Signal transduction leading to calcium release in echinoderm eggs at fertilization requires phospholipase Cγ-mediated production of inositol trisphosphate (IP₃), indicating that a tyrosine kinase is a likely upstream regulator. Because previous work has shown a fertilization-dependent association between the Src homology 2 (SH2) domains of phospholipase Cγ and a Src family kinase, we examined whether a Src family kinase was required for Ca²⁺ release at fertilization. To inhibit the function of kinases in this family, we injected starfish eggs with the SH2 domains of Src and Fyn kinases. This inhibited Ca²⁺ release in response to fertilization but not in response to injection of IP₃. We further established the specificity of the inhibition by showing that the SH2 domains of several other tyrosine kinases (Abl, Syk, and ZAP-70), and the SH3 domain of Src, were not inhibitory. Also, a point-mutated Src SH2 domain, which has reduced affinity for phosphotyrosine, was a correspondingly less effective inhibitor of fertilization-induced Ca²⁺ release. These results indicate that a Src family kinase, by way of its SH2 domain, links sperm-egg interaction to IP₃-mediated Ca²⁺ release at fertilization in starfish eggs.

At fertilization, inositol trisphosphate (IP₃) releases calcium from the egg’s endoplasmic reticulum (1–4), which signals many events including resumption of the cell cycle (5, 6). In echinoderm eggs, the IP₃ is produced by phospholipase Cγ (PLCγ), as indicated by the inhibition of Ca²⁺ release by microinjection of the PLCγ Src homology 2 (SH2) domains (7, 8, 37). Because PLCγ is activated by phosphorylation that occurs when its SH2 domains interact with a specific binding site on a tyrosine kinase (9), a tyrosine kinase is a likely upstream regulator. Consistently, tyrosine kinase activity in sea urchin eggs increases by 15 s post-insemination (10), and the tyrosine kinase inhibitor genistein delays Ca²⁺ release at fertilization (11). In starfish eggs, an increase in tyrosine kinase activity associated with PLCγ SH2 domains in vitro occurs by 15 s post-insemination (12).

The Src family is among the several tyrosine kinase families thought to participate in activation of PLCγ, based on studies using cells from mice deficient in the Src family kinase Fyn (13) and on studies using Src inhibitory antibodies (14, 15), as well as on evidence for molecular associations between PLCγ and Src family kinases (16, 17). Evidence that a Src family kinase might activate PLCγ at fertilization is provided by the finding that a Src family protein in starfish eggs associates with PLCγ SH2 domains in vitro by 15 s post-insemination (12).

Progress in understanding the cellular functions of Src family kinases has been limited by the lack of convincingly specific inhibitors. However, it was recently shown that serum stimulation of cell division in fibroblasts was partially inhibited by injection of recombinant SH2 domains of Fyn (18). Injection of SH2 domains of three unrelated enzymes had no effect, indicating that SH2 domain injection is likely to be a specific way to inhibit Src family kinases. In the present study, we establish the specificity of the inhibitory action of Src family kinase SH2 domains and use them to demonstrate a requirement for this kinase family in initiating Ca²⁺ release at fertilization in starfish eggs.

EXPERIMENTAL PROCEDURES

GST Fusion Proteins—GST fusion proteins of SH2 domains from various tyrosine kinases were synthesized in bacteria as described previously (7). DNA encoding the indicated amino acids was obtained as follows. For wild-type chicken cSrc SH2, amino acids 148–246 were amplified from a full-length cSrc cDNA (provided by S. A. Courtneidge, Sugen, Inc., Redwood City, CA) using the polymerase chain reaction and oligonucleotides that introduced the restriction sites BamH1 (5') and EcoRI (3'). Amplification products were then cloned in-frame into pGEX2. For the chicken c-Src SH2 R175K mutant, arginine 175 of the SH2 domain was mutated to lysine by primer-mediated mutagenesis using the Transformer site-directed mutagenesis kit (CLONTECH). Chicken cSrc SH3 (amino acids 82–167) and chicken Fyn SH2 (amino acids 148–251) were provided by K. Vuori (The Burnham Institute, La Jolla, CA). Murine cAbl SH2 (amino acids 120–220) was from B. J. Mayer (Harvard Medical School, Boston, MA); human ZAP-70 SH2 (two tandem SH2 domains, amino acids 1–263) was from L. E. Samel (National Institutes of Health, Bethesda, MD); and rat Syk SH2 (two tandem SH2 domains, amino acids 1–269) was from R. P. Siragaguna (National Institutes of Health, Bethesda, MD). Sequences of all DNA constructs were confirmed by double-strand cycle sequencing by the Iowa State sequencing facility (Ames, IA).

