Focus on Fertilization

Evidence that activation of Src family kinase is not required for fertilization-associated $[Ca^{2+}]_i$ oscillations in mouse eggs

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Abstract

Recent evidence in marine invertebrate, frog, and zebrafish eggs suggests the involvement of a Src family kinase (SFK) in fertilization-induced Ca^{2+} release. In the present study, we have investigated whether activation of an SFK is required for initiation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in mouse fertilization. We detected a Hck-like protein and tyrosine-phosphorylated proteins in soluble and insoluble sperm fractions, respectively. However, the presence of these proteins did not correspond to the active fractions of porcine sperm extracts (pSE). Moreover, $[Ca^{2+}]_i$ oscillations induced by pSE in mouse eggs were unaltered by pre-incubation of pSE with specific SFK inhibitors such as 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazol[3,4-*d*]pyrimidine (PP2) or lavendustin A, despite the fact that the inhibitors were shown to be active both *in vivo* and *in vitro*. Another SFK inhibitor, peptide A, blocked oscillations when incubated with pSE prior to injection into eggs, but this inhibition required more than ten times the concentration reportedly required to inhibit SFK activity. In addition, pre-injection or pre-incubation of eggs with these inhibitors did not affect the ability of pSE to trigger $[Ca^{2+}]_i$ oscillations in mouse eggs. Microinjection of a recombinant c-Src protein or mRNAs encoding constitutively active Src proteins did not induce $[Ca^{2+}]_i$ release. Finally, when sperm and eggs, both of which were pre-treated with PP2, were fertilized, $[Ca^{2+}]_i$ oscillations occurred normally. We can therefore conclude that activation of an SFK is neither necessary nor sufficient for triggering fertilization-induced $[Ca^{2+}]_i$ oscillations.

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Introduction

At fertilization the sperm initiates a series of cellular and biochemical events in the egg that are collectively referred to as egg activation. It has been demonstrated that the intracellular Ca^{2+} ($[Ca^{2+}]_i$) release induced by the sperm is essential and sufficient for initiation of egg activation. An increasing body of evidence suggests that, upon gamete fusion, the sperm delivers a factor(s), termed sperm factor, into the egg's cytoplasm to initiate the Ca^{2+} signal. In support of this notion, the $[Ca^{2+}]_i$ release induced by injection of sperm extracts is spatially and temporally similar to that at fertilization and is highly

conserved across species (Wu *et al.* 1997, Oda *et al.* 1999, Parrington *et al.* 1999, Dong *et al.* 2000, Jones *et al.* 2000, Stricker *et al.* 2000, Li *et al.* 2001, Coward *et al.* 2003), although the exact mechanism utilized by sperm extracts still remains elusive. Previous studies have shown that the $[Ca^{2+}]_i$ release induced by sperm or by injection of sperm extracts involves production of inositol 1,4,5-trisphosphate (IP_3) via activation of a phospholipase C (PLC) (Stith *et al.* 1993, Dupont *et al.* 1996, Snow *et al.* 1996, Lee & Shen 1998, Sato *et al.* 2000, Kuroda *et al.* 2001). IP_3 , in turn, leads to $[Ca^{2+}]_i$ release from the endoplasmic reticulum by interacting with IP_3 receptors (Miyazaki *et al.* 1992, Wu *et al.* 1997).

Given this known requirement for stimulation of the IP₃ pathway by the sperm or injection of sperm extracts, significant research has logically focused on the mechanism by which activation of a PLC occurs in the ooplasm. It is known that mammalian eggs express the $\beta 1$, $\beta 3$, $\gamma 1$, and $\gamma 2$ PLC isozymes, and the case for activation of an egg PLC by the fertilizing sperm was first made by Dupont *et al.* (1996), when they demonstrated that fertilization-induced $[Ca^{2+}]_i$ oscillations are sensitive to antagonism by U-73122, a specific pan-PLC inhibitor. A similar inhibition was later demonstrated for sperm extract-induced oscillations (Wu *et al.* 2001b). However, these experiments failed to demonstrate whether the active PLC is native to the egg or is delivered by the sperm or sperm extract injection, due to ambiguity over the exact site of action of U-73122 (Yule & Williams 1992, Walker *et al.* 1998). It has also been shown that sperm express multiple PLC isoforms (Mehlmann *et al.* 1998, Fukami *et al.* 2001, Wu *et al.* 2001b, Parrington *et al.* 2002 and *in vitro* assays have demonstrated PLC activity in sperm extracts (Jones *et al.* 1998, 2000, Rice *et al.* 2000). The case for a sperm-borne PLC as the key factor during fertilization-induced Ca^{2+} signaling was recently bolstered by the discovery of PLC ζ , a novel, sperm-specific isoform that has been detected in mouse, human, and cynomolgus monkey sperm (Cox *et al.* 2002, Saunders *et al.* 2002, Swann *et al.* 2004 (this issue)). These studies further demonstrated that injection of mRNA encoding PLC ζ induces $[Ca^{2+}]_i$ oscillations in mouse eggs similar to those generated by fertilization, and that immunodepletion of PLC ζ protein from sperm extracts abrogates the ability of those extracts to induce $[Ca^{2+}]_i$ release when injected into mouse eggs. Thus, strong evidence supports the notion that PLC ζ represents the Ca^{2+} -active factor of sperm extracts; however, the question remains as to whether PLC ζ is also the physiological factor responsible for triggering oscillations during natural fertilization. Furthermore, these findings do not rule out the possibility that a concurrent pathway(s) leading to activation of an egg PLC(s) may also function during fertilization.

One candidate pathway that may result in activation of an egg PLC is that involving protein tyrosine kinases. Recently, significant progress has been made in the study of the involvement of tyrosine kinases in triggering sperm- and/or sperm extract-induced $[Ca^{2+}]_i$ release in echinoderm, ascidian, *Xenopus*, and zebrafish eggs, raising the possibility that a similar mechanism may also be in operation during mammalian fertilization (Giusti *et al.* 1999, 2000, Glahn *et al.* 1999, Sato *et al.* 1999, 2000, Abassi *et al.* 2000, Runft & Jaffe 2000, Wu & Kinsey 2000). In these studies, injection of GST fusion proteins containing the SH2 domains of a Src family kinase (SFK), which act as dominant negative inhibitors, blocked the sperm-initiated Ca^{2+} signal, suggesting a role for SFKs in initiating the fertilization-induced Ca^{2+} signal in echinoderm eggs (Giusti *et al.* 2000). In addition, similar to fertilization, the $[Ca^{2+}]_i$ release in response to Src protein

injection was blocked by prior injection of PLC γ SH2 domains (Carroll *et al.* 1997, 1999, Shearer *et al.* 1999), which implies that the sperm initiates $[Ca^{2+}]_i$ release in these eggs by sequential activation of an SFK and PLC γ . Using a similar strategy in ascidian eggs, it has been shown that both fertilization and injection of sperm extracts trigger $[Ca^{2+}]_i$ release by activating the same pathway that involves PLC γ and an SFK (Runft & Jaffe 2000). In *Xenopus*, pharmacological and specific SFK inhibitors have been reported to block fertilization-induced Ca^{2+} release by inhibiting the SFK-dependent PLC γ activity (Glahn *et al.* 1999, Sato *et al.* 2000, Tokmakov *et al.* 2002). However, differences among species as to how PLC γ is activated by the SFK pathway have been documented. For example, it has been shown that injection of PLC γ SH2 fusion proteins, which inhibit sperm- or sperm extract-triggered $[Ca^{2+}]_i$ signals in echinoderm and ascidian eggs, do not block the sperm-initiated Ca^{2+} signal in *Xenopus* (Runft *et al.* 1999). Therefore, although the same signaling pathway may participate at fertilization in most species, the activation and regulation of it may be species specific.

