was prepared in parallel for use in control injections.

cGMP and cADPR were obtained from Calbiochem and dissolved in ultrapure water. Eggs were injected with either 100 μM cADPR or 2 mM cGMP to 1% of the total egg volume, resulting in a final concentration of 1 μM cADPR or 20 μM cGMP in the egg cytoplasm.

GST Fusion Proteins

Plasmid DNAs encoding GST fusion proteins of bovine PLCγSH2 (N+C), PLCγSH2 (N), and PLCγSH2 (C) in the vector pGEX2T′6 (Roche *et al.*, 1996) were obtained from S. Courtneidge (Sugen, Inc., Redwood City, CA). Plasmid DNA encoding a human point-mutated PLCγSH2 (N+C) (R586/694K) was obtained originally from P. Huang (Merck Research Laboratories, West Point, PA) and was constructed as described (Carroll *et al.*, 1997). DNA for the GST fusion protein consisting of the tandem SH2 domains from the phosphatase SHP2 in the vector pGEX-2T (Carroll *et al.*, 1997) was originally obtained from T. Pawson (Mt. Sinai Hospital, Toronto, Canada).

GST fusion proteins were prepared as described by Gish *et al.* (1995), purified using glutathione agarose (Sigma Chemical Co., St. Louis, MO) or glutathione Sepharose (Pharmacia Biotech, Piscataway, NJ), dialyzed extensively in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4), and concentrated using a 10-kDa cutoff Ultrafree-4 centrifugal filter (Millipore Corp., Bedford, MA). Protein concentrations were determined using the BCA protein determination assay (Pierce Chemical Co., Rockford, IL) with BSA as the standard.

Ca^{2+} Measurements

The eggs were injected with 10 μM Ca^{2+} green 10-kDa dextran (Molecular Probes, Eugene, OR) alone, or in combination with the indicated GST fusion protein, while being held between two coverslips as described by Kiehart (1982); the eggs were positioned in the chambers so that they were within one egg diameter (~110 μm) of the coverslip edge. This was performed so that sperm access to the eggs was kept relatively constant. The eggs were kept at 16–18°C before, during, and for 10 min after microinjection. To measure the Ca^{2+} green fluorescence of *L. pictus* eggs, the chambers containing the injected eggs were moved to an Olympus BX60 microscope equipped with a photomultiplier. The eggs were fertilized by replacing the entire volume of seawater in the chamber with the indicated sperm dilution; the fertilization and fluorescence recording were done at 20–23°C. Total light intensity gathered by the photomultiplier was collected every 100 ms using the ACE-MCS system, DOS software, Version 1.6X (EG&G ORTEC, Oak Ridge, TN). ACE-MCS data files were converted to Macintosh text files using MCS file conversion software written by Dr. Steven Haddock (Marine Science Institute, UCSB, Santa Barbara, CA). This software is available for download at http://lifesci.ucsb.edu/~haddock. Data were plotted and analyzed using the graphing software KaleidaGraph Version 3.0.2 (Abelbeck Software). Ca^{2+} measurements from *L. variegatus eggs* were made at 18°C, using equipment as described by Carroll *et al.* (1997).

pH Measurements

BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) 10-kDa dextran fluorescence was used to monitor intracellular pH (Shen and Buck, 1990). Eggs were injected with 30 μM BCECF 10-kDa dextran (Molecular Probes) or co-injected with 30 μM BCECF dextran and 250 μg/ml PLCγSH2 (N+C). After incubation for 10 min, BCECF fluorescence was recorded as described above for Ca^{2+} green fluorescence.

Phosphorylated MAP Kinase Immunoblots

L. pictus eggs were injected with 250 μg/ml PLCγSH2 (N+C) or were left uninjected. Unfertilized PLCγSH2 (N+C)-injected eggs and uninjected eggs were removed from the microinjection chamber and placed into separate 0.5-ml microfuge tubes. The remainder of the eggs were inseminated in the microinjection chamber with a

1:5000 dilution of sperm. Twenty to thirty minutes after insemination, the eggs were removed from the injection chambers and placed into 0.5-ml microfuge tubes and collected by brief (~15- to 20-s) centrifugation at 2000*g*. The excess seawater was removed and the number of eggs remaining in the tubes (25–30 per sample) was counted using a stereomicroscope. Gel samples were prepared either by lysing the eggs in 50 mM Tris, pH 8.0, 1% SDS, and 1 μM NaV_3O_4 or by lysing eggs in 1% NP-40, 150 mM NaCl, 10 mM Tris, pH 8.0, 5 μM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml benzamidine, 2 μg/ml SBTI, and 1 μM NaV_3O_4 followed by a 15-min centrifugation at 16,000*g* to pellet insoluble material. The soluble material was transferred to a new tube and used to prepare the sample.

Samples were diluted in Laemmli SDS sample buffer (Laemmli, 1970) and heated at 90°C for 10 min. The proteins were separated by electrophoresis in 10% polyacrylamide Tris–glycine SDS gels and then transferred to nitrocellulose. Polyclonal anti-phosphoMAP kinase antibody (New England BioLabs, Inc., Beverly, MA) was used to identify phosphorylated MAP kinase in the samples. Antibody binding was detected by HRP-conjugated anti-rabbit IgG (Transduction Labs, Lexington, KY) and Enhanced Chemiluminescence (Pierce Chemical Co.).

DNA Synthesis

DNA synthesis was measured using a new technique; the principle of the method is described under Results. *L. variegatus* eggs were injected at 18–20°C with 1 μM Oregon Green 488-5-dUTP (C-7630; Molecular Probes) or co-injected with either 300 μg/ml PLCγSH2 (N+C) and 1 μM Oregon Green dUTP or 300 μg/ml SHP2 (N+C) and 1 μM Oregon Green dUTP (final cytoplasmic concentration). Sperm were added 10 min after microinjection. Eggs were visualized using a confocal microscope (MRC600; Bio-Rad Laboratories, Hercules, CA) with a 40×/1.3 NA Plan Neofluar lens and a Zeiss Axioskop (Zeiss, Inc., Thornwood, NY). Fluorescence of Oregon Green dUTP that was not incorporated into DNA was reduced by photobleaching the live eggs. The eggs were positioned so that a 46 × 31-μm region of egg cytoplasm exposed to the photobleaching was at least 20 μm from the nucleus (see Fig. 6). The confocal laser was set to normal power, and neutral-density filters were removed from the light path. The eggs were scanned on zoom 6, with two sets of 36 passes on the slow scan rate. Images of the eggs before and after photobleaching were taken with laser power set to low, zoom 1, 10% neutral-density filter, and normal scan rate and were saved to the hard disk. Adobe Photoshop 4.0 (Adobe Systems, Inc., Seattle, WA) was used to make Fig. 6.

Observations of Pronuclei

Pronuclei were visualized 20–45 min after insemination in live eggs that had been incubated in seawater containing 5 μg/ml Hoechst 33258 (Molecular Probes). Pronuclei were also visualized using Oregon Green dUTP (see above) or by injection of 70-kDa fluorescein dextran (Molecular Probes). The 70-kDa fluorescein dextran was injected into the oocyte cytoplasm at a final concentration of 200 μg/ml, allowing nuclei to be seen as dark areas that excluded the fluorescent marker (see Cameron and Poccia, 1994; Terasaki, 1994).

RESULTS

Characterization of Intracellular Ca^{2+} Increase during Fertilization of Sea Urchin Eggs

During fertilization of echinoderms, intracellular free Ca^{2+} increases in two discrete phases: a small initial increase due to an influx of Ca^{2+} through voltage-gated Ca^{2+} channels in the egg plasma membrane (a Ca^{2+} action potential), followed by a larger, longer increase resulting from the release of Ca^{2+} from the endoplasmic reticulum (Oberdorf *et al.*, 1986; Terasaki and Sardet, 1991; McDougall *et al.*, 1993; Shen and Buck, 1993; Carroll *et al.*, 1997). The small initial Ca^{2+} increase due to the Ca^{2+} action potential is likely occurring simultaneously with sperm–egg fusion (McCulloh and Chambers, 1992).