Calcium Measurements—Experiments were performed as described previously (7) using oocytes and sperm from the starfish Asterina miniata. To measure calcium, the oocytes were injected with calcium green 10-kDa dextran (Molecular Probes, Eugene, OR). See Ref. 38 for a description of procedures used for quantitative injection. Calcium green fluorescence was measured with a photodiode (71182; Oriol Instruments, Stratford, CT) or imaged with a confocal microscope (MRC600;
Bio-Rad Laboratories, Hercules, CA. SH2 domain GST fusion proteins were mixed with calcium green dextran, and oocytes were injected with 90 picoliters (3% of their 3100-piculiter volume) to give the indicated cytoplasmic protein concentrations and 10 μM calcium green. 1–5 min later, the oocytes were treated with 1 μM 1-methyladenine to induce meiotic maturation. 50–75 min after injection, when the eggs were at first metaphase, they were inseminated with a 1:20,000 dilution of the sperm suspension from the testes. All experiments were performed at 18 °C. In other experiments (not shown), injections of the SRC SH2 protein were made after oocyte maturation, 10–30 min before insemination, with similar results to those shown in Table I.

RESULTS

Inhibition of Ca$^{2+}$ Release at Fertilization by SRC SH2 Domains—GST fusion proteins comprising the SH2 domains of chicken SRC and other vertebrate tyrosine kinases (Fig. 1) were injected into starfish eggs, and fertilization-induced Ca$^{2+}$ release was monitored using the fluorescent Ca$^{2+}$ indicator, calcium green dextran. Fertilization of eggs injected with calcium green dextran alone or with control proteins resulted in a small increase seen in the confocal images (Fig. 3A). Two of six showed a Ca$^{2+}$ wave that propagated over the entire cell, but this occurred with an increased delay after the Ca$^{2+}$ action potential. In one of three of these eggs, the Ca$^{2+}$ action potential was followed by local Ca$^{2+}$ rise only (Fig. 3B), and in one of six eggs, Ca$^{2+}$ release after the action potential was not detected. Five of five control eggs showed a normal Ca$^{2+}$ action potential and Ca$^{2+}$ wave (Fig. 3A).

Eggs that were fertilized after injection of 1 mg/ml SRC SH2 domains showed multiple pronuclei in their cytoplasm, indicating that they were polyspermy (data not shown). The multiple sperm entries may correspond to the multiple rises seen in the Ca$^{2+}$ traces (Fig. 2B) and the multiple sites of local Ca$^{2+}$ increase seen in the confocal images (Fig. 3B).

The inhibition of Ca$^{2+}$ release by SRC SH2 domains was concentration-dependent; weaker inhibition was seen with 0.1 mg/ml (2.5 μM) of the SRC SH2 domains (Table I). No inhibition of Ca$^{2+}$ release was seen in eggs injected with 1 mg/ml (25 μM) of the SH3 domain of SRC (Table I).

Requirement for an Active Phosphotyrosine Binding Region—To test whether the inhibition of Ca$^{2+}$ release at fertilization required the presence of an active phosphotyrosine binding region in the SRC SH2 domain, we injected eggs with a point-mutated form of the SRC SH2 domain, in which arginine 175 in the phosphotyrosine binding pocket was changed to lysine. This mutation reduces the in vitro association of the v-Src SH2 domain with phosphotyrosyl proteins to 35% of the wild-type SH2 domain (20). Correspondingly, eggs injected with 1 mg/ml R175K SRC SH2 domains showed weaker inhibition of Ca$^{2+}$ release at fertilization compared with the wild-type protein (Fig. 2C and Table I). These results indicate that the inhibition of Ca$^{2+}$ release by SRC SH2 domains requires the ability to bind phosphotyrosine.

Specificity among Different Tyrosine Kinases—Similar concentration-dependent inhibition of Ca$^{2+}$ release at fertilization was observed in eggs injected with SH2 domains of another SRC family kinase, Fyn (Fig. 2D and Table I). This finding was as expected, because SRC and FYN SH2 domains have similar binding preferences for peptide sequences containing phosphotyrosine (21). In starfish eggs, 2.5 μM SRC and FYN SH2 domains was sufficient to delay Ca$^{2+}$ release at fertilization and reduce its amplitude. The concentration of FYN SH2 domains that was used to inhibit serum stimulation of cell division in mammalian fibroblasts is not known precisely but has been estimated to be in a similar range of about 1–2 μM (Ref. 18 and footnote 2).

