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# Sperm extract injection into ascidian eggs signals $Ca^{2+}$ release by the same pathway as fertilization

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## SUMMARY

**Injection of eggs of various species with an extract of sperm cytoplasm stimulates intracellular $Ca^{2+}$ release that is spatially and temporally like that occurring at fertilization, suggesting that $Ca^{2+}$ release at fertilization may be initiated by a soluble factor from the sperm. Here we investigate whether the signalling pathway that leads to $Ca^{2+}$ release in response to sperm extract injection requires the same signal transduction molecules as are required at fertilization. Eggs of the ascidian *Ciona intestinalis* were injected with the Src-homology 2 domains of phospholipase Cγ or of the Src family kinase Fyn (which act as specific dominant negative inhibitors of the activation of these enzymes), and the effects on $Ca^{2+}$ release at fertilization or in response to injection of a sperm extract were compared. Our findings indicate that both fertilization and sperm extract injection initiate $Ca^{2+}$ release by a pathway requiring phospholipase Cγ and a Src family kinase. These results support the hypothesis that, in ascidians, a soluble factor from the sperm cytoplasm initiates $Ca^{2+}$ release at fertilization, and indicate that the activating factor from the sperm may be a regulator, directly or indirectly, of a Src family kinase in the egg.**

Key words: Fertilization, Calcium, Egg activation, Phospholipase Cγ, Src family kinase

## INTRODUCTION

At fertilization, eggs of most if not all species undergo a transient rise in cytosolic free $Ca^{2+}$ (see Stricker, 1999). In eggs of ascidians (Speksnijder et al., 1989, 1990a,b; Brownlee and Dale, 1990; Kyozuka et al., 1998) as well as mammals (Miyazaki et al., 1993; Kline et al., 1999), the initial $Ca^{2+}$ rise at fertilization begins at the point of sperm-egg fusion, spreads across the egg in the form of a wave, and is followed by additional $Ca^{2+}$ transients that also occur in the form of waves. These $Ca^{2+}$ rises cause a decrease in cyclin-dependent kinase activity, which stimulates the egg to reenter the meiotic cell cycle (Kline and Kline, 1992; Collas et al., 1995; Sensui and Morisawa, 1996; Albrieux et al., 1997; McDougall and Levasseur, 1998; Levasseur and McDougall, 2000); however, the signalling pathway leading to the initiation of the $Ca^{2+}$ rises is only beginning to be understood.

In eggs of echinoderms (Whitaker and Irvine, 1984; Ciapa and Whitaker, 1986; Terasaki and Sardet, 1991; Mohri et al., 1995; Carroll et al., 1997, 1999; Lee and Shen, 1998; Shearer et al., 1999), frogs (Nuccitelli et al., 1993; Runft et al., 1999) and mammals (Miyazaki et al., 1992, 1993), the $Ca^{2+}$ rise at fertilization results from the release of $Ca^{2+}$ from the endoplasmic reticulum, which is mediated, at least in part, by inositol trisphosphate ($IP_3$). In ascidian eggs, $IP_3$ is produced at fertilization (Toratani and Yokosawa, 1995), and introduction of $IP_3$ causes $Ca^{2+}$ release (McDougall and Sardet, 1995; Albrieux et al., 1997; Yoshida et al., 1998), but the question of whether $IP_3$ is required for $Ca^{2+}$ release at fertilization has not been definitively answered (Russo et al., 1996; Yoshida et al., 1998; Wilding et al., 1999).

$IP_3$ is produced by the phospholipase C (PLC) family of enzymes, which cleave phosphatidylinositol 4,5-bisphosphate to generate $IP_3$ and diacylglycerol (Singer et al., 1997). The PLC family includes three subgroups: β, γ and δ. PLCγ is activated when its two tandem Src-homology 2 (SH2) domains interact with a specific phosphotyrosine-containing binding site present on an activated tyrosine kinase. This association allows the tyrosine kinase to phosphorylate and activate PLCγ. Recombinant proteins containing the SH2 domains of PLCγ have been used as specific dominant negative inhibitors of PLCγ activation in vitro (Bae et al., 1998) and in vivo (Roche et al., 1996; Carroll et al., 1997, 1999; Wang et al., 1998; Mehlmann et al., 1998; Shearer et al., 1999; Runft et al., 1999). The SH2 domains are believed to block the association of endogenous full-length PLCγ with its activating tyrosine kinase. Injection of PLCγ SH2 domains into echinoderm eggs inhibits the $Ca^{2+}$ rise at fertilization, demonstrating that, in starfish and sea urchin eggs, this $Ca^{2+}$ rise is initiated by SH2 domain-mediated activation of PLCγ (Carroll et al., 1997, 1999; Shearer et al., 1999). Injection of PLCγ SH2 domains into mouse or frog eggs, however, does not inhibit the $Ca^{2+}$ rise at fertilization, indicating that, unlike echinoderms, these vertebrate eggs do not require SH2 domain-mediated activation of PLCγ to initiate the $Ca^{2+}$ rise at fertilization (Mehlmann et al., 1998; Runft et al., 1999). Here we investigate whether the

$Ca^{2+}$ release signalling pathway in ascidians (an evolutionary intermediate deuterostome) resembles that in echinoderms or vertebrates.

In echinoderm fertilization, PLCγ activation (Rongish et al., 1999) relies, directly or indirectly, on the activation of a Src family kinase (Giusti et al., 1999a,b, 2000; Abassi et al., 2000; Kinsey and Shen, 2000), and studies in frog eggs indicate that a Src family kinase may also be required in vertebrate fertilization (Sato et al., 1996, 1998, 1999, 2000; Glahn et al., 1999). Evidence that a Src family kinase plays a role in echinoderm egg activation includes the finding that injection of SH2 domains of Src family kinases into sea urchin and starfish eggs inhibits $Ca^{2+}$ release at fertilization (Giusti et al., 1999b; Abassi et al., 2000; Kinsey and Shen, 2000). In this study, we use SH2 domains to investigate the role of a Src family kinase in ascidian fertilization.

How the sperm initiates the signal transduction cascade that leads to the $Ca^{2+}$ rise in the egg at fertilization is unknown. One possibility is that influx of extracellular $Ca^{2+}$ might initiate this process (see Créton and Jaffe, 1995). This hypothesis, however, is not consistent with observations that $Ca^{2+}$ release at fertilization can be initiated in the absence of extracellular $Ca^{2+}$ in eggs of mammals (Jones et al., 1998), echinoderms (Schmidt et al., 1982), and ascidians (Speksnijder et al., 1989, 1990a; Sensui and Morisawa, 1996). Other possibilities are that the sperm contacts a receptor on the egg plasma membrane that initiates the signal transduction pathway, or that the fusion of sperm and egg introduces a factor from the sperm into the egg membrane or cytoplasm that induces egg activation. In favor of contact-initiated egg activation, externally applied proteases, lectins and sperm proteins can cause egg activation events in several species: the echiuroid worm *Urechis* (Jaffe et al., 1979; Gould and Stephano, 1987; Stephano and Gould, 1997), echinoderms (Steinhardt et al., 1971; Carroll and Jaffe, 1995), ascidians (Zalokar, 1980; Speksnijder et al., 1990a; Flannery and Epel, 1998), and amphibians (Shilling et al., 1998; Mizote et al., 1999). In favor of fusion-initiated egg activation, injection of a sperm extract into eggs causes $Ca^{2+}$ release and other egg activation events in various species: the nemertean worm *Cerebratulus* (Stricker, 1997), sea urchins (Dale et al., 1985), ascidians (Dale, 1988; Wilding and Dale, 1998; Kyozuka et al., 1998) and mammals (Swann, 1990; Oda et al., 1999; Perry et al., 2000). The $Ca^{2+}$ release induced by sperm extract injection is spatially and temporally like that occurring at fertilization (Swann, 1990; Stricker, 1997; Kyozuka et al., 1998; Wilding and Dale, 1998; Oda et al., 1999; Perry et al., 2000), and in mouse eggs, both fertilization and sperm extract-induced $Ca^{2+}$ release are inhibited by an antibody against the $IP_3$ receptor (Miyazaki et al., 1993; Oda et al., 1999). Here we investigate whether the sperm extract initiates intracellular $Ca^{2+}$ release in ascidian eggs by activating the same signal transduction molecules that function at fertilization.

## MATERIALS AND METHODS

### Collecting *Ciona intestinalis* eggs and sperm

*Ciona intestinalis* were obtained from the Marine Biological Laboratory (Woods Hole, MA, USA). To obtain gametes, the tunic was removed, the egg or sperm duct was punctured, and the gametes were collected. Eggs were washed twice with natural sea water and observed under a microscope to make sure no sperm were present. Sperm were kept on ice. For fertilization, eggs and sperm were collected from separate animals to facilitate rapid and synchronous fertilization (Rosati and De Santis, 1978; De Santis and Pinto, 1991). Insemination was performed by replacing the solution in the injection chamber (see below) with a suspension of sperm. In most experiments, the suspension of sperm collected from the sperm duct was diluted 1:100 in natural sea water. However, the final sperm concentration was somewhat variable, because the concentration of the suspension collected from the sperm duct was not constant.