In mammalian eggs, due to their small size and the limited sensitivity of kinase assays, the role of Src kinases following fertilization or sperm extract injection has not been characterized. In rat eggs, it has been reported that several proteins become tyrosine phosphorylated shortly after fertilization, and that c-Fyn is expressed in the egg before and after following fertilization (Ben-Yosef *et al.* 1998, Talmor *et al.* 1998). Moreover, incubation of mouse eggs with tyrosine kinase inhibitors has been shown to have limited (maybe due to the poor permeability of mouse egg membranes for these inhibitors) but significant inhibitory effects on Ca^{2+} responses at fertilization, suggesting the possible involvement of a tyrosine kinase(s) in mammalian fertilization (Dupont *et al.* 1996). Interestingly, and similar to *Xenopus* eggs, $[Ca^{2+}]_i$ release at fertilization was not inhibited in mouse eggs by injection of excess PLC γ SH2 domains, suggesting that if PLC γ participates in the sperm-initiated pathway, it is not activated by an SH2 domain-related mechanism (Mehlmann *et al.* 1998). Thus, given the limited investigation into the possible role of a tyrosine kinase-regulated pathway in mammalian fertilization and in light of the strong evidence in favor of such a pathway in several non-mammalian species, a thorough investigation into the possible role of tyrosine kinases, and specifically Src-related kinases, in sperm/sperm extract-induced $[Ca^{2+}]_i$ release in mammalian eggs is warranted.

Materials and Methods

Gamete collection

MII eggs were obtained from B6D2F1 (C57BL/6J $\times$ DBA/2J) and CD1 female mice (8–12 weeks old)

superovulated by injection of 5 IU pregnant mare serum gonadotropin (Sigma, St Louis, MO, USA) followed 48 h later by 5 IU human chorionic gonadotropin (hCG; Sigma). Eggs were recovered 14 h post-hCG into a Hepes-buffered tyrode-lactate solution supplemented with 5% heat-treated calf serum (TL-Hepes). Cumulus cells were removed by brief treatment with 0.1% bovine testes hyaluronidase (Sigma). Mouse sperm (12- to 24-week-old B6D2F1 and CD1 male mice) collected from cauda epididymides and boar sperm collected from freshly ejaculated semen were washed twice with injection buffer (75 mM KCl and 20 mM Hepes, pH 7.0) before use.

All procedures involving live animal handling and euthanasia were performed according to standard animal protocols approved by the University of Massachusetts Animal Care Committee.

Preparation of porcine sperm extracts (pSE)

Cytosolic pSE (pSE^C) were prepared as previously described (Wu *et al.* 2001b). Briefly, after washing, the sperm suspension was sonicated (XL2020; Heat Systems Inc., Farmingdale, NY, USA) in sperm buffer (75 mM KCl, 20 mM Hepes, 1 mM EDTA, 10 mM glycerophosphate, 1 mM dithiothreitol, 200 μ M phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml pepstatin, and 10 μ g/ml leupeptin, pH 7.0) at 4°C and the lysate ultracentrifuged. The clear supernatant was concentrated with ultrafiltration membranes (Centriprep-30; Amicon, Beverly, MA, USA). These extracts were then mixed for 30 min at 4°C with ammonium sulfate at 50% final saturation; the precipitates were collected by centrifugation and kept at -80°C. Upon use, the pellets were resuspended in injection buffer, washed, and re-concentrated with ultrafiltration membranes.

Triton X-100 soluble sperm extracts (pSE^{TX}) were prepared as following. After preparation of pSE^C, the resulting sperm pellets were thoroughly washed with sperm buffer, and then subjected to incubation with 0.1% Triton X-100 (Sigma) in sperm buffer for 30 min at 4°C. After the treatment, the sperm suspension was centrifuged at 100 000 g for 1 h at 4°C. The clear supernatant was collected and concentrated with ultrafiltration membranes. Following pSE^{TX} extraction, the sperm pellets were washed and further subjected to alkaline carbonate extraction (100 mM Na₂CO₃, pH 11.5, 10 min at 4°C; Fujiki *et al.* 1982) to collect high pH-soluble sperm extracts (pSE^{pH}). After the treatment, the sperm suspension was neutralized, centrifuged, and concentrated. Samples were aliquoted and kept at -80°C.

To solubilize insoluble sperm fractions in certain experiments (see Fig. 2A), the porcine sperm pellets following sonication (see above) were thoroughly mixed with five strokes using a small needle (26 gauge) in phosphate-buffered saline containing 0.5% SDS. The supernatant obtained following brief centrifugation (10 000 r.p.m., 10 min) was used for protein assays.

Fast protein liquid chromatography columns

pSEs were subjected to Superose 12 columns (Superose 12 HR 10/30; Amersham Biosciences Corp., Piscataway, NJ, USA) using an FPLC system as described previously (Wu *et al.* 1998a, 2001b). Proteins obtained from the ammonium sulfate precipitation were loaded at 4°C onto a Superose 12 column. Proteins were eluted with elution buffer (75 mM KCl, 20 mM Hepes, 1 mM EDTA, 200 μ M PMSF, 20 μ g/ml pepstatin, and 20 μ g/ml leupeptin, pH 7.0) at a flow rate of 0.1 ml/min and detected at OD₂₈₀ by a UV-M monitor (Pharmacia Biotech Inc., Piscataway, NJ, USA). Fractions (0.25 ml) were collected and concentrated for testing the Ca²⁺-releasing activity and for performing Western blots.

Microinjection

Microinjection procedures were as previously described (Wu *et al.* 1997, 1998a,b, 2001b). In brief, eggs were microinjected using Narishige manipulators (Medical Systems Corp., Great Neck, NY, USA) mounted on a Nikon Diaphot microscope (Nikon Inc., Garden City, NY, USA). Glass micropipettes were filled by suction from a microdrop containing pSE, a recombinant c-Src protein (Cat. No. 14-117, 3 μ g/ μ l, 75 units/25 μ l; Upstate Biotechnology, Lake Placid, NY, USA), or mRNAs. Solution was expelled into the cytoplasm of eggs by pneumatic pressure (PLI-100 picoinjector; Harvard Apparatus, Cambridge, MA, USA). The injection volume was approximately 5–10 pl and resulted in a final intracellular concentration of the injected compounds of approximately 1–5% of the concentration in the injection pipette.

