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Role of phospholipase Cγ at fertilization and during mitosis in sea urchin eggs and embryos

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SUMMARY

It is well known that stimulation of egg metabolism after fertilization is due to a rise in intracellular free calcium concentration. In sea urchin eggs, this first calcium signal is followed by other calcium transients that allow progression through mitotic control points of the cell cycle of the early embryo. How sperm induces these calcium transients is still far from being understood. In sea urchin eggs, both $InsP_3$ and ryanodine receptors contribute to generate the fertilization calcium transient, while the $InsP_3$ receptor generates the subsequent mitotic calcium transients. The identity of the mechanisms that generate $InsP_3$ after fertilization remains an enigma.

In order to determine whether PLCγ might be the origin of the peaks of $InsP_3$ production that punctuate the first mitotic cell cycles of the fertilized sea urchin egg, we have amplified by RT-PCR several fragments of sea urchin PLCγ containing the two SH2 domains. The sequence shares similarities with SH2 domains of PLCγ from mammals. One fragment was subcloned into a bacterial expression plasmid and a GST-fusion protein was produced and purified. Antibodies raised to the GST fusion protein demonstrate the presence of PLCγ protein in eggs. Microinjection of the fragment into embryos interferes with mitosis. A related construct made from bovine PLCγ also delayed or prevented entry into mitosis and blocked or prolonged metaphase. The bovine construct also blocked the calcium transient at fertilization, in contrast to a tandem SH2 control construct which did not inhibit either fertilization or mitosis. Our data indicate that PLCγ plays a key role during fertilization and early development.

Key words: Phospholipase C gamma, Fertilization, Egg, Tyrosine phosphorylation, Calcium, Sea urchin

INTRODUCTION

The quiescent unfertilized sea urchin egg waits in G_1 of the first mitotic cell cycle. This arrest is overcome at fertilization by a propagated rise in the intracellular free calcium concentration ($[Ca^{2+}]_i$) that is necessary and sufficient for activation of the egg's metabolism (Whitaker and Steinhardt, 1985; Berger, 1992). This first large increase in intracellular $[Ca^{2+}]_i$ is followed by other calcium transients that are correlated with the succeeding cell-cycle events: pronuclear migration, nuclear envelope breakdown, metaphase-anaphase transition and cytokinesis (Poenie et al., 1985; Steinhardt and Alderton, 1988; Twigg et al., 1988; Ciapa et al., 1994; Wilding et al., 1996; Groigno and Whitaker, 1998). The $[Ca^{2+}]_i$ signals are due to release of calcium from intracellular stores (for review see Shen, 1995). However, mitotic cell-cycle transients are induced by an endogenous mechanism, a fundamental difference from the fertilization $[Ca^{2+}]_i$ signal that is generated at the plasma membrane level. The mechanisms that underlie both types of calcium signals are incompletely understood.

Sea urchin eggs can be activated after microinjection of inositol trisphosphate ($InsP_3$: Whitaker and Irvine, 1984; Swann and Whitaker, 1986) or after treatment with phorbol esters (Nishizuka, 1986 for review) that mimic the effects of diacyglycerol (DAG) in the egg (Swann and Whitaker, 1985; Ciapa et al., 1988, 1989). Measurements of polyphosphoinositide (PPI) turnover and $InsP_3$ production (Turner et al., 1984, 1986; Ciapa and Whitaker, 1986; Ciapa et al., 1991; Lee and Shen, 1998) strongly suggest that PPI metabolism is activated at fertilization. Besides the $InsP_3$ receptor, the activation of ryanodine receptor by cADPr also contributes to the $[Ca^{2+}]_i$ rise at fertilization of sea urchin egg (Galione et al., 1993; Lee et al., 1993). Nonetheless, activation of the $InsP_3$ receptor may be the primary event, since Mohri et al. (1995) obtained a complete inhibition of calcium release at fertilization using pentosan polyphosphate, an inhibitor of the $InsP_3$ receptor. However, like heparin, pentosan polyphosphate

is not specific for the $InsP_3$ receptor (Bezprozvanny et al., 1993). More recently, it was shown that U73122, an inhibitor of PLC, blocked the sperm-induced calcium release in the sea urchin egg (Lee and Shen, 1998). Given these results, it is likely that the production of $InsP_3$ by activation of PLC is turned on as sperm and egg interact and is the primary event. However, whether PLC stimulation occurs alone or whether the sperm also independently triggers another pathway, such as that involving cADPR, is still debated. Moreover, U73122 has also been shown to block cGMP-mediated calcium release in sea urchin eggs (Lee et al, 1998), a mechanism that is thought to be $InsP_3$-independent (Whalley et al, 1992; Galione et al, 1993), suggesting that the inhibitor cannot be used to distinguish these two pathways.

Recently, we suggested that transient increases in the production of $InsP_3$, generated by cyclic oscillations in the turnover of PPI, might also be the trigger for the mitotic $[Ca^{2+}]_i$ transients in the sea urchin embryo (Ciapa et al., 1994; Pesando et al., 1995). Mitotic transients were absent in eggs microinjected with heparin, the $InsP_3R$ antagonist (Ciapa et al., 1994). Therefore, PLC activation might be involved not only in the awakening of egg metabolism at fertilization, but might also govern the transition through the different cell cycle check points (Whitaker and Patel, 1990). The question, then, is the identity of the PLC that is activated to generate $InsP_3$ at these different points in the cell cycle.

Several PLC isoforms have been identified and can be divided into three types β, γ and δ (Lee and Rhee, 1995; Rhee and Bae, 1997). The regulation of PLCδ still remains unclear but results suggest that this PLC isoform is the most sensitive to calcium. It is not known whether this PLC isoform is present in eggs. PLCβ is activated by heterotrimeric G-proteins and data indicate that it could play a role at fertilization in invertebrate eggs. For example, in starfish oocytes, expression of receptors known to release calcium by a G-protein/PLCβ pathway allows intracellular calcium release when the corresponding agonist is applied (Shilling et al., 1994). This suggests that functional PLCβ and corresponding G-proteins are present and can be stimulated. Furthermore, microinjection of GTPγS, an activator of G-protein, triggers fertilization-like responses (Turner et. al., 1986) including the rise in $[Ca^{2+}]_i$ and intracellular pH (Crossley et al., 1991) that can be abolished by U73122 (Lee and Shen, 1998). However, the ability of this inhibitor to distinguish PLCβ from PLCγ has been questioned (see Lee and Shen, 1998) and the fact that microinjection of GDPβs did not inhibit the fertilization $[Ca^{2+}]_i$ rise (Whitaker et al., 1989) argues against PLCβ.

PLCγ contains two src homology-2 (SH2) domains and one SH3 domain that govern protein-protein interactions. The SH2 domains allow the interaction of PLCγ with phosphotyrosine residues of receptor and nonreceptor tyrosine kinases. In most of cases, PLCγ is activated by tyrosine phosphorylation. Tyrosine kinase activity is stimulated in sea urchin eggs during the latent period (that is, before the fertilization $[Ca^{2+}]_i$ signal: Ciapa and Epel, 1991), suggesting the likely presence of a signalling pathway involving tyrosine kinase and PLCγ. Similar experiments to those described above for PLCβ have also been performed to reinforce the hypothesis that PLCγ could be involved in the generation of the fertilization calcium wave. For example, application of EGF and PDGF in eggs that express the tyrosine kinase receptors of these agonists leads to a release of intracellular $[Ca^{2+}]_i$ that does not occur when the expressed receptors are point-mutated and cannot activate PLCγ (Shilling et al., 1994). It has also recently been reported that injection of a glutathione-S-transferase (GST) fusion protein containing the two SH2 domains of a bovine PLCγ abolished the fertilization $[Ca^{2+}]_i$ signal in starfish eggs (Carroll et al., 1997). Finally, a role of PLCγ at fertilization has been proposed in mouse oocytes, which can be activated by injection of a truncated c-kit, a receptor tyrosine kinase expressed in the sperm (Albanesi et al., 1996) that activates PLCγ (Sette et al., 1997, 1998). From all these results, it is clear that the roles of PLCβ and of PLCγ need to be distinguished.