L. pictus eggs injected with 10 μM Ca^{2+} green dextran and fertilized exhibited a Ca^{2+} action potential followed by a larger Ca^{2+} increase. The Ca^{2+} action potential appeared as a small but rapid rise in Ca^{2+} green fluorescence that reached a peak and then began to fall (arrowhead in Fig. 1A); the fluorescence did not return to prefertilization levels before the larger Ca^{2+} release from the endoplasmic reticulum began (Fig. 1A). The magnitude of the fluorescence signal due to the Ca^{2+} action potential varied from 7 to 12% of the peak amplitude of the larger Ca^{2+} release. The delay between the rise of the Ca^{2+} action potential and the beginning of the Ca^{2+} release from the endoplasmic reticulum was 13 ± 3 s (mean ± SD; $n = 11$). The Ca^{2+} green fluorescence increased to a peak amplitude of 1.1 ± 0.3, expressed as the ratio of the change in fluorescence after fertilization to the baseline fluorescence in the unfertilized egg; therefore, a doubling in the level of fluorescence after fertilization would be equal to a peak amplitude of 1.

Inhibition of Ca^{2+} Release during Fertilization by the PLCγ Tandem SH2 Domain Fusion Protein

Microinjection of the tandem SH2 domains of PLCγ inhibited Ca^{2+} release from the endoplasmic reticulum after fertilization in *L. pictus* eggs in a dose-dependent fashion. In eggs injected with 125–1000 μg/ml (2.6–21 μM) PLCγSH2 (N+C) fusion protein (Figs. 1B and 1C; Table 1), 14/17 exhibited no Ca^{2+} release after the Ca^{2+} action potential. In 8 of these eggs there was more than one Ca^{2+} action potential, suggesting that they were polyspermic. In 3/17 eggs injected with 125–1000 μg/ml PLCγSH2 (N+C) fusion protein, there was a small and slow increase to an average peak amplitude of 0.09 ± 0.04 (mean ± SD) following the Ca^{2+} action potential, which probably indicated a small Ca^{2+} release from the endoplasmic reticulum.

In eggs injected with 31–62 μg/ml (0.6–1.2 μM) PLCγSH2 (N+C) (Fig. 1D; Table 1), 9/13 exhibited some Ca^{2+} increase beginning an average of 65 ± 73 s (mean ± SD) after the first Ca^{2+} action potential. The peak amplitude of the Ca^{2+} increase (0.2 ± 0.2; mean ± SD) was less than that seen in eggs injected with Ca^{2+} green alone. In many cases, the Ca^{2+} increase was composed of multiple, small increases rather

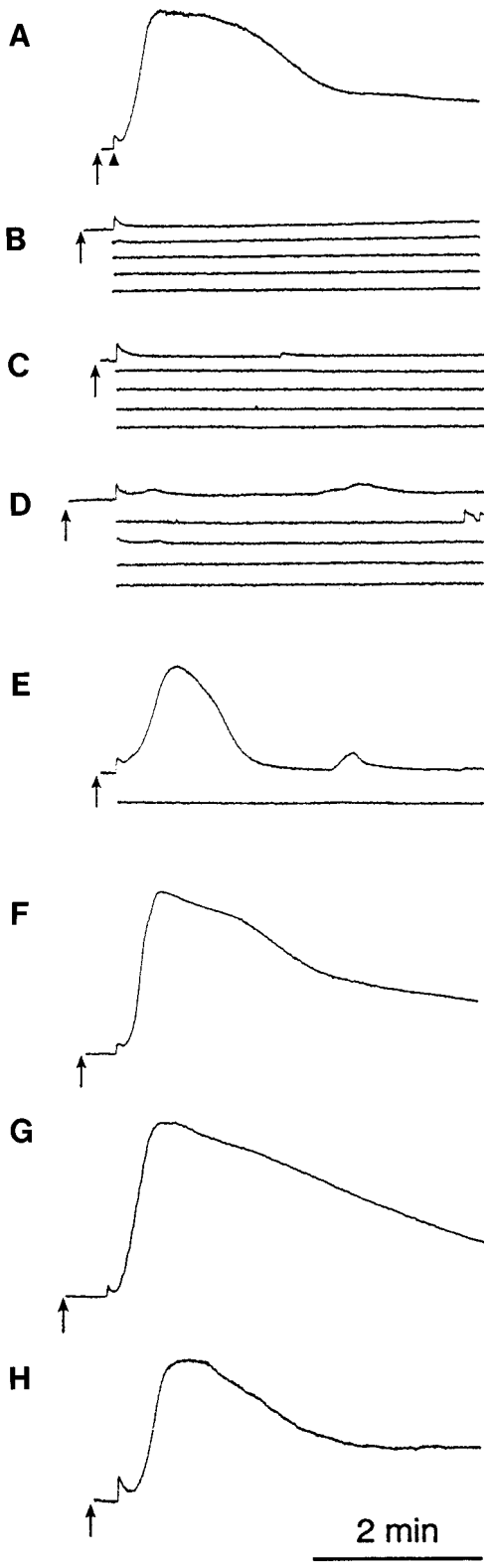

FIG. 1. Concentration-dependent inhibition of Ca^{2+} release by PLCγSH2 (N+C) during fertilization of sea urchin eggs. Eggs were injected with 10 μM Ca^{2+} green dextran only (A) or with 10 μM Ca^{2+} green dextran and the indicated fusion proteins (B–H): (B) 1000 μg/ml PLCγSH2 (N+C). Ca^{2+} increase after the Ca^{2+} action potential was completely inhibited. (C) 125 μg/ml PLCγSH2 (N+C). A second, smaller Ca^{2+} action potential was seen in this egg and may represent an independent sperm–egg interaction. (D) 62 μg/ml PLCγSH2 (N+C). Three Ca^{2+} action potentials occurred in this egg as well as the larger, slower Ca^{2+} increases typical of Ca^{2+} release from internal stores. (E) 15 μg/ml PLCγSH2 (N+C). (F) 5 μg/ml PLCγSH2 (N+C). At this concentration of PLCγSH2 (N+C), Ca^{2+} release during fertilization was normal. (G) 170 μg/ml PLCγ mutant. (H) 1000 μg/ml SHP2 SH2 (N+C). Arrows represent the time of sperm addition. Arrowhead shows the beginning of the Ca^{2+} action potential in (A). Scale bar, 2 min.

than a single increase, and there was more than one Ca^{2+} action potential occurring in the same fertilized egg. In the remaining 4/13 eggs injected with 31–62 μg/ml PLCγSH2 (N+C), more than one Ca^{2+} action potential was seen after fertilization; however, no further Ca^{2+} increase was observed in any of these eggs.

All eight of the eggs injected with 15 μg/ml (0.3 μM) PLCγSH2 (N+C) had significant Ca^{2+} release following the Ca^{2+} action potential (Fig. 1E; Table 1), with a peak amplitude (0.8 $\pm$ 0.2) that was similar to that of control eggs injected with Ca^{2+} green dextran only. The delay between the Ca^{2+} action potential and the beginning of the second Ca^{2+} increase was also the same in these eggs as in control eggs. The eggs injected with 15 μg/ml PLCγSH2 (N+C) had a shorter duration of Ca^{2+} release, measured from the beginning of the second Ca^{2+} increase until the time the fluorescence returned to 50% of the peak amplitude, than eggs injected with Ca^{2+} green only. In three of these eight eggs, there was more than one phase of Ca^{2+} release, rather than the single Ca^{2+} release seen in five of the eight eggs injected with 15 μg/ml PLCγSH2 (N+C) and in all of the controls. At 5 μg/ml (0.1 μM), PLCγSH2 (N+C) had no effect on Ca^{2+} release during fertilization of sea urchin eggs (Fig. 1F; Table 1).

