Focus on Fertilization

Evidence that activation of Src family kinase is not required for fertilization-associated [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+} release. In the present study, we have investigated whether activation of an SFK is required for initiation of intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{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)pyrazolo[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\textsuperscript{2+}]\textsubscript{i} oscillations in mouse eggs. Microinjection of a recombinant c-Src protein or mRNAs encoding constitutively active Src proteins did not induce [Ca\textsuperscript{2+}]\textsubscript{i} release. Finally, when sperm and eggs, both of which were pre-treated with PP2, were fertilized, [Ca\textsuperscript{2+}]\textsubscript{i} oscillations occurred normally. We can therefore conclude that activation of an SFK is neither necessary nor sufficient for triggering fertilization-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations.

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\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+} signal. In support of this notion, the [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{i} release induced by sperm or by injection of sperm extracts involves production of inositol 1,4,5-trisphosphate (IP\textsubscript{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\textsubscript{3}, in turn, leads to [Ca\textsuperscript{2+}]\textsubscript{i} release from the endoplasmic reticulum by interacting with IP\textsubscript{3} receptors (Miyazaki et al. 1992, Wu et al. 1997).
Given this known requirement for stimulation of the IP$_3$ 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 β1, β3, γ1, and γ2 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$_{2+}$], 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 PLCz, 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 PLCz induces [Ca$_{2+}$], oscillations in mouse eggs similar to those generated by fertilization, and that immunodepletion of PLCz protein from sperm extracts abrogates the ability of those extracts to induce [Ca$_{2+}$], release when injected into mouse eggs. Thus, strong evidence supports the notion that PLCz represents the Ca$_{2+}$-active factor of sperm extracts; however, the question remains as to whether PLCz 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$_{2+}$], 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$_{2+}$], release in response to Src protein injection was blocked by prior injection of PLCγ SH2 domains (Carroll et al. 1997, 1999, Shearer et al. 1999), which implies that the sperm initiates [Ca$_{2+}$], release in these eggs by sequential activation of an SFK and PLCγ.

Using a similar strategy in ascidian eggs, it has been shown that both fertilization and injection of sperm extracts trigger [Ca$_{2+}$], release by activating the same pathway that involves PLCγ 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 PLCγ activity (Glahn et al. 1999, Sato et al. 2000, Tokmakov et al. 2002). However, differences among species as to how PLCγ is activated by the SFK pathway have been documented. For example, it has been shown that injection of PLCγ SH2 fusion proteins, which inhibit sperm- or sperm extract-triggered [Ca$_{2+}$] 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$_{2+}$], release at fertilization was not inhibited in mouse eggs by injection of excess PLCγ SH2 domains, suggesting that if PLCγ 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$_{2+}$], release in mammalian eggs is warranted.

Materials and Methods

Gamete collection

MII eggs were obtained from B6D2F1 (C57BL/6J × DBA/2J) and CD1 female mice (8–12 weeks old).
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 Heps, 1 mM EDTA, 10 mM glycerophosphate, 1 mM dithiothreitol, 200 µM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml pepstatin, and 10 µg/ml leupeptin, pH 7.0) at 4°C and the lysate ultracentrifuged. The clear supernatant was concentrated 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 Na$_2$CO$_3$, 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 aliquotted 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 Heps, 1 mM EDTA, 200 µM PMSF, 20 µg/ml pepstatin, and 20 µg/ml leupeptin, pH 7.0) at a flow rate of 0.1 ml/min and detected at OD$_{280}$ by a UV-M monitor (Pharmacia Biotech Inc., Piscataway, NJ, USA). Fractions (0.25 ml) were collected and concentrated for testing the Ca$^{2+}$-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 µg/µl, 75 units/25 µl; 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 Heps-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 (Piezodril; 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. 1998b); sonication resulted in more than 90% of sperm heads separated from their tails. In certain experiments, porcine

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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 \( \text{H}_2\text{O}_2 \), 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% \( \text{CO}_2 \). 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-\((\tau\text{-butyl})\)pyrazolo[3,4-\(d\)]pyrimidine (PP2) and lavendustin A, and an inactive analogue, 4-amino-7-phenylpyrazolo[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 \(-20^\circ\text{C}\) until use. Peptide A (VAPDSIQAEWWYFGKitRRE) was purchased from Calbiochem (Sato et al. 1990, Glahn et al. 1999). Peptide A9 (SDSIQAEEWYFGK) 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 \(-80^\circ\text{C}\). 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.