Sea urchin eggs contain a 150 kDa protein that cross-reacts with commercial anti-PLCγ antibodies (De Nadai et al., 1998a,b). We therefore undertook the present study to investigate the role of PLCγ at fertilization of sea urchin eggs. By using a reverse-transcriptase PCR (RT-PCR) based strategy, we have demonstrated that sea urchin eggs have mRNA coding for PLCγ. A GST-fusion protein containing the SH2 domains of PLCγ was produced. Detection with antibodies raised against this protein showed that PLCγ is expressed in the unfertilized egg. We report here the effects on fertilization and early development of microinjection of this GST-PLCγ fusion protein and of its bovine homologue into eggs.

MATERIALS AND METHODS

Handling of gametes

Gametes were collected from sea urchins *Paracentrotus lividus* or *Lytechinus pictus* and prepared as described previously (Ciapa et al., 1989; Ciapa and Epel, 1991). Eggs were kept in filtered sea water (FSW) at 13°C and sperm was stored dry at 4°C until used.

Cloning of PLCγ cDNA fragments from sea urchin

cDNA fragments coding for sea urchin PLCγ were isolated by RT-PCR. Total RNA was prepared from the unfertilized eggs of *Paracentrotus lividus* as described by Lepage and Gache (1990) using the method of Cathala et al. (1983). Reverse transcription was carried out using oligo(dT) (15) and the Super Script II polymerase. For PCR amplification, we used several degenerate primers: a forward primer, X1, encoding amino acid sequence HNTYLTGDQ (positions 335-343 in the human PIP4 sequence) and two reverse primers, S3, encoding ES(A,E)TF(V,P)(G,N)DY (positions 587-595) and S6, encoding WW(R,K,I)GDYG(G,T) (positions 828-835). Conditions for amplification with the Taq polymerase were: 95°C for 5 minutes, followed by 40 cycles at 95°C for 2 minutes, 40°C for 2 minutes and 63°C for 3 minutes, and then 63°C for 10 minutes. Amplification using X1 and S3 produced fragment I, which had the expected length. Due to the presence of an internal restriction site used for cloning, only the 3′ part was cloned and sequenced. Sequence information was used to synthesize the non-degenerate forward primer S8 encoding (DGTFLVR) just upstream of the S3 sequence. Amplification using S8 and S6 produced fragment II. Amplification with X1 and S6 produced fragment III. Fragments II and III were cloned in Bluescript by blunt ligation and sequenced. Fragment II was used to construct a fusion protein to raise antibodies.

Construction and purification of GST-PLCγ fusion protein from sea urchin

cDNA fragment II was inserted into the glutathione S-transferase (GST) gene fusion vector pGEX-4T3 (Pharmacia Biotech) using the restriction sites *Eco*RI and *Xho*I. *E.coli* BL21 cells (Stratagene) were

then transformed with the construct. Bacteria were grown at 37°C and induced with 0.1 mM IPTG for 3 hours in order to express the recombinant protein. The culture was then centrifuged (4000 rpm, 10 minutes) and the pellet resuspended in a buffer comprising 25 mM Hepes (pH 7.6), 12.5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, 2 mg/ml aprotinin, 1 mg/ml leupeptin and 0.1 mM PMSF. All subsequent steps were carried out at 4°C. Bacteria were lysed at high pressure in a French press and incubated for 1 hour with 1% NP-40 before centrifugation at 12000 rpm for 30 minutes. Aliquots of the supernatent and pellet were analysed by SDS-PAGE. The supernatent (containing soluble protein) was then applied to glutathione-sepharose 4B (Pharmacia Biotech) for purification. The glutathione-sepharose 4B was first equilibrated with the same buffer as described above. The loaded column was then washed with 25 mM Hepes, pH 7.6, before elution with 10 mM reduced glutathione in 50 mM Tris, pH 8.0. Glutathione was removed by dialysis against 0.5× PBS. Collected fractions were analyzed by SDS-PAGE and the more concentrated fractions were pooled and lyophilized. GST alone was purified in the same way (produced by expression of the native pGEX vector) for use as a control. Identity of GST and fusion protein were confirmed by western blot with an anti-GST antibody.

Expression and purification of GST-PLCγ fusion protein from bovine

Plasmid DNAs encoding the bovine PLCγ-SH2(N+C) and murine SYP-SH2(N+C) were obtained from S. Courtneidge (Sugen, Inc. Redwood City, CA). The fragments were excised from the original constructs and subcloned into pGEX6p1 (Amersham-Pharmacia Biotech, UK) before being used to transform BL21-DE3 bacterial cells for expression of the GST-fusion proteins. An overnight culture of the bacteria was diluted 1:10 and grown for 30 minutes at 37°C before being induced with 0.05 mM IPTG for 2 hours. After harvest and lysis, purification was carried out on glutathione sepharose (Amersham-Pharmacia Biotech) according to the manufacturers protocol. Subsequent to purification, the glutathione elution buffer was substituted with PBS and the proteins were concentrated using Microcon® 10 microconcentrators (Amicon, Inc. MA) to 10 mg/ml (PLCγ-SH2) and 20 mg/ml (SYP-SH2). Protein concentrations were determined using the BCA reagent assay (Pierce Chemical Co, Illinois). Constructs were injected to a volume of 0.1-1% egg volume with a pipette concentration of 5-20 mg/ml. The molecular weight of the tandem SH2 domain was calculated to be 50 kDa. Thus a typical 0.5% injection volume, at a pipette concentration of 10 mg/ml results in a cytoplasmic concentration of 1 μM (50 μg/ml).

Immunoprecipitations and western blots

After induction, bacterial proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with Ponceau red. The band corresponding to the insoluble GST/PLCγ protein was cut and used to immunize rabbits (Eurogentec). A polyclonal serum was obtained after a 3 months immunization protocol.

Immunoprecipitates (Ip) were prepared using eggs from the sea urchin *Paracentrotus lividus* as previously described (Ciapa and Epel, 1991). 1 ml samples containing around 10 mg protein/ml were incubated 4 hours at 4°C with rabbit polyclonal serum raised against the sea urchin GST-PLCγ construct (2 μl/sample) and then 30 minutes with a 2nd antibody linked to agarose (Sigma). IPs were treated as described in Ciapa and Epel (1991), run on a 10% SDS polyacrylamide gel and then transfered to nitrocellulose membrane. The membrane was blocked with 5% skimmed milk for 1 hour and incubated for 3 hours with the rabbit polyclonal serum raised against the GST-PLCγ (sea urchin) construct. After washing, the blot was incubated for 1 hour with a horseradish peroxidase-linked anti-rabbit secondary antibody (Biorad) before being detcted by ECL (Amersham). All immunoprecipitates contained proteins of approximately 60 kDa, corresponding to the primary antibody.

Microinjection procedures

In microinjection experiments with the sea urchin contruct, eggs from *Paracentrotus lividus* were dejellied by successive filtrations, treated for 1 minute with FSW pH 5.0, and then rinsed with FSW pH 8.0. Eggs were stuck to Petri dishes coated with 1% polylysine. The fusion protein was diluted to a final concentration of 2 mg/ml in an injection buffer (480 mM KCl, 0.1 mM EGTA, 20 mM Pipes, pH 7.0) containing 0.4 mM carboxyfluorescein. The microinjection system used resulted in the injection of approximately 1% of the egg volume (Rees et al., 1995). Elevated fertilization envelopes, formation of the streak stage and rates of cleavage were scored by observation using a light microscope.

Microinjection experiments with the bovine construct and the 13-mer peptides were performed using eggs from the sea urchin *Lytechinus pictus*. 0.1-1% egg volume of up to 10 mg/ml pipette concentration was microinjected using pressure pulses (Swann and Whitaker, 1986). All cell cycle data regarding the use of the bovine tandem SH2 domain and the peptides has been normalized such that the uninjected embryos underwent Nuclear Envelope Breakdown (NEB) at 70 minutes postfertilization. This was to allow comparison and collation of data from different experiments and took into account any biological variation between females and any differences in experimental temperature.