### Preparation of sperm extract

Sperm extract was prepared based on methods described by Kyozuka et al. (1998). Sperm were washed 3 times in $Ca^{2+}$-free sea water, and then after centrifugation, the volume of packed sperm was estimated, and the sperm were resuspended in 2.5 μl of extraction buffer (140 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.0) per μl of packed sperm. Resuspended sperm were then homogenized 50 μl at a time for approx. 10-15 minutes in a microfuge tube on ice using a conical teflon pestle (PGC Scientific, Frederick, MD, USA, #63-5430). The homogenate was observed under a microscope to check that intact sperm or sperm heads were not present. The homogenate was then centrifuged at 4°C at 16,000 ***g*** for 10 minutes, or at 100,000 ***g*** for 30 minutes. The supernatant was collected as sperm extract, divided into portions, frozen in liquid nitrogen, and stored at −70°C. The protein yield in these extracts was 0.7-2.4 pg per sperm, out of a total protein content of 11 pg per sperm. The total protein content of the sperm was determined after sonication in 1% SDS. Protein concentrations were measured using a BCA assay (Pierce Chemical Co., St Louis, MO, USA) with BSA standards. For the experiments involving SH2 domain injections, the sperm extract was prepared using a 16,000 ***g*** centrifugation and the protein concentration was 6 mg/ml.

The sperm extract could be thawed and refrozen up to 3 times without losing activity. However, extract kept at 16-18°C for over an hour showed a detectable loss of activity. The activity of the extract made with the high speed centrifugation was the same as that made with the low speed centrifugation, indicating that the activity is cytosolic rather than an integral membrane component. Heat-inactivated sperm extract was prepared by incubating the extract at 96°C for 10 minutes. This extract was then centrifuged for 2 minutes at 16,000 ***g*** and the supernatant was collected and stored at −70°C. Control extract from ascidian ovary was prepared by homogenizing minced ovary as described above for sperm, and collecting the supernatant after centrifugation at 100,000 ***g*** for 30 minutes.

### Recombinant proteins

Plasmid DNA encoding glutathione-S-transferase (GST)-SH2 domain fusion constructs was obtained from the following sources: bovine GST-PLCγ1 SH2 (N + C) from S. A. Courtneidge (Sugen, Inc., Redwood City, CA, USA; see Carroll et al., 1997), murine GST-SHP2 SH2 (N + C) from T. Pawson (Mount Sinai Hospital, Toronto, Ontario, Canada; see Feng et al., 1993; Carroll et al., 1997), chicken GST-Fyn SH2 from K. Vuori (The Burnham Institute, La Jolla, CA, USA; see Giusti et al., 1999b), and murine GST-Abl SH2 from B. J. Mayer (Harvard Medical School, Boston, MA, USA; see Giusti et al., 1999b). GST fusion proteins were made as previously described (Gish et al., 1995; Carroll et al., 1997), then spin-dialyzed and concentrated in 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.2.

### Microinjection

*Ciona* eggs arrested at metaphase of meiosis I, with chorion and follicle cells intact, were placed in a microinjection chamber between two coverslips separated by two pieces of double stick tape (Kiehart, 1982). All experiments were performed with the eggs in natural sea water. The eggs were microinjected using mercury-filled micropipets, which allows injection of precise picoliter volumes (Hiramoto, 1962). The micropipet tips were broken to a diameter of 2-4 μm and

calibrated by expelling a drop of oil in sea water and calculating the volume of the drop based on its diameter. Injected volumes were 1-3% of the egg volume (1800 pl). All injections were performed at 16°C on the stage of an upright microscope with a 20×, 0.5 numerical aperture Plan Neofluar objective (Carl Zeiss, Inc., Thornwood, NY, USA) and a micrometer reticle in the eyepiece.

To prevent the eggs from lysing during the injection, the micropipet was first pushed through the chorion and positioned so that its tip faced the egg equator, and then pushed into the egg until the tip was approximately at the egg center. The injection solution was expelled and the micropipet was then quickly pulled out of the egg to prevent the micropipet from sticking to the chorion and follicle cells. Each micropipet was used only once. Protein and $IP_3$ concentrations given in the text refer to the final concentration in the egg cytoplasm.

### Calcium measurements

$Ca^{2+}$ measurements were made using calcium green-1 10-kDa dextran (Molecular Probes, Eugene, OR, USA) at a final concentration of 10 μM in the egg cytoplasm. Fluorescence was detected using a 20×, 0.5 numerical aperture Plan Neofluar objective, 485 nm excitation and 535 nm emission filters, and a photomultiplier tube connected through a current-to-voltage converter to a chart recorder as previously described (Chiba et al., 1990). For imaging calcium green fluorescence, we used a laser scanning confocal microscope (MRC600; BioRad Laboratories, Hercules, CA, USA) with a 20×, 0.5 numerical aperture Plan Neofluar objective. The video output from the confocal microscope was stored on an optical memory disk recorder (see Terasaki et al., 1997; Carroll et al., 1997). Eggs to be used for $Ca^{2+}$ measurements were coinjected with calcium green dextran and SH2 domain protein (3% of the egg volume), incubated at 16°C for 1-2 hours, and then inseminated or injected with sperm extract (1% of the egg volume) or $IP_3$ (Calbiochem, La Jolla, CA, USA; 1% of the egg volume). Experiments were done at 16-18°C.

### Detection of sperm entry

Sperm entry was detected by injecting eggs with 4'6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes). The DAPI was dissolved in distilled water at 1 mg/ml, and injected to obtain a final concentration of 10 μg/ml. Fluorescence was detected using a 20×, 0.5 numerical aperture Plan Neofluar objective, and 330 nm excitation and 400 nm long-pass emission filters, and photographed using TMAX 400 film (Eastman Kodak Co., Rochester, NY, USA).

## RESULTS

### The release of $Ca^{2+}$ from intracellular stores at fertilization is inhibited by injection of SH2 domains of PLCγ or Fyn

The $Ca^{2+}$ rise at fertilization in *Ciona* eggs consists of several components. The initial response that is seen in records of total calcium green fluorescence is a $Ca^{2+}$ action potential (Figs 1A, 2A, 4A, asterisks), resulting from $Ca^{2+}$ entry through voltage-gated channels in the egg plasma membrane (Goudeau et al., 1992). The action potential is triggered by the depolarization that occurs at the time of sperm-egg fusion (McCulloh and Chambers, 1992), and serves to amplify the depolarization and establish an electrical block to polyspermy (Goudeau and Goudeau, 1993; Goudeau et al., 1994). It is followed by a much larger $Ca^{2+}$ rise (Figs 1A, 2A, 4A), which crosses the egg in the form of a wave (Fig. 5A) and is due to release of $Ca^{2+}$ from intracellular stores (Speksnijder et al., 1989, 1990a,b; Brownlee and Dale, 1990; Yoshida et al., 1998; Kyozuka et al., 1998). $Ca^{2+}$ remains high for several minutes and then begins to oscillate. We used the action potential as a marker of when fertilization occurred and measured the timing of intracellular $Ca^{2+}$ release with respect to this.

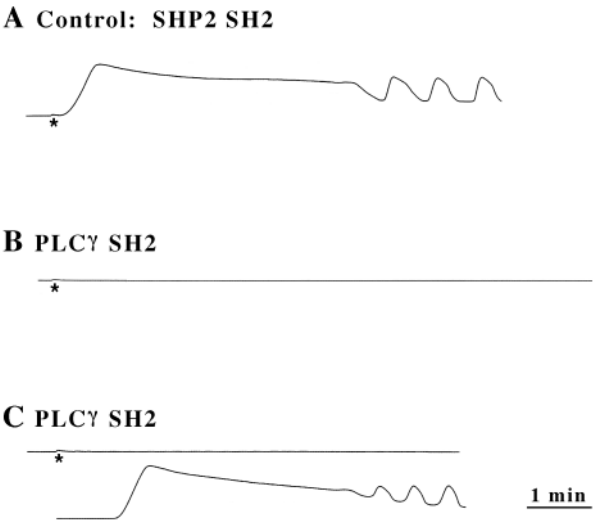


**Fig. 1.** PLCγ SH2 protein inhibits intracellular $Ca^{2+}$ release at fertilization in ascidian eggs. Eggs were coinjected with 10 μM calcium green dextran and the indicated protein and then inseminated 1-2 hours later. Traces show calcium green fluorescence as a function of time. Asterisks indicate the action potential. (A) SHP2 SH2, 1 mg/ml (control). $Ca^{2+}$ was released from the intracellular stores beginning 12 seconds after the action potential rise. (B) PLCγ SH2, 1 mg/ml. No $Ca^{2+}$ rise was detected after the action potential during the 8 minute recording period (5 of 7 eggs tested). (C) PLCγ SH2, 1 mg/ml. A $Ca^{2+}$ rise occurred 7 minutes after the action potential rise (2 of 7 eggs tested).

To investigate the role of PLCγ at fertilization in ascidian eggs, *Ciona* eggs were coinjected with calcium green dextran and PLCγ SH2 domains, or SH2 domains from a control protein, the phosphatase SHP2. These eggs were then inseminated while total calcium green fluorescence was monitored using a photomultiplier. All eggs injected with the control SH2 domains at a final concentration of 1 mg/ml in the egg cytoplasm showed $Ca^{2+}$ rises similar to those that normally occur at fertilization, with the release of intracellular $Ca^{2+}$ starting at an average of 18 seconds after the action potential rise (Fig. 1A, Table 1). In contrast, 5 out of 7 eggs injected with 1 mg/ml (20 μM) PLCγ SH2 domains showed an action potential but no subsequent $Ca^{2+}$ release (Fig. 1B). The other 2 eggs injected with 1 mg/ml PLCγ SH2 domains did release $Ca^{2+}$ at fertilization, but with a delay of several minutes after the action potential (Fig. 1C, Table 1). In these eggs, the peak amplitude of the $Ca^{2+}$ increase, and its rate of rise, were the same as in control eggs, although the duration of the initial $Ca^{2+}$ elevation was shorter (Table 1). The three subsequent $Ca^{2+}$ oscillations appeared to be normal, but recordings were not continued beyond this point. All eggs injected with 0.1 mg/ml (2 μM) PLCγ SH2 domains showed a $Ca^{2+}$ increase of normal amplitude, rate of rise and duration, but the delay between the action potential and $Ca^{2+}$ release was about 1 minute longer than in controls (Table 1). These data indicate that, as in echinoderm eggs, SH2 domain-mediated activation of PLCγ is needed to initiate the release of $Ca^{2+}$ from intracellular stores at fertilization in *Ciona* eggs.