Specificity of the Inhibition by PLCγSH2 (N+C) Fusion Protein

PLCγ activity is regulated by the interaction of its SH2 domains with upstream kinases. As a control, we injected a nonfunctional, point-mutated form of the PLCγ tandem SH2 domain protein (Huang *et al.*, 1995). Each SH2 domain of this mutant carries an arginine (R) to lysine (K) mutation in the FLVRES sequence, which eliminates binding of the SH2 domain to its upstream kinase (Huang *et al.*, 1995). Injection of the mutant protein to 170 μg/ml (3 μM; $n = 5$) final concentration had no effect on the release of Ca^{2+} during fertilization (Table 1; Fig. 1G).

Because several other signaling molecules also use SH2 domains for interacting with other proteins, it is possible that the effect observed with the PLCγSH2 (N+C) fusion

TABLE 1
PLCγSH2 (N+C) Inhibits Ca^{2+} Release during Fertilization of Sea Urchin Eggs

Protein injected	Fraction of eggs with Ca^{2+} release	Delay (s)[a,b]	Duration (s)[b,c]	Peak amplitude[b,d]	*N* (eggs, animals)
Ca^{2+} green only	15/15	13 ± 3	181 ± 41	1.1 ± 0.3	15, 11
125–1000 μg/ml PLCγ (N+C)	3/17	94 ± 132	39 ± 23	0.09 ± 0.04	17, 6
31–62 μg/ml PLCγ (N+C)	9/13	65 ± 73	79 ± 23	0.2 ± 0.2	13, 4
15 μg/ml PLCγ (N+C)	8/8	13 ± 12	107 ± 33	0.8 ± 0.2	8, 2
5 μg/ml PLCγ (N+C)	5/5	14 ± 2	181 ± 22	1.0 ± 0.1	5, 1
170 μg/ml PLC mutant	5/5	11 ± 5	211 ± 90	1.0 ± 0.3	5, 1
1000 μg/ml SHP2 (N+C)	9/9	15 ± 4	210 ± 54	1.0 ± 0.1	9, 4

[a] Delay indicates the time between the rise of the Ca^{2+} action potential and the beginning of the second Ca^{2+} rise. Mean ± SD.
[b] Only measured in eggs defined to have a Ca^{2+} release after the Ca^{2+} action potential; not an average of all the eggs recorded.
[c] Ca^{2+} rise duration is defined as the time from the initiation of Ca^{2+} release after the Ca^{2+} action potential to the time when the fluorescence falls to 50% of the peak amplitude. Mean ± SD.
[d] Peak amplitude represents the ratio of the change in fluorescence after fertilization to the baseline fluorescence in the unfertilized egg. Mean ± SD.

proteins is nonspecific. We therefore injected the tandem SH2 domains of the phosphatase SHP2 as a control for SH2 domain specificity. The amino acid sequences of the SH2 domains of PLCγ and SHP2 are 51% identical when analyzed using the BESTFIT program of the GCG sequence analysis software package (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI). Microinjection of 1000 μg/ml (20 μM) SHP2 SH2 (N+C) had no effect on the Ca^{2+} action potential or on subsequent Ca^{2+} release during fertilization in sea urchin eggs (see Table 1; Fig. 1H). These results indicate that the inhibition seen with the PLCγSH2 (N+C) fusion protein is not due to a nonspecific effect of excess recombinant SH2 domain protein in the egg and that these SH2 domain fusion proteins do retain specificity in the egg.

Single SH2 domains are known to mediate protein–protein interactions by binding to specific phosphorylated tyrosine motifs, but their affinity for binding to relevant targets is less than that of the tandem SH2 domains (Anderson *et al.*, 1990; Rotin *et al.*, 1992; Larose *et al.*, 1995; Roche *et al.*, 1996; Ottinger *et al.*, 1998). The individual N- or C-terminal SH2 domains of PLCγ inhibited Ca^{2+} release during fertilization of sea urchin eggs, although the effect was less than with the tandem N+C domains (Fig. 2; Table 2). Four of seven eggs injected with 250 μg/ml (6.5 μM) PLC (N) exhibited an increase of Ca^{2+} green fluorescence after the Ca^{2+} action potential, but the response was delayed and reduced in amplitude compared with controls. In 3/7 eggs injected with 250 μg/ml PLC (N), no Ca^{2+} increase following the Ca^{2+} action potential was observed. In 4/6 eggs injected with 250 μg/ml (7 μM) PLC (C), some Ca^{2+} increase occurred after the initial Ca^{2+} action potential, while in 2/6 of these eggs no further Ca^{2+} increase after the action potential was observed. In eggs injected with 125 μg/ml (3.3 μM) PLCγ SH2 (N) or (C) individual domains, Ca^{2+} release occurred, but was delayed. At 62 μg/ml (1.6 μM), Ca^{2+} release occurred with normal kinetics and reached a normal peak amplitude (Table 2). These results indicate that, as expected, the individual PLCγSH2 domains do inhibit Ca^{2+} release during fertilization, but they are less effective than the tandem PLCγSH2 domain fusion protein.

To examine the possibility that the PLCγSH2 (N+C) fusion proteins might be interfering with IP_3-induced Ca^{2+} release directly rather than inhibiting PLCγ function, we injected IP_3 into eggs that had been injected with the PLCγSH2 (N+C) fusion protein (Table 3). Using close to the minimal amount of IP_3 needed for Ca^{2+} release in starfish eggs (1–2% injection of 1 μM IP_3; Chiba *et al.*, 1990), we found that injection of IP_3 into sea urchin eggs that had been injected with 250 μg/ml PLCγSH2 (N+C) caused a normal Ca^{2+} release, bypassing the PLCγSH2 (N+C) inhibition ($n = 8$; Fig. 3A). This indicated that the fusion protein is inhibiting a step prior to the production of IP_3 during fertilization.

The possibility that the PLCγSH2 domains might be inhibiting PLCβ was also considered (Table 3). Since PLCβ is activated by heterotrimeric G proteins, we used the G-protein activator cholera toxin as a means of stimulating PLCβ (see Discussion). Sea urchin eggs injected with Ca^{2+}

