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REVIEW

Egg Activation at Fertilization: Where It All Begins

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A centrally important factor in initiating egg activation at fertilization is a rise in free Ca^{2+} in the egg cytosol. In echinoderm, ascidian, and vertebrate eggs, the Ca^{2+} rise occurs as a result of inositol trisphosphate-mediated release of Ca^{2+} from the endoplasmic reticulum. The release of Ca^{2+} at fertilization in echinoderm and ascidian eggs requires SH2 domain-mediated activation of a Src family kinase (SFK) and phospholipase C (PLC)γ. Though some evidence indicates that a SFK and PLC may also function at fertilization in vertebrate eggs, SH2 domain-mediated activation of PLCγ appears not to be required. Much work has focused on identifying factors from sperm that initiate egg activation at fertilization, either as a result of sperm–egg contact or sperm–egg fusion. Current evidence from studies of ascidian and mammalian fertilization favors a fusion-mediated mechanism; this is supported by experiments indicating that injection of sperm extracts into eggs causes Ca^{2+} release by the same pathway as fertilization.

Key Words: **fertilization; calcium; egg activation.**

There is perhaps no phenomenon in the field of biology that touches so many fundamental questions as the union of the germ cells in the act of fertilization; in this supreme event all the strands of the webs of two lives are gathered in one knot, from which they diverge again and are re-woven in a new individual life-history.

–Lillie, *Problems of Fertilization*, 1919

INTRODUCTION

At fertilization, the sperm activates the egg to reenter the cell cycle and begin embryonic development. The cell cycle stage at which the egg is paused until it is fertilized varies among species; for example, first meiotic prophase in the clam *Spisula* and in the marine worm *Urechis*, first meiotic metaphase in ascidians, second meiotic metaphase in almost all vertebrates, and G_1 of the first mitosis in sea urchins (Stricker, 1999). In plants, the cell cycle pause and subsequent resumption at fertilization always occur after the egg has completed meiosis (Mogensen *et al.*, 1995; Friedman, 1999). An analogous activation of the cell cycle also occurs during mating in the yeast *Saccharomyces cerevisiae*; the haploid cells are paused at G_1, and mitosis resumes after the two cells fuse (Kim *et al.*, 2000).

A universal feature of the egg-activation process is an increase in cytosolic free Ca^{2+} within the egg. This was first demonstrated 25 years ago in fish and sea urchin eggs (Ridgway *et al.*, 1977; Steinhardt *et al.*, 1977), and has subsequently been observed at fertilization in eggs of all other animal and plant species studied (Jaffe, 1985; Stricker, 1999; Antoine *et al.*, 2000; Samuel *et al.*, 2001). Ca^{2+} levels in the egg increase from ~0.1 to 1 μM, and in almost all species, this occurs as a wave or waves that cross the egg (Gilkey *et al.*, 1978; Jaffe, 1985; Stricker, 1999).

The Ca^{2+} increase at fertilization is necessary and sufficient for restarting cell cycle events in eggs of ascidians (Sensui and Morisawa, 1996; Russo *et al.*, 1996), vertebrates (Steinhardt *et al.*, 1974; Kline, 1988; Kline and Kline, 1992; Yamamoto *et al.*, 1999), and echinoderms (Steinhardt and Epel, 1974; Nomura and Nemoto, 1998; Carroll *et al.*, 1999,

Abbreviations used: ADAM, a disintegrin and metalloprotease; ER, endoplasmic reticulum; ICSI, intracytoplasmic sperm injection; IP_3, inositol 1,4,5-trisphosphate; NO, nitric oxide; PDGF, platelet derived growth factor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; SFK, Src family kinase; SH2, Src homology 2.

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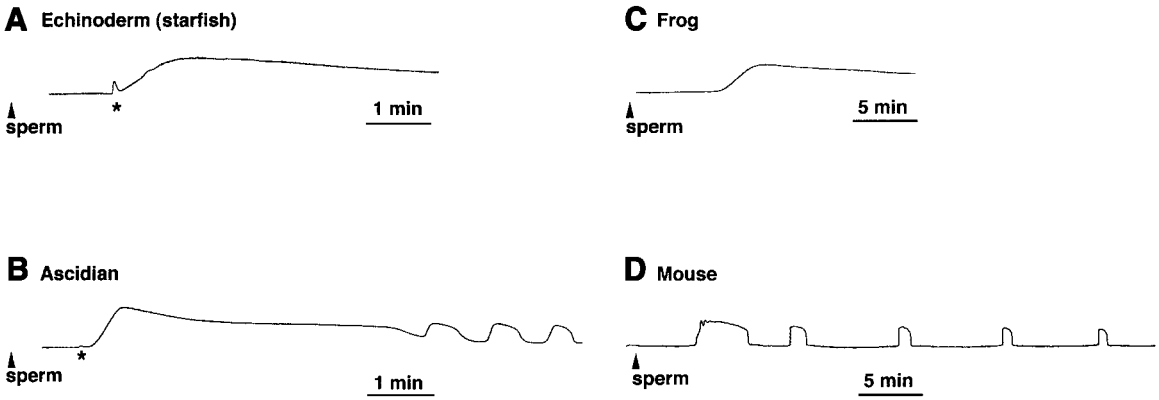

FIG. 1. Ca^{2+} rises at fertilization in echinoderm, ascidian, frog, and mouse eggs. Eggs were injected with calcium green dextran and then inseminated (arrowheads). Records show calcium green fluorescence as a function of time. (A) Echinoderm (the starfish *Asterina miniata*). The Ca^{2+} rise at fertilization consists of two phases. The first rise, marked by an asterisk, represents the entry of extracellular Ca^{2+} and occurs as a result of the opening of voltage-sensitive Ca^{2+} channels in response to depolarization of the egg plasma membrane. This entry of extracellular Ca^{2+} further depolarizes the egg membrane, which is significant for the electrical block to polyspermy (Jaffe, 1976; Chambers and de Armendi, 1979); however, this small Ca^{2+} rise does not stimulate further events of egg activation (Jaffe, 1976; Mohri *et al.*, 1995). The action potential occurs at the time of sperm–egg fusion (McCulloh and Chambers, 1992), and is followed about 12 s later by a large Ca^{2+} rise that is due to a propagating wave of Ca^{2+} release from the ER. Following the Ca^{2+} wave, Ca^{2+} levels remain high for several minutes before decreasing (from Carroll *et al.*, 1997). (B) Ascidian (*Ciona intestinalis*). The Ca^{2+} elevation at fertilization consists of an action potential (marked by an asterisk) and, about 15 s later, release of Ca^{2+} from the ER which crosses the egg in the form of a wave. After the initial Ca^{2+} wave, Ca^{2+} levels remain high for several minutes and then begin to oscillate. These Ca^{2+} oscillations (which also occur as waves) last for ~10 min, stop while the first polar body is extruded, resume for another 10–20 min, and then terminate before extrusion of the second polar body. In this figure, only three Ca^{2+} oscillations are shown following the initial Ca^{2+} wave (from Runft and Jaffe, 2000). (C) Frog (*Xenopus laevis*). The Ca^{2+} rise at fertilization consists of a single elevation, which represents the release of Ca^{2+} from the ER in the form of a wave. Following the Ca^{2+} wave, Ca^{2+} levels remain high for several minutes before decreasing (from Runft *et al.*, 1999). (D) Mouse (*Mus musculus*). The initial Ca^{2+} rise at fertilization consists of a wave of Ca^{2+} release from the ER, and is followed by Ca^{2+} oscillations that also occur as waves. These oscillations continue for several hours and