**Table 1. $Ca^{2+}$ release from eggs injected with SH2 domain proteins and then inseminated**

| SH2 domain protein injected (mg/ml) | % of eggs with $Ca^{2+}$ release | Time to the $Ca^{2+}$ release (seconds)[1] | Peak amplitude[2] | Number of eggs |
|---|---|---|---|---|
| None | 100 | 13±11 | 1.6±0.4 | 14 |
| SHP2 SH2 | | | | |
| (1) | 100 | 18±13 | 2.0±0.5 | 7 |
| (0.1) | 100 | 11±1 | 1.5±0.3 | 6 |
| PLCγ SH2 | | | | |
| (1) | 29[3] | 426, 355 | 1.9, 2.3[5] | 7 |
| (0.1) | 100 | 73±32[4] | 1.7±0.5 | 7 |
| Abl SH2 (1) | 100 | 10±1 | 1.5±0.1 | 8 |
| Fyn SH2 (1) | 38[3] | 268±171[4] | 1.6±0.4[5] | 8 |

Eggs were coinjected with 10 μM calcium green dextran and the indicated protein. 1-2 hours later, the eggs were inseminated and calcium green fluorescence was monitored as in Figs 1 and 2.

[1]Values refer to the time between the rise of the action potential and the time at which the fluorescence versus time trace started to rise as $Ca^{2+}$ was released from intracellular stores (mean ± s.d.). Values include only those eggs that released intracellular $Ca^{2+}$ (only two eggs injected with PLCγ SH2 domains showed $Ca^{2+}$ release).

[2]Values are expressed as the peak increase in fluorescence after fertilization divided by the fluorescence of the unfertilized egg (mean ± s.d.). Values include only those eggs that released intracellular $Ca^{2+}$.

[3]These values are significantly different from controls (Chi-square test, $P<0.01$).

[4]These values are significantly different from controls (Mann-Whitney test, $P<0.02$).

[5]In the two eggs injected with 1 mg/ml PLCγ SH2 domains that activated, the peak amplitude of the $Ca^{2+}$ increase and its rate of rise were the same as in SHP2 SH2 controls, but the time from the peak to the return to half maximum was 198±9 seconds compared to 330±87 seconds in controls. In the three eggs injected with Fyn SH2 domains that activated, the peak amplitude of the $Ca^{2+}$ rise and its rate of rise were the same as in Abl SH2 controls, but the time from the peak to the return to half maximum was 127±59 seconds compared to 345±84 seconds in controls.

To examine if SH2 domain-mediated activity of a Src family tyrosine kinase is needed for release of intracellular $Ca^{2+}$ at fertilization in ascidian eggs, *Ciona* eggs were coinjected with calcium green dextran and SH2 domains from the Src family kinase Fyn, or control SH2 domains from the non-Src family kinase Abl. The SH2 domain of Fyn acts as a specific dominant negative inhibitor of the activation of Src family kinases (Roche et al., 1995; Giusti et al., 1999b; Abassi et al., 2000; Kinsey and Shen, 2000). The activation of several members of the Src kinase family is probably inhibited by the Fyn SH2 domain, since the SH2 domains of the mammalian Src family kinases that have been studied have similar phosphopeptide binding specificities (Songyang et al., 1995). Which particular Src family kinases are present in ascidian eggs is unknown.

All control eggs injected with 1 mg/ml Abl SH2 domains showed $Ca^{2+}$ rises similar to those that normally occur at fertilization (Fig. 2A, Table 1), while 5 out of 8 eggs injected with 1 mg/ml (25 μM) Fyn SH2 domains showed no $Ca^{2+}$ release following the action potential (Fig. 2B, Table 1). 3 of the 8 eggs injected with Fyn SH2 domains did release intracellular $Ca^{2+}$ at fertilization, but the time from the action potential to intracellular $Ca^{2+}$ release was increased by several minutes (Fig. 2C, Table 1). In these eggs, the peak amplitude of the $Ca^{2+}$ increase, and its rate of rise, were the same as in control eggs; the duration of the initial $Ca^{2+}$ elevation was shorter, but the subsequent three oscillations appeared to be normal (Fig. 2C, Table 1). These results indicate that SH2

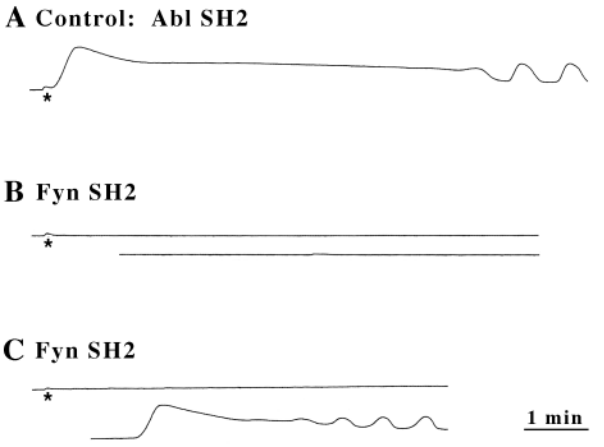


**Fig. 2.** Fyn SH2 protein inhibits intracellular $Ca^{2+}$ release at fertilization in ascidian eggs. Eggs were coinjected with 10 μM calcium green dextran and the indicated protein and then inseminated 1-2 hours later. Traces show calcium green fluorescence as a function of time. Asterisks indicate the action potential. (A) Abl SH2, 1 mg/ml (control). $Ca^{2+}$ was released from intracellular stores beginning 10 seconds after the action potential rise. (B) Fyn SH2, 1 mg/ml. No $Ca^{2+}$ rise was detected after the action potential during the 14 minute recording period (5 of 8 eggs tested). (C) Fyn SH2, 1 mg/ml. A $Ca^{2+}$ rise occurred 7 minutes after the action potential rise (3 of 8 eggs tested).

domain-mediated activity of a Src family tyrosine kinase is required for initiating intracellular $Ca^{2+}$ release at fertilization in ascidian eggs. The action potential component of the $Ca^{2+}$ rise still occurs in eggs injected with PLCγ or with Fyn SH2 domains, indicating that this $Ca^{2+}$ rise does not depend on PLCγ or Src family kinases.

To confirm that the inhibition of $Ca^{2+}$ release at fertilization by PLCγ or Fyn SH2 domains is upstream of $IP_3$ production, *Ciona* eggs were injected with 1 mg/ml PLCγ SH2 or Fyn SH2 domains followed by 50 nM $IP_3$ (close to the minimum amount of $IP_3$ needed to initiate $Ca^{2+}$ release). Control eggs injected with 50 nM $IP_3$, without SH2 domains, showed a $Ca^{2+}$ rise immediately after injection (within the several seconds required to open the photomultiplier shutter), which was followed by 2-4 oscillations ($n$=3). A longer series of $Ca^{2+}$ transients like that at fertilization is seen only with constant perfusion of $IP_3$ (Albrieux et al., 1997). In response to injection of 50 nM $IP_3$, all eggs that had been preinjected with PLCγ SH2 domains ($n$=4) or Fyn SH2 domains ($n$=4) showed an immediate $Ca^{2+}$ rise followed by 2-4 oscillations, as in controls (data not shown).

To confirm that sperm entry occurred in the PLCγ and Fyn SH2 domain injected eggs, we used the DNA stain DAPI. The DAPI was injected, rather than applied externally, in order to stain only internalized sperm. The DAPI injection was made 2-3 minutes after insemination, and the egg was observed 1-7 minutes after the injection, before the dye began to leak out of the egg (Kaji et al., 2000). A calcium green recording was made in each of these eggs, to be sure that no $Ca^{2+}$ release had occurred at the time sperm nuclei were first observed in the cytoplasm. 1-3 nuclei were detected in the cytoplasm of eggs injected with 1 mg/ml of the SH2 domains of PLCγ ($n$=4) or Fyn ($n$=2) (Fig. 3). Due to the optical density of the egg cytoplasm, it is possible that additional sperm entries occurred

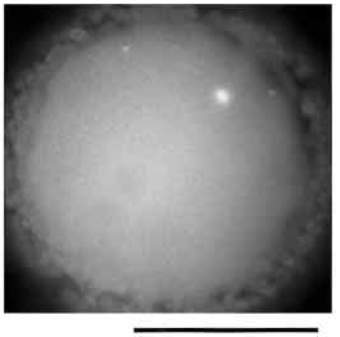

**Fig. 3.** Fyn SH2 protein does not inhibit sperm entry in ascidian eggs. The egg was injected with 1 mg/ml Fyn SH2 and 10 μM calcium green dextran, and then inseminated while recording calcium green fluorescence, as described for Fig. 2. 2.6 minutes after insemination, the egg was injected with DAPI (10 μg/ml final concentration), and observed as described in the Materials and Methods to determine that sperm entry had occurred (first seen at 1.4 minutes after the DAPI injection). After sperm entry was detected, we checked the calcium green signal to be sure no $Ca^{2+}$ rise had occurred, and then took the photograph. The egg is seen within the surrounding layer of autofluorescent test cells. Within the egg cytoplasm, the large bright spot indicates the egg chromosomes, and the two smaller spots, at 11:30 and 2:00, are sperm nuclei. Scale bar, 100 μm.

but were not detected. The occurence of polyspermy in the SH2 domain-injected eggs suggests that $Ca^{2+}$ release may be necessary for the establishment of a polyspermy block.