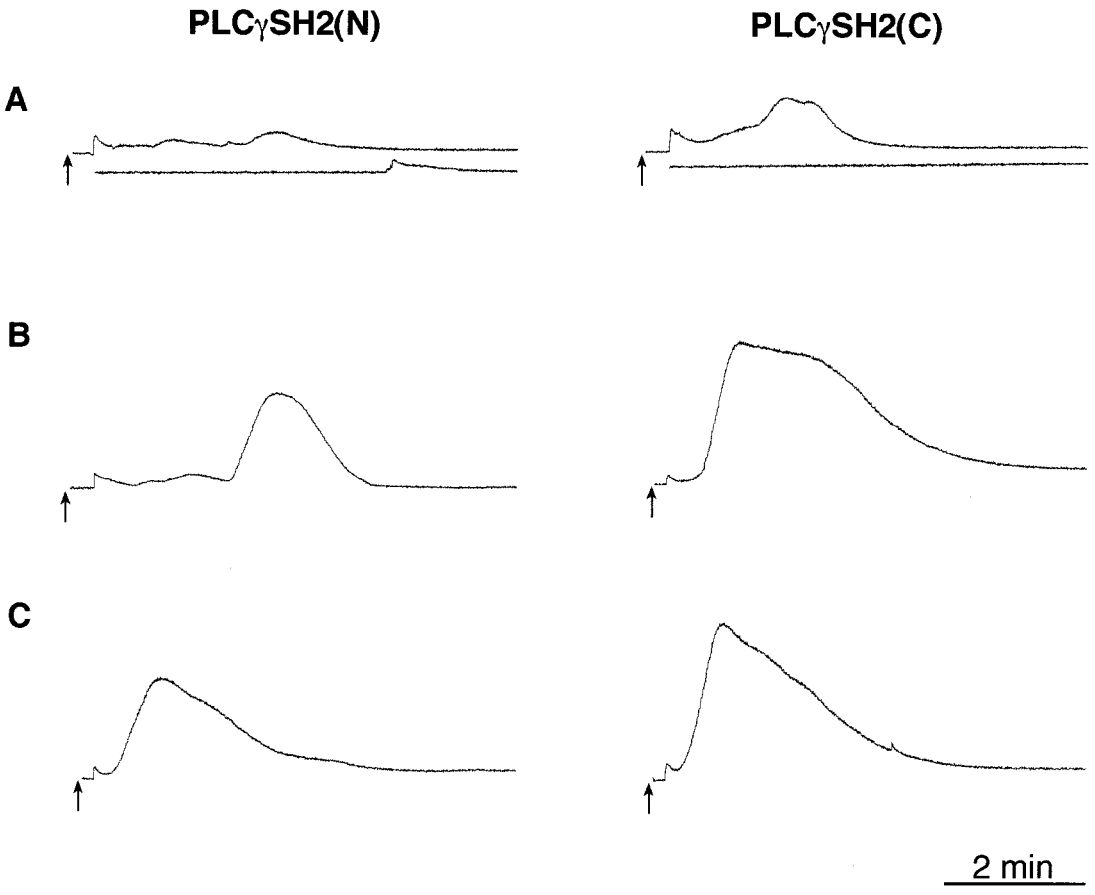

FIG. 2. Individual SH2 domains of PLCγ inhibit Ca^{2+} release during fertilization of sea urchin eggs. Eggs were co-injected with 10 μM Ca^{2+} green dextran and (A) 250 μg/ml of the individual N- or C-terminal PLCγSH2 domain, (B) 125 μg/ml of the individual N- or C-terminal PLCγSH2 domain, or (C) 62 μg/ml of the individual N- or C-terminal PLCγSH2 domain. The column on the left shows records from eggs injected with the N-terminal PLCγSH2 domain, and the column on the right shows eggs injected with the equivalent concentration of the C-terminal PLCγSH2 domain. Sperm were added at the arrows. Scale bar, 2 min.

green dextran responded to injection of 30 μg/ml cholera toxin by releasing Ca^{2+} (Fig. 3B); 250 μg/ml PLCγSH2 (N+C) did not inhibit this response (Fig. 3B). This indicates that the PLCγSH2 domains inhibit PLCγ specifically and do not inhibit G-protein-mediated Ca^{2+} release.

In sea urchin eggs, both cyclic ADP ribose and cGMP have been shown to induce Ca^{2+} release (Whalley *et al.*, 1992; Shen and Buck, 1993; Table 3). Injection of 500 μg/ml PLCγSH2 fusion protein had no effect on Ca^{2+} release in response to either 20 μM cGMP (n = 5; Fig. 3C) or 1 μM cADPR (n = 5; Fig. 3D). These concentrations of cGMP and cADPR are close to the minimal amount needed to cause consistent activation of sea urchin eggs (Whalley *et al.*, 1992; Shen and Buck, 1993). These data provide additional evidence that the PLCγ SH2 domains inhibit PLCγ specifically and do not interfere with the eggs' ability to release Ca^{2+} through alternative pathways.

Inhibition of Cytoplasmic Alkalinization by PLCγ SH2 (N+C) Fusion Protein

BCECF-dextran fluorescence was used to monitor intracellular pH during fertilization in eggs injected with PLCγSH2 domains (Fig. 4). During fertilization of control eggs, BCECF fluorescence increased to a peak amplitude of 0.091 ± 0.022 (n = 8 eggs, 4 animals). Peak amplitude represents the change in BCECF fluorescence intensity divided by baseline BCECF fluorescence in the unfertilized egg. Therefore, a peak amplitude of 0.091 represents a 9.1% change in BCECF fluorescence intensity after fertilization in the control eggs. In the eggs injected with BCECF only, the fluorescence increase began at 1.1 ± 0.4 min after insemination and reached the peak at 5.0 ± 2.2 min (n = 8 eggs from 4 animals). Of seven eggs co-injected with 250 μg/ml PLCγSH2 (N+C) and BCECF, six showed no increase in BCECF fluorescence over baseline. One egg showed a

TABLE 2

Individual N or C Domains of PLCγ Inhibit Ca^{2+} Release during Fertilization of Sea Urchin Eggs to a Lesser Extent than the Tandem PLCγSH2 Domains

Protein injected (μg/ml)	Fraction of eggs with Ca^{2+} release	Delay (s)[a]	Duration (s)[a]	Peak amplitude[a]	*N* (eggs, animals)
PLC (N)					
62	4/4	11 ± 2	101 ± 23	1.0 ± 0.4	4, 1
125	4/4	75 ± 66	105 ± 41	0.5 ± 0.4	4, 1
250	4/7	138 ± 194	84 ± 40	0.4 ± 0.3	7, 2
PLC (C)					
62	4/4	11 ± 3	165 ± 17	1.4 ± 0.4	4, 2
125	4/4	20 ± 5	140 ± 53	1.2 ± 0.4	4, 1
250	4/6	172 ± 178	82 ± 22	0.6 ± 0.5	6, 2

[a] See Table 1.

small increase that began at 0.83 min after insemination and reached a peak amplitude of 0.004 at 1.2 min after insemination. Sperm pronuclei were visible after fertilization in all of the eggs injected with PLCγSH2 (N+C) (Fig. 4C). This demonstrates that the lack of a pH increase in these eggs was not due to a failure of fertilization.

Inhibition of the Dephosphorylation of MAP Kinase after Fertilization by PLCγSH2 (N+C) Fusion Protein

To examine whether PLCγ activation and thus the intracellular Ca^{2+} and/or pH increase is required for the dephosphorylation of MAP kinase at fertilization, we determined whether inhibition of PLCγ activation would prevent MAP kinase dephosphorylation after fertilization. Eggs were microinjected with 250 μg/ml (5.2 μM) PLCγSH2 domain fusion protein and then fertilized. The eggs were lysed and subjected to SDS–PAGE and immunoblot analysis with an antibody specific for phosphorylated MAP kinase (Fig. 5). The PLCγSH2-injected eggs, which did not elevate FEs, retained the phosphorylated form of MAP kinase (Fig. 5, lane 4). Fertilized, uninjected control eggs from the same chamber had very little detectable phosphorylated MAP kinase (Fig. 5, lane 3). Identical results were obtained in four independent experiments.

Inhibition of DNA Synthesis and Cleavage by PLCγSH2 (N+C) Fusion Protein

We next examined whether inhibition of PLCγ activation, by microinjection of the PLCγSH2 (N+C) fusion protein, would also inhibit the initiation of DNA synthesis in *L. variegatus* eggs after fertilization (Fig. 6). In order to assay the occurrence of DNA synthesis in injected eggs, we developed a technique for detecting DNA synthesis in live eggs, thus avoiding the fixation and processing involved in the bromodeoxyuridine method (Nomura *et al.*, 1991; Shilling *et al.*, 1994). For the bromodeoxyuridine method, eggs are incubated with the thymidine analog bromodeoxyuridine, which is subsequently detected with an antibody. We used a fluorescent nucleotide in place of bromodeoxyuridine and detected DNA synthesis by observation of fluorescence in the (pro)nuclei. Unfertilized eggs were injected with Oregon Green dUTP (1 μM) and then inseminated. After injection, the pronucleus of unfertilized eggs appeared brighter than the surrounding cytoplasm when viewed with a confocal microscope, due to the greater amount of dye present in the yolk-free nuclear space (Fig. 6A). After fertilization, the fluorescence in the nucleus increased slightly, as Oregon Green dUTP was incorporated into DNA (Fig. 6C). At 18–20°C, the increase in brightness had occurred by 40 min postinsemination, consistent with the

TABLE 3

PLCγSH2 Fusion Protein Does Not Inhibit Ca^{2+} Release via IP_3, Cholera Toxin, cGMP, or cAMP Injection[a]

	IP_3 (20 nM)[b]	Cholera toxin (30 μg/ml)	cGMP (20 μM)	cADP ribose (1 μM)
Control (Ca^{2+} green only)	0.9 ± 0.4 (*n*=4)	0.6 ± 0.1 (*n*=9)	0.3 ± 0.1 (*n*=5)	0.6 ± 0.2 (*n*=5)
PLCγSH2 (250–500 μg/ml)	1.0 ± 0.4 (*n*=4)	0.8 ± 0.1 (*n*=8)	0.3 ± 0.1 (*n*=5)	0.6 ± 0.2 (*n*=5)

[a] The values reported are the peak amplitude (mean ± SD), which represents the ratio of the change in fluorescence after fertilization to the baseline fluorescence in the unstimulated egg. In all cases, Ca^{2+} release occurred immediately following injection (see Fig. 3).