Calcium measurements

Changes in intracellular free calcium concentration after microinjection of the bovine construct into *L. pictus* eggs were monitored using calcium green dextran dye and confocal microscopy as described previously (Wilding et al., 1996). Confocal sections were imaged at 128×128 pixel resolution. The images displayed were low-pass filtered ten times using a 3×3 filter matrix.

Calcium was also monitored with fura-2 dye (10 μM), using a CCD-based imaging system (Princeton Instruments, UK) capable of simultaneous measurements of up to ten eggs.

RESULTS

Presence of PLCγ mRNA in the unfertilized sea urchin egg

All isoforms of PLC share highly conserved regions, designated X and Y, that constitute the PLC catalytic domain, whereas only PLCγ isoforms have two SH2 and one SH3 domains. Therefore, from alignment of published PLCγ amino acid sequences, we designed degenerate primers corresponding to conserved sequence blocks within region X, first SH2 domain and SH3 domain. Using various combination of primers (see Materials and Methods), we have cloned several overlapping cDNA fragments (Fig. 1A). The amino acid sequences encoded by these fragments strongly resemble the expected PLCγ sequences. The longest fragment cloned (fragment III) is 1515 bp long. It codes for a 505 amino acid peptide, which comprises most of the X domain (except for the first 15 amino acids), the two SH2 domains and a large part of the SH3 domain (except for the 11 last amino acids). Alignment of the predicted amino acid sequence from fragment III with that of human and bovine PLCγ is shown in Fig. 1B. The sea urchin sequence has about 55% identity and 70% similarity with the vertebrate sequences (with 3% gap). However, crucial residues in the extended hydrophobic groove binding region characteristic of the PLCγ C-terminal SH2 domain (Pascal et al., 1994) are very highly conserved (Fig. 1). This region is absent in otherwise homologous SH2 domains (for example those of Syp phosphatase) that also recognise the pTyr-hydrophobic-X-hydrophobic motif (Pascal et al., 1994). Similarity is of the

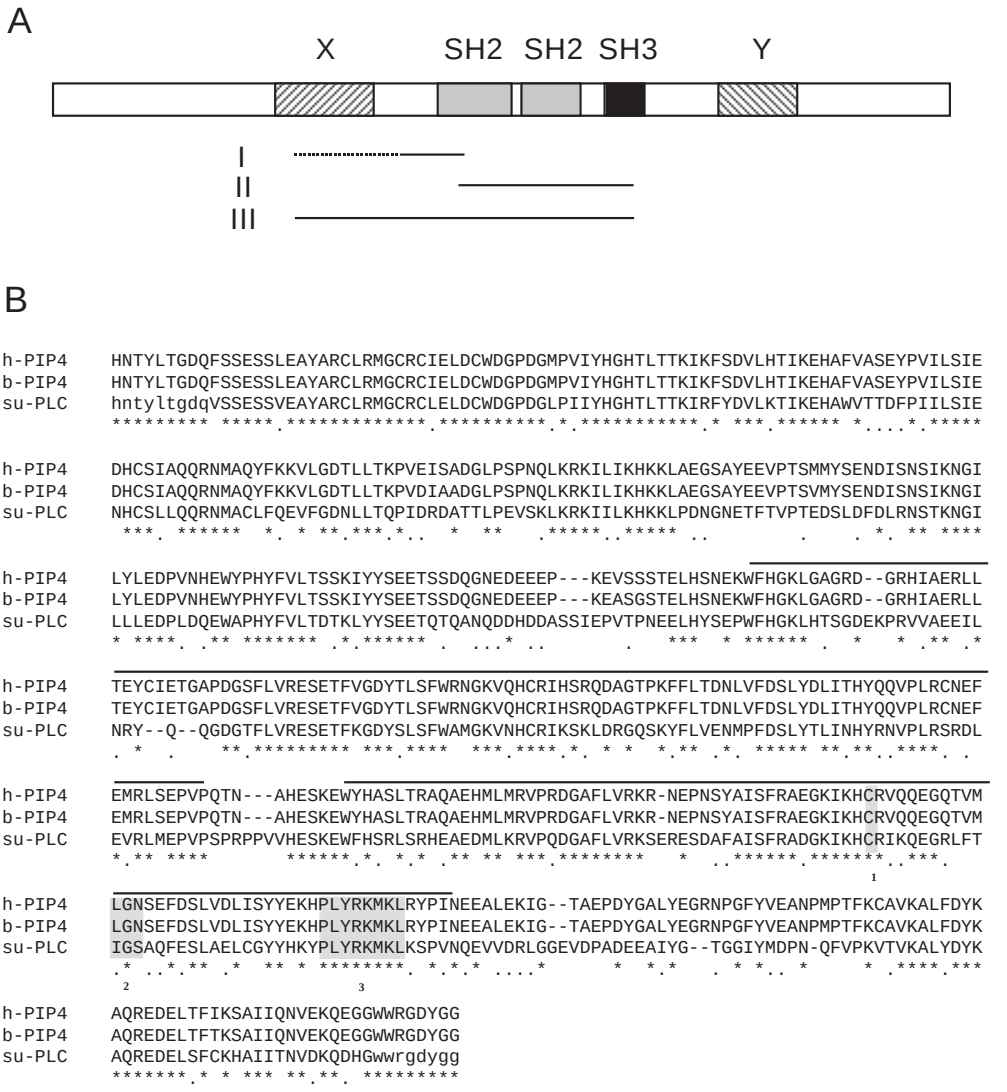


Fig. 1. Cloning of the sea urchin PLCγ. (A) Domain organisation of PLCγ and RT-PCR cloning of cDNA fragments from PLCγ of the sea urchin *Paracentrotus lividus*. I, II and III designate three overlapping fragments amplified from RNA extracted from unfertilized sea urchin eggs. The left part of fragment I (dotted line) was lost during the cloning procedure. (B) Similarities between sea urchin and vertebrate PLCγ. Alignment of partial sequences from human (h), bovine (b) and sea urchin *P.lividus* (su, fragment III), using Clustal w. Positions with identical (*) or similar (.) residues in the three sequences are indicated. The two SH2 domains are indicated by the solid line above the sequence. Amino acids coded for by the PCR primers are in lower case. The crucial residues that constitute the extended groove binding region of the PLCγ C-terminal SH2 domain (Pascal et al. 1994) are indicated by shading and are highly conserved. Highlighted are (1) the cysteine residue that opens out the groove, (2) the hydrophobic leucine/isoleucine residue and the EF loop that together with (3) the BG loop and a second leucine, coordinate the deep binding of proline from the YIIP recognition motif. The partial nucleotide sequence of the sea urchin PLCγ will appear in the EMBL, GenBank and DDJB Sequence Databases under the accession number AJ012336.

same order for the SH2 domains, the second SH2 domain being slightly better conserved (SH2.1: 58% identity, 68% similarity, 5% gap; SH2.2: 59% identity, 81% similarity, 1% gap). The X and SH3 domains appear highly conserved (Fig. 1B).

Purification of GST-PLCγ fusion protein

cDNA fragment II was inserted in frame downstream of the GST coding sequence. The construct was used to transform competent *E. coli* BL21 cells. After induction, the bacterial proteins were analysed on SDS-PAGE (Fig. 2A). A Coomassie blue staining of the gel revealed that the protein of interest was produced (lane TI) but was poorly soluble. 10 litres of bacterial culture were then necessary to purify 100 μg of this fusion protein. The 60 kDa soluble protein and the 30 kDa GST control were purified on a glutathione-sepharose column (lane P and lane C, respectively). Western blotting with an anti-GST antibody (not shown) indicated that this protocol was more efficient than one that involved treatment of inclusion bodies with 8 M urea; this induced protein degradation during renaturation.

