Focus on Fertilization

Are Src family kinases involved in cell cycle resumption in rat eggs?

A Talmor-Cohen, R Tomashov-Matar, E Eliyahu, R Shapiro and R Shalgi

Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel Aviv University, Ramat-Aviv 69978, Tel-Aviv, Israel

Correspondence should be addressed to R Shalgi; Email: shalgir@post.tau.ac.il

Abstract

The earliest visible indications for the transition to embryos in mammalian eggs, known as egg activation, are cortical granules exocytosis (CGE) and resumption of meiosis (RM); these events are triggered by the fertilizing spermatozoon through a series of Ca^{2+} transients. The pathways, within the egg, leading to the intracellular Ca^{2+} release and to the downstream cellular events, are currently under intensive investigation. The involvement of Src family kinases (SFKs) in Ca^{2+} release at fertilization is well supported in marine invertebrate eggs but not in mammalian eggs. In a previous study we have shown the expression and localization of Fyn, the first SFK member demonstrated in the mammalian egg. The purpose of the current study was to identify other common SFKs and resolve their function during activation of mammalian eggs. All three kinases examined: Fyn, c-Src and c-Yes are distributed throughout the egg cytoplasm. However, Fyn and c-Yes tend to concentrate at the egg cortex, though only Fyn is localized to the spindle as well. The different localizations of the various SFKs imply the possibility of their different functions within the egg. To examine whether SFKs participate in the signal transduction pathways during egg activation, we employed selective inhibitors of the SFKs activity ((PP2 and SU6656). The results demonstrate that RM, which is triggered by Ca^{2+} elevation, is an SFK-dependent process, while CGE, triggered by either Ca^{2+} elevation or protein kinase C (PKC), is not. The possible involvement of SFKs in the signal transduction pathways that lead from the sperm–egg fusion site downstream of the Ca^{2+} release remains unclear.

Introduction

Ovulated mammalian eggs remain arrested at the second meiotic metaphase (MII) until fertilization. At fertilization the spermatozoon initiates a sequence of biochemical events, collectively referred to as egg activation. The initial observable change within the activated egg is a transient rise in intracellular Ca^{2+} concentration (\([Ca^{2+}]_i\)) that leads to cortical granule exocytosis (CGE) and to resumption of meiosis (RM). Two main hypotheses, the sperm factor and the receptor hypotheses, have been suggested for explaining the manner by which the spermatozoon activates Ca^{2+} release in the mammalian egg. According to the first hypothesis, the sperm introduces a factor into the egg to trigger Ca^{2+} release, whereas according to the second hypothesis the binding of the spermatozoon to a receptor on the egg's plasma membrane leads, eventually, to Ca^{2+} release. The fact that inositol 1,4,5-trisphosphate (IP_{3}) is required for initiating Ca^{2+} release indicates the activation of enzymes of the phospholipase C (PLC) family. The in vivo cleavage products of phosphatidylinositol (4,5) bisphosphate (PIP_{2}) are IP_{3}, required for Ca^{2+} release from the endoplasmic reticulum, and diacylglycerol (DAG), which activates the enzyme protein kinase C (PKC; Eliyahu & Shalgi 2002). The Ca^{2+} transients actuate the resumption of the cell cycle by decreasing the activity of both the M-phase promoting factor (MPF) and the cytostatic factor (CSF; see the review by Dupont 1998). The Ca^{2+} transients and/or PKC lead to CGE (Eliyahu & Shalgi 2002) by an, as yet, undefined mechanism.