[b] All concentrations indicate the final concentration in the egg cytoplasm (*n* = number of eggs).

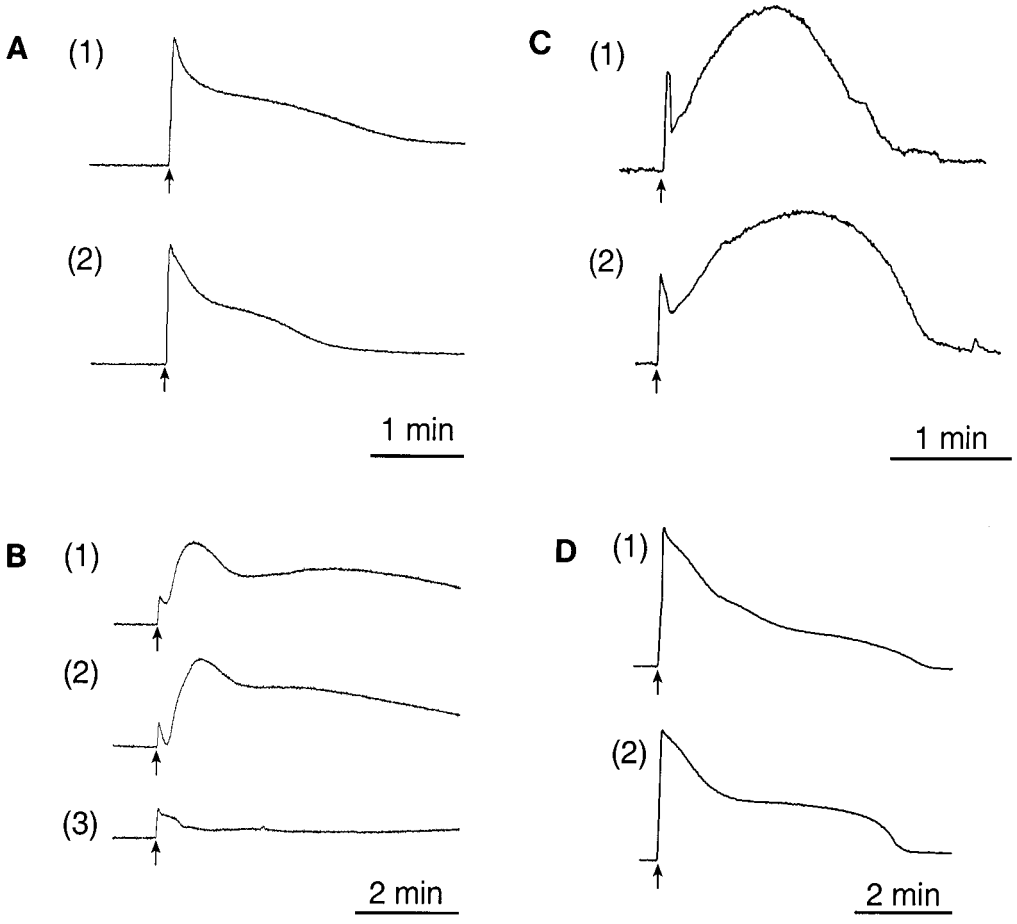

FIG. 3. Specificity of inhibition by PLCγSH2 (N+C) in sea urchin eggs. (A) Eggs were (1) injected with 10 μM Ca^{2+} green dextran or (2) co-injected with 10 μM Ca^{2+} green dextran and 250 μg/ml PLCγSH2 (N+C). The eggs were then injected (arrows) with 20 nM IP_3 (final concentration in the egg cytoplasm). Scale bar, 1 min. (B) Eggs were (1,3) injected with 10 μM Ca^{2+} green dextran or (2) co-injected with 10 μM Ca^{2+} green dextran and 250 μg/ml PLCγSH2 (N+C). The eggs were then injected (arrows) with (1,2) 30 μg/ml cholera toxin or (3) buffer control. The small Ca^{2+} green fluorescence increase seen in the buffer-injected egg (3) is presumably due to a Ca^{2+} increase in response to the pressure of the injection. Scale bar, 2 min. (C) Eggs were (1) injected with 10 μM Ca^{2+} green dextran or (2) co-injected with 10 μM Ca^{2+} green dextran and 250 μg/ml PLCγSH2 (N+C). The eggs were then injected with 20 μM cGMP (final concentration in the egg cytoplasm). Scale bar, 1 min. (D) Eggs were (1) injected with 10 μM Ca^{2+} green dextran or (2) co-injected with 10 μM Ca^{2+} green dextran and 250 μg/ml PLCγSH2 (N+C). The eggs were then injected with 1 μM cADPR (final concentration in the egg cytoplasm). Scale bar, 2 min.

previously reported time course of DNA synthesis in *L. pictus* eggs at 16–18°C (Steinhardt and Epel, 1974). By 60–70 min, fluorescently labeled chromosomes were visible.

Although the increase in nuclear fluorescence was seen consistently, it was not a practical indicator of the increase in DNA synthesis, because the fluorescence increase was small compared with background fluorescence and was detected reliably only by analyzing time lapse recordings in which the focus was kept on the nucleus. In order to eliminate background fluorescence due to free nucleotides, allowing for a practical method of scoring whether DNA synthesis had occurred, eggs were photobleached in a region away from the nucleus. Since free nucleotides can diffuse within the cytoplasm and in and out of the nucleus, the random movement of these molecules into the photobleaching region resulted in the loss of their fluorescence over a period of a few minutes of exposure to the high-intensity light. In 3/3 unfertilized eggs, photobleaching eliminated the Oregon Green fluorescence in the nucleus as well as the cytoplasm, since only free nucleotides were present (Fig. 6B). However, in 9/9 fertilized eggs that were photobleached at 37–83 min after insemination, the nucleus remained bright. This indicated that DNA synthesis had occurred, since nucleotides that had been incorporated into DNA could not diffuse out of the nucleus (Fig. 6D). DNA synthesis occurred normally in 5/5 eggs injected with SH2 domains of the control protein SHP2 (300 μg/ml)

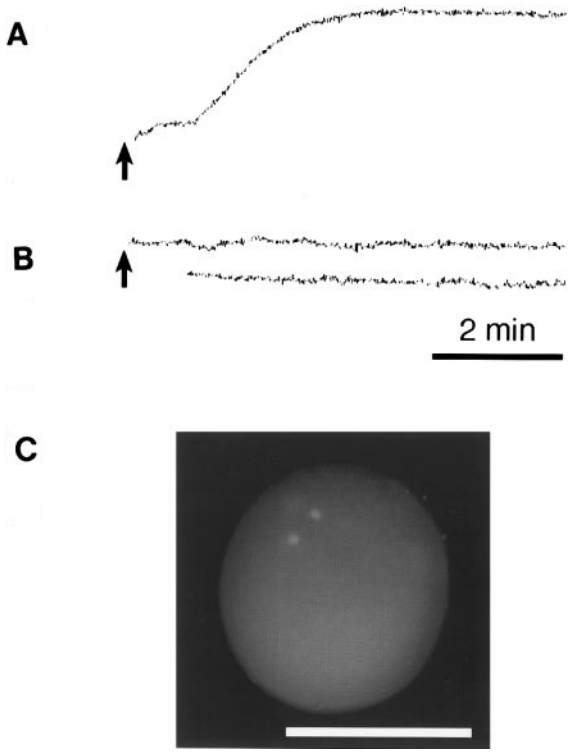

FIG. 4. PLCγSH2 (N+C) inhibits the pH increase after fertilization in sea urchin eggs. (A) An egg injected with 30 μM BCECF dextran and inseminated (arrow). (B) An egg co-injected with 30 μM BCECF dextran and 250 μg/ml PLCγSH2 (N+C) and inseminated (arrow). Scale bar (A, B), 2 min. (C) The PLCγSH2-injected egg from (B) was labeled with Hoechst DNA stain and photographed 45 min after fertilization. The presence of the two decondensed sperm pronuclei confirms that this egg was fertilized. Scale bar, 100 μm.

and photobleached at 49–70 min after insemination (Fig. 6F).