### Injection of sperm extract into ascidian eggs stimulates $Ca^{2+}$ release like that which occurs at fertilization

Studies of the ascidian species *Ciona savignyi* (Kyozuka et al., 1998) and *Ciona intestinalis* (Wilding and Dale, 1998) have demonstrated that injecting ascidian sperm extract into these eggs stimulates a series of $Ca^{2+}$ rises similar to that at fertilization. We confirmed these findings using *Ciona intestinalis*; eggs injected with sperm extract produced a series of $Ca^{2+}$ rises resembling that at fertilization, except that no action potential was seen (Fig. 4A,B, Tables 1 and 2). As reported for *C. savignyi* (Kyozuka et al., 1998), the sperm extract initiated a $Ca^{2+}$ rise in *C. intestinalis* that originated at the egg surface and propagated across the egg in the form of a wave like that at fertilization (Fig. 5). Control eggs injected with heat-inactivated sperm extract ($n$=5) did not show $Ca^{2+}$ rises, indicating that the activating factor is most likely a protein (Fig. 4C).

To quantitate the amount of sperm extract required to release $Ca^{2+}$, various amounts of sperm extract were injected into eggs. All eggs injected with 35-135 pg of sperm extract protein showed $Ca^{2+}$ rises, starting 84±149 seconds after injection (mean ± s.d., $n$=18). With a 2-12 pg injection, $Ca^{2+}$ release did not occur ($n$=4). A single *Ciona intestinalis* sperm contains approx. 11 pg of total protein. Therefore, 12-35 pg of sperm extract corresponds to the amount of protein present in approx. 1-3 sperm, indicating that, under our experimental conditions, microinjection of between 1 and 3 sperm equivalents of extract protein is needed to cause $Ca^{2+}$ release. Since some $Ca^{2+}$ releasing activity may be lost in the process of preparing the sperm extract (see Materials and Methods), these observations are consistent with the idea that the protein present in a single sperm is sufficient to cause $Ca^{2+}$ release. Furthermore, microinjection of the sperm extract into the center of the egg cytoplasm differs from the process of sperm-egg fusion, which

**Table 2. $Ca^{2+}$ release from eggs injected with SH2 domain proteins and then injected with sperm extract**

| SH2 domain protein injected (1 mg/ml) | % of eggs with $Ca^{2+}$ release | Time to the $Ca^{2+}$ release (seconds)[1] | Peak amplitude[2] | Number of eggs |
|---|---|---|---|---|
| None | 100 | 54±54 | 1.5±0.3 | 10 |
| SHP2 SH2 | 90 | 113±78 | 1.7±0.2 | 10 |
| PLCγ SH2 | 0[3] | – | – | 7 |
| Abl SH2 | 86 | 66±25 | 1.6±0.2 | 7 |
| Fyn SH2 | 17[3] | 975 | 1.1 | 6 |

Eggs were coinjected with 10 μM calcium green dextran and the indicated protein. 1-2 hours later, the eggs were injected with sperm extract (108 pg), and calcium green fluorescence was monitored as in Figs 6 and 7.

[1]Values refer to the time between sperm extract injection and the time at which the fluorescence versus time trace first started to rise as $Ca^{2+}$ was released from intracellular stores (mean ± s.d.). Values include only those eggs that released intracellular $Ca^{2+}$ (only one egg injected with Fyn SH2 domains showed $Ca^{2+}$ release).

[2]Values are expressed as the peak increase in fluorescence after sperm extract injection divided by the fluorescence of the unstimulated egg (mean ± s.d.). Values include only those eggs that released intracellular $Ca^{2+}$.

[3]These values are significantly different from controls (Chi-square test, $P$<0.02).

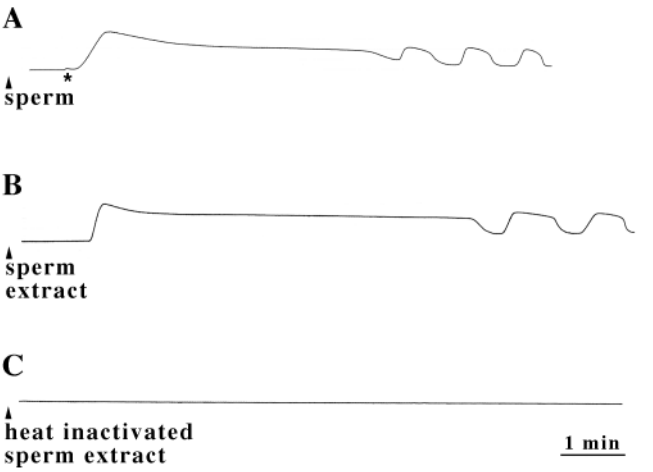


**Fig. 4.** Injection of sperm extract into ascidian eggs stimulates $Ca^{2+}$ release like that which occurs at fertilization. Traces show calcium green fluorescence as a function of time. The asterisk indicates the action potential at fertilization. Arrowheads represent the time of either sperm addition or sperm extract injection. (A) An egg injected with 10 μM calcium green dextran and then inseminated 1-2 hours later. A $Ca^{2+}$ rise occurred beginning 10 seconds after the action potential rise (14 of 14 eggs tested). (B) An egg injected with 10 μM calcium green dextran and then injected with sperm extract (108 pg protein) 1-2 hours later. A $Ca^{2+}$ rise occurred 1.3 minutes after the extract injection (10 of 10 eggs tested). (C) A control egg injected with 10 μM calcium green dextran and then injected with heat-inactivated sperm extract 1-2 hours later. The heat-inactivated extract was prepared by heating the same extract as used in B; see Materials and Methods. No $Ca^{2+}$ rise was detected during the 10 minute recording period (5 of 5 eggs tested).

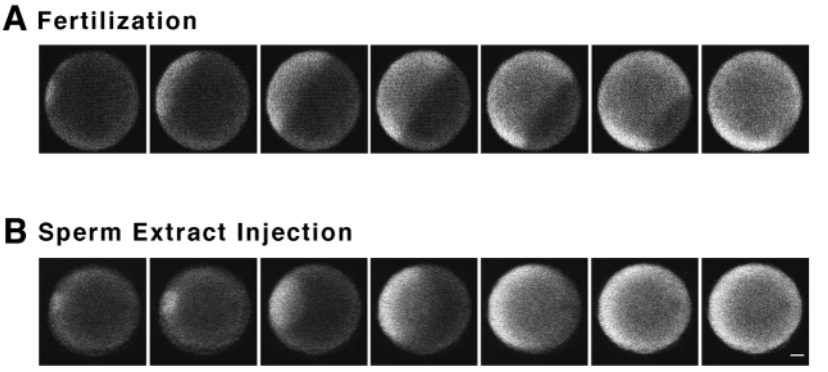


**Fig. 5.** Imaging of intracellular $Ca^{2+}$ release at fertilization and in response to sperm extract injection. Eggs were injected with calcium green dextran and 1-2 hours later either inseminated or injected with sperm extract. Fluorescence was monitored using a confocal microscope. Images are shown at 3 second intervals. Only the first $Ca^{2+}$ wave is shown. (A) An egg injected with calcium green dextran and then inseminated (representative of 6 similar records). (B) An egg injected with calcium green dextran and then injected with 108 pg sperm extract protein (representative of 5 similar records). Bar, 20 μm.

introduces sperm proteins in a concentrated form near the egg plasma membrane.

To investigate if an extract made from a tissue other than sperm could initiate $Ca^{2+}$ rises in the ascidian egg, we prepared an extract from a *Ciona* ovary. Of three eggs injected with the ovary extract (216 pg protein), none showed a $Ca^{2+}$ rise during a 15 minute recording period. This result indicates that the activating factor present in sperm is not present in ovary.

To test if the sperm extract could initiate $Ca^{2+}$ rises in the egg when applied externally, 70 pl of sperm extract (98 pg protein) was injected into the approx. 700 pl space between the chorion (external egg envelope) and the egg plasma membrane. Of three eggs tested, none showed a $Ca^{2+}$ rise during a 15 minute recording period. Injection of fluorescent 10 kDa dextran into the perichorion space showed that fluorescence was retained in this space for at least 30 minutes, indicating that sperm extract probably remained in this space during the recording period. These results show that the sperm extract is only effective when injected into the egg cytoplasm, and does not initiate $Ca^{2+}$ rises in the egg by an external action on the egg surface.

### The release of $Ca^{2+}$ from intracellular stores in response to sperm extract injection is inhibited by injection of SH2 domains of PLCγ or Fyn

If a soluble sperm factor initiates $Ca^{2+}$ release at fertilization, then sperm extract injection should cause $Ca^{2+}$ release by the same pathway as operates at fertilization. To determine if sperm extract-induced $Ca^{2+}$ release requires SH2-mediated activation of PLCγ, *Ciona* eggs were coinjected with calcium green dextran and 1 mg/ml PLCγ SH2 domains or control SHP2 SH2 domains. After 1-2 hours, these eggs were injected with sperm extract (108 pg protein) and calcium green fluorescence was monitored. While 9 out of 10 eggs preinjected with control SH2 domains showed a normal series of $Ca^{2+}$ rises in response to sperm extract injection, none of the eggs preinjected with PLCγ SH2 domains showed any $Ca^{2+}$ release in response to sperm extract injection (Fig. 6A,B, Table 2). These results indicate that, like $Ca^{2+}$ release at fertilization, sperm extract-induced $Ca^{2+}$ release requires SH2 domain-mediated activation of PLCγ.