Src family kinases (SFKs) have been suggested as possible inducers of some aspects of egg activation (see reviews by Kinsey 1997, Runft et al. 2002, Sato et al. 2000b). SFKs are the products of nine known genes: c-Src, c-Fgr, c-Yes, Fyn, Lck, Lyn, Hck, Blk and Yrk (Erpel & Courtneidge 1995, Superti-Furga & Courtneidge 1995, Bjorge et al. 2000, Schlessinger 2000). They fall into two classes: those found in a broad range of tissue types, namely c-Src, Fyn, c-Yes and Yrk, and those whose expression is restricted to cells of specific hematopoietic lineages, namely Blk, c-Fgr, Hck, Lck and Lyn. Current
evidence indicates that the Ca\textsuperscript{2+} rise at fertilization in marine invertebrate eggs is initiated by a process that requires the sequential activation of SFK, PLC gamma (PLC\gamma) and IP\textsubscript{3} receptors at the endoplasmic reticulum (Carroll et al. 1999, Giusti et al. 1999a,b, 2000a,b, 2003, Abassi et al. 2000, Kinsey & Shen 2000, Runft & Jaffe 2000, Jaffe et al. 2001, Runft et al. 2002). In vertebrate eggs, the mechanism leading to IP\textsubscript{3} production and to Ca\textsuperscript{2+} rise during fertilization is less established. The SFKs are known to be expressed in vertebrate eggs as: c-Src kinase, c-Yes kinase and a 57 kDa Src-related kinase in Xenopus laevis (Scharl & Barnekow 1984, Steele et al. 1989, Sato et al. 1996); Fyn kinase in Xenopus, zebrafish, rat and mouse (Kinsey 1996, Talmor et al. 1998, Wu & Kinsey 2001, Sette et al. 2002).

There is evidence that SFK is activated within 30s of insemination of zebrafish eggs (Wu & Kinsey 2001). A co-immunoprecipitation study has identified receptor-like protein tyrosine phosphatase-a (rPTPa) as the phosphatase complexed with Fyn in the zebrafish egg, thus raising the possibility that PT alpha is part of the regulatory mechanism responsible for activating Fyn at fertilization (Wu & Kinsey 2002). Other studies have indicated that an Src-related protein tyrosine kinase (PTK) may act upstream of the Ca\textsuperscript{2+} release during fertilization of frog eggs (Sato et al. 1999, 2001, Iwao 2000). Moreover, Src-related PTK-dependent PLC\gamma activity, that acts upstream of the Ca\textsuperscript{2+} rise is required for fertilization of Xenopus eggs (Sato et al. 2000a). The tyrosine kinase inhibitor lavendustin, inhibits Ca\textsuperscript{2+} release at fertilization of frog eggs (Glahn et al. 1999), as do two other SFK-specific inhibitors, the peptide A7 and the pharmacological inhibitor pyrazolopyrimidin (PP1; Sato et al. 1999, 2000a,b). However, since A7 and PP1 inhibit sperm-egg fusion as well, it is still debatable whether they inhibit egg activation or a former sperm-related function.

The purpose of the current study was to identify other common SFKs and resolve their function during activation of mammalian eggs.

Materials and Methods

Animals and gametes

Wistar-derived rats were housed in air-conditioned, light-controlled rooms, in the animal housing facilities of the Sackler Medical School. Food and water were provided ad libitum. Female rats (23–25 days old) were primed with a subcutaneous injection of 10 UI pregnant mares’ serum gonadotropin (PMSG; Syncro-Part, France). An i.p. injection of 10 UI human chorionic gonadotropin (hCG; Sigma) was administered 48–54 h after PMSG.

Oocytes resuming meiosis

Cumulus-enclosed oocytes at the germinal vesicle breakdown (GVBD) or metaphase I (MI) stages were isolated from antral follicles into culture medium (Toyoda Hepes (TH) medium supplemented with 0.1% BSA; Talmor et al. 1998), 4 or 8 h after hCG administration respectively. At all times, the medium temperature was kept at 36 ± 1°C. All manipulations were performed on a warm plate (37°C) placed in a temperature-controlled hood.

MI eggs

MI ovulated eggs were isolated, 14 h after hCG injection, from the oviductal ampullae into TH medium and their cumulus cells were removed by hyaluronidase (Sigma). The eggs were parthenogenetically activated by either calcium ionophore (ionomycin 407950, Calbiochem, San Diego, CA, USA) or phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA; Sigma). Stock solutions of 10 mM ionomycin and of 1 µg/ml TPA in dimethyl sulfoxide (DMSO) were prepared and kept at 4°C or at −20°C respectively. MI eggs were incubated for 3 min in the presence of 2 µM ionomycin followed by an additional 0, 2, 7, or 17 min incubation in fresh medium lacking the activator. Other MI eggs were incubated for 5 min in the presence of 30 or 50 ng/ml TPA followed by an additional 0, 5, or 15 min incubation in fresh medium lacking the activator.