**[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 acetoxyethyl ester (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 Photoscan 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/F380). 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\(^{Y416}\), 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 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (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 mM MgCl2, 5 \( \mu \text{L} \) 100 mM KCl, 50 \( \mu \text{L} \) Tris (pH 7.5), 2.5 \( \mu \text{L} \) 10 mM ATP, and 7 \( \mu \text{L} \) H2O). 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\(^{Y416}\).

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\( \gamma \) (q360 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 PLCζ (accession no. AK006672, a gift from RIKEN; Kouchi et al. 2004). Full-length mouse PLCζ was cloned into pBluescript KS(−) vector and complete insertion of full-length PLCζ 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 mMessageMachine 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 pSEC into mouse MII eggs is able to trigger [Ca2+]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 pSEC contains an SFK and, further, to begin to address the possibility that an SFK may represent the Ca2+-releasing factor in pSEC, we performed Western blotting on pSEC 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 pSEC (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-SrcpY416 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 pSEC, pSEC 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-SrcpY416 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 pSEC (Fig. 1A) represents Hck kinase. Interestingly, however, this Hck-like protein only localized in fractions 48 and 49, whereas Ca2+-releasing activity was found in fractions 47–50, indicating that, if the [Ca2+]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 pSEC. (A) A protein band of approximately 60 kDa from pSEC is detectable by immunoblotting (IB) with an anti-SrcpY416 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). 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 pSEC (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-SrcpY416 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 pSEC, pSEC 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-SrcpY416 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 pSEC (Fig. 1A) represents Hck kinase. Interestingly, however, this Hck-like protein only localized in fractions 48 and 49, whereas Ca2+-releasing activity was found in fractions 47–50, indicating that, if the [Ca2+]i oscillation-inducing factor is a single molecule, then this Hck-like protein is unlikely to be the candidate.

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Since the presence of Src family kinases can also be indirectly measured by detection of tyrosine-phosphorylated proteins, we conducted in vitro phosphotyrosine
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 \([\text{Ca}^{2+}]\); oscillation-inducing sperm extracts.

Interestingly, however, we found that the sperm pellets that result from the pSE\(^C\) extraction procedure still 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 \([\text{Ca}^{2+}]\); 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 \([\text{Ca}^{2+}]\); oscillation-inducing factor still remains associated with the sperm pellet.

To fully extract the \([\text{Ca}^{2+}]\);-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 \([\text{Ca}^{2+}]\); oscillations with similar specific activity (a protein concentration of 0.5 \(\mu\)g/\(\mu\)l) to that of pSE\(^C\). More importantly, only after treatment with high pH did the sperm completely lose the ability to induce \([\text{Ca}^{2+}]\); oscillations (Fig. 3A). Taken together, these results demonstrated that \([\text{Ca}^{2+}]\); 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 \([\text{Ca}^{2+}]\); 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 \([\text{Ca}^{2+}]\); oscillation-inducing activity, did not contain tyrosine-phosphorylated proteins.

**SFK inhibitor-treated pSE\(^C\) induces \([\text{Ca}^{2+}]\); 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 \([\text{Ca}^{2+}]\); 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 \([\text{Ca}^{2+}]\); oscillations (Fig. 4A) and egg activation. As shown in Fig. 4B, incubation and co-injection of pSE\(^C\) with 200 \(\mu\)M PP2 failed to block oscillations induced by injection of pSE\(^C\). Likewise, treatment of pSE\(^C\) with 200 \(\mu\)M lavendustin A, a tyrosine kinase inhibitor, did not have any effects on initiation of \([\text{Ca}^{2+}]\); oscillations and activation of the eggs (data not shown).
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-SrcpY416 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\(^{2+}\)] 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\(^{2+}\)] oscillations in mouse eggs when pre-incubated with pSEC (Fig. 4C).
pSEC 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 pSEC-induced 
\[\text{Ca}^{2+}\] 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 pSEC-induced 
\[\text{Ca}^{2+}\] oscillations, 200 \(\mu\)M 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 \[\text{Ca}^{2+}\] oscillations in mouse eggs**