We next investigated if, as at fertilization, sperm extract-induced $Ca^{2+}$ release requires SH2 domain-mediated activity of a Src family kinase. *Ciona* eggs were coinjected with calcium green dextran and with 1 mg/ml Fyn SH2 domains or control Abl SH2 domains and then, 1-2 hours later, injected with sperm extract (108 pg protein). Although 6 out of 7 eggs

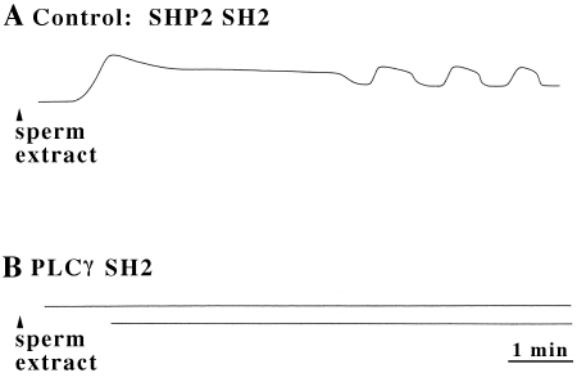


**Fig. 6.** PLCγ SH2 protein inhibits sperm extract-induced $Ca^{2+}$ release in ascidian eggs. Eggs were coinjected with 10 μM calcium green dextran and the indicated protein and then injected with sperm extract (108 pg protein) 1-2 hours later. Traces show calcium green fluorescence as a function of time. Arrowheads indicate the time of sperm extract injection. A. SHP2 SH2, 1 mg/ml (control). $Ca^{2+}$ was released 1.2 minutes after the sperm extract injection (9 of 10 eggs tested). B. PLCγ SH2, 1 mg/ml. No $Ca^{2+}$ rise was detected after the sperm extract injection during the 16 minute recording period (7 of 7 eggs tested).

preinjected with control SH2 domains showed a normal series of $Ca^{2+}$ rises in response to sperm extract injection, only 1 of 6 eggs preinjected with Fyn SH2 domains showed $Ca^{2+}$ release in response to sperm extract injection (Fig. 7A,B, Table 2), and in the one egg that released intracellular $Ca^{2+}$, the release was delayed (Table 2). These results indicate that, like $Ca^{2+}$ release at fertilization, sperm extract-induced $Ca^{2+}$ release also requires SH2 domain-mediated activity of a Src family kinase.

## DISCUSSION

### Signal transduction leading to $Ca^{2+}$ release at fertilization in ascidian eggs requires phospholipase Cγ and a Src family kinase

While a $Ca^{2+}$ rise appears to be an essentially universal signal for restarting the cell cycle in eggs at fertilization, the pathway leading to this $Ca^{2+}$ rise varies among different species (see Introduction). Along the evolutionary branch that includes echinoderms, ascidians and vertebrates, the $Ca^{2+}$ rise results from $Ca^{2+}$ release from the endoplasmic reticulum, and this process requires $IP_3$ (see Table 3). In echinoderms and

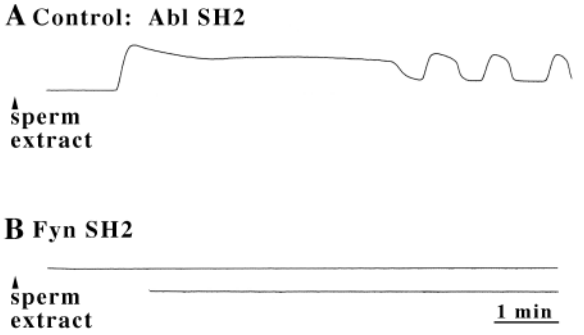


**Fig. 7.** Fyn SH2 protein inhibits sperm extract-induced $Ca^{2+}$ release in ascidian eggs. Eggs were coinjected with 10 μM calcium green dextran and the indicated protein and then injected with sperm extract (108 pg protein) 1-2 hours later. Traces show calcium green fluorescence as a function of time. Arrowheads indicate the time of sperm extract injection. (A) Abl SH2, 1 mg/ml (control). $Ca^{2+}$ was released 1.6 minutes after the sperm extract injection (6 of 7 eggs tested). (B) Fyn SH2, 1 mg/ml. No $Ca^{2+}$ rise was detected after the sperm extract injection during the 15 minute recording period (5 of 6 eggs tested).

ascidians, $IP_3$ production results from an SH2 domain-mediated activation of a Src like kinase and PLCγ, whereas in vertebrates (frog and mouse), a different and as yet unidentified pathway leads to $IP_3$ production at fertilization (see Table 3).

In this study of ascidian eggs, we demonstrate that PLCγ is required for the release of intracellular $Ca^{2+}$ at fertilization, by showing that injection of eggs with the SH2 domains of PLCγ, which act as specific dominant negative inhibitors of PLCγ activation, inhibits $Ca^{2+}$ release at fertilization. Likewise, we demonstrate that a Src-like kinase is required in this pathway, by showing that injection of the SH2 domain of the Src family kinase Fyn inhibits $Ca^{2+}$ release at fertilization. That tyrosine kinase activity is required for ascidian egg activation is also indicated by studies showing that the tyrosine kinase inhibitor erbstatin inhibits the surface contraction that occurs at about 5 minutes after insemination (Ueki and Yokosawa, 1997).

The specific action of PLCγ and Fyn SH2 domains was demonstrated by the lack of effect of control SH2 domains from other proteins on $Ca^{2+}$ release, by the ability of $IP_3$ to bypass the PLCγ and Fyn SH2 domain inhibition, and by the lack of inhibition of sperm entry by PLCγ and Fyn SH2 domains. Additional specificity controls have been done in echinoderm eggs, where it was found that injecting SH2 domains of several other kinases and phosphatases, or point-mutated forms of PLCγSH2 and SrcSH2, had no effect on $Ca^{2+}$ release at fertilization (Carroll et al., 1997, 1999; Shearer et al., 1999; Giusti et al., 1999b; Abassi et al., 2000; Kinsey and Shen, 2000). Specificity was further established by the findings in echinoderm eggs that PLCγ SH2 domains do not inhibit $Ca^{2+}$ release in response to PLCβ stimulation, cholera toxin, cGMP or cADP ribose (Carroll et al., 1997, 1999), and that Fyn SH2 domains have only a small effect on $Ca^{2+}$ release in response to cGMP (Kinsey and Shen, 2000).

The concentration dependence of the inhibitory effects of SH2 domains is in the low micromolar range, and shows some variability among different animal species. In sea urchin eggs, 1-2 μM of the mammalian PLCγ SH2 domains is sufficient to completely inhibit $Ca^{2+}$ release at fertilization (Carroll et al., 1999; Shearer et al., 1999), and a significant delay is seen at 0.1-0.3 μM (Shearer et al., 1999). In contrast, in starfish and ascidian eggs, 20 μM of the PLCγ SH2 domains is required to inhibit $Ca^{2+}$ release completely, and 2 μM results in a delay but not complete inhibition (Carroll et al., 1997; present results). This difference in sensitivity between species is not understood, but could be related to differences in the amino acid sequences of the endogenous PLCγ proteins in these organisms (see Shearer et al., 1999). Fyn SH2 domains completely inhibit or substantially delay $Ca^{2+}$ release at fertilization in ascidian, sea urchin and starfish eggs at 25 μM (Giusti et al., 1999b; Abassi et al., 2000; Kinsey and Shen, 2000; present results). Although lower concentrations have not been examined in ascidian eggs, 2.5 μM Fyn SH2 domains significantly delay $Ca^{2+}$ release at fertilization in sea urchin and starfish eggs (Giusti et al., 1999b; Abassi et al., 2000).

**Table 3. Signalling molecules required for $Ca^{2+}$ release at fertilization in eggs of various animals**

| Signalling molecule | Echinoderm | Ascidian | Frog | Mouse |
|---|---|---|---|---|
| $IP_3$[1] | + | + | + | + |
| PLCγ SH2[2] | + | + | – | – |
| Tyrosine kinase[3] | + | + | + | ? |
| Src family kinase SH2[4] | + | + | ? | ? |

[1]Echinoderm: Whitaker and Irvine, 1984; Ciapa and Whitaker, 1986; Mohri et al., 1995; Carroll et al., 1997, 1999; Lee and Shen, 1998; Shearer et al., 1999. Ascidian: this paper. Frog: Nuccitelli et al., 1993; Runft et al., 1999. Mouse: Miyazaki et al., 1993.

[2]Echinoderm: Carroll et al., 1997, 1999; Shearer et al., 1999. Ascidian: this paper. Frog: Runft et al., 1999. Mouse: Mehlmann et al., 1998.

[3]Echinoderm: Shen et al., 1999. Ascidian: Ueki and Yokosawa, 1997. Frog: Glahn et al., 1999; Sato et al., 2000. Mouse: Dupont et al., 1996.

[4]Echinoderm: Giusti et al., 1999a,b, 2000; Abassi et al., 2000; Kinsey and Shen, 2000. Ascidian: this paper. Frog and mouse: not tested.

Our finding that PLCγ SH2 domains can completely inhibit $Ca^{2+}$ release at fertilization indicates that $IP_3$ is required to initiate $Ca^{2+}$ release at fertilization in ascidians, and that the other known $Ca^{2+}$ release channel in the endoplasmic reticulum, the ryanodine receptor, does not initiate this $Ca^{2+}$ release. This is consistent with previous studies showing that ruthenium red, an inhibitor of the ryanodine receptor, does not inhibit $Ca^{2+}$ release at fertilization in ascidians (Wilding and Dale, 1998; Yoshida et al., 1998).