Fertilized eggs

Fertilized eggs at different stages of development were isolated either from the oviductal ampullae of superovulated immature females at various time points after mating, or from the oviducts of mated rats, 40 h after hCG injection (Kaplan-Kraicer et al. 1995). The developmental stages of zygotes were classified as: sperm binding (SB), fertilization cone (FC), second polar body (PBII) and pronuclear stage (PN) (at 0–0.25, 0.25–1, 1–3 or 6–8 h after sperm attachment respectively (Eliyahu & Shalgi 2002)), followed by mitosis and two-cell embryos.

Antibodies and drugs

Anti-Yes (sc-14), anti-Src (sc-19) and anti-Fyn (sc-16) rabbit polyclonal antibodies (pAb; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Src mouse monoclonal antibody (mAb; kindly contributed by Dr Joan S Brugge, Harvard Medical School, Boston, MA, USA), goat anti-rabbit immunoglobulin G (IgG) peroxidase (Sigma), donkey anti-rabbit IgG-Cy and donkey anti-mouse IgG-Cy (Jackson Immunoresearch Laboratories, West Grove, PA, USA), anti-B-tubulin mAb (Sigma).

Stock solutions of 13 mM 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), PP3 and of 10 mM SU6656 (529573, 529574 and 572635 respectively; Calbiochem-Novabiochem, CA, USA) were prepared in DMSO and diluted before use in TH medium to final concentrations of either 10–100 µM (PP2, PP3) or 1–5 µM (SU6656).
**SDS-PAGE and Western blot analysis of proteins**

Samples of 100–300 eggs were collected in 1–3 μl TH medium, mixed with 10 μl of NP-40 lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 1 mM Na3VO4, 5 mM NaF and 10 μg/ml aprotinin (Sigma)) and stored at −70°C until use. Upon thawing, lysates of eggs were boiled for 5 min and subjected to separation by 10% SDS-PAGE under reducing conditions. Proteins were transferred onto a Millipore membrane (Millipore Co., Bedford, MA, USA) using a wet blotting apparatus (Hoeffer, San Francisco, CA, USA). Approximate molecular masses were determined by comparison with the migration of prestained protein standards (Amersham). Western blot analysis was performed with either anti-Yes IgG or anti-Src IgG at a dilution of 1:250 or anti-Fyn IgG at a dilution of 1:1000 in blocking buffer (100 mM NaCl, 10 mM Tris, 0.1% Tween 20 and 5% non-fat dry milk, pH 7.4). Bound antibodies were recognized by horseradish peroxidase conjugated to anti-rabbit antibodies (1:1000) and then washed with 0.005% NP-40 in DPBS/FCS solution and washed again. The plasma membranes of the eggs were permeabilized by 0.05% NP-40 in DPBS/FCS and then washed with 0.005% NP-40 in DPBS/FCS (blocking solution). All further manipulations were performed in blocking solution.

Eggs were incubated in the presence of specific primary antibodies for 1–2 h, washed in blocking solution and reincubated for 30 min in the presence of secondary, fluorescent antibodies. SFKs were detected within the eggs by specific antibodies: anti-c-Src mAb and anti-c-Yes pAb (1:10); anti-Fyn pAb (1:100). Microtubules were detected by anti-β-tubulin mAb (1:200). Primary antibodies were detected by fluorescent-labeled Cy secondary antibodies: donkey anti-rabbit IgG-Cy (1:1000) for pAb and donkey anti-mouse IgG-Cy (1:1000) for mAb. The chromosomal stage was detected by a DNA-specific fluorochrome (Hoechst 33342, Sigma).

**Immunofluorescent staining for SFK localization**

Eggs were fixed for 10 min at room temperature in 3% paraformaldehyde, supplemented with 0.01% glutaraldehyde, and then washed with Dulbecco’s phosphate-buffered saline (DPBS/FCS solution (3% FCS in DPBS; Biological Industries, Beit-Haemek, Israel). After fixation, the zona pellucidae (ZP) were removed by a brief exposure to 0.25% pronase (Sigma) prepared in DPBS/FCS solution and washed again. The plasma membranes of the eggs were permeabilized by 0.05% NP-40 in DPBS/FCS and then washed with 0.005% NP-40 in DPBS/FCS (blocking solution). All further manipulations were performed in blocking solution.