Intracytoplasmic sperm injection (ICSI)

ICSI was carried out as previously described (Kimura & Yanagimachi 1995, Kurokawa & Fissore 2003) using Narishige manipulators mounted on a Nikon Diaphot microscope. ICSI was performed in Hepes-buffered CZB (Chatof, Ziomek and Bavister) medium (Kimura & Yanagimachi 1995). One part sperm suspension was mixed with one part injection buffer containing 12% polyvinyl pyrrolidone (molecular weight 360 kDa; Sigma). Sperm were delivered into the eggs' cytosol using a piezo micropipette-driving unit (Piezodrill; Burleigh Instruments Inc., Rochester, NY, USA) as described elsewhere (Kimura & Yanagimachi 1995, Kurokawa & Fissore 2003); a few piezo pulses were applied to puncture the egg plasma membrane following penetration of the zona pellucida. Mouse sperm heads were separated from tails by applying a few piezo pulses at the mid-piece of the sperm immediately prior to injection into the egg. In experiments using boar sperm, the heads were separated from tails by a 45–60 s sonication period (at 4°C) since piezo pulses are ineffective at disrupting these sperm (Kimura *et al.* 1998); sonication resulted in more than 90% of sperm heads separated from their tails. In certain experiments, porcine

sperm were subjected to Triton X-100 or high pH extraction (see section on Preparation of porcine sperm extracts) following sonication. After such treatments, sperm were thoroughly washed with injection buffer before ICSI.

Following ICSI, injection of pSE, or treatment of eggs with H_2O_2 , eggs were either used for $[\text{Ca}^{2+}]_i$ monitoring or cultured in KSOM (potassium Simplex Optimization Medium) to evaluate activation at 36.5°C in a humidified atmosphere containing 5.5% CO_2 . Activation was assessed from the number of zygotes with extrusion of the second polar body and formation of pronuclei at 6 h post-ICSI or pSE injection.

Inhibitor preparations and loading conditions

Tyrosine kinase inhibitors, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) and lavendustin A, and an inactive analogue, 4-mino-7-phenylpyrazol[3,4-*ad*]pyrimidine (PP3), were obtained from Calbiochem (San Diego, CA, USA). These compounds were dissolved in dimethyl sulfoxide at 5 mM and kept at -80°C until use. Peptide A (VAPSDSIQAEWYFGKITRRE) was purchased from Calbiochem (Sato *et al.* 1990, Glahn *et al.* 1999). Peptide A9 (SDSIQAEWYFGKIT) was prepared as previously described (Fukami *et al.* 1993, Sato *et al.* 2003). The inhibitory peptides were dissolved in injection buffer at 1 mM and kept at -80°C . Upon use, the compounds were further diluted in KSOM, TL-Hepes, or injection buffer to working concentrations, and eggs and/or sperm were loaded by 30-min incubations. Pre-incubation and culture of eggs/sperm with PP2, PP3, or lavendustin A were performed in the absence of oil to avoid the possible permeability of these compounds to the oil.

$[\text{Ca}^{2+}]_i$ measurements

$[\text{Ca}^{2+}]_i$ measurements were carried out as previously described (Kurokawa & Fissore 2003). Eggs were loaded with $1\ \mu\text{M}$ fura-2 acetoxymethylester (Molecular Probes, Eugene, OR, USA) supplemented with 0.02% pluronic acid (Molecular Probes) for 20 min at room temperature. $[\text{Ca}^{2+}]_i$ values were monitored using a Nikon Diaphot microscope fitted for fluorescence measurements. Eggs were either individually monitored using modified Phoscan 3.0 software (Nikon Inc.) or measured simultaneously (2 to 15 eggs) using the software Image 1/FL (Universal Imaging, Downingtown, PA, USA) and an SIT camera (Dage-MTI, Michigan City, IN, USA) coupled to an amplifier (Video Scope International Ltd, Sterling, VA, USA). $[\text{Ca}^{2+}]_i$ values are reported as the ratios of 340/380 nm fluorescence (F340/380). Fluorescence ratios were obtained every 4–20 s depending on the experiments.

Western blotting

Western blotting was carried out as previously described (He *et al.* 1997, Jellerette *et al.* 2000). Rabbit polyclonal antibodies raised against Blk (sc-329), Fgr (sc-130), Hck

(sc-72), Lyn (sc-15), Src (sc-19), Yes (sc-14), and anti-phosphotyrosine mouse monoclonal antibody (PY99, sc-7020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Lck rabbit polyclonal antibody (06-135) was obtained from Upstate Biotechnology. Rabbit polyclonal antibodies against Abl (PC-01) and Fyn (PC-32), and mouse monoclonal antibodies against c-Src (mAb327, OP-07) and phosphorylated tyrosine 416 of c-Src (Src^{PY416}, 44-660) were from Oncogene Science (San Diego, CA, USA). The anti-pepY antibody was raised against a synthetic peptide, pepY, which corresponds to residues 410–428 of chicken c-Src (Fukami *et al.* 1993). Mouse monoclonal antibody raised against the unphosphorylated C-terminus of Src/Fyn/Yes, termed clone 12, was a kind gift from Dr Owada, Kyoto Pharmaceutical University, Japan.

Samples of 40 eggs were collected after treatment with $10\ \mu\text{M}$ H_2O_2 (10 min post-treatment) or injection of mRNAs encoding the Src mutants (6 h post-injection) into SDS sample buffer. For analysis of pSE, $10\ \mu\text{g}$ protein samples were loaded to each lane. Protein samples were subjected to 10% SDS-PAGE and then transferred onto nitrocellulose membranes (Micron Separation, Westboro, MA, USA) for 1 h at 4°C . Following incubation with dry milk or bovine serum albumin, the membranes were probed with a primary antibody (see above) and then an appropriate horseradish peroxidase-labeled secondary antibody (170-6515/170-6516; BioRad, Hercules, CA, USA). Immunoreactivity was detected using chemiluminescence reagents (NEN Life Science Products, Boston, MA, USA) and the Kodak Image Station 440CF (NEN Life Science Products).

In vitro protein kinase assays

In vitro SFK assays were performed based on immunoblotting analysis. Eight microliters (24 ng) of recombinant Src (Cat. no. 14–117; Upstate Biotechnology) was mixed with $2\ \mu\text{l}$ $100\ \mu\text{M}$ compound (lavendustin A, PP2, or PP3) in the presence of $15\ \mu\text{l}$ kinase buffer ($0.5\ \mu\text{l}$ $250\ \text{mM}$ MgCl_2 , $5\ \mu\text{l}$ $100\ \text{mM}$ KCl, $50\ \mu\text{M}$ Tris (pH 7.5), $2.5\ \mu\text{l}$ $10\ \text{mM}$ ATP, and $7\ \mu\text{l}$ H_2O). The reaction was allowed to proceed for 10 min at 30°C , after which SDS-PAGE and Western blotting were carried out to detect Src protein and Src^{PY416}.