To evaluate whether activation of a Src family kinase per se is sufficient to induce \[\text{Ca}^{2+}\] oscillations in mouse eggs, we microinjected an active recombinant c-Src protein or mRNAs encoding two different constitutively active mutants of c-Src, Src\(^{\text{Y527F}}\) and Src\(^{\text{E378G}}\). As shown in Fig. 5, injection of neither the recombinant c-Src (3.0 \(\mu\)g/\(\mu\)l) nor the mRNAs (Fig. 5D and C) induced \[\text{Ca}^{2+}\] release in mouse eggs, whereas injection of pSEC \(0.5 \mu\text{g}/\mu\text{l}\) (Fig. 5A) or a single mouse sperm (Fig. 5B) triggered \[\text{Ca}^{2+}\] oscillations. Moreover, none of the eggs injected with the recombinant protein or the mRNAs...
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 SrcE378G 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 PLCz, a novel candidate of the sperm’s [Ca^{2+}]i oscillation-inducing factor (Saunders et al. 2002). As shown in Fig. 5F, microinjection of PLCz 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 μM PP2, lavendustin A, or peptide A (concentration in the pipette; results in an intracellular concentration of approximately 5-10 μM) followed by injection of 0.5 μg/μl pSEC. As shown in Fig. 6A, C, D and E injection of these compounds did not affect the ability of pSE to induce [Ca^{2+}]i oscillations. Moreover, when eggs were pre-incubated in 5 μM PP2 for 60 min prior to pSE injection, pSE-induced [Ca^{2+}]i oscillations occurred normally (Fig. 6A and B).
Previously, we have demonstrated using Xenopus eggs that H$_2$O$_2$ is a strong trigger of tyrosine phosphorylation of several egg proteins, including an Src protein. Further, brief treatment of Xenopus eggs with H$_2$O$_2$ leads to Ca$^{2+}$ release and egg activation via activation of PLC$\gamma$ 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$_2$O$_2$ at 1, 10, and 100 $\mu$M for 10, 20 and 30 min. Importantly, mouse eggs treated with H$_2$O$_2$ 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 $\mu$M H$_2$O$_2$ for 10 min, and that this phosphorylation was fully blocked in the presence of PP2 (Fig. 5G).

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

The above results indicated that activation of an SFK is neither necessary nor sufficient for triggering [Ca$^{2+}$]; 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$^{2+}$]; 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 triggered upon fertilization, which in turn activates PLC$\gamma$ 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$\gamma$1 and 2 isoforms are expressed at the protein level in mouse eggs (Dupont et al. 1996, Mehlmann et al. 1998); thus, a similar mechanism could operate in mammals. In the present study, we have investigated whether activation of an SFK(s) is required for initiation of [Ca$^{2+}$]; 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) \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), oscillations in mouse eggs; (iii) microinjection of a recombinant c-Src protein or expression of mRNAs encoding constitutively active Src proteins did not induce \([\text{Ca}^{2+}]\) release or egg activation; (iv) when ICSI was performed using sperm and eggs, both of which were pre-treated with PP2, \([\text{Ca}^{2+}]\), oscillations occurred normally. Our results indicated that activation of an SFK is neither necessary nor sufficient for triggering \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), 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\(_X\) and pSE\(_P\). 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 \([\text{Ca}^{2+}]\)-active pSE fractions following Superose 12 column elution. Therefore, our results suggested that if the \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), oscillations, confirming the result that a sperm SFK(s) is not essential for \([\text{Ca}^{2+}]\), oscillations induced by injection of pSE. When mouse eggs were pre-injected or pre-incubated with PP2, lavendustin A, or peptide A, pSE\(_C\)-induced \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), 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 egg SFK is not necessary for initiation of \([\text{Ca}^{2+}]\), oscillations**