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**Detection of c-Src and c-Yes kinases**

The expression of c-Src and c-Yes kinases in rat eggs was investigated using Western blot analysis (Fig. 1). We detected a c-Src band at 60 kDa (Fig. 1A), which is in keeping with the predicted relative molecular mass, Mr, of the protein (Steele 1985). A minor band of 200 kDa was often detected by this method as well. c-Yes appeared as a single band with an apparent Mr of 62 kDa (Fig. 1A) which is consistent with the reported Mr expected for c-Yes kinase (Zhao et al. 1990). Both SFKs were detected in Jurkat cells that served as positive control (Fig. 1B; c-Src 60 kDa, c-Yes 62 kDa). Our observations demonstrated, for the first time, the expression of c-Src and c-Yes in mammalian eggs. Fyn had already been demonstrated to be expressed in mammalian eggs at the predicted Mr of 59 kDa (Talmor et al. 1998).

**Localization of SFKs shortly after sperm penetration**

The intracellular distribution of the SFKs in unfertilized and fertilized eggs could imply their possible functions. In this part of the study we followed the distribution of the SFKs before, and shortly after, sperm penetration. Localization of the kinases was visualized by immunohistochemistry using confocal microscopy.

**Inhibition of SFK activation**

To determine the effect of selective inhibitors of SFKs (PP2 and SU6656) on parthenogenetic activation, MI eggs were incubated for 30 min in the presence of 10–100 μM PP2 or 1–5 μM SU6656, then subjected to parthenogenetic activation by either ionomycin or TPA as described previously, followed by an additional 0–15 min incubation in the presence of the inhibitors only. Eggs were fixed and monitored for morphological criteria that indicate progression through the egg activation process: RM and CGE (see previous subsection). Eggs incubated in the presence of either PP3 (a negative control for PP2), DMSO or medium devoid of PP2, served as controls.

**Statistical analysis**

Data are expressed as the percentage of eggs which resumed meiosis or demonstrated CGE, per total number of treated eggs. The significance of differences between treated and control eggs was determined by one-way ANOVA; P < 0.01 was considered significant.

**Results**

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were further incubated in the absence of ionomycin (Fig. 3). Exposing the eggs to ionomycin for 1–10 min induced RM, although the 10 min exposure presented different dynamics of RM. A 1 min exposure to the activator plus an additional 9 min in fresh culture medium caused a transition of 76.5 ± 13.7% (mean±s.e.) of the eggs to the anaphase stage, as compared with 19.3 ± 5.6% (mean±s.e.) of the eggs that were exposed to the activator for 10 min (Fig. 3A). The results infer that a short exposure to ionomycin is advantageous. A 1 min exposure to ionomycin is too short for manipulation of a large number of eggs. We chose to expose the eggs to ionomycin for 3 min, as the Ca²⁺ transient within the eggs is already detectable after a 1–2 min exposure to 2 µM ionomycin (Raz & Shalgi 1998). Under these conditions (Fig. 3B), 91% of the eggs reached the anaphase stage within 15 min of culture (3 min exposure to ionomycin plus an additional 12 min incubation in fresh medium). After 30 min of culture (3 min exposure to ionomycin plus an additional 27 min), 41% were still at anaphase whereas 51% had already progressed to telophase and the rest extruded the PBII. After 45 min (3 min exposure to ionomycin plus an additional 42 min), the majority of eggs had already extruded PBII.

A 3 min exposure to ionomycin, regardless of additional incubation in fresh medium, caused no change in the subcellular distribution of the three SFKs (data not shown), similar to in vivo fertilization.

To examine the possible role of SFKs in CGE and/or in RM we used SFK inhibitors (PP2 and SU6656). Exposing the eggs to 100 µM PP2 caused a dose-dependent inhibition of ionomycin-induced cell cycle resumption in 75% of the eggs, but had no effect on CGE (Fig. 4). PP3 had no effect on ionomycin-induced RM or CGE. In order to verify the effect of PP2 on egg activation we used another specific SFK inhibitor, SU6656 (Blake et al. 2000, Bowmann et al. 2001). Exposing the eggs to 5 µM SU6656 significantly inhibited ionomycin-induced cell cycle resumption, in a dose-dependent manner with no effect on CGE, compared with controls (Fig. 5).

