Sperm extract injection into ascidian eggs signals Ca\textsuperscript{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\textsuperscript{2+} release that is spatially and temporally like that occurring at fertilization, suggesting that Ca\textsuperscript{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\textsuperscript{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 C\textgamma 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\textsuperscript{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\textsuperscript{2+} release by a pathway requiring phospholipase C\textgamma and a Src family kinase. These results support the hypothesis that, in ascidians, a soluble factor from the sperm cytoplasm initiates Ca\textsuperscript{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 C\textgamma, Src family kinase

INTRODUCTION

At fertilization, eggs of most if not all species undergo a transient rise in cytosolic free Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} transients that also occur in the form of waves. These Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} rise at fertilization results from the release of Ca\textsuperscript{2+} from the endoplasmic reticulum, which is mediated, at least in part, by inositol trisphosphate (IP\textsubscript{3}). In ascidian eggs, IP\textsubscript{3} is produced at fertilization (Toratani and Yokosawa, 1995), and introduction of IP\textsubscript{3} causes Ca\textsuperscript{2+} release (McDougall and Sardet, 1995; Albrieux et al., 1997; Yoshida et al., 1998), but the question of whether IP\textsubscript{3} is required for Ca\textsuperscript{2+} release at fertilization has not been definitively answered (Russo et al., 1996; Yoshida et al., 1998; Wilding et al., 1999).

IP\textsubscript{3} is produced by the phospholipase C (PLC) family of enzymes, which cleave phosphatidylinositol 4,5-bisphosphate to generate IP\textsubscript{2} and diacylglycerol (Singer et al., 1997). The PLC family includes three subgroups: \(\beta\), \(\gamma\) and \(\delta\). PLC\textgamma 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 PLC\textgamma. Recombinant proteins containing the SH2 domains of PLC\textgamma have been used as specific dominant negative inhibitors of PLC\textgamma 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 PLC\textgamma with its activating tyrosine kinase. Injection of PLC\textgamma SH2 domains into echinoderm eggs inhibits the Ca\textsuperscript{2+} rise at fertilization, demonstrating that, in starfish and sea urchin eggs, this Ca\textsuperscript{2+} rise is initiated by SH2 domain-mediated activation of PLC\textgamma (Carroll et al., 1997, 1999; Shearer et al., 1999). Injection of PLC\textgamma SH2 domains into mouse or frog eggs, however, does not inhibit the Ca\textsuperscript{2+} rise at fertilization, indicating that, unlike echinoderms, these vertebrate eggs do not require SH2 domain-mediated activation of PLC\textgamma to initiate the Ca\textsuperscript{2+} rise at fertilization (Mehlmann et al., 1998; Runft et al., 1999). Here we investigate whether the
Ca2+ release signalling pathway in ascidians (an evolutionary intermediate deuterostome) resembles that in echinoderms or vertebrates.

In echinoderm fertilization, PLCγ activation (Rongish et al., 1999) relays, 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 starfish eggs inhibits Ca2+ 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 Ca2+ rise in the egg at fertilization is unknown. One possibility is that influx of extracellular Ca2+ might initiate this process (see Créton and Jaffe, 1995). This hypothesis, however, is not consistent with observations that Ca2+ release at fertilization can be initiated in the absence of extracellular Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ release are inhibited by an antibody against the IP3 receptor (Miyazaki et al., 1993; Oda et al., 1999). Here we investigate whether the sperm extract initiates intracellular Ca2+ 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 Ca2+-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 MgCl2, 5 mM Heps, 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. V. K. (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 Neofluor 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 IP3 concentrations given in the text refer to the final concentration in the egg cytoplasm.

**Calcium measurements**

Ca²⁺ 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 Neofluor 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 Neofluor 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 Ca²⁺ 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 IP3 (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 Neofluor 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 Ca²⁺ from intracellular stores at fertilization is inhibited by injection of SH2 domains of PLCγ or Fyn

The Ca²⁺ 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 Ca²⁺ action potential (Figs 1A, 2A, 4A, asterisks), resulting from Ca²⁺ 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 Ca²⁺ rise (Figs 1A, 2A, 4A), which crosses the egg in the form of a wave (Fig. 5A) and is due to release of Ca²⁺ from intracellular stores (Speksnijder et al., 1989, 1990a; Brownlee and Dale, 1990; Yoshida et al., 1998; Kyozuka et al., 1998). Ca²⁺ 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 Ca²⁺ release with respect to this.

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 Ca²⁺ rises similar to those that normally occur at fertilization, with the release of intracellular Ca²⁺ 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 Ca²⁺ release (Fig. 1B). The other 2 eggs injected with 1 mg/ml (20 μM) PLCγ SH2 domains did release Ca²⁺ 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 Ca²⁺ increase, and its rate of rise, were the same as in control eggs, although the duration of the initial Ca²⁺ elevation was shorter (Table 1). The three subsequent Ca²⁺ 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 Ca²⁺ increase of normal amplitude, rate of rise and duration, but the delay between the action potential and Ca²⁺ 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 Ca²⁺ from intracellular stores at fertilization in Ciona eggs.
To examine if SH2 domain-mediated activity of a Src family tyrosine kinase is required for release of intracellular Ca\textsuperscript{2+} at fertilization in ascidian eggs, Ciona eggs were coinjected with calcium green dextran and SH2 domains from the Src family kinase Abl, or control SH2 domains from the non-Src family kinase Abl. The SH2 domain of Abl 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 Src 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\textsuperscript{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\textsuperscript{2+} release following the action potential (Fig. 2B, Table 1). 3 of the 8 eggs injected with Fyn SH2 domains did release intracellular Ca\textsuperscript{2+} at fertilization, but the time from the action potential to intracellular Ca\textsuperscript{2+} release was increased by several seconds (Fig. 2C, Table 1). In these eggs, the peak amplitude of the Ca\textsuperscript{2+} increase 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.