Before testing the effect of PLCγSH2 (N+C) on DNA synthesis, we established that PLCγSH2 (N+C) inhibited Ca^{2+} release in *L. variegatus*. Microinjection of 300 μg/ml (6 μM) PLCγSH2 (N+C) completely inhibited Ca^{2+} release after the Ca^{2+} action potential in 4/4 eggs tested, during a recording period of 30–49 min. Eggs from the same chambers, which had been co-injected with Oregon Green dUTP and 300 μg/ml PLCγSH2 (N+C), were assayed for incorporation of the Oregon Green dUTP into newly synthesized DNA at the conclusion of the Ca^{2+} green recording period.

In 9/9 eggs injected with PLCγSH2 domains (300 μg/ml), inseminated, and then photobleached at 55–74 min after insemination, Oregon Green fluorescence did not remain in the nucleus, indicating that DNA synthesis had not occurred (Fig. 6H). These experiments demonstrate that inhibition of PLCγ activation prevents DNA synthesis at fertilization.

In Oregon Green dUTP-injected eggs that were not injected with the PLCγSH2 (N+C) protein, or were injected with the control protein SHP2 SH2, chromosomes began to appear at 60–70 min after fertilization (18°C), and cleavage occurred normally. No chromosomes were visible in the PLCγSH2-injected eggs when observed at times at which chromosomes were clearly visible in control eggs in the same chamber. No cleavage occurred in the PLCγSH2-injected eggs.

Sperm Entry and Pronuclear Formation in the Presence of PLCγSH2 (N+C) Fusion Protein

As mentioned above, it was possible to observe the nuclei of unfertilized or fertilized eggs using Oregon Green dUTP. Eggs injected with Oregon Green dUTP and observed at 37–83 min after fertilization all showed a single nucleus, ranging in diameter from 12 to 14 μm ($n = 9$) (Figs. 6C and 6D). By 60–70 min after fertilization, condensed chromosomes were clearly visible in these eggs, and cleavage occurred normally. Four of five eggs co-injected with 300 μg/ml control fusion protein SHP2 (N+C) and Oregon Green dUTP and observed 49–70 min after fertilization were also apparently monospermic, because only a single 12- to 14-μm-diameter nucleus was visible in each. Chromosomes began to appear in these eggs at 60–70 min after fertilization, and cleavage occurred normally. In one SHP2 (N+C)-injected egg, there were two nuclei, suggesting that this zygote was polyspermic.

Many of the PLCγSH2 (N+C)-injected eggs were polyspermic, with a range of two to five nuclei being visible in an individual Oregon Green-injected egg at 55–74 min

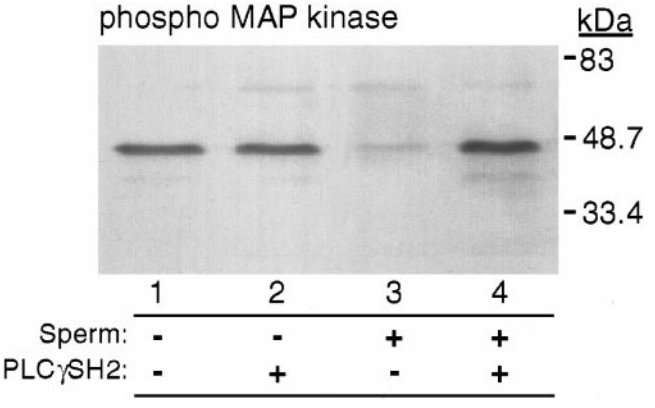

FIG. 5. PLCγSH2 (N+C) inhibits the dephosphorylation of MAP kinase after fertilization. Samples were prepared before and 45 min after fertilization from uninjected eggs and from eggs that had been injected with 250 μg/ml PLCγSH2 (N+C). The proteins were resolved by gel electrophoresis and transferred to nitrocellulose. The nitrocellulose blot was probed with an anti-phosphorylated MAP kinase antibody. Samples of 25–30 eggs were loaded onto the gel as follows: (1) uninjected, unfertilized eggs; (2) PLCγSH2 (N+C)-injected, unfertilized eggs; (3) uninjected, fertilized eggs; and (4) PLCγSH2 (N+C)-injected, fertilized eggs.

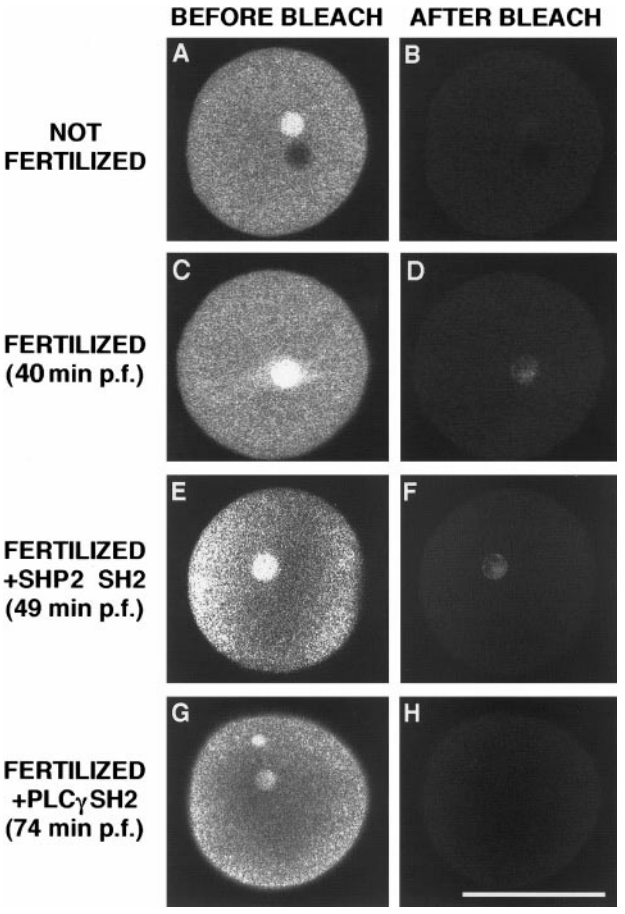

FIG. 6. PLCγSH2 (N+C) inhibits the initiation of DNA synthesis after fertilization. Eggs were injected with 1 μM Oregon Green dUTP (A–D), co-injected with 1 μM Oregon Green dUTP and 300 μg/ml SHP2 SH2 (N+C) (E and F), or co-injected with 1 μM Oregon Green dUTP and 300 μg/ml PLCγSH2 (N+C) (G and H). C–H were fertilized 10 min after injection. After a 35- to 81-min incubation at 18–20°C, the eggs were imaged by confocal microscopy (A, C, E, and G). To visualize DNA synthesis, the Oregon Green dUTP in the egg cytoplasm was photobleached as described under Materials and Methods. The photobleached eggs were again imaged by confocal microscopy (B, D, F, and H). The Oregon Green dUTP is retained only in nuclei in which DNA synthesis has occurred. The dark circle visible just below the nucleus in (A) is an oil droplet that was introduced during microinjection. The oil drops are present in all of the eggs, but depending upon the plane of focus, they are not always visible. Two nuclei are visible in the PLCγSH2-injected egg (G); the smaller, more peripheral, nucleus is the sperm pronucleus. Scale bar, 100 μm.

after insemination (before photobleaching). There were always one large (10–12 μm diameter) nucleus, presumably the egg pronucleus, and multiple smaller nuclei (3–8 μm diameter), presumably sperm pronuclei (Figs. 6G and 6H). Decondensed chromatin could be seen when these nuclei were stained with the Hoechst DNA stain (Fig. 4C). The smaller nuclei were located 2–22 μm from the egg plasma membrane. No chromosomes were visible in the nuclei of these eggs, even when observed at times at which chromosomes were clearly visible in control eggs in the same chamber. No cleavage occurred in the PLCγSH2-injected eggs.