As previously demonstrated, CGE can be triggered by either [Ca²⁺], rise or PKC activation (Eliyahu & Shalgi 2002). Thus, we examined the possible involvement of SFKs in the signaling pathways leading to the CGE via PKC. Eggs were parthenogenetically activated by the PKC activator, TPA. TPA (50 ng/ml) is known to have no effect on RM but does induce CGE (Raz et al. 1998). In two separate experiments, eggs were preincubated for 30 min in TH medium or in TH medium containing either PP2 (100 µM; n = 58) or PP3 (100 µM; n = 71), activated for 5 min by TPA in the presence of PP2 or PP3 respectively; the eggs were then incubated for an additional 15 min in TH medium devoid of TPA but in the presence of PP2 or PP3 (respectively). The presence of the SFK inhibitor (PP2) did not affect the degree of CGE (91.3%), as compared with PP3 (87.3%) or TPA alone (89.4%).
Figure 2 Immunolocalization of c-Src, c-Yes and Fyn kinases. (I) Unfertilized MII eggs; fertilized eggs at the sperm binding stage (0–0.25 h after sperm attachment); fertilized eggs at the fertilization cone stage (0.25–1 h after sperm attachment). (II) Control, unfertilized MII eggs incubated with secondary antibody only. (A–C) Light microscopy of eggs and DNA staining. (A’–C’) Confocal microscopy of Src family members. Panels show anti-Src (A'), anti-Yes (B') and anti-Fyn (C') antibodies (1:10). Inset in C' is an egg photographed at the spindle plan. Primary antibodies were detected by fluorescent-labeled Cy secondary antibodies (donkey anti-rabbit IgG for anti-Yes (1:250) and for anti-Fyn (1:500) antibodies; donkey anti-mouse IgG for anti-Src antibody (1:100)). At least three independent experiments were performed for each kinase. Each image was taken at the equatorial plane of the egg. DNA was labeled by Hoechst (2 μg/ml). Scale bar, 10 μm. Localization of Fyn in MII eggs and in eggs at the fertilization cone stage had already been demonstrated in our previous work (Talmor et al. 1998).
The aim of our study was to examine the expression and localization of the SFKs in the mammalian egg, and their potential role in egg activation. SFKs are cytoplasmic enzymes capable of associating with the plasma membrane when myristoylated (Cooper 1990). The localization of SFKs to the perinuclear membrane, to the endosomes and possibly even to the nucleus, suggests that they are involved in signal transduction events that do not necessarily involve only the plasma membrane. However, only limited information exists regarding the function of the SFKs at these intracellular locations, and in particular, regarding the specific substrates with which they may interact. Association of SFKs with the plasma membrane had been described in platelets (Stenberg et al. 1997) but not in Jurkat T cell lymphoma cells (Ley et al. 1994). Other studies had implicated a role for PTKs in microtubule (MT) function (Wright & Schatten 1995). Src kinase has been localized to the spindle poles in mitotic 3T3 cells (David-Pfeuty & Nouvian-Dooghe 1990, Kaplan et al. 1992), and Fyn was found to be associated with the spindle MTs in Jurkat T cells (Ley et al. 1994).

Our study has demonstrated that c-Yes and c-Src are distributed throughout the egg cytoplasm, but c-Yes, like Fyn (Talmor et al. 1998), tends to concentrate at the egg cortex as well. The egg cortex is known to be rich in cortical structures such as actin cytoskeleton and cortical vesicles. Of...
the three SFKs studied, Fyn kinase is unique in that it is also localized to the meiotic and mitotic spindles (Talmor et al. 1998). Localization of c-Src, c-Yes and Fyn to different compartments within the egg indicates that these proteins may have different functions within the egg. No change in the subcellular distribution of the three kinases has been observed throughout the stages of the fertilization process, or after parthenogenetic activation. It is possible that the intracellular distribution of c-Src, c-Yes and Fyn imply their association with the cytoskeleton. SFKs have been shown to be associated with a wide range of cytoskeletal components and/or to phosphorylate them (Thomas & Brugge 1997). Microfilaments play a role in many dynamic events that take place during mammalian egg maturation and fertilization, such as sperm incorporation, CGE, PB emission, etc. These processes, among others, are accompanied by reorganization of the actin and tubulin cytoskeleton (Di-maggio et al. 1997). We may speculate that SFKs are involved in signaling events that implicate cytoskeletal reorganization via association with activated cytoskeletal proteins. The reorganization of the cytoskeleton could play a physiological role during fertilization. Although our findings may hint towards involvement of SFKs in reorganization of the cytoskeleton, further studies are needed to determine whether and how this might occur.