Immunodetection of PLCγ in sea urchin eggs

Proteins from eggs of *P. lividus* were immunoprecipitated with the polyclonal serum produced using the GST/PLCγ protein. Separation by SDS-PAGE followed by western blot using the same serum (Fig. 2B) permitted the detection of a 150 kDa protein in unfertilized (lane 3) and fertililized (lane 4) eggs. An immunoprecipitation control was performed with rabbit preimmune serum followed by a western blot with the polyclonal serum containing anti-GST/PLCγ. No signal was detected on blotting the immunoprecipitates with anti-phosphotyrosine antibodies (not shown).

Role of PLCγ at fertilization and during mitosis tested with the sea urchin tandem SH2 construct

The GST/PLCγ fusion protein was lyophilized and solubilized in an injection buffer (Ciapa and Epel, 1996). The pipette concentration was 2 mg/ml, and 1% egg volume was injected into *P. lividus* eggs, giving a final cytoplasmic concentration of 20 μg/ml. We observed that injection of the fusion protein did not induce any spontaneous activation (Fig. 3). At this concentration, fertilization was not altered in the injected eggs, since elevation of the fertilization envelope occurred, just as it did in eggs injected with the GST alone (not shown).

However, although eggs injected with a buffer containing

GST alone cleaved normally, a large proportion of eggs injected with GST/PLCγ fusion protein did not divide. We observed that most of them remained at metaphase stage, some showed abnormal division and a small proportion showed some cytolysis (Fig. 3). These results suggested that PLCγ might play an important role during mitosis.

Effects of bovine tandem SH2 construct and its control on fertilization

Because of the difficulty in obtaining the sea urchin tandem SH2 construct at high concentration, we used a bovine PLCγ1 tandem SH2 construct that shows close homology with the sea urchin sequence (Fig. 1). This construct has previously been shown to block the fertilization calcium transient in starfish oocytes (Carroll et al., 1997) and to prevent PDGF-induced activation of NIH 3T3 cells (Roche et al., 1996). Fig. 4 shows that the construct prevents the calcium transient at fertilization. Fertilization envelope (FE) elevation is blocked completely at a cytoplasmic concentration of 1 μM (50 μg/ml; Fig. 4A). The murine Syp phosphatase tandem SH2 domain containing the same generic SH2 domain FLVRES-binding sequence but lacking the PLCγ-specific targetting sequences was used as a control (Roche et al., 1996) and was ineffective at a 2-fold higher concentration. FE elevation was comparable to controls at a concentration of 50 nM PLCγ-SH2, while 100 nM concentrations prevented full or partial FE elevation in about half the eggs. The magnitude of the fertilization calcium transient was halved at concentrations of 100-300 nM and blocked completely at 1 μM. (Fig. 4B). The latency of the fertilization response was also affected, increasing 3-fold at a concentration of 100-300 nM PLCγSH2 and 20-fold at a concentration of 600 nM (Fig. 4C). These data indicate that, as proposed in starfish oocytes (Carroll et al., 1997), the interaction of PLCγ with phosphotyrosine via its SH2 domains is essential for generation of the fertilization calcium signal. The lengthening of the latent period indicates that PLCγ is involved in the early stages of the signal transduction response. We found the bovine PLCγSH2 construct to be 20 times more effective in sea urchin than was reported in starfish (Carroll et al., 1997).

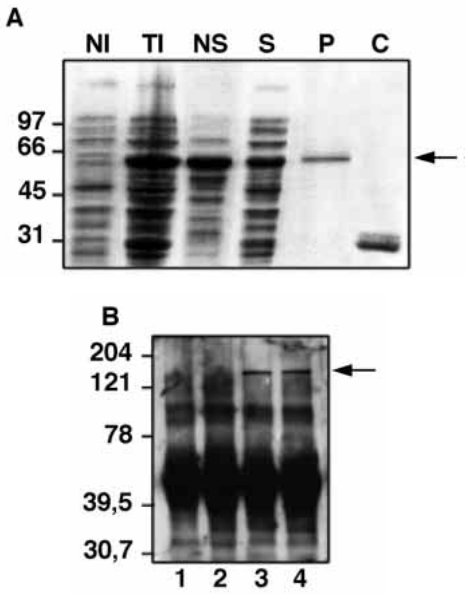


Fig. 2. Production of the GST/PLCγ and detection of PLCγ in sea urchin eggs (*P. lividus)*. (A) Identification of GST/PLCγ after SDS-page and staining with Coomassie Blue. NI, total bacterial proteins before induction; TI, total bacterial proteins after 4 hours of 0.1 mM IPTG induction at 37°C; NS, nonsoluble proteins; S: soluble proteins; P, protein purified on gluthatione-sepharose beads and eluted with 10 mM of free gluthatione; C, a 30 kDa GST protein control producted and purified with the same protocol as GST/PLCγ. The arrow indicates the 60 kDa protein which was purified. Molecular markers (kDa) are shown on the left of the panel. (B) Immunodetection of PLCγ in sea urchin egg. Immunoprecipitation with anti-PLCγ polyclonal serum followed by a western blot with the same serum was performed on proteins from unfertilized oocytes (lane 3) or eggs arrested 5 minutes postfertilization (lane 4). A control was performed with a rabbit preimmune serum and proteins from unfertilized oocytes (lane 1) or fertilized eggs (lane 2). The arrow indicates PLCγ. Molecular markers (kDa) are shown on the left of the panel.

The bovine PLCγSH2 construct does not prevent sperm egg interaction or sperm incorporation

We used confocal calcium imaging microscopy to determine whether small and local changes in $[Ca^{2+}]_i$ occurred in PLCγ construct-injected eggs. At a concentration of 2 μM, small transient elevations of $[Ca^{2+}]_i$ were seen at the periphery of the egg (Fig. 5), corresponding to episodes of depolarisation-induced calcium influx that occur when sperm and egg fuse (McDougall et al., 1993). All ten eggs tested at these concentrations showed these small transient calcium blips and all showed multiple sperm entry. Up to eight sperm in the egg cytoplasm could be observed after Hoechst dye H 33342 labelling ten minutes after fertilization (not shown). The control Syp construct did not induce polyspermy. At the lower concentration of 600 nM PLCγ construct, small calcium transients of very long latency were observed (see Fig. 4). Confocal imaging at this concentration revealed depolarisation events but, in addition, showed small and spatially localised calcium signalling events (Fig. 5B) that we took to represent sperm-induced calcium release at the point of sperm-egg fusion that failed to grow and propagate as full-blown calcium waves. In three eggs injected with 2 μM PLCγSH2 construct, we subsequently injected 10 μM $InsP_3$. These eggs (not shown) raised fertilization envelopes, demonstrating that the construct did not affect the $InsP_3$ receptor and calcium-release mechanism. The data indicate that inhibition of PLCγ activation by the SH2 construct occurs after sperm-egg fusion; at the step at which a local calcium increase is generated close to the point of this fusion.

The bovine PLCγ tandem SH2 construct delays or prevents passage through mitosis

The effects of microinjection of the bovine PLCγ1 tandem SH2 construct on passage through first mitosis is shown in Fig. 6. The construct was microinjected at various concentrations 40 minutes after fertilization, around 20-30 minutes before entry into mitosis. Embryos were observed throughout and scored three hours after fertilization, when the controls were at the 4- to 8-cell stage. A variety of cell cycle states were seen. Some embryos showed a single nucleus in a single cell, others a metaphase arrest, others two nuclei within a single cell, others again two nuclei within two cells. These cytological states

represented block at first nuclear envelope breakdown (NEB: that is, entry into mitosis), at the first metaphase/anaphase transition, at cleavage or at second NEB. No embryo had progressed beyond NEB of the second cell cycle. The data are shown quantitatively in Fig. 6. Mitotic arrest was seen at a concentration of 400 nM PLCγSH2 construct. The Syp control was without effect: at 3 hours the Syp-injected embryos were at the same 4- to 8-cell stage as controls. The effect of the construct on mitosis is reminiscent of the effects of lithium, a phosphoinositide pathway inhibitor, where embryos also show arrest at one of the cell cycle control points in the first and second cell cycles (Becchetti and Whitaker, 1997). We have previously shown that both $InsP_3$ and calcium levels increase at these stages in the cell cycle (Ciapa et al., 1994).