Injection of 1 mg/ml SH2 domains of three non-SRC-family tyrosine kinases (ABL, SYK, and ZAP-70) did not inhibit Ca$^{2+}$ release (Fig. 2E and Table I). Like SRC and FYN, ABL has a single SH2 domain, whereas SYK and ZAP-70 have two tandem SH2 domains. All of these SH2 domains are structurally similar (22, 23). Compared with SRC and FYN, the SH2 domains of ABL, SYK, and ZAP-70 share some but not all preferences for phosphotyrosine-containing peptides (21, 23). The lack of any effect on Ca$^{2+}$ release of these control SH2 domains indicates that the inhibition of Ca$^{2+}$ release at fertilization is specific for the SH2 domain of SRC family kinases. These results are consistent with the finding that injection of sea urchin eggs with SH2 domains of FYN, but not ABL, results in polyspermy.

Lack of Inhibition of IP$_3$-induced Ca$^{2+}$ Release by SRC and FYN SH2 Domains—To determine whether the inhibition of Ca$^{2+}$ release at fertilization was upstream or downstream of IP$_3$ production, we tested whether SRC or FYN SH2 domains inhibited Ca$^{2+}$ release in response to injection of IP$_3$. These experiments showed no inhibition of IP$_3$-induced Ca$^{2+}$ release (Fig. 4) when using an IP$_3$ concentration close to the minimum needed to cause Ca$^{2+}$ release (24). This result indicates that a SRC family kinase acts on an early step in the fertilization signaling pathway, upstream of IP$_3$ production.

DISCUSSION

We have used the SH2 domains of SRC family kinases to specifically inhibit the cellular function of these kinases and to demonstrate a requirement for their function in initiating Ca$^{2+}$ release at fertilization of starfish eggs. Evidence for the specificity of the inhibitory action of the SH2 domains is as follows.

1) SRC and FYN SH2 domains have similar effects, as expected from their similar phosphopeptide binding specificities.

2) The SH2 domains of three other tyrosine kinases with similar protein structure but different phosphopeptide binding specificities (ABL, SYK, ZAP-70) have no effect.

3) A point-mutated SRC SH2 domain with reduced ability to bind phosphotyrosine has a correspondingly reduced inhibitory effect.

4) Injection of a downstream component of the pathway (IP$_3$) bypasses the inhibition. Based on our results, SRC family SH2 domains are

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2 S. Roche, personal communication.

W. H. Kinsey, personal communication.
among the best characterized means available to investigate the requirement of these proteins for cellular functions.

Although our results demonstrate that a Src family kinase is required for Ca\(^{2+}\) release at fertilization, it is not known which particular Src family kinase is involved and whether the function of the Src family protein depends on its kinase activity. The function of Src family proteins does not always depend on their kinase activity (25–27), suggesting that in some cases they function as adapter proteins. In sea urchin eggs, the kinase activity immunoprecipitated by antibodies directed against mammalian Fyn was found to increase by 5 min post-insemination (28). No significant increase was detected at earlier time points preceding Ca\(^{2+}\) release, but local activation of a kinase at the site of fertilization might produce only a small increase, below the limit of the method of detection. Alternatively, the primary role of the kinase activity associated with these particular antibodies may occur in later events. Only one Src family kinase present in echinoderm eggs has been cloned and sequenced (29). Characterization of which Src family kinases are present in echinoderm eggs will allow determination of which if any are activated within the first minute after fertilization.

Directly or indirectly, the target of the Src family kinase in the signaling pathway at fertilization is very likely phospholipase C\(_\gamma\), because PLC\(_\gamma\) is necessary for Ca\(^{2+}\) release at fertilization in starfish and sea urchin eggs (7, 8, 37) and because PLC\(_\gamma\) is activated by tyrosine phosphorylation (9). In vitro, PLC\(_\gamma\) SH2 domains associate with a 58-kDa protein in fertilized starfish egg lysates, which is recognized by a vertebrate Src family kinase antibody (12). This association is not seen in unfertilized egg lysates but is detected by 15 s post-insemination. A Src family kinase might activate PLC\(_\gamma\) by directly phosphorylating it (16, 17), or alternatively, the Src family kinase might be coupled to PLC\(_\gamma\) activation by intermediate kinases and/or adapter proteins as in T cell receptor signaling (30). As noted above, the Src family kinase might itself be an adapter protein (25–27).