To demonstrate that the nuclei seen in the PLCγSH2-injected eggs were true pronuclei with a reformed nuclear envelope, we injected *L. variegatus* eggs with fluorescein dextran of 70 kDa molecular weight, along with the PLCγSH2 (N+C) fusion protein (300 μg/ml; 6 μM). These eggs were fertilized and then observed with confocal microscopy 28–82 min later. The 70-kDa dextran did not enter the sperm or egg pronuclei (Fig. 7), indicating the presence of a nuclear envelope impermeable to 70-kDa molecules (Cameron and Poccia, 1994; Terasaki, 1994). Similar results were obtained from three eggs. These results indicate that apparently normal sperm pronuclei can form in the absence of PLCγ activation; however, we did not compare the precise kinetics of pronuclear formation in control eggs to eggs injected with the PLCγSH2 fusion protein.

DISCUSSION

PLCγ Mediates Intracellular Ca^{2+} Release at Fertilization in Sea Urchin Eggs

Because the sea urchin egg possesses both the ryanodine receptor (McPherson *et al.*, 1992) and the IP_3 receptor (Parys *et al.*, 1994), it has been proposed that both Ca^{2+} channels contribute to the intracellular Ca^{2+} increase that occurs during fertilization in the sea urchin egg (Galione *et al.*,

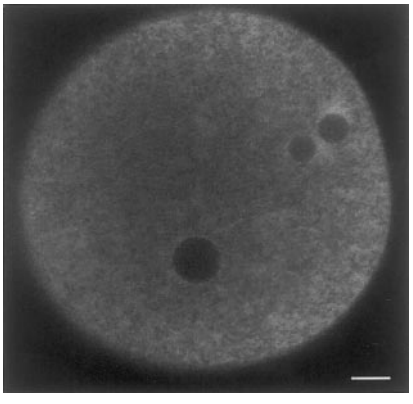

FIG. 7. PLCγSH2 (N+C) does not inhibit sperm pronuclear formation. This egg was co-injected with 300 μg/ml PLCγSH2 (N+C) and 200 μg/ml fluorescein 70-kDa dextran and fertilized 66 min later. After a 74-min incubation at 24°C, the egg was imaged by confocal microscopy. The 70-kDa dextran was excluded from the large egg pronucleus as well as the two smaller sperm pronuclei, indicating the presence of nuclear envelopes. Scale bar, 10 μm.

1993; Lee *et al.*, 1993). Although studies with heparin and pentosan polysulfate, which inhibit IP_3 receptor activation, suggest that Ca^{2+} release during fertilization is mediated primarily by IP_3 (Mohri *et al.*, 1995; Lee *et al.*, 1996; Lee and Shen, 1998), and studies using the PLC inhibitor, U73122, suggest that the IP_3 responsible for this Ca^{2+} increase requires the action of a PLC isozyme (Lee and Shen, 1998), these inhibitors have some nonspecific effects on other cellular processes (Willems *et al.*, 1994; Muto *et al.*, 1997; Lee and Shen, 1998). In addition, U73122 does not distinguish between the different isoforms of PLC (Chen *et al.*, 1994; Dupont *et al.*, 1996; Lee and Shen, 1998). In this paper, we used recombinant SH2 domains of PLCγ as a specific inhibitor of SH2-domain-mediated activation of PLCγ to demonstrate that PLC-mediated generation of IP_3 accounts for Ca^{2+} release at fertilization in sea urchin eggs and to differentiate between the β, the γ, and the δ isoforms of PLC.

Experiments in which recombinant SH2 domains have been used to disrupt PLCγ function *in vivo* indicate that they act as specific inhibitors in mammalian cells (Roche *et al.*, 1996; Wang *et al.*, 1998; Mehlmann *et al.*, 1998). In mouse eggs in which PDGF receptors are experimentally expressed, injection of the PLCγSH2 fusion protein blocks a known PLCγ signaling pathway, Ca^{2+} release in response to PDGF (Mehlmann *et al.*, 1998). Injection of 2 μM, but not 0.2 μM, PLCγSH2 protein is inhibitory, which is reasonable considering that the concentration of endogenous PLCγ in the mouse egg is ~0.1 μM (Mehlmann *et al.*, 1998). The concentration of PLCγSH2 fusion protein required to inhibit this well-established mammalian pathway is similar to that required for inhibition of Ca^{2+} release in sea urchin eggs (~0.6 μM) or starfish eggs (~1.8 μM). The reason for the threefold difference in sensitivity between starfish and sea urchin eggs is not known, but the similar dose dependence between the mammalian and the echinoderm species provides further evidence that the PLCγSH2 domains are acting specifically in the echinoderm eggs.

Evidence that the SH2 domains of PLCγ act as specific inhibitors of PLCγ activation also comes from *in vitro* studies in which the binding of the tandem SH2 domains from different signaling molecules to peptides representing phosphorylated tyrosine activation motifs (TAMs) from biologically relevant sites of various receptors was examined (Ottinger *et al.*, 1998). This study found that each tandem SH2 domain bound with higher affinity to its respective biological TAM than to alternate TAMs and that, in general, tandem SH2 domains bind with higher affinity to their targets than do single SH2 domains (100- to 1000-fold difference in k_d; Ottinger *et al.*, 1998). These peptide binding studies are supported by studies in which binding of single and tandem SH2 domains to full-length receptor tyrosine kinases were compared directly or in cells (Anderson *et al.*, 1990; Rotin *et al.*, 1992; Larose *et al.*, 1995). These results demonstrate that isolated, tandem SH2 domains retain the ability to bind specifically to their natural substrates even when separated from the rest of the PLCγ molecule and that the tandem SH2 domains are more effective than the single SH2 domains. Our finding that the tandem SH2 domains of PLCγ are more effective inhibitors of Ca^{2+} release during fertilization than are the individual N or C SH2 domains is in good agreement with these previous studies. Based upon the lowest molar concentration of fusion protein required to see an effect on the initiation of Ca^{2+} release during fertilization of sea urchin eggs, the tandem PLCγSH2 (N+C) domains were approximately 6-fold more effective than the PLCγSH2 individual (N) or (C) domains.

Other arguments for the specificity of the tandem SH2 domains of PLCγ come from experiments done in this study as well as others. In starfish eggs, microinjection of the same PLCγSH2 (N+C) fusion protein used in the present study inhibits Ca^{2+} release during fertilization, but does not inhibit Ca^{2+} release in response to microinjection of IP_3 or activation of the serotonin 2c receptor, a G-protein/PLCβ-coupled receptor (Carroll *et al.*, 1997). As in the sea urchin egg, microinjection of mutant PLCγSH2 fusion protein that is defective in binding to phosphotyrosine has no effect on Ca^{2+} release during fertilization of starfish eggs (Carroll *et al.*, 1997), providing further support for the specificity of the inhibitory action of the PLCγSH2 domain protein. Further, we found that the Ca^{2+} increase at fertilization was not affected in eggs injected with the tandem N+C-terminal SH2 domains of a control protein, the phosphatase SHP-2, even at concentrations ~30 times higher than that needed to see inhibition with PLCγ SH2 domains. This result demonstrates that the inhibition of Ca^{2+} increase seen with the PLCγSH2 (N+C) fusion protein is not simply a nonspecific inhibition due to the presence of tandem SH2 domains.