The timing of NEB and cleavage are also affected by the PLCγSH2 construct. Figure 7 indicates that 600 nM to 2 μM construct delays both NEB and cleavage, by a factor of 2. The Syp control was without effect at these concentrations. It was possible to induce NEB in embryos injected with 0.6 μM PLCγSH2 construct by treatment with ionomycin (10 μM) or by microinjecting $InsP_3$ (200 nM final injected concentration), though we did not pursue these observations quantitatively. We confirmed the importance of phosphotyrosine signalling to PLCγ at mitosis by constructing a 13-mer peptide using the sequence of the human PDGFβ receptor (NEGDNDYIIPLPD) to which the C-terminal SH2 domain of PLCγ binds (Larose et al., 1993). The phosphotyrosine form of the peptide (Y^p1021) delayed NEB in a dose-dependent way, while a higher concentration of the control (Y1021), unphosphorylated peptide was without effect (Fig. 7C). These observations indicate that a phosphotyrosine interaction mediated by PLCγSH2 domains is essential for entry into and progression through mitosis and cleavage. Interaction of PLCγ with phosphotyrosines can involve both N and C-terminal SH2 domains (Ottinger et al, 1998), perhaps explaining why a 50-fold higher concentration of the C-terminal recognition phosphotyrosine peptide is required for inhibition as compared to the tandem SH2 domain construct.

Pharmacological inhibition of PLC activity prevents mitosis

The PLC inihibitor U73122 has been shown to prevent the fertilization calcium transient in sea urchin eggs (Lee and Shen 1996, 1998). It also blocks NEB (Fig. 8). The inactive analogue U73343 is without effect. These data offer further confirmation that a PLC activity is essential for mitosis entry, though the inhibitor affects both PLCβ and PLCγ activity (see Lee and Shen, 1998). Our results overall suggest that, in blocking both fertilization and mitosis, U73122 is affecting PLCγ, though it is also capable of inhibiting PLCβ and may also inhibit the cGMP signalling pathway (Lee et al., 1998).

DISCUSSION

Our results indicate that unfertilized eggs contain mRNA coding for a PLCγ isoenzyme with a tandem SH2 domain sequence highly homologous to those of bovine and human PLCγ1. Furthermore, immunodetection of PLCγ using polyclonal antibodies raised against the SH2 domains of the sea urchin protein revealed the presence of the PLCγ protein, indicating that the gene was not only transcribed but also expressed in the unfertilized sea urchin egg, confirming our previous results with commercial anti-PLCγ antibodies (De Nadai et al., 1998a).

A role for PLCγ at fertilization has been inferred in mouse

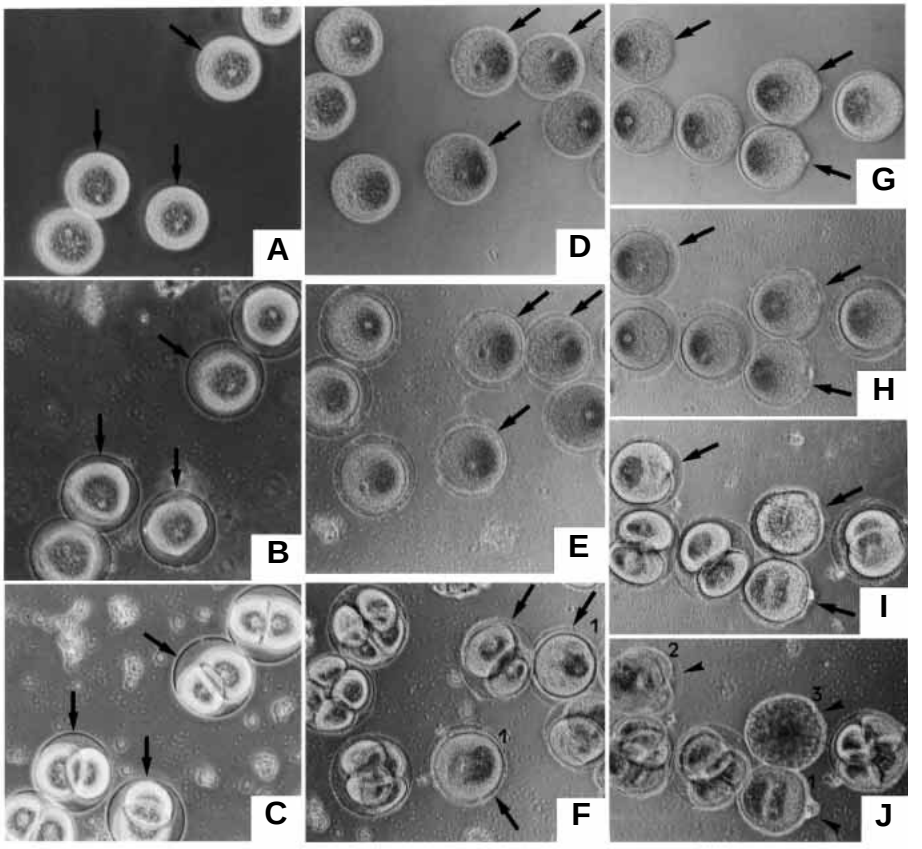


Fig. 3. Effect of the sea urchin GST/PLCγ fusion protein on progression through the first cell cycle. GST alone (A-C) or the sea urchin GST-PLCγ fusion protein (D-J, representing two different batches of eggs) was microinjected into unfertilized eggs (A,D,G). Of the 5 or 6 eggs shown for each group, 3 were injected and are indicated by arrows; those remaining were observed as uninjected controls. Eggs were then fertilized (B,E,H) and allowed to develop (C,F,I, controls at 2-cell stage; J, controls at 4-cell stage). Both GST injected (B) and GST-PLCγ injected (E,H) fertilized successfully as judged by the elevation of a fertilization envelope. All uninjected or GST control injected eggs (C) cleaved normally. Conversely, eggs injected with the sea urchin GST-PLCγ fusion protein were either blocked at metaphase, (marked '1' in F,I,J), showed abnormal division ('2' in J) or lysed ('3' in J). *Paracentrotus lividus*. 18°C.

where this protein was also detected in oocytes (Dupont et al., 1996) and starfish (Carroll et al., 1997). We found that injection of the GST-fusion protein containing the SH2 domains of the sea urchin PLCγ (with a truncated N terminal SH2 domain) or of a bovine PLCγ tandem SH2 domain led to inhibition of fertilization and/or mitotic interruption. This raises several interesting questions about the role of PLCγ in the generation of the fertilization and subsequent endogenous mitotic calcium transients observed during the first cell cycle of the sea urchin egg.

PLCγ at fertilization

We observed that injection of the sea urchin PLCγ construct did not prevent egg activation at a cytoplasmic concentration of 400 nM (20 μg/ml), but nonetheless resulted in an arrest of early development at first mitosis. A higher concentration of the sea urchin fusion protein may be necessary to block PLCγ-mediated activation at fertilization than during mitosis. Because we were unable to solubilize the sea urchin GST fusion protein at high concentration, we tested this idea using the bovine tandem SH2 homologue. A complete block of the calcium transient at fertilization was obtained at a concentration of 1 μM. A concentration of 200 nM of the bovine construct was effective as a blocker of mitosis, but showed more variable results at fertilization, often merely delaying egg activation. Given the approximately 2-fold uncertainty in injected volume, the fact that the sea urchin PLCγ construct had a truncated N-terminal SH2 domain and uncertainty over the activity of the expressed construct, it is reasonable to conclude that the effects of the sea urchin and bovine constructs are quantitatively comparable. An inhibition of the fertilization calcium signal in starfish egg was obtained by Carroll et al. (1997) by injecting the GST-fusion protein containing the SH2 domains of a bovine PLCγ at a 20-times higher concentration than that used in our experiments. It is conceivable that the GST-PLCγ fusion protein, when injected at high concentration, may interact with phosphorylated tyrosine residues that normally associate with SH2 domains of proteins other than PLCγ. However, the Syp SH2 domain control construct was ineffective at twice the blocking concentration of the PLCγSH2 construct, indicating that the construct is interacting specifically at these concentrations. We therefore concur with earlier results in starfish (Carroll et al., 1997) in concluding that activation of PLCγ is an essential step in the signal transduction pathway at fertilization