As in echinoderms (11), tyrosine kinase inhibitors also inhibit or delay Ca\(^{2+}\) release at fertilization in vertebrate eggs (31, 32). However, only general chemical tyrosine kinase inhibitors have been used, for which the specificity is uncertain. Signaling at fertilization of mammalian eggs appears to differ somewhat from that in echinoderms, because although the IP\(_3\) receptor is required for Ca\(^{2+}\) release (4), the Ca\(^{2+}\) release is not inhibited by PLC\(_\gamma\) SH2 domains (33).

What upstream events in starfish fertilization might involve the SH2 domain of a Src family kinase? One possibility is suggested by findings in other cells that receptor cross-linking can lead to Src activation; this can occur by way of the tyrosine phosphorylation of Fak family kinases, which provides a high affinity binding site for the SH2 domain of Src (34). Similarly, receptor cross-linking at the site where the sperm contacts the

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**Fig. 2. Inhibition of Ca\(^{2+}\) release at fertilization in starfish eggs injected with Src or Fyn SH2 domains.** A, Calcium green dextran only, 10 \(\mu\)M. B, Src SH2 domain, 1 mg/ml (cytoplasmic concentration). C, Src SH2, R175K mutation, 1 mg/ml. D, Fyn SH2, 1 mg/ml. E, Abl SH2, 1 mg/ml. Traces show calcium green fluorescence as a function of time. The asterisk in A indicates the action potential. The amplitude of the action potential in eggs from different starfish was variable but was not affected by any of the injected proteins. Arrowheads indicate the time of insemination.
FIG. 3. Imaging of the inhibition of Ca\(^{2+}\) release at fertilization in a starfish egg injected with Src SH2 domains. A, control, calcium green dextran only, 10 \(\mu\)M. B, Src SH2, 1 mg/ml (cytoplasmic concentration). Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration. Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration. Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration. Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration. Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration. Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration. Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration. Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration. Confocal images are arranged horizontally at 5-s intervals. Increasing Ca\(^{2+}\) concentration.

A images. Quicktime movies corresponding to these still images show Ca\(^{2+}\) release in control and Src SH2-injected eggs at 30\(\times\) real time. Each movie is composed of confocal images taken at 5-s intervals (played back at 6 frames/s). For the control, the total duration is 2 min; for SrcSH2, the total duration is 6 min.

TABLE I

Calcium release in starfish eggs injected with SH2 domains

| Injection                  | Delay | Peak amplitude (% increase over unfertilized egg) | No. of eggs |
|----------------------------|-------|--------------------------------------------------|-------------|
| Calcium green dextran      | 8 ± 1 | 117 ± 3                                          | 17          |
| Src SH2, 1 mg/ml           | 200 ± 54* | 35 ± 4*                                          | 21          |
| Src SH2, 0.1 mg/ml         | 21 ± 1* | 76 ± 8*                                          | 5           |
| Src SH2 R175K, 1 mg/ml     | 30 ± 2* | 52 ± 4*                                          | 12          |
| Src SH3, 1 mg/ml           | 8 ± 1  | 167 ± 4*                                         | 5           |
| Fyn SH2, 1 mg/ml           | 397 ± 182* | 33 ± 11*                                       | 4           |
| Fyn SH2, 0.1 mg/ml         | 42 ± 18* | 85 ± 6*                                          | 3           |
| Abi SH2, 1 mg/ml           | 8 ± 1  | 114 ± 4*                                         | 5           |
| Syk SH2, 1 mg/ml           | 7 ± 1  | 136 ± 9*                                         | 5           |
| ZAP SH2, 1 mg/ml           | 7 ± 1  | 124 ± 4*                                         | 6           |

Values for delay indicate the time between the rise of the action potential and the time at which the fluorescence versus time trace started its second rise. Values for peak amplitude are expressed as change in fluorescence after fertilization fluorescence of the unfertilized egg \(\times 100\). Data are expressed as mean ± S.E. Points marked with asterisks are significantly different from eggs injected with calcium green dextran only \((p < 0.01)\). Data were analyzed using the two-tailed \(t\) test (Instat), comparing each set of data in Table I with the complete set of calcium green dextran controls \((n = 17)\). Calcium green dextran controls were done on the same days and with the same starfish as the experimental eggs. Points marked with asterisks were also found to be significantly different from the pooled values for the three control SH2 domain proteins \((\text{Abi SH2, Syk SH2, ZAP SH2}) (p < 0.01)\). In addition, data for the delays seen with Src SH2-injected eggs, and 58 ± 4\% \((n = 3)\) for Fyn SH2-injected eggs. Values are not statistically different from control values \((\text{two-tailed} t\) test, Instat).}

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