Table 1. Ca\textsuperscript{2+} release from eggs injected with SH2 domain proteins and then inseminated.

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

Eggs were cooinjected 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\textsuperscript{2+} was released from intracellular stores (mean ± s.d.). Values include only those eggs that released intracellular Ca\textsuperscript{2+} (only two eggs injected with PLCγ SH2 domains showed Ca\textsuperscript{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\textsuperscript{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, \(P<0.02\)).

In the two eggs injected with 1 mg/ml PLCγ SH2 domains that activated, the peak amplitude of the Ca\textsuperscript{2+} increase 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 198±9 seconds compared to 530±87 seconds in controls. In the three eggs injected with Fyn SH2 domains that activated, the peak amplitude of the Ca\textsuperscript{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 confirm that the inhibition of Ca\textsuperscript{2+} release at fertilization by PLCγ or Fyn SH2 domains is upstream of IP\textsubscript{3} production, Ciona eggs were injected with 1 mg/ml PLCγ SH2 or Fyn SH2 domains followed by 50 nM IP\textsubscript{3} (close to the minimum amount of IP\textsubscript{3} needed to initiate Ca\textsuperscript{2+} release). Control eggs injected with 50 nM IP\textsubscript{3}, without SH2 domains, showed a Ca\textsuperscript{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\textsuperscript{2+} transients like that at fertilization is seen only with constant perfusion of IP\textsubscript{3} (Albrieux et al., 1997). In response to injection of 50 nM IP3, all eggs that had been preinjected with PLCγ SH2 domains (\(n=4\)) or Fyn SH2 domains (\(n=4\)) showed an immediate Ca\textsuperscript{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\textsuperscript{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...
but were not detected. The occurrence 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**

<table>
<thead>
<tr>
<th>SH2 domain protein injected (1 mg/ml)</th>
<th>% of eggs with Ca$^{2+}$ release</th>
<th>Time to the Ca$^{2+}$ release (seconds)$^1$</th>
<th>Peak amplitude$^2$</th>
<th>Number of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>54±54</td>
<td>1.5±0.3</td>
<td>10</td>
</tr>
<tr>
<td>SHP2 SH2</td>
<td>90</td>
<td>113±78</td>
<td>1.7±0.2</td>
<td>10</td>
</tr>
<tr>
<td>PLCy SH2</td>
<td>6$^3$</td>
<td>66±25</td>
<td>1.6±0.2</td>
<td>7</td>
</tr>
<tr>
<td>Abl SH2</td>
<td>86</td>
<td>975</td>
<td>1.1</td>
<td>6</td>
</tr>
<tr>
<td>Fyn SH2</td>
<td>17$^3$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.).

$^3$These values are significantly different from controls (Chi-square test, P<0.02).
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 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 IP3 (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 (1-2 hours later). (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 PLC\(\gamma\), 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 PLC\(\gamma\) is required for the release of intracellular Ca\(^{2+}\) at fertilization, by showing that injection of eggs with the SH2 domains of PLC\(\gamma\), which act as specific dominant negative inhibitors of PLC\(\gamma\) 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 PLC\(\gamma\) 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 PLC\(\gamma\) and Fyn SH2 domain inhibition, and by the lack of inhibition of sperm entry by PLC\(\gamma\) 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 PLC\(\gamma\)SH2 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 PLC\(\gamma\)SH2 domains do not inhibit Ca\(^{2+}\) release in response to PLC\(\beta\) 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 PLC\(\gamma\) 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 PLC\(\gamma\) 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 PLC\(\gamma\) 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).

Our finding that PLC\(\gamma\) 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 PLC\(\gamma\). The Src family kinases are among the tyrosine kinases known to participate in the activation of PLC\(\gamma\) 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 PLC\(\gamma\) at fertilization. Recombinant SH2 domains of PLC\(\gamma\) 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, PLC\(\gamma\) from fertilized sea urchin eggs can form a stable complex with recombinant domains of Fyn (Kinsey and Shen, 2000). Injection of starfish eggs with PLC\(\gamma\) SH2 domains delays Ca\(^{2+}\) release in response to injection of recombinant Src protein, indicating that Src acts upstream of PLC\(\gamma\) (Giusti et al., 2000).

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

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

<table>
<thead>
<tr>
<th>Signalling molecule</th>
<th>Echinoderm</th>
<th>Ascidian</th>
<th>Frog</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP(_3)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLC(\gamma) SH2(^2)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tyrosine kinase(^3)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Src family kinase SH2(^2)</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

\(^1\)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.


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 PLC\(\gamma\), 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 PLC\(\gamma\), which produces the IP\(3\) which stimulates Ca\(^{2+}\) release from the endoplasmic reticulum.

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