Likewise, *in vitro* tyrosine kinase assays were carried out as follows. Ten microliters of pSE ($0.5\ \mu\text{g}/\mu\text{l}$) were mixed with $10\ \mu\text{l}$ kinase buffer and $5\ \mu\text{l}$ of either $50\ \mu\text{M}$ PP2 or PP3. Following the reaction (10 min, 30°C), the samples were subjected to SDS-PAGE and Western blot using the anti-phosphotyrosine antibody (PY99).

In vitro transcription

The plasmids encoding constitutively active mutants of chicken c-Src (Y527F and E378G) were kind gifts from Dr Joan S Brugge (Harvard Medical School, Boston, MA, USA). The DNA sequence containing the 5' end of PLC ζ ($\sim 360\ \text{bp}$) was isolated from mouse testis by RT-PCR. The

amplified 5' fragment was then subcloned into a vector containing a truncated construct of mouse PLC ζ (accession no. AK006672, a gift from RIKEN; Kouchi *et al.* 2004). Full-length mouse PLC ζ was cloned into pBluescript KS(–) vector and complete insertion of full-length PLC ζ was confirmed by DNA sequencing. The mRNAs were produced as previously described (Fissore *et al.* 1996). In brief, the cDNAs were linearized with the appropriate restriction enzyme, and transcribed *in vitro* using the mMessage-Machine capping kit (Ambion, Austin, TX, USA). Poly(A) tails of approximately 150–200 bp were added to the full-length transcripts using the Poly(A) Kit (Ambion), and capped and tailed mRNAs were purified from the reaction mixture using the MEGAclear Kit (Ambion).

Results

A Hck-like protein is present in pSE

We and others have previously shown that injection of pSE^C into mouse MII eggs is able to trigger $[Ca^{2+}]_i$ oscillations that are similar to those observed at fertilization (Wu *et al.* 1997) (see Fig. 4A for an example). To ascertain whether or not pSE^C contains an SFK and, further, to begin to address the possibility that an SFK may represent the Ca^{2+} -releasing factor in pSE^C, we performed Western blotting on pSE^C using anti-Src family kinase antibodies. Among the various antibodies tested, including anti-Lyn, Blk, Fgr, Lck, Abl (non-SFK), Src, Yes, Lyn, Fyn, and Hck (see Materials and Methods), only the anti-Hck antibody detected a protein band of around 60 kDa in pSE^C (Fig. 1A); the same antibody recognized a Hck-like protein in mouse somatic cells (not shown). Importantly, the molecular size of this protein corresponded to that of a protein band recognized by anti-Src^{pY416} antibody, a specific antibody against phosphotyrosine 416 of c-Src (Fig. 1A), suggesting that this 60 kDa protein is a Src family protein, most likely Hck. To determine whether the localization of this Hck-like protein is specific to active pSE^C, pSE^C was fractionated through a Superose 12 column (Fig. 1B), and Western blotting was performed using the anti-Hck antibody. Western blotting results showed that the Hck-like protein was present in fractions 48 and 49, and that the anti-Src^{pY416} antibody also detected the 60 kDa band in the same fractions (Fig. 1C; arrows), further supporting the idea that the 60 kDa protein present in pSE^C (Fig. 1A) represents Hck kinase. Interestingly, however, this Hck-like protein only localized in fractions 48 and 49, whereas Ca^{2+} -releasing activity was found in fractions 47–50, indicating that, if the $[Ca^{2+}]_i$ oscillation-inducing factor is a single molecule, then this Hck-like protein is unlikely to be the candidate.

Tyrosine-phosphorylated proteins mainly localize in less soluble sperm fractions

Since the presence of Src family kinases can also be indirectly measured by detection of tyrosine-phosphorylated proteins, we conducted *in vitro* phosphotyrosine

Figure 1 The Src family kinase Hck is detectable in pSE^C. (A) A protein band of approximately 60 kDa from pSE^C is detectable by immunoblotting (IB) with an anti-Src^{pY416} antibody, which recognizes phosphotyrosine 416 of c-Src (left panel). This protein is likely to be Hck, since a band of the same size is also recognized by an anti-Hck antibody (right panel). (B) Superose 12 fractionation of pSE^C, followed by Western blotting (C) reveals that the 60 kDa protein recognized by anti-Hck (upper panel) and anti-Src^{pY416} antibodies (middle panel) elutes in fractions 48 and 49 (arrows indicate the locations of the 60 kDa bands). In contrast an anti-phosphotyrosine antibody (pTyr) recognized a lower molecular weight band of approximately 55 kDa in fractions 47 and 48 (lower panel). The lowermost line indicates Superose 12 fractions that exhibited Ca^{2+} -releasing activity when injected into mouse eggs (indicated by +). Ca^{2+} -active fractions are also indicated by a solid bar in the elution profile in (B). Note that fractions with Ca^{2+} activity do not necessarily correspond with fractions that contain either the 60 kDa or the 55 kDa protein bands (i.e. fraction 50).

assays using pSE^C and porcine sperm pellets that resulted after soluble pSE^C preparation (see Materials and Methods). As shown in Fig. 2A, miniscule amounts of tyrosine-phosphorylated proteins were detected in pSE^C. When further examined in Superose 12 column-eluted pSE^C, a tyrosine-phosphorylated protein was found in fractions 47 and 48 (Fig. 1C, bottom panel). However, the size of this molecule (approximately 55 kDa) was smaller than that of Src kinases (approximately 60 kDa; also see Fig. 1C, top and middle panels). Moreover, similar to the 60 kDa protein, the tyrosine-phosphorylated protein of 55 kDa was not found in all active pSE^C fractions. Taken together, these results indicate that a tyrosine-phosphorylated protein present in sperm, including a Src kinase and substrate, may not be an active component of [Ca²⁺]_i oscillation-inducing sperm extracts.