Our results support the hypothesis that in ascidian eggs, a Src family kinase, directly or through intermediate molecules, phosphorylates and activates PLCγ. The Src family kinases are among the tyrosine kinases known to participate in the activation of PLCγ in other cells (Arkinstall et al., 1995; Melford et al., 1997; Clements and Koretsky, 1999; Schlesinger et al., 1999), and studies in echinoderms indicate that activation of a Src family kinase leads to activation of PLCγ at fertilization. Recombinant SH2 domains of PLCγ associate with a starfish egg Src family kinase in a fertilization-dependent manner (Giusti et al., 1999a), and this association occurs by 15 seconds post-insemination. In addition, PLCγ from fertilized sea urchin eggs can form a stable complex with recombinant domains of Fyn (Kinsey and Shen, 2000). Injection of starfish eggs with PLCγ SH2 domains delays $Ca^{2+}$ release in response to injection of recombinant Src protein, indicating that Src acts upstream of PLCγ (Giusti et al., 2000).

As in most species, the release of $Ca^{2+}$ at fertilization of

ascidian eggs is a regenerative process that proceeds across the egg in a wave; $Ca^{2+}$ then remains high for several minutes before beginning to oscillate (see Introduction). Our results with ascidian eggs indicate that the SH2 domains of PLCγ and the Src family kinase function primarily in the initiation of $Ca^{2+}$ release, and have only a minor effect on the subsequent pattern of $Ca^{2+}$ elevation. This conclusion is based on the observation that in the few cases where fertilization caused a $Ca^{2+}$ response in the presence of PLCγ or Fyn SH2 domains, its initiation was delayed but its peak amplitude and rate of rise were unaffected. The only effect of the PLCγ and Fyn SH2 domains that we observed on the $Ca^{2+}$ response was that the duration of the initial $Ca^{2+}$ plateau was somewhat shorter. As in many other cells, such as HeLa cells responding to histamine (Bootman et al., 1997), amplification of $Ca^{2+}$ release in the ascidian egg probably occurs when $Ca^{2+}$ and/or $IP_3$ at the sperm interaction site rises to a critical threshold, leading to an almost 'all-or-none' response. In echinoderm eggs, the initiation and amplification steps in $Ca^{2+}$ release appear to be somewhat more interdependent. In these eggs, the injection of PLCγ or Src family kinase SH2 domains both delays and reduces the amplitude of the $Ca^{2+}$ release (Carroll et al., 1997, 1999; Shearer et al., 1999; Giusti et al., 1999b; Abassi et al., 2000; Kinsey and Shen, 2000). Whether an initiating signal results in a graded or all-or-none response may depend on the balance between positive feedback components that raise $Ca^{2+}$, such as $Ca^{2+}$ activation of phospholipase C and the $IP_3$ receptor channel, and other factors that lower $Ca^{2+}$, such as degradation of $IP_3$, $Ca^{2+}$ pump activity, and the inhibition of some $IP_3$ receptor isoforms by high $Ca^{2+}$ (see Miyazaki et al., 1993; Bootman et al., 1997; Hagar et al., 1998).

### Fertilization and sperm extract injection activate a similar signalling pathway to stimulate $Ca^{2+}$ release in ascidian eggs

In eggs of various species, injection of a sperm extract causes intracellular $Ca^{2+}$ release like that at fertilization (see Introduction). Our present results show that for ascidians, the signalling pathway initiated by sperm extract injection requires the same components, a Src family kinase and PLCγ, that are required at fertilization. These findings support the hypothesis that during fertilization, intracellular $Ca^{2+}$ release is initiated by a factor from the sperm that enters the egg as a consequence of sperm-egg fusion.

The component in the ascidian sperm extract that stimulates egg activation is heat labile, and is between 30 and 100 kDa in molecular mass (Kyozuka et al., 1998). Sperm extract activity is not affected by high speed centrifugation (Wilding and Dale 1998; present results) or by the presence of protease inhibitors in the extraction buffer (Wilding and Dale, 1998), indicating that the factor is soluble and is probably not a protease. The activating factor found in sperm is not present in ovary (present results).

In mammals, several specific sperm proteins have been investigated as possible candidates for the egg activating factor, but none has been definitively established (Sette et al., 1997; Wolosker et al., 1998; Swann and Parrington, 1999; Perry et al., 2000). Our findings indicate that, in ascidians, the activating factor in the sperm extract may be a regulator, directly or indirectly, of a Src family kinase in the egg. Src family kinases can be regulated by kinases or phosphatases that affect the phosphorylation state of two regulatory tyrosines and also by molecules that bind to their SH2 and SH3 domains (Erpel and Courtneidge, 1995; Boerner et al., 1996; Brown and Cooper, 1996; Xu et al., 1999; Thomas, 1999). Our evidence that the SH2 domain of a Src family kinase is required for $Ca^{2+}$ release at fertilization suggests that the Src activator that functions at fertilization may act by way of the Src SH2 domain. Examples of such regulators in other signalling systems include the focal adhesion kinase FAK (Thomas et al., 1998; Schaller et al., 1999), the platelet-derived growth factor receptor (see Erpel and Courtneidge, 1995), and the 'immune receptor tyrosine activation motifs' of antigen receptors (Johnson et al., 1995); these regulatory proteins bind to the SH2 domains of Src family kinases, causing them to adopt an active conformation.

In summary, our results support the hypothesis that activation of ascidian eggs at fertilization is initiated when sperm-egg fusion introduces a soluble factor from the sperm into the egg cytosol, which directly or indirectly activates a Src family kinase. This Src family kinase then directly or indirectly activates PLCγ, which produces the $IP_3$ that stimulates $Ca^{2+}$ release from the endoplasmic reticulum.

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## REFERENCES

**Abassi, Y. A., Carroll, D. J., Giusti, A. F., Belton, R. J. and Foltz, K. R.** (2000). Evidence that Src-type tyrosine kinase activity is necessary for initiation of $Ca^{2+}$ release at fertilization in sea urchin eggs. *Dev. Biol.* **218**, 206-219.

**Albrieux, M., Sardet, C. and Villaz, M.** (1997). The two intracellular $Ca^{2+}$ release channels, ryanodine receptor and inositol 1,4,5-trisphosphate receptor, play different roles during fertilization in ascidians. *Dev. Biol*. **189**, 174-185.

**Arkinstall, S., Payton, M. and Maundrell, K.** (1995). Activation of phospholipase Cγ in *Schizosaccharomyces pombe* by coexpression of receptor or nonreceptor tyrosine kinases. *Mol. Cell Biol.* **15**, 1431-1438.

**Bae, Y. S., Cantley, L. G., Chen, C. S., Kim, S. R., Kwon, K. S. and Rhee, S. G.** (1998). Activation of phospholipase C-γ by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **273**, 4465-4469.

**Boerner, R. J., Kassel, D. B., Barker, S. C., Ellis, B., DeLacy, P. and Knight, W. B.** (1996). Correlation of the phosphorylation states of $pp60^{c\text{-}src}$ with tyrosine kinase activity: The intramolecular pY530-SH2 complex retains significant activity if Y419 is phosphorylated. *Biochemistry* **35**, 9519-9525.

**Bootman, M. D., Berridge, M. J. and Lipp, P.** (1997). Cooking with calcium: the recipes for composing global signals from elementary events. *Cell* **91**, 367-373.

**Brown, M. T. and Cooper, J. A.** (1996). Regulation, substrates and functions of src. *Biochim. Biophys. Acta* **1287**, 121-149.

**Brownlee, C. and Dale, B.** (1990). Temporal and spatial correlation of fertilization current, calcium waves, and cytoplasmic contraction in eggs of *Ciona intestinalis*. *Proc. R. Soc. Lond*. B **239**, 321-328.

**Carroll, D. J. and Jaffe, L. A.** (1995). Proteases stimulate fertilization-like responses in starfish eggs. *Dev. Biol.* **170**, 690-700.

**Carroll, D. J., Ramarao, C. S., Mehlmann, L. M., Roche, S., Terasaki, M. and Jaffe, L. A.** (1997). Calcium release at fertilization in starfish eggs is mediated by phospholipase Cγ. *J. Cell Biol*. **138**, 1303-1311.

**Carroll, D. J., Albay, D. T., Terasaki, M., Jaffe, L. A. and Foltz, K. R.** (1999). Identification of PLCγ-dependent and independent events during fertilization of sea urchin eggs. *Dev. Biol.* **206**, 232-257.

**Chiba, K., Kado, R. T. and Jaffe, L. A.** (1990). Development of calcium release mechanisms during starfish oocyte maturation. *Dev. Biol*. **140**, 300-306.

**Ciapa, B. and Whitaker, M.** (1986). Two phases of inositol polyphosphate and diacylglycerol production at fertilization. *FEBS Lett.* **195**, 347-351.

**Clements, J. S. and Koretzky, G. A.** (1999). Recent developments in lymphocyte activation: linking kinases to downstream signaling events. *J. Clin. Invest.* **103**, 925-929.

**Collas, P., Chang, T., Long, C. and Robl, J. M.** (1995). Inactivation of histone H1 kinase by $Ca^{2+}$ in rabbit oocytes. *Mol. Reprod. Dev.* **40**, 253-258.

**Créton, R. and Jaffe, L. F.** (1995). Role of calcium influx during the latent period in sea urchin fertilization. *Dev. Growth Differ*. **37**, 703-709.