Fertilization is known to stimulate the SFKs in eggs of some vertebrates such as Xenopus and zebrafish (Sato et al. 1996, 1999, Wu & Kinsey 2001, 2002). Furthermore, SFKs inhibitors interrupt sperm–egg fusion in frogs (Sato et al. 1999, 2000a,b). In the mouse, several tyrosine kinase inhibitors delay Ca2+ release at fertilization and reduce the number of Ca2+ oscillations (Dupont et al. 1996). A recent study has demonstrated the existence of a sperm protein capable of inducing activation of a Src-like kinase in mouse eggs (Sette et al. 2002). However, Kurokawa et al. (2004, this issue) suggest that activation of an SFK is neither necessary, nor sufficient for triggering fertilization-induced [Ca2+]i oscillations.

To examine whether SFKs participate in the signal transduction pathways during egg activation, we have employed selective inhibitors of the SFKs activity. PP1 is a potent, selective inhibitor (Hanke et al. 1996) that has been used to study a number of Src-type mediated signaling processes in a variety of cells (Briddon et al. 1999, Mocsai et al. 1999). SU6656, a potent SFK specific inhibitor (Blake et al. 2000), inhibits c-Src (concentration giving 50% of maximum inhibition, IC50 = 280 nM) as well as the closely related kinases, Fyn (IC50 = 170 nM) and c-Yes (IC50 = 20 nM). Both inhibitors bind the kinase at its ATP binding site and insert a methylphenyl group into an adjacent hydrophobic pocket, thus inhibiting the kinase activity (Schindler et al. 1999, Bowman et al. 2001). Incubation of sea urchin eggs in 10 µM PP1 had resulted in a significant delay in Ca2+ release, implying the importance of SFKs for the initiation of Ca2+ release (Abassi et al. 2000). Recent evidence, from studies engaging domain-specific fusion proteins and constitutively active Src kinase mutants, have indicated a requirement for SFKs, such as c-Src or Fyn, for initiating Ca2+ release at fertilization of marine invertebrate eggs (Giusti et al. 1999b, 2000b, Kinsey & Shen 2000). PP2, similar to PP1, can selectively inhibit the Src-family tyrosine kinase activity (Hanke et al. 1996). An Src-like kinase inhibitor, PP2, has been demonstrated to suppress tr-kit-induced resumption of the cell cycle in mouse eggs (Sette et al. 2002). We found that PP2 and SU6656 inhibit RM, in a dose-dependent manner, in ionomycin-activated MII eggs, implying the dependency of RM, at least in part, on the activity of SFKs (Fig. 6).

Fertilization in mammals triggers a train of Ca2+ spikes which is responsible for CGE and for the decrease in activity of both CSF and MPF, thus leading to the resumption of the cell cycle (Carroll 2001). Although there is evidence for a role of SFK in initiating a Ca2+ rise (through PLCγ) at fertilization of the eggs of marine invertebrates, their role in mammalian eggs remains doubtful (Mehlman et al. 1998). PLC zeta (PLCζ), a suspected sperm factor that has recently been discovered, initiates Ca2+ oscillations during mammalian egg fertilization (Cox et al. 2002, Saunders et al. 2002, Swann et al. 2004 (this issue)). The fact that PLCζ lacks Src-homology domains, implies that any involvement of SFKs in mammalian egg activation is downstream of Ca2+ release. We have demonstrated that SFKs play a role downstream of Ca2+ elevation leading to resumption of the cell cycle, but are not required

![Figure 6](image-url)
for the completion of CGE. It has been shown that CGE can be triggered, independently, either by Ca\(^{2+}\) release or by PKC activation (Eliyahu & Shalgi 2002). In the current work, CGE could be induced either by TPA, which activates PKC (Nishizuka 1986), or by ionomycin, even in the presence of a SFKs inhibitors. These findings imply that under the conditions described, the CGE is not dependent on SFKs when it is triggered either by Ca\(^{2+}\) elevation, or by PKC (Fig. 6). The possibility that SFKs play a role, at some step upstream of the Ca\(^{2+}\) transient, during egg activation by a spermatozoon, remains plausible (Fig. 6).

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