In addition, Ca^{2+} release in response to microinjection of sea urchin eggs with small amounts of IP_3 (1–2% injection of the total egg volume with 1 μM IP_3, bringing the final cytoplasmic concentration of IP_3 to 10–20 nM in these eggs) is not affected by the PLCγSH2 (N+C) fusion protein. This demonstrates that the fusion protein-injected eggs are still capable of responding to physiological levels of IP_3 and suggests that the fusion protein acts by inhibiting the production of IP_3, as would be expected if it inhibits the activation of PLCγ.

As further evidence for the specificity of the PLCγSH2 fusion protein as an inhibitor of PLCγ, we showed that the PLCγSH2 (N+C) domains do not inhibit the Ca^{2+} rise in response to injection of cholera toxin (CTX). CTX injection has been found to cause cortical granule exocytosis in sea urchin eggs (Turner *et al.*, 1987), and in the present paper, we show directly that CTX injection into sea urchin eggs causes a Ca^{2+} rise. CTX ADP-ribosylates a specific arginine residue that is conserved in the α subunits of all heterotrimeric G proteins (Hepler and Gilman, 1992; Spiegel, 1994), and for certain α subunits (α_t, α_s, α_{olf}, α_{gust}), this modification can cause constitutive activation and, coincidentally, the release of free βγ subunits (Hepler and Gilman, 1992; Spiegel, 1994; Wieland *et al.*, 1997). How CTX causes a Ca^{2+} rise in sea urchin eggs is not known, but a likely explana-

tion is that it activates $G\alpha_s$, causing the release of $\beta\gamma$ subunits, which activate PLCβ (Lim *et al.*, 1995; Park *et al.*, 1993; Smrcka and Sternweis, 1993). Our finding that PLCγ SH2 domains do not inhibit the Ca^{2+} rise in response to CTX injection indicates that the PLCγSH2 domains do not inhibit G-protein-mediated Ca^{2+} release.

Finally, we find that sea urchin eggs which have been injected with the PLCγSH2 domain fusion protein are still capable of releasing Ca^{2+} in response to injection of cADPR or cGMP, further indicating the specificity of the PLCγSH2 domain as an inhibitor of PLCγ function. These results show that complete inhibition of Ca^{2+} release at fertilization by PLCγSH2 domains cannot be explained by an action that involves joint inhibition of the PLCγ/IP_3 receptor and ryanodine receptor pathways. Our results support the hypothesis that Ca^{2+} release at fertilization in sea urchin eggs is initiated by stimulation of PLCγ and the resulting production of IP_3.

PLCγ Mediates the pH Change, the Dephosphorylation of MAP Kinase, and the Initiation of DNA Synthesis and Cleavage at Fertilization in Sea Urchin Eggs

The cytoplasmic alkalinization that occurs during fertilization of sea urchin eggs is thought to depend upon the stimulation of a Na^+/H^+ antiporter by Ca^{2+}-calmodulin kinase and/or PKC (Shen, 1989; Shen and Buck, 1990), consistent with the involvement of a PLC isozyme in this pathway. PLC enzymes hydrolyze PIP_2 to produce IP_3, which mobilizes Ca^{2+} from the endoplasmic reticulum, and DAG, which activates PKC (Rhee and Bae, 1997). We find that microinjection of the PLCγSH2 (N+C) fusion protein inhibits the increase in cytoplasmic pH that is normally seen during fertilization of sea urchin eggs. This indicates that SH2 domain-mediated activation of PLCγ is required for the intracellular pH increase during fertilization of sea urchin eggs.

The exact contribution of the Ca^{2+} increase and/or pH increase to later egg activation events in the sea urchin is unclear. Several important "late" egg activation events occur after the Ca^{2+} and pH increase in sea urchins, including the dephosphorylation of MAP kinase and the initiation of DNA synthesis and cleavage. Our results from injections of the dominant negative SH2 domains of PLCγ indicate that PLCγ activation is required for MAP kinase dephosphorylation and the initiation of DNA synthesis, but do not distinguish which branch of the PLCγ pathway (IP_3/Ca^{2+} or DAG/PKC) leads to these events.

PLCγ Activity Is Not Required for the Ca^{2+} Action Potential, Sperm Entry, or Sperm Pronuclear Formation

After the sperm nucleus enters the egg, its nuclear envelope breaks down, its chromatin decondenses, and a new nuclear envelope forms (Longo and Anderson, 1968; Poccia and Collas, 1996, 1997). Sperm pronuclei were visible in the fertilized eggs that were injected with the PLCγSH2 fusion protein, indicating that Ca^{2+} release from the endoplasmic reticulum and the cytoplasmic pH increase are not required for sperm entry and pronuclear formation. Inhibition of the pH change at fertilization by use of Na-free seawater caused a pronounced slowing of pronuclear formation in observations made up to 40 min postinsemination (Carron and Longo, 1980; Luttmer and Longo, 1987). In our observations, made at 28–82 min postinsemination, kinetic changes might not have been detected. Alternatively, use of Na-free seawater may cause unknown effects. In particular, Na-free seawater results in a change in cytoplasmic pH to a level lower than that of the unfertilized egg (Rees *et al.*, 1995) and, as discussed by Carron and Longo (1980), Na-free seawater may have effects other than preventing the pH change at fertilization.

Our findings that the Ca^{2+} action potential, the initial entry of the sperm into the egg cytoplasm, and the formation of the sperm pronucleus can occur in the absence of PLCγ activation raise the question of what the initial signaling pathways leading to these events may involve. The action potential results from the opening of voltage-dependent Ca^{2+} channels in the egg plasma membrane (Chambers and de Armendi, 1979), and it has been proposed that the depolarization leading to the opening of these channels may be due to the incorporation of sperm membrane channels into the egg membrane during the process of sperm–egg fusion (McCulloh and Chambers, 1992). The entry of the sperm into the egg cytoplasm is microtubule independent (Bestor and Schatten, 1981) and cytochalasin sensitive (Longo, 1978) and is associated with actin filament accumulation (Tilney and Jaffe, 1980), indicating that actin may mediate this process in some unknown way. The stimulation of sperm nuclear envelope breakdown and chromatin decondensation is due in part to factors present in the egg prior to fertilization, since sperm nuclei injected into the unfertilized eggs undergo partial decondensation (Cothren and Poccia, 1993). However, such nuclei do not complete their decondensation, in contrast to sperm nuclei injected into fertilized eggs, which decondense completely. These results indicate that signaling at fertilization is required for complete male pronuclear formation.

In conclusion, we find that SH2 domain-mediated activation of PLCγ is necessary for the release of Ca^{2+} from the endoplasmic reticulum, cytoplasmic alkalinization, dephosphorylation of MAP kinase, and the initiation of DNA synthesis and cleavage after fertilization in sea urchin eggs. We also find that the Ca^{2+} action potential, entry of the sperm nucleus, and formation of the sperm pronucleus can occur in the absence of PLCγ activation. These findings indicate that multiple signaling pathways are stimulated in sea urchin eggs very early during fertilization. The upstream components of the signaling pathway that activates PLCγ in sea urchin eggs are unknown, but it is likely that a tyrosine kinase is involved, since this is the most common mechanism of regulating PLCγ in all systems studied to

date (Rhee and Bae, 1997). This tyrosine kinase could be derived from the egg or the sperm. Tyrosine kinase activity increases at fertilization (see Kinsey, 1997, for review). The tyrosine kinase inhibitor, genistein, delays release of Ca^{2+} at fertilization (Shen *et al.*, 1999) and causes polyspermy (Moore and Kinsey, 1995). Interestingly, under the conditions used, this inhibitor does not affect the amplitude of Ca^{2+} release; this could result if the inhibitor did not completely block the activity of the tyrosine kinase which targets PLCγ. This may be the case because, although tyrosine kinase activity is decreased in eggs incubated with the inhibitor genistein, some activity remains (Livingston *et al.*, 1998; Shen *et al.*, 1999). Conceivably a kinase that activates PLCγ could also phosphorylate and activate proteins involved in the actin polymerization associated with sperm entry and proteins involved in the formation of the sperm pronucleus.

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