A number of signal transduction pathways appear to be activated at fertilization in sea urchin eggs (see review by Ciapa and De Nadai, 1996). PLCγ activation thus belongs to one

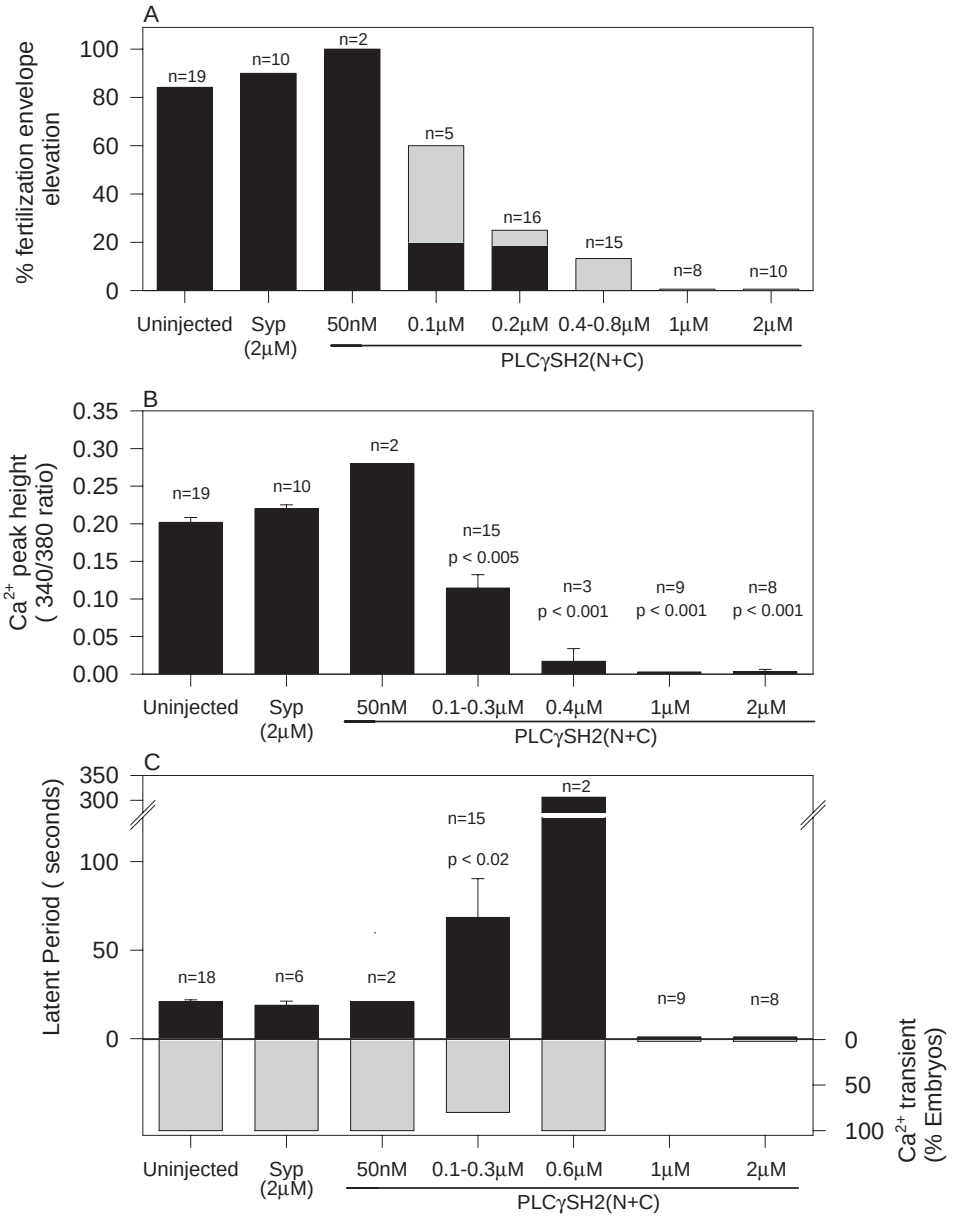
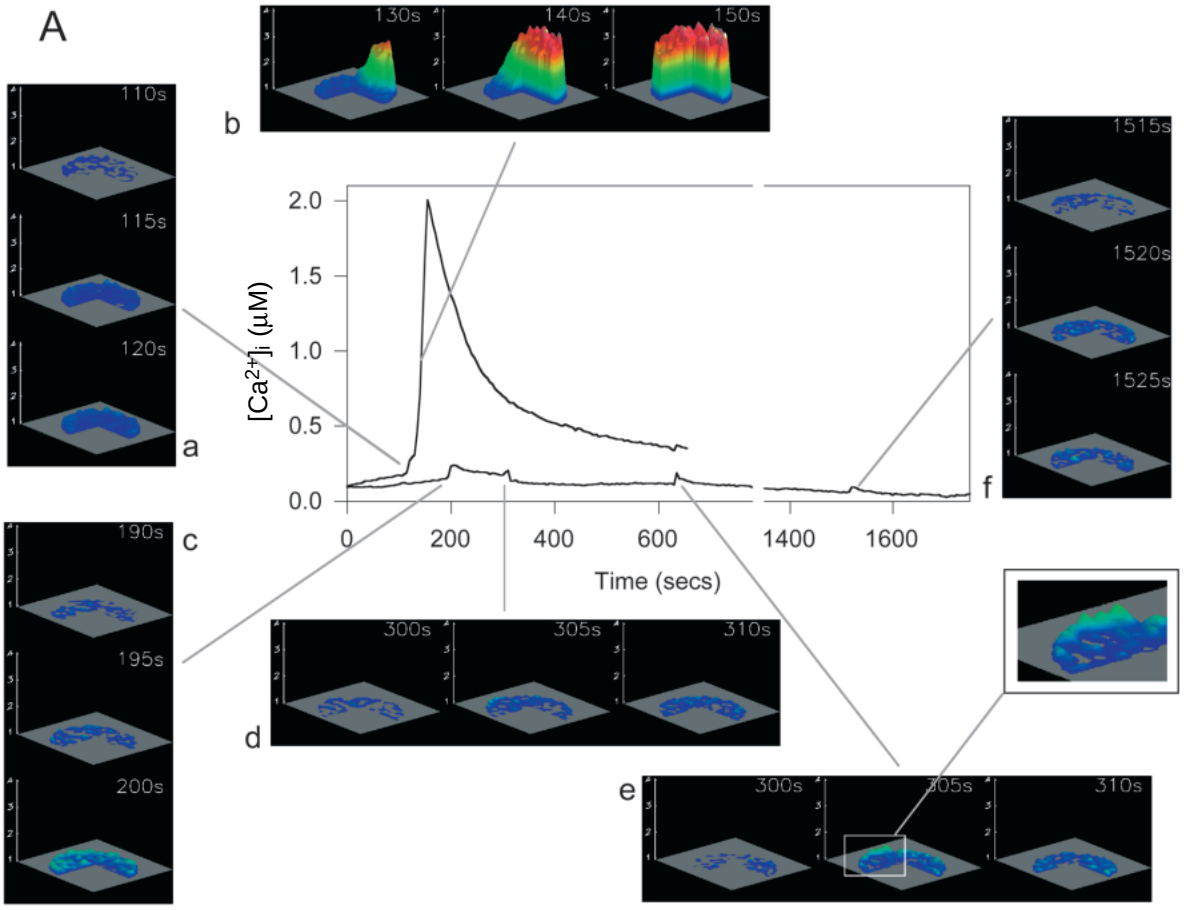


Fig. 4. Inhibition of fertilization by bovine PLCγ tandem SH2 construct. Dose-response relationships for fertilization envelope elevation (A), the magnitude of the fertilization calcium transient (B) and the latent period (C). Full envelope elevation (A) is depicted as a solid black bar whilst partial envelope elevation is shown as a grey bar. The latent period (C) was assessed as described previously (Whitaker et al., 1989). Also shown are the % embryos which exhibited a calcium transient. In these experiments, a calcium transient was scored if it was at least 0.03 ratio units above baseline at its peak. The tandem SH2 domain construct was injected up to 30 min before fertilization. Error bars indicate the s.e.m. and statistical analysis was by ANOVA: Single factor test. *Lytechinus pictus*. 16°C.



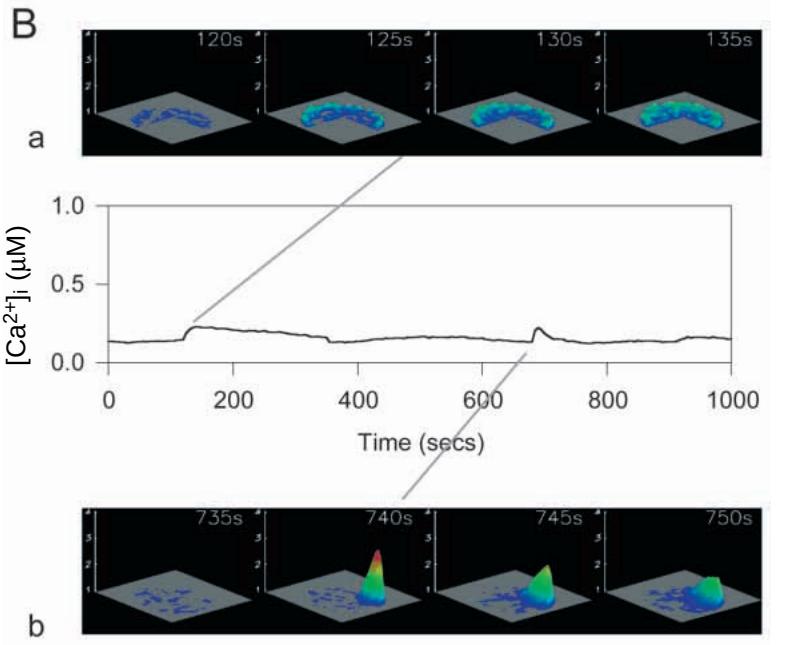


Fig. 5. Confocal calcium imaging of PLCγSH2-injected eggs. (A) Calcium levels in 2 μM bovine PLCγSH2-injected eggs show small transient blips that have an identical spatial profile to the action potential elicited by the sperm at fertilization. The centre panel shows the average $[Ca^{2+}]_i$ within a confocal section measured using calcium green dextran. The satellite panels show topographical plots of selected confocal sections to illustrate the pattern of $[Ca^{2+}]_i$ at various times during the calcium response. The upper trace (centre) shows the calcium transient during a control fertilization. The initial small increase in $[Ca^{2+}]_i$ occurs rapidly throughout the egg (a) and is due to a calcium-dependent action potential triggered by sperm egg fusion. It is followed immediately by the calcium wave that begins at the point of sperm entry and crosses the egg in around 20s (b). The lower trace and satellites (c-f) shows the small calcium increases that occur in eggs injected with 2 μM bovine PLCγSH2 construct. They closely resemble the pattern of $[Ca^{2+}]_i$ induced by depolarization-mediated calcium influx (a). Episode (e) shows the faint suggestion of a more localized calcium release event (see inset). (B) Localized calcium release events clearly occurred in eggs injected with 600 nM of the construct. While the first episode of calcium increase is relatively spatially uniform and due to calcium influx as a result of sperm-egg fusion (a), the second episode, occuring after a significant amount of time (10 minutes), is strongly spatially localized and can be identified as a local sperm-induced release of calcium that fails to propagate across the egg as a wave (b). The local release may have been caused by the sperm that induced the depolarization event (episode (a)), but we cannot be certain of this: at the end of this experiment (30 minutes after fertilization and 19 minutes after the local release episode) the egg was observed to be heavily polyspermic and only a partial envelope had been elevated. *Lytechinus pictus.* 16°C.

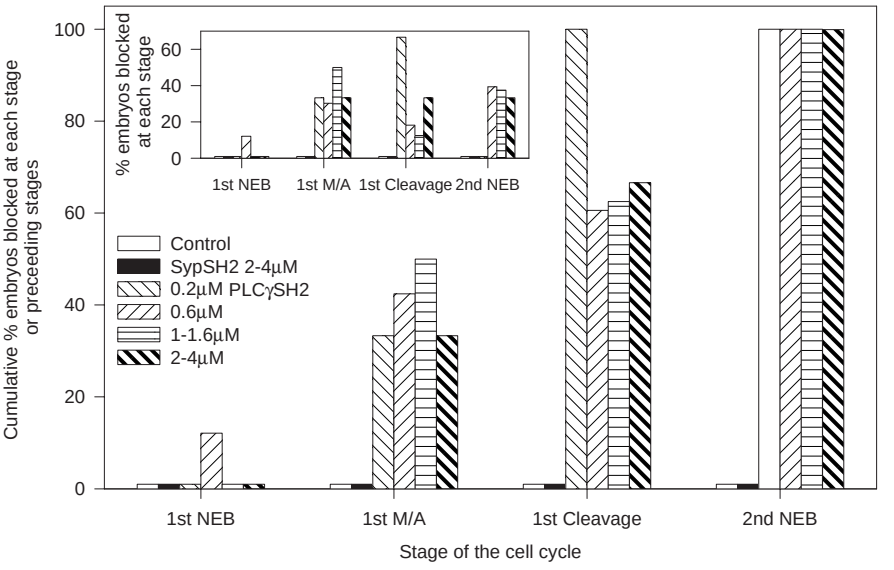


Fig. 6. Effects of bovine PLCγ tandem SH2 construct and its Syp control on entry into and exit from mitosis. Embryos were microinjected with constructs to the final cytoplasmic concentration shown and observed at 3 hours postfertilization. The cumulative block is shown for each concentration, with the proportion of embryos at each cell cycle stage shown as an inset. 1st NEB (1st M/A; 1st cleavage): arrested prior to nuclear envelope breakdown (metaphase/anaphase transition; cleavage) in the first cell cycle. 2nd NEB: arrested prior to nuclear envelope breakdown in the second cell cycle. *Lytechinus pictus.* 16°C.

among several pathways including cADPr production (Galione et al., 1993; Lee et al., 1993), PLCβ activation (Lee and Shen, 1998) and activation by a sperm-derived factor (Parrington et al., 1996; Stricker, 1997). Can we identify any of these pathways as primary? Our data add to those of Carroll et al. (1997) in identifying the production of $InsP_3$ by PLCγ as the initiating event at fertilization in echinoderms. These results imply that the cADPR/ryanodine receptor pathway is dependent on the initial increase in $InsP_3$ for its activation; a hypothesis consistent with the observation (Becchetti, Galione and Whitaker, unpublished) that the latency of the fertilization response is determined by the $InsP_3$ pathway, while the cADPR pathway governs the time course of rise and decay of the fertilization calcium signal, with no effect on latency at all. This hypothesis necessarily supposes that the effect of U73122 (Lee and Shen, 1998) is to inhibit PLCγ (Chen et al., 1994, 1996; Heemskerk et al., 1997), not PLCβ, as Lee and Shen believed. Moreover, U73122 also blocks the cGMP-mediated cyclic ADPribose calcium release pathway, making the effects of the inhibitor on fertilization very difficult to interpret. In addition, it has recently been found that the soluble sperm factor that activates eggs has a phospholipase activity that uses $PtdinsP_2$ as a substrate (Jones et al., 1998). The molecular identity of this spermatic PLC activity is unknown. It is conceivable that the PLCγSH2 constructs are blocking interaction of both the egg and a sperm PLCγ linked with a

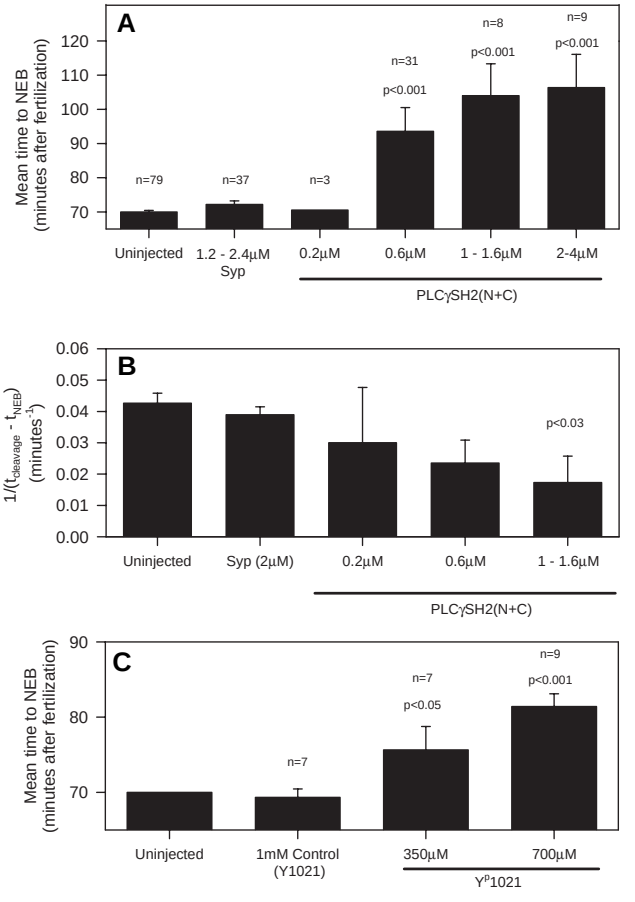


Fig. 7. Effects of bovine PLCγ tandem SH2 construct on the timing of NEB and cleavage and the effects of the phosphotyrosine peptide. (A) Mean time to NEB after microinjection of the bovine PLCγ tandem SH2 construct and its Syp control. The experiment was ended at 3 hours at which time five of the embryos injected to a final concentration of 600 nM or higher had failed to undergo NEB. The bovine PLCγ tandem SH2 construct caused a signifcant delay to NEB (ANOVA: Single factor test). (B) Effect of microinjection of the bovine PLCγ tandem SH2 construct and its Syp control on the transit time of mitosis. Mitotic transit time was taken as the time from NEB to the time of initiation of the cleavage furrow. As some embryos did not complete cleavage, the reciprocal of the transit time is plotted (Becchetti and Whitaker, 1997). The bovine PLCγ tandem SH2 construct significantly decreases the reciprocal transit time at a concentration of 1-1.6 μM (ANOVA: Single factor test). (C) Mean time to NEB after microinjection of the 13-mer phosphotyrosine peptide based on the PLCγ C-terminal SH2 binding site of the human PDGFβ receptor to the final cytoplasmic concentrations shown. The phosphopeptide (Y^p1021) significantly delays NEB (ANOVA: Single factor test), wheareas the unphosphorylated control peptide (Y1021) was without effect. *Lytechinus pictus.* 16°C.

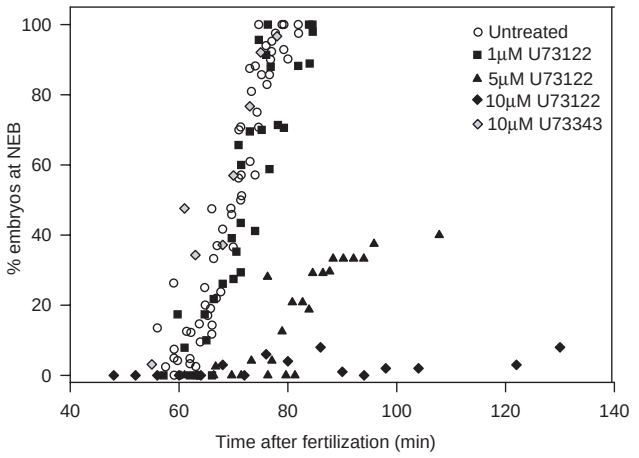


Fig. 8. Effects of the phospholipase inhibitor U73122. U73122 or its inactive analogue (U73343) were added to embryos 30 minutes postfertilization, that is approximately 30-40 minutes before the expected time of entry into mitosis, and embryos were scored for NEB alone. *Lytechinus pictus.* 16°C.

phosphotyrosine activator contained in sperm, for example tr-kit (Sette et al., 1998).

In many cases, the stimulation of PLCγ is associated with its phosphorylation on several tyrosine residues, so we might expect to detect this after fertilization. Our attempts to determine whether or not PLCγ was phosphorylated on tyrosine in the first minutes following fertilization by performing immunoprecipitation followed by western blotting using anti-phosphotyrosine antibodies and either commercial anti-PLCγ antibodies (De Nadai et al., 1998) or the polyclonal serum reported here were unsuccessful. Similar negative results were reported in mouse oocytes, where no evidence of PLCγ phosphorylation after fertilization was obtained (Dupont et al., 1996). It is possible that the active PLCγ becomes associated with the cytoskeleton after fertilization; the insoluble fraction was poorly solubilized in our immunoprecipitation procedure. For example, translocation of PLCγ to the cytoskeleton has been reported in other types of cells such as hepatocytes (Yang et al., 1994). Another possibility is that activation of PLCγ occurs independently of tyrosine phosphorylation (review by Rhee and Bae, 1997). For example, PLCγ isozymes can be stimulated by arachidonic acid (Hwang et al., 1996), the metabolism of which increases at fertilization (Perry and Epel, 1985) and which has been proposed to play a role in regulating free calcium concentration and pH (Ciapa et al., 1995). Lipid second messengers generated by PI 3-kinase could also stimulate PLCγ by interacting with its SH2 domains (Falasca et al., 1998b). However, this hypothesis does not fit with our recent results. We showed that PI 3-kinase activity is indeed stimulated after fertilization but that treatment of unfertilized eggs with wortmannin, a potent inhibitor of PI 3-kinase, did not inhibit fertilization events, including fertilization envelope elevation and thus, the calcium signal (De Nadai et al., 1998). Finally, phospholipase D (PLD) might also activate PLCγ (review by Rhee and Bae, 1997), though it is not known whether this enzyme is present and stimulated at fertilization in sea urchin eggs.

PLCγ at mitosis

The block of cell division in eggs injected with the sea urchin GST-PLCγ fusion protein supports the hypothesis that PLCγ plays a role during mitosis, at NEB, anaphase and cleavage. This fits with results published earlier showing that $InsP_3$ production and calcium release occured at these particular times of the cell cycle (Ciapa et al., 1994), and that heparin, an $InsP_3$ receptor inhibitor, blocks mitosis in sea urchin embryos (Ciapa et al., 1994; Wilding et al., 1996; Groigno and Whitaker, 1998). The observation also agrees with the finding that lithium, a PPI antagonist, also arrests the fertilized sea urchin egg at these precise stages of the mitotic cycle (Becchetti and Whitaker, 1997).

Using the bovine tandem SH2 PLCγ construct we have determined the timing of the block to mitosis. Entry into mitosis (NEB) is markedly delayed or blocked. In embryos that ultimately enter mitosis, anaphase is itself markedly delayed, as is cleavage. These observations mirror those obtained with the PPI antagonist lithium (Becchetti and Whitaker, 1997) and are consistent with the view that PLCγ generates an $InsP_3$-driven calcium transient first to cause NEB (Wilding et al., 1996), then further calcium transients to control chromosome disjunction and cleavage (Groigno and Whitaker, 1998). It has been reported that disruption of the PLCγ1 gene leads to early mouse embryonic lethality (Ji et al., 1997). In the absence of PLCγ activation, it has been suggested, redundant signaling pathways may compensate for the loss of PLCγ (Schlessinger, 1997). However, the early embryonic cell cycles are almost certainly dependent on maternally derived PLCγ. Our hypothesis is that fertilization turns on an endogenous clock (probably regulated by cyclin synthesis and cell cycle kinases: Elledge, 1996 for review) that triggers PLCγ activation at certain times of the succeeding mitotic cycles, making PLCγ an important key regulator of early embryonic development.

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