Although a sperm SFK(s) is unlikely to be responsible for \([\text{Ca}^{2+}]\), 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\(_\gamma\) 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), release and egg activation via a PLC\(_\gamma\)-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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), oscillations**

Lastly, we tested the possibility that activation of an SFK(s) is sufficient to trigger \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), release and egg activation via PLC\(_\gamma\) 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 \([\text{Ca}^{2+}]\), 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_{2}O_{2} is a strong trigger of protein tyrosine phosphorylation and that H_{2}O_{2} treatment of Xenopus eggs triggers [Ca^{2+}], 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_{2}O_{2} did not cause any [Ca^{2+}], release or egg activation, even though different H_{2}O_{2} concentrations and incubation times were applied. Nevertheless, the eggs treated with H_{2}O_{2} exhibited a significant increase in protein phosphotyrosine content that was inhibited in the presence of PP2, indicating that H_{2}O_{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 [Ca^{2+}], 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 PLCγ1 and subsequent [Ca^{2+}], 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, [Ca^{2+}], 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 [Ca^{2+}], 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, PLCζ, was discovered and has been proposed to be the active Ca^{2+}-releasing component of sperm factor (Saunders et al. 2002). PLCζ mRNA is able to trigger [Ca^{2+}], 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 PLCζ 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 [Ca^{2+}], 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, 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 PLCζ 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 [Ca^{2+}], 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 [Ca^{2+}], oscillations in mouse fertilization. A possible explanation for this is that the active [Ca^{2+}], 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 [Ca^{2+}], oscillations. Currently, we are investigating whether or not PLCζ 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 [Ca^{2+}], oscillations in the mouse. However, it should be noted that our results do not necessarily discount the possibility that activation of PLCζ isoforms is required for fertilization-associated [Ca^{2+}], oscillations in the mouse. Although PLCζ 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 [Ca^{2+}], oscillations. Therefore, the mechanism leading to initiation of [Ca^{2+}], 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-zeeta has high Ca^{2+}-sensitivity and induces Ca^{2+} oscillations in mouse eggs.” The authors showed that injections of high concentration of recombinant PLCζ protein into mouse eggs triggered [Ca^{2+}], oscillations, whereas injection of PLCζ protein failed to do so.

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References


Dong JB, Tang TS & Sun FZ 2000 Xenopus and chicken sperm contain a cytosolic soluble protein factor which can trigger calcium oscillations in mouse eggs. Biochemical and Biophysical Research Communications 268 947–951.


Fissore RA, Gordo AC & Wu H 1998 Activation of development in mammals: is there a role for a sperm cytosolic factor? Theriogenology 49 43–52.


Jellerette T, He CL, Wu H, Parys JB & Fissore RA 2000 Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilization or parthenogenetic activation. Developmental Biology 223 238–250.


Li ST, Huang XY & Sun FZ 2001 Flowering plant sperm contains a cytosolic soluble protein factor which can trigger calcium oscillations in mouse eggs. Biochemical and Biophysical Research Communications 287 56–59.


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

Nishio H, Tokuda M, Itano T, Matsui H, Takeuchi Y & Hatase O 1995 pp60
t expression in rat spermogenesis. Biochemical and Biophysical Research Communications 206 502–510.


Rice A, Parrington J, Jones KT & Swann K 2000 Mammalian sperm contain a Ca2+-sensitive phospholipase C activity that can generate Ins(1,4,5)P3 from PIP2, associated with intracellular organelles. Developmental Biology 228 125–135.

Runft LL & Jaffe LA 2000 Sperm extract injection into ascidian eggs signals Ca2+ release by the same pathway as fertilization. Development 127 3227–3236.


Talmor A, kinsey WH & Shalgi R 1998 Expression and immunolocalization of p59
 tyrosine kinase in rat eggs. Developmental Biology 194 38–46.