Interestingly, however, we found that the sperm pellets that result from the pSE^C extraction procedure still

Figure 2 The pellet resulting from extraction of pSE^C contains significant phosphotyrosine residues and exhibits Ca²⁺-releasing activity. (A) Phosphorylated proteins, as detected by the anti-pTyr antibody, are only weakly detectable in pSE^C (lanes 1–4), whereas multiple protein bands are readily visible in the pellet that results from pSE^C preparation (lanes 5–8). Moreover, addition of ATP to the pellet increases the intensities of several of these bands (lane 6, see arrows), and these increases are significantly reduced by addition of 10 μ M PP2 (lane 7), or 10 μ M peptide A (lane 8). (B) Injection of a single pig sperm isolated from the pellet into a mouse egg induces high frequency [Ca²⁺]_i oscillations. The arrow indicates the time of sperm injection; the graph is representative of nine replicates.

contained a significant amount of tyrosine-phosphorylated proteins (Fig. 2A). Moreover, in some proteins, tyrosine phosphorylation appeared to be up-regulated in an ATP-dependent manner and down-regulated in the presence of PP2 or peptide A, specific Src kinase inhibitors (Fig. 2A, arrows), suggesting the presence of active Src kinases in the pellets. Importantly, when microinjected into mouse eggs, a single sperm head from the pellet was still capable of inducing [Ca²⁺]_i oscillations (Fig. 2B) that were similar to those triggered by injection of an intact sperm head (data not shown), indicating that a significant amount of [Ca²⁺]_i oscillation-inducing factor still remains associated with the sperm pellet.

To fully extract the Ca²⁺-releasing activity from the sperm, we sequentially treated porcine sperm with sonication, Triton X (TX), and high pH buffer, and the resulting supernatants collected after each treatment were named pSE^C (see above), TX-soluble extracts (pSE^{TX}) and high pH-soluble extracts (pSE^{pH}) respectively. As shown in Fig. 3A, injection of pSE^{TX} and pSE^{pH} into mouse eggs induced [Ca²⁺]_i oscillations with similar specific activity (a protein concentration of 0.5 μ g/ μ l) to that of pSE^C. More importantly, only after treatment with high pH did the sperm completely lose the ability to induce [Ca²⁺]_i oscillations (Fig. 3A). Taken together, these results demonstrated that [Ca²⁺]_i oscillation-inducing sperm factor, including both soluble and less soluble components, was fully released from the sperm and recovered into the supernatant by our extraction procedure.

Western blot analysis revealed that tyrosine-phosphorylated proteins were exclusively present in pSE^{TX} and pSE^{pH} (Fig. 3B). To examine whether or not any tyrosine-phosphorylated proteins localize in the active fractions, pSE^{pH} was eluted into five fractions (F1–F5) through a Superose 12 column (Fig. 3C), and [Ca²⁺]_i oscillation-inducing activity in each fraction was tested by microinjection into mouse eggs. As shown in Fig. 3D, tyrosine-phosphorylated proteins only localized in inactive F1 and F2, whereas F3 and F4, which had [Ca²⁺]_i oscillation-inducing activity, did not contain tyrosine-phosphorylated proteins.

SFK inhibitor-treated pSE^C induces [Ca²⁺]_i oscillations

In the above experiments we could not detect SFKs or tyrosine-phosphorylated proteins specifically in the active pSE fractions. To further evaluate the requirement of activation of a Src family kinase for initiation of [Ca²⁺]_i oscillations and egg activation in the mouse, we tested whether the addition of specific Src/tyrosine kinase inhibitors is capable of inhibiting pSE^C-induced [Ca²⁺]_i oscillations (Fig. 4A) and egg activation. As shown in Fig. 4B, incubation and co-injection of pSE^C with 200 μ M PP2 failed to block oscillations induced by injection of pSE^C. Likewise, treatment of pSE^C with 200 μ M lavendustin A, a tyrosine kinase inhibitor, did not have any effects on initiation of [Ca²⁺]_i oscillations and activation of the eggs (data not

Figure 3 Full extraction of Ca^{2+} -releasing activity from porcine sperm indicates that activity does not correlate with the presence of tyrosine-phosphorylated proteins. (A) Porcine sperm were sequentially treated with sonication, Triton X, and alkaline pH of 11.5, and in each case the resulting supernatant and a single sperm from the pellet were injected into mouse eggs. A representative $[Ca^{2+}]_i$ graph for each injection is shown. Note that only in the case of high pH treatment was the Ca^{2+} -releasing activity fully released from the sperm pellet into the supernatant. (B) Immunoblotting with the anti-pTyr antibody of supernatants resulting from sonicated, Triton X-100- and high pH-treated sperm indicate that tyrosine-phosphorylated proteins are present predominantly in the latter two fractions, with the highest levels detectable in the alkaline pH extraction. (C) Superose 12 fractionation of the high pH extraction, followed by Western blotting with the anti-pTyr antibody (D) indicates that tyrosine-phosphorylated proteins elute in fractions F1 and F2, in contrast to fractions F3 and F4 which exhibited Ca^{2+} -releasing activity when injected into mouse eggs (indicated by + in the lowermost line; fractions with more + symbols indicate higher frequency of oscillations).

shown). To confirm that these inhibitors were indeed active, *in vitro* kinase assays were performed. As shown in Fig. 4F, a recombinant Src protein was *in vitro* auto-phosphorylated at Tyr⁴¹⁶ in the presence of ATP as revealed by the anti-Src^{pY416} antibody. PP2 and lavendustin A, but not PP3, the inactive analog of PP2, were both capable of inhibiting this phosphorylation, indicating that the inhibitors used were active. It is worth noting that these inhibitors were shown to prevent fertilization-induced Ca^{2+} release and egg activation in *Xenopus laevis*, and that the

concentrations of these compounds used in our studies were equal to, or much higher than, those reported in the *Xenopus* studies (Glahn *et al.* 1999, Sato *et al.* 2002). Therefore, these results suggested that activation of SFKs present in sperm is unlikely to be necessary for initiation of $[Ca^{2+}]_i$ oscillations and egg activation in mouse eggs.

In contrast, however, we found that a specific Src kinase inhibitory peptide, peptide A, significantly blocked the ability of pSEC to trigger $[Ca^{2+}]_i$ oscillations in mouse eggs when pre-incubated with pSEC (Fig. 4C). Pre-incubation of

Figure 4 pSE^C-induced Ca²⁺ oscillations are blocked by pre-incubation with peptide A, but not with PP2. pSE^C treated with 200 µM PP2 induces oscillations when injected into mouse eggs (B) similar to those induced by untreated pSE^C (A). In contrast, treatment of pSE^C with 200 µM peptide A prevents the ability of pSE^C to generate oscillations (C), whereas the control peptide, A9, is ineffective (D). Arrows indicate time of injection; numbers of replicates are shown in each graph. (E) Percentage of eggs that exhibited [Ca²⁺]_i oscillations when injected with pSE^C treated with peptide A at various concentrations. (F) Recombinant Src protein was autophosphorylated *in vitro* in the presence of ATP, as revealed by immunoblotting with the anti-Src^{pY416} antibody (upper panel). This phosphorylation was significantly reduced in the presence of 10 µM PP2 and, to a lesser extent, 10 µM lavendustin A. Immunoblotting with the anti-Src antibody (lower panel) indicates equal loading of Src protein in each lane.

pSE^C with the control peptide A9, which lacks three amino acids of both C- and N-terminus present in peptide A and does not inhibit Src kinase activity, failed to prevent pSE^C-induced [Ca²⁺]_i oscillations (Fig. 4D), indicating that the inhibitory effect of peptide A is specific and is not a result of toxicity or high protein concentrations. As shown in Fig. 4E, the inhibitory effect of peptide A was concentration dependent and, to fully inhibit pSE^C-induced [Ca²⁺]_i oscillations, 200 µM peptide A was required. This contradicts the above results obtained by the other SFK inhibitors. Nevertheless, it is possible that there is a homologous domain in the sperm factor molecule(s) to the peptide A-binding site in SFKs so that this peptide could inhibit the activity of the putative active molecule in the extracts (see Discussion).

Injection of a c-Src protein or mRNAs encoding active Src mutants is unable to induce [Ca²⁺]_i oscillations in mouse eggs

To evaluate whether activation of a Src family kinase *per se* is sufficient to induce [Ca²⁺]_i oscillations in mouse eggs, we microinjected an active recombinant c-Src protein or mRNAs encoding two different constitutively active mutants of c-Src, Src^{Y527F} and Src^{E378G}. As shown in Fig. 5, injection of neither the recombinant c-Src (3.0 µg/µl; it was the highest concentration that we could possibly inject) nor the mRNAs (Fig. 5D and C) induced [Ca²⁺]_i release in mouse eggs, whereas injection of pSE^C (0.5 µg/µl) (Fig. 5A) or a single mouse sperm (Fig. 5B) triggered [Ca²⁺]_i oscillations. Moreover, none of the eggs injected with the recombinant protein or the mRNAs

Figure 5 Src is not sufficient to induce $[Ca^{2+}]_i$ oscillations in mouse eggs. Injection of mouse eggs with pSE^C (A) or a single mouse sperm (B) triggers $[Ca^{2+}]_i$ oscillations, whereas injection of either recombinant c-Src (C), Src^{Y527F} mRNA (D), or Src^{E378G} mRNA (E) is unable to do so. Injection of PLC ζ mRNA (F) induces oscillations similar to those generated by a single mouse sperm. Arrows indicate time of injections; numbers of replicates are shown in each graph. (G) Lysates of eggs injected with Src^{E378G} mRNA or treated with 10 μ M H₂O₂ for 10 min, in the presence of 5 μ M PP2 or PP3, were subjected to Western blotting with the anti-pTyr antibody. In the presence of PP3, the phosphotyrosine content of several protein bands (see arrows) increased as a result of Src^{E378G} mRNA injection or H₂O₂ treatment compared with MII controls, and this increase was prevented in the presence of PP2. Note that the arrows point at the bands that are likely substrates of SFKs, but they do not denote SFKs themselves.

exhibited signs of egg activation, such as extrusion of the second polar body or pronuclear formation (data not shown). As shown in Fig. 4F, the recombinant Src protein was auto-phosphorylated at Tyr⁴¹⁶ *in vitro*, indicating that the recombinant protein used was functional. In addition, mouse eggs injected with mRNA encoding Src^{E378G} exhibited an increase in protein phosphotyrosine content, and this phosphorylation was inhibited in the presence of PP2, but not PP3 (Fig. 5G), suggesting that these introduced mRNAs were translated and generated active proteins in the eggs. As a positive control, we microinjected into mouse eggs mRNA encoding PLC ζ , a novel candidate of the sperm's $[Ca^{2+}]_i$ oscillation-inducing factor (Saunders *et al.* 2002). As shown in Fig. 5F, microinjection of PLC ζ mRNA initiated persistent $[Ca^{2+}]_i$ oscillations similar to those induced by fertilization (Fig. 5B), and the injected

eggs extruded the second polar body and formed a pronucleus ($n = 10$).

pSE induces $[Ca^{2+}]_i$ oscillations in mouse eggs treated with an SFK inhibitor

To investigate the possible involvement of SFKs present in eggs, mouse eggs were first injected with 500 μ M PP2, lavendustin A, or peptide A (concentration in the pipette; results in an intracellular concentration of approximately 5–10 μ M) followed by injection of 0.5 μ g/ μ l pSE^C. As shown in Fig. 6A, C, D and E injection of these compounds did not affect the ability of pSE^C to induce $[Ca^{2+}]_i$ oscillations. Moreover, when eggs were pre-incubated in 5 μ M PP2 for 60 min prior to pSE^C injection, pSE^C-induced $[Ca^{2+}]_i$ oscillations occurred normally (Fig. 6A and B).

Figure 6 Treatment of eggs with Src/tyrosine kinase inhibitors does not prevent induction of $[Ca^{2+}]_i$ oscillations by pSE^C. Injection of pSE^C into eggs pre-incubated with PP2 generates $[Ca^{2+}]_i$ oscillations (B) comparable with those seen in controls (A). To avoid drug permeability, in the next series of experiments the inhibitors were microinjected. Pre-injection of eggs with PP2 (C), lavendustin A (D), or peptide A (E) does not prevent induction of $[Ca^{2+}]_i$ oscillations by pSE^C injection. The first arrow in each graph indicates the time of inhibitor injection, while the second arrow indicates pSE^C injection. The injection of pSE^C occurred within 30 min of the inhibitor injection. The numbers of replicates are shown in each graph.

Previously, we have demonstrated using *Xenopus* eggs that H_2O_2 is a strong trigger of tyrosine phosphorylation of several egg proteins, including an Src protein. Further, brief treatment of *Xenopus* eggs with H_2O_2 leads to Ca^{2+} release and egg activation via activation of PLC γ in the eggs (Sato *et al.* 2001). To examine whether a similar signaling pathway is operational in mouse eggs, we treated mouse eggs with H_2O_2 at 1, 10, and 100 μ M for 10, 20 and 30 min. Importantly, mouse eggs treated with H_2O_2 under any of these conditions did not exhibit Ca^{2+} release or signs of egg activation (data not shown). However, when Western blotting was performed, it was found that tyrosine phosphorylation was greatly enhanced in eggs treated with 10 μ M H_2O_2 for 10 min, and that this phosphorylation was fully blocked in the presence of PP2 (Fig. 5G).

Sperm-induced $[Ca^{2+}]_i$ oscillations are not prevented by PP2

The above results indicated that activation of an SFK is neither necessary nor sufficient for triggering $[Ca^{2+}]_i$ oscillations and egg activation in mouse fertilization. To further support this conclusion, we performed mouse ICSI using sperm and eggs that were both pre-incubated with PP2 or PP3 prior to injection. As expected, when PP2-treated eggs were fertilized by PP2-treated sperm and further cultured in the presence of PP2, $[Ca^{2+}]_i$ oscillations were not different from those seen with PP3 treatment (Fig. 7A and B), and egg activation also took place normally in both PP2- and PP3-treated eggs (data not shown).

Discussion

Recent evidence in echinoderm, ascidian, *Xenopus*, and zebrafish eggs suggests that activation of an SFK(s) is

Figure 7 PP2 does not prevent $[Ca^{2+}]_i$ oscillations generated by sperm. Injection of a single mouse sperm into eggs pre-incubated with 5 μ M PP3 (A) or PP2 (B) for 30 min results in $[Ca^{2+}]_i$ oscillations that are similar to those seen in untreated control eggs (see Fig. 6B). Pre-incubation was performed in the absence of oil. ICSI and $[Ca^{2+}]_i$ monitoring were carried out in the presence of the inhibitors. Arrows indicate time of injection; numbers of replicates are shown in each graph.

triggered upon fertilization, which in turn activates PLC γ in the eggs, leading to Ca^{2+} release and egg activation (Abassi *et al.* 2000, Giusti *et al.* 2000, Runft & Jaffe 2000, Sato *et al.* 2000, Wu & Kinsey 2000). Previous studies showed that PLC γ 1 and 2 isoforms are expressed at the protein level in mouse eggs (Dupont *et al.* 1996, Mehlmann *et al.* 1998); thus, a similar mechanism could operate in mammals. In the present study, we have investigated whether activation of an SFK(s) is required for initiation of $[Ca^{2+}]_i$ oscillations in mammalian fertilization using mouse gametes and porcine sperm as models. Our results have shown the following: (i) although a Hck-like protein and various tyrosine-phosphorylated proteins were detected in porcine sperm fractions, the presence of these proteins did not correspond to the active fractions of pSE;

(ii) $[Ca^{2+}]_i$ oscillations induced by pSE^C in mouse eggs were unaltered by pre-incubation of pSE with specific tyrosine kinase inhibitors such as PP2 or lavendustin A, despite the fact that these inhibitors were shown to be active both *in vivo* and *in vitro*. Likewise, pre-injection or pre-incubation of eggs with these inhibitors did not affect the ability of pSE^C to trigger $[Ca^{2+}]_i$ oscillations in mouse eggs; (iii) microinjection of a recombinant c-Src protein or expression of mRNAs encoding constitutively active Src proteins did not induce Ca^{2+} release or egg activation; (iv) when ICSI was performed using sperm and eggs, both of which were pre-treated with PP2, $[Ca^{2+}]_i$ oscillations occurred normally. Our results indicated that activation of an SFK is neither necessary nor sufficient for triggering $[Ca^{2+}]_i$ oscillations during mammalian fertilization.

Hck-like protein and tyrosine-phosphorylated proteins in porcine sperm fractions

It is well established that injection of sperm extracts from mouse or other species into mouse eggs can trigger fertilization-like $[Ca^{2+}]_i$ oscillations and egg activation (Fissore *et al.* 1998, 2002, Swann *et al.* 2001). However, the identity of the molecule(s) that is delivered from the sperm and that is responsible for initiation of $[Ca^{2+}]_i$ oscillations still remains to be fully elucidated. In support of the possibility that the fertilizing sperm delivers an SFK to the egg, to date expression of three SFKs, c-Abl, c-Src, and c-Yes, has been reported in human and rat sperm (Zhao *et al.* 1993, Nishio *et al.* 1995, Naz 1998, Leclerc & Goupil 2002). Therefore, we first sought to determine which, if any, SFKs are present in pSE^C. We detected a Hck-like protein in soluble pSE^C and significant amounts of tyrosine-phosphorylated proteins in the less soluble fractions, pSE^{TX} and pSE^{PH}. Moreover, *in vitro* kinase assays showed that there was an ATP-dependent increase in protein phosphotyrosine content in the less soluble sperm fractions, and this increase was antagonized by PP2, further suggesting the presence of an active SFK(s) in porcine sperm. However, the localization of this Hck-like protein and other tyrosine-phosphorylated proteins did not correspond with all of the Ca^{2+} -active pSE fractions following Superose 12 column elution. Therefore, our results suggested that if the $[Ca^{2+}]_i$ oscillation-inducing sperm factor is a single molecule, then this molecule is unlikely to be an SFK. Furthermore, we showed that inhibition of the sperm SFK(s) by PP2 or lavendustin A did not affect the ability of pSE^C to trigger $[Ca^{2+}]_i$ oscillations, confirming the result that a sperm SFK(s) is not essential for $[Ca^{2+}]_i$ oscillations induced by injection of pSE. It is well documented that protein tyrosine phosphorylation is increased during sperm capacitation and the acrosome reaction, and is a critical process that regulates these events in many species (Evans & Florman 2002, Visconti *et al.* 2002). Therefore, although our results indicated the presence of a Hck-like protein and, potentially, other active SFKs in porcine sperm fractions, it is more likely that these proteins,

together with other SFKs that reportedly are present in sperm, are involved in the capacitation/acrosome reaction-related increase in protein phosphotyrosine content rather than in fertilization-associated $[Ca^{2+}]_i$ oscillations (Leclerc & Goupil 2002, Dorval *et al.* 2003).

Activation of an egg SFK is not necessary for initiation of $[Ca^{2+}]_i$ oscillations

Although a sperm SFK(s) is unlikely to be responsible for $[Ca^{2+}]_i$ oscillations triggered by injection of pSE, it is possible that an egg SFK(s) is directly or indirectly activated by a factor present in pSE which, in turn, stimulates activation of PLC γ isoforms in the egg. It should be noted that activation of an egg SFK(s) is observed soon after fertilization and is essential for sperm-induced $[Ca^{2+}]_i$ release and egg activation in sea urchin, zebrafish, and *Xenopus* (Kinsey 1996, Sato *et al.* 1999, 2000, Abassi *et al.* 2000, Wu & Kinsey 2000). The importance of activation of an egg SFK(s) has also been demonstrated in starfish and ascidian (Giusti *et al.* 1999, 2000, Runft & Jaffe 2000). It has been shown that in ascidian, starfish, and *Xenopus* eggs, inhibition of SFK signaling by the SFK SH2 domain or various SFK inhibitors, such as PP2, lavendustin A, and peptide A, prevents fertilization-associated $[Ca^{2+}]_i$ release and egg activation via a PLC γ -dependent mechanism (Glahn *et al.* 1999, Sato *et al.* 2000). Importantly, the presence of the SFK members c-Abl, c-Fyn, and c-Yes has been reported in rat and mouse eggs (Talmor *et al.* 1998, Sutovsky *et al.* 2001, Talmor-Cohen *et al.* 2004 (this issue)). Moreover, in rat eggs, an increase in tyrosine phosphorylation of several proteins was observed shortly after fertilization (Ben-Yosef *et al.* 1998). However, in the present study, we have demonstrated that activation of an egg SFK(s) is not necessary for $[Ca^{2+}]_i$ oscillations in mouse eggs induced by injection of pSE. When mouse eggs were pre-injected or pre-incubated with PP2, lavendustin A, or peptide A, pSE^C-induced $[Ca^{2+}]_i$ oscillations occurred normally, despite the fact that these inhibitors were proven to function *in vitro* (Fig. 4F) and *in vivo* (Fig. 5G). In addition, it should be noted that these inhibitors were reported to completely block sperm-induced $[Ca^{2+}]_i$ release in *Xenopus* at the same, or even lower, concentrations than those that we used (Glahn *et al.* 1999, Sato *et al.* 2002).

Activation of an SFK is not sufficient for initiation of $[Ca^{2+}]_i$ oscillations

Lastly, we tested the possibility that activation of an SFK(s) is sufficient to trigger $[Ca^{2+}]_i$ oscillations and/or egg activation in the mouse. It has been demonstrated that, in starfish and *Xenopus* eggs, activation of an egg SFK(s) *per se* is sufficient to induce Ca^{2+} release and egg activation via PLC γ activation (Giusti *et al.* 2000, Tokmakov *et al.* 2002, Sato *et al.* 2003). Our results show that neither injection of active Src protein nor expression of mRNAs encoding constitutively active Src mutants induced $[Ca^{2+}]_i$ release or egg activation in mouse eggs. *In vitro* kinase assays proved

that the recombinant protein used was active (Fig. 4F). Moreover, the eggs injected with the mRNAs showed an increase in protein phosphotyrosine content that was sensitive to inhibition by PP2, indicating that the introduced mRNAs were properly translated to active Src proteins in the eggs. It was recently demonstrated that H_2O_2 is a strong trigger of protein tyrosine phosphorylation and that H_2O_2 treatment of *Xenopus* eggs triggers $[\text{Ca}^{2+}]_i$ release and egg activation via activation of the egg SFK, Xyk (Sato *et al.* 2001, 2003, Tokmakov *et al.* 2002). In contrast to *Xenopus*, however, our results showed that incubation of eggs with H_2O_2 did not cause any $[\text{Ca}^{2+}]_i$ release or egg activation, even though different H_2O_2 concentrations and incubation times were applied. Nevertheless, the eggs treated with H_2O_2 exhibited a significant increase in protein phosphotyrosine content that was inhibited in the presence of PP2, indicating that H_2O_2 treatment triggered tyrosine phosphorylation of mouse egg proteins via activation of an egg SFK(s). Taken together, our results demonstrated that activation of an egg SFK(s) is not sufficient for initiation of $[\text{Ca}^{2+}]_i$ oscillations or egg activation.

Nevertheless, contrary to our results, two groups have recently reported activation of mouse eggs via stimulation of an egg SFK. Sette *et al.* (2002) have proposed a hypothesis that the truncated c-kit tyrosine kinase (tr-kit) is delivered from the fertilizing sperm and activates c-Fyn in mouse eggs, leading, potentially, to stimulation of PLC γ 1 and subsequent $[\text{Ca}^{2+}]_i$ responses and egg activation. Sette *et al.* (2002) showed that injection of the active c-Fyn mutant or tr-kit-expressing cell extracts into mouse eggs caused egg activation. Likewise, it was reported by another group that a novel protein in the sperm perinuclear theca, PT32, is able to induce egg activation in mouse eggs by interacting with and activating eggs' c-Yes (Sutovsky *et al.* 2001, Wu *et al.* 2001a). However, $[\text{Ca}^{2+}]_i$ measurements were not performed in either of those two studies. Thus, it is possible that excessive amounts of an active SFK(s) may be able to trigger egg activation independently of the Ca^{2+} signal (Talmor-Cohen *et al.* 2004 (this issue)). It should be noted that PP2 at the same concentration as that used to inhibit egg activation induced by the active c-Fyn mutant and tr-kit (Sette *et al.* 2002) did not block fertilization-triggered $[\text{Ca}^{2+}]_i$ oscillations or egg activation in the present study. Therefore, the physiological significance of tr-kit- and PT32-induced signaling pathways remains unclear and requires further investigation.

What is sperm factor?

Recently, a novel sperm-specific PLC, PLC ζ , was discovered and has been proposed to be the active Ca^{2+} -releasing component of sperm factor (Saunders *et al.* 2002). PLC ζ mRNA is able to trigger $[\text{Ca}^{2+}]_i$ oscillations in mouse eggs that are similar to those at fertilization (Saunders *et al.* 2002, present study). Nevertheless, it still remains to be fully elucidated whether PLC ζ represents all of the active components of sperm factor since the localization of this

protein in sperm as well as the ability of the recombinant protein, instead of mRNA, to induce $[\text{Ca}^{2+}]_i$ oscillations has not been shown (Swann *et al.* 2004 (this issue)). In the present study, we developed a new extraction method of pSE, whereby less soluble pSE (pSE^{TX} and pSE^{PH}), which remains in the sperm head after extraction of conventional soluble pSE^C, could be recovered. These less soluble pSE fractions appear to have different protein profiles from those of conventional soluble pSE^C since both pSE^{TX} and pSE^{PH} contained significantly greater amounts of protein phosphotyrosine content. It would therefore be of particular interest to determine whether or not PLC ζ is equally present in soluble and less soluble pSEs.

We also found that peptide A, originally created as an inhibitor of SFKs, was able to prevent the $[\text{Ca}^{2+}]_i$ oscillation-inducing activity of pSE^C only when incubated with the extracts, despite the discrepancy that activation of an SFK(s) is unlikely to be required for the initiation of $[\text{Ca}^{2+}]_i$ oscillations in mouse fertilization. A possible explanation for this is that the active $[\text{Ca}^{2+}]_i$ oscillation-inducing molecule(s) in pSE^C may contain a domain that is highly similar to the peptide A-binding domain present in SFKs. Nevertheless, regardless of the lack of involvement of an SFK(s) in mouse fertilization, it is significant that peptide A is the first reagent capable of inhibiting pSE-induced $[\text{Ca}^{2+}]_i$ oscillations. Currently, we are investigating whether or not PLC ζ activity is inhibited by this peptide.

In summary, our results have demonstrated that, in contrast to the mechanisms in invertebrates and *Xenopus*, activation of an SFK(s) is neither necessary nor sufficient for fertilization-induced $[\text{Ca}^{2+}]_i$ oscillations in the mouse. However, it should be noted that our results do not necessarily discount the possibility that activation of PLC γ isoforms is required for fertilization-associated $[\text{Ca}^{2+}]_i$ oscillations in the mouse. Although PLC ζ certainly is a strong candidate as the trigger of the oscillations, it is not clear whether it is the sole molecule required for initiation of $[\text{Ca}^{2+}]_i$ oscillations. Therefore, the mechanism leading to initiation of $[\text{Ca}^{2+}]_i$ oscillations at fertilization still remains to be fully elucidated.

Note added in proof

While this paper was in preparation, a paper appeared in the *Journal of Biological Chemistry* in the form of papers in press: Kouchi *et al.* "Recombinant phospholipase C-zeta has high Ca^{2+} -sensitivity and induces Ca^{2+} oscillations in mouse eggs". The authors showed that injections of high concentration of recombinant PLC ζ protein into mouse eggs triggered $[\text{Ca}^{2+}]_i$ oscillations, whereas injection of PLC δ 1 protein failed to do so.

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