**Dale, B.** (1988). Primary and secondary messengers in the activation of ascidian eggs. *Exp. Cell Res.* **177**, 205-211.

**Dale, B., De Felice, L. J. and Ehrenstein, G.** (1985). Injection of soluble sperm extract into sea urchin eggs triggers the cortical reaction. *Experientia* **41**, 1068-1070.

**De Santis, R. and Pinto, M. R.** (1991). Gamete self-discrimination in ascidians: A role for the follicle cells. *Mol. Reprod. Dev.* **29**, 47-50.

**Dupont, G., McGuinness, O. M., Johnson, M. H., Berridge, M. J. and Borgese, F.** (1996). Phospholipase C in mouse ooctyes: Characterization of β and γ isoforms and their possible involvement in sperm-induced $Ca^{2+}$ spiking. *Biochem. J.* **316**, 583-591.

**Erpel, T. and Courtneidge, S.** (1995). Src family protein tyrosine kinases and cellular signal transduction pathways. *Curr. Opin. Cell Biol*. **7**, 176-182.

**Feng, G., Hui, C. and Pawson, T.** (1993). SH2-containing phospho-tyrosine phosphatase as a target of protein-tyrosine kinases. *Science* **259**, 1607-1611.

**Flannery, B. and Epel, D.** (1998). Effects of wheat germ agglutinin on tunicate egg activation and fertilization: Is there a plasma membrane sperm receptor system on *Ascidia ceratodes* eggs? *Dev. Growth Differ*. **40**, 297-306.

**Gish, G., Larose, L., Shen, R. and Pawson, T.** (1995). Biochemical analysis of SH2 domain-mediated protein interactions. *Meth. Enzymol*. **254**, 503-523.

**Giusti, A. F., Carroll, D. J., Abassi, Y. A. and Foltz, K. R.** (1999a). Evidence that a starfish egg src family tyrosine kinase associates with PLC-γ1 SH2 domains at fertilization. *Dev. Biol*. **208**, 189-199.

**Giusti, A. F., Carroll, D. J., Abassi, Y. A., Terasaki, M., Foltz, K. R. and Jaffe, L. A.** (1999b). Requirement of an Src family kinase for initiating calcium release at fertilization of starfish eggs. *J. Biol. Chem.* **274**, 29318-29322.

**Giusti, A. F., Xu, W., Hinkle, B., Terasaki, M. and Jaffe, L.A.** (2000). Evidence that fertilization activates starfish eggs by sequential activation of an Src-like kinase and phospholipase Cγ. *J. Biol. Chem.* **275**, 16788-16794.

**Glahn, D., Mark, D. S., Behr, R. K. and Nuccitelli, R.** (1999). Tyrosine kinase inhibitors block sperm-induced egg activation in *Xenopus laevis*. *Dev. Biol*. **205**, 171-180.

**Goudeau, M. and Goudeau, H.** (1993). In the egg of the ascidian *Phallusia mammillata*, removal of external $Ca^{2+}$ modifies the fertilization potential, induces polyspermy, and blocks the resumption of meiosis. *Dev. Biol*. **160**, 165-177.

**Goudeau, H., Goudeau, M. and Guibourt, N.** (1992). The fertilization potential and associated membrane potential oscillations during the resumption of meiosis in the egg of the ascidian *Phallusia mammillata*. *Dev. Biol*. **153**, 227-241.

**Goudeau, H., Depresle, Y., Rosa, A. and Goudeau, M.** (1994). Evidence by a voltage clamp study of an electrically mediated block to polyspermy in the egg of the ascidian *Phallusia mammillata*. *Dev. Biol*. **166**, 489-501.

**Gould, M. and Stephano, J. L.** (1987). Electrical responses of eggs to acrosomal protein similar to those induced by sperm. *Science* **235**, 1653-1656.

**Hagar, R. E., Burgstahler, A. D., Nathanson, M. H. and Ehrlich, B. E.** (1998). Type III $InsP_3$ receptor channel stays open in the presence of increased calcium. *Nature* **396**, 81-84.

**Hiramoto, Y.** (1962). Microinjection of live spermatozoa into sea urchin eggs. *Exp. Cell Res.* **27**, 416-426.

**Jaffe, L. A., Gould-Somero, M. and Holland, L.** (1979). Ionic mechanism of the fertilization potential of the marine worm, *Urechis caupo* (Echiura). *J. Gen. Physiol.* **73**, 469-492.

**Johnson, S. A., Pleiman, C. M., Pao, L., Schneringer, J., Hippen, K. and Cambier, J. C.** (1995). Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. *J. Immunol*. **155**, 4596-4603.

**Jones, K. T, Soeller, C. and Cannell, M. B.** (1998). The passage of $Ca^{2+}$ and fluorescent markers between the sperm and egg after fusion in the mouse. *Development* **125**, 4627-4635.

**Kaji, K., Oda, S., Shikano, T., Ohnuki, T., Uematsu, Y., Sakagami, J., Tada, N., Miyazaki, S. and Kudo, A.** (2000). The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nature Genet.* **24**, 279-282.

**Kiehart, D. P.** (1982). Microinjection of echinoderm eggs: Apparatus and procedures. *Meth. Cell Biol.* **25**, 13-31.

**Kinsey, W. H. and Shen, S. S.** (2000). Role of the Fyn kinase in calcium release during fertilization of the sea urchin egg. *Dev. Biol.* (in press).

**Kline, D. and Kline, J. T.** (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80-89.

**Kline, D., Mehlmann, L., Fox, C. and Terasaki, M.** (1999). The cortical endoplasmic reticulum (ER) of the mouse egg: localization of ER clusters in relation to the generation of repetitive calcium waves. *Dev. Biol.* **215**, 431-442.

**Kyozuka, K., Deguchi, R., Mohri, T. and Miyazaki, S.** (1998). Injection of sperm extract mimics spatiotemporal dynamics of $Ca^{2+}$ responses and progression of meiosis at fertilization of ascidian oocytes. *Development* **125**, 4099-4105.

**Lee, S. J. and Shen, S. S.** (1998). The calcium transient in sea urchin eggs during fertilization requires the production of inositol 1,4,5-trisphosphate. *Dev. Biol.* **193**, 195-208.

**Levasseur, M. and McDougall, A.** (2000). Sperm-induced calcium oscillations at fertilisation in ascidians are controlled by cyclin B1-dependent kinase activity. *Development* **127**, 631-641.

**McCulloh, D. H. and Chambers, E. L.** (1992). Fusion of membranes during fertilization: Increases of sea urchin egg's membrane capacitance and membrane conductance at the site of contact with the sperm. *J. Gen. Physiol.* **99**, 137-175.

**McDougall, A. and Levasseur, M.** (1998). Sperm-triggered calcium oscillations during meiosis in ascidian oocytes first pause, restart, then stop: correlations with cell cycle kinase activity. *Development* **125**, 4451-4459.

**McDougall, A. and Sardet, C.** (1995). Function and characteristics of repetitive calcium waves associated with meiosis. *Curr. Biol.* **5**, 318-328.

**Mehlmann, L. M., Carpenter, G., Rhee, S. G. and Jaffe, L. A.** (1998). SH2 domain-mediated activation of phospholipase Cγ is not required to initiate $Ca^{2+}$ release at fertilization of mouse eggs. *Dev. Biol*. **203**, 221-232.

**Melford, S. K., Turner, M., Briddon, S. J., Tybulewicz, V. L. J. and Watson, S. P.** (1997). Syk and fyn are required by mouse megakaryocytes for the rise in intracellular calcium induced by a collagen-related peptide. *J. Biol. Chem.* **272**, 27539-27542.

**Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S. and Mikoshiba, K.** (1992). Block of $Ca^{2+}$ wave and $Ca^{2+}$ oscillation by antibody to the inositol 1,4,5- trisphosphate receptor in fertilized hamster eggs. *Science* **257**, 251-255.

**Miyazaki, S., Shirakawa, H., Nakada, K. and Honda, Y.** (1993). Essential role of the inositol 1,4,5-trisphosphate receptor/$Ca^{2+}$ release channel in $Ca^{2+}$ waves and $Ca^{2+}$ oscillations at fertilization of mammalian eggs. *Dev. Biol.* **158**, 62-78.

**Mizote, A., Okamoto, S. and Iwao, Y.** (1999). Activation of *Xenopus* eggs by proteases: possible involvement of a sperm protease in fertilization. *Dev. Biol.* **208**, 79-92.

**Mohri, T., Ivonnet, P. I. and Chambers, E. L.** (1995). Effect on sperm-induced activation current and increase of cytosolic $Ca^{2+}$ by agents that modify the mobilization of $[Ca^{2+}]_i$. I. Heparin and pentosan polysulfate. *Dev. Biol*. **172**, 139-157.

**Nuccitelli, R., Yim, D. L. and Smart, T.** (1993). The sperm-induced $Ca^{2+}$ wave following fertilization of the *Xenopus* egg requires the production of $Ins(1,4,5)P_3$. *Dev. Biol*. **158**, 200-212.

**Oda, S., Deguchi, R., Mohri, T., Shikano, T., Nakanishi, S. and Miyazaki, S.** (1999). Spatiotemporal dynamics of the $[Ca^{2+}]$ rise induced by microinjection of sperm extract into mouse eggs: Preferential induction of a $Ca^{2+}$ wave from the cortex mediated by the inositol 1,4,5-trisphosphate receptor. *Dev. Biol.* **209**, 172-185.

**Perry, A. C. F., Wakayama, T., Cooke, I. M. and Yanagimachi, R.** (2000). Mammalian oocyte activation by the synergistic action of discrete sperm head components: induction of calcium transients and involvement of proteolysis. *Dev. Biol.* **217**, 386-393.

**Roche, S., Fumagalli, S. and Courtneidge, S. A.** (1995). Requirement for

Src family protein tyrosine kinases in $G_2$ for fibroblast cell division. *Science* **269**, 1567-1569.

**Roche, S., McGlade, J., Jones, M., Gish, G. D., Pawson, T. and Courtneidge, S. A.** (1996). Requirement of phospholipase Cγ, the tyrosine phosphatase Syp and the adaptor proteins Shc and Nck for PDGF-induced DNA synthesis: Evidence for the existence of Ras-dependent and Ras-independent pathways. *EMBO J.* **15**, 4940-4948.

**Rongish, B. J., Wu, W. and Kinsey, W. H.** (1999). Fertilization-induced activation of phospholipase C in the sea urchin egg. *Dev. Biol.* **215**, 147-154.

**Rosati, F. and De Santis, R.** (1978). Studies on fertilization in the ascidians. I. Self-sterility and specific recognition between gametes of *Ciona intestinalis*. *Exp. Cell Res.* **112**, 111-119.

**Runft, L. L., Watras, J. and Jaffe, L. A.** (1999). Calcium release at fertilization of *Xenopus* eggs requires type I $IP_3$ receptors, but not SH2 domain-mediated activation of PLCγ or $G_q$ mediated activation of PLCβ. *Dev. Biol.* **214**, 399-411.

**Russo, G. L., Kyozuka, K., Antonazzo, L., Tosti, E. and Dale, B.** (1996). Maturation promoting factor in ascidian oocytes is regulated by different intracellular signals at meiosis I and II. *Development* **122**, 1995-2003.

**Sato, K., Aoto, M., Mori, K., Akasofu, S., Tokmakov, A. A., Sahara, S. and Fukami, Y.** (1996). Purification and characterization of an Src-related p57 protein-tyrosine kinase from *Xenopus* oocytes. *J. Biol. Chem.* **271**, 13250-13257.

**Sato, K., Iwasaki, T., Tamaki, I., Aoto, M., Tokmakov, A. A. and Fukami, Y.** (1998). Involvement of protein-tyrosine phosphorylation and dephosphorylation in sperm-induced *Xenopus* egg activation. *FEBS Lett.* **424**, 113-118.

**Sato, K., Iwao, Y., Fujimura, T., Tamaki, I., Ogawa, K., Iwasaki, T., Tokmakov, A. A., Hatano, O. and Fukami, Y.** (1999). Evidence for the involvement of an Src-related tyrosine kinase in *Xenopus* egg activation. *Dev. Biol.* **209**, 308-320.

**Sato, K., Tokmakov, A. A., Iwasaki, T. and Fukami, Y.** (2000). Tyrosine kinase-dependent activation of phospholipase Cγ is required for calcium transient in *Xenopus* egg fertilization. *Dev. Biol.* (in press).

**Schaller, M. D., Hildebrand, J. D. and Parsons, J. T.** (1999). Complex formation with focal adhesion kinase: A mechanism to regulate activity and subcellular localization of Src kinases. *Mol. Biol. Cell* **10**, 3489-3505.

**Schlesinger, T. K., DeMali, K. A., Johnson, G. L. and Kazlauskas, A.** (1999). Platelet-derived growth factor-dependent association of the GTPase-activating protein of Ras and Src. *Biochem. J.* **344**, 519-526.

**Schmidt, T., Patton, C. and Epel, D.** (1982). Is there a role for the $Ca^{2+}$ influx during fertilization of the sea urchin egg? *Dev. Biol.* **90**, 284-290.

**Sensui, N. and Morisawa, M.** (1996). Effect of $Ca^{2+}$ on deformation, polar body extrusion and pronucleus formation in the egg of the ascidian, *Ciona savignyi*. *Dev. Growth Differ.* **38**, 341-350.

**Sette, C., Bevilacqua, A., Bianchini, A., Mangia, F., Geremia, R. and Rossi, P.** (1997). Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. *Development* **124**, 2267-2274.

**Shearer, J., De Nadai, C., Emily-Fenouil, F., Gache, C., Whitaker, M. and Ciapa, B.** (1999). Role of phospholipase Cγ at fertilization and during mitosis in sea urchin eggs and embryos. *Development* **126**, 2273-2284.

**Shen, S. S., Kinsey, W. H. and Lee, S. J.** (1999). Protein tyrosine kinase-dependent release of intracellular calcium in the sea urchin egg. *Dev. Growth Differ.* **41**, 345-355.

**Shilling, F. M., Magie, C. R. and Nuccitelli, R.** (1998). Voltage-dependent activation of frog eggs by a sperm surface disintegrin peptide. *Dev. Biol.* **202**, 113-124.

**Singer, W. D., Brown, H. A. and Sternweis, P. C.** (1997). Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Ann. Rev. Biochem.* **66**, 475-509.

**Songyang, A. and Cantley, L. C.** (1995). Recognition and specificity in protein tyrosine kinase-mediated signalling. *Trends Biochem. Sci.* **20**, 470-475.

**Speksnijder, J. E., Corson, D. W., Sardet, C. and Jaffe, L. F.** (1989). Free calcium pulses following fertilization in the ascidian egg. *Dev. Biol.* **135**, 182-190.

**Speksnijder, J. E, Sardet, C. and Jaffe, L. F.** (1990a). The activation wave of calcium in the ascidian egg and its role in ooplasmic segregation. *J. Cell Biol.* **110**, 1589-1598.

**Speksnijder, J. E, Sardet, C. and Jaffe, L. F.** (1990b). Periodic calcium waves cross ascidian eggs after fertilization. *Dev. Biol.* **142**, 246-249.

**Steinhardt, R. A., Lundin, L. and Mazia, D.** (1971). Bioelectric responses of the echinoderm egg to fertilization. *Proc. Nat. Acad. Sci. USA* **68**, 2426-2430.

**Stephano, J. L. and Gould, M. C.** (1997). The intracellular calcium increase at fertilization in *Urechis caupo* oocytes: Activation without waves. *Dev. Biol.* **191**, 53-68.

**Stricker, S. A.** (1997). Intracellular injections of a soluble sperm factor trigger calcium oscillations and meiotic maturation in unfertilized oocytes of a marine worm. *Dev. Biol.* **186**, 185-201.

**Stricker, S. A.** (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* **211**, 157-176.

**Swann, K.** (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development* **110**, 1295-1302.

**Swann, K. and Parrington, J.** (1999). Mechanism of $Ca^{2+}$ release at fertilization in mammals. *J. Exp. Zool.* **285**, 267-275.

**Terasaki, M. and Sardet, C.** (1991). Demonstration of calcium uptake and release by sea urchin egg cortical endoplasmic reticulum. *J. Cell Biol.* **115**, 1031-1037.

**Terasaki, M., Miyake, K. and McNeil, P. L.** (1997). Large plasma membrane disruptions are rapidly resealed by $Ca^{2+}$- dependent vesicle-vesicle fusion events. *J. Cell Biol.* **139**, 63-74.

**Thomas, M. L.** (1999). The regulation of antigen-receptor signaling by protein tyrosine phosphatases: a hole in the story. *Curr. Opin. Immunol.* **11**, 270-276.

**Thomas, J. W., Ellis, B., Boerner, R. J., Knight, W. B., White, G. C. and Schaller, M. D.** (1998). SH2- and SH3- mediated interactions between focal adhesion kinase and Src. *J. Biol. Chem.* **273**, 577-583.

**Toratani, S. and Yokosawa, H.** (1995). Evidence for the involvement of the Rho GTP-binding protein in egg activation of the ascidian *Halocynthia roretzi*. *Dev. Growth Differ.* **37**, 31-17.

**Ueki, K. and Yokosawa, H.** (1997). Evidence for an erbstatin-sensitive tyrosine kinase functioning in ascidian egg activation. *Biochem. Biophys. Res. Commun.* **238**, 130-133.

**Wang, Z., Gluck, S., Zhang, L. and Moran, M. F.** (1998). Requirement for phospholipase C-γ1 enzymatic activity in growth factor-induced mitogenesis. *Mol. Cell Biol.* **18**, 590-597.

**Whitaker, M. and Irvine, R. F.** (1984). Inositol 1,4,5-trisphosphate microinjection activates sea urchin eggs. *Nature (Lond.)* **312**, 636-639.

**Wilding, M. and Dale, B.** (1998). Soluble extracts from ascidian spermatozoa trigger intracellular calcium release independently of the activation of the ADP ribose channel. *Zygote* **6**, 149-154.

**Wilding, M., Marino, M. and Dale, B.** (1999). Nicotinamide alters the calcium release pattern and the degradation of MPF activity after fertilization in ascidian oocytes. *Zygote* **7**, 255-260.

**Wolosker, H., Kline, D., Bian, Y., Blackshaw, S., Cameron, A. M., Fralich, T. J., Schnaar, R. L. and Snyder, S. H.** (1998). Molecularly cloned mammalian glucosamine-6-phosphate deaminase localizes to transporting epithelium and lacks oscillin activity. *FASEB J.* **12**, 91-99.

**Xu, W., Doshi, A., Lei, M., Eck, M. J. and Harrison, S. C.** (1999). Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol. Cell* **3**, 629-638.

**Yoshida, M., Sensui, N., Inoue, T., Morisawa, M. and Mikoshiba, K.** (1998). Role of two series of $Ca^{2+}$ oscillations in activation of ascidian eggs. *Dev. Biol.* **203**, 122-133.

**Zalokar, M.** (1980). Activation of ascidian eggs with lectins. *Dev. Biol.* **79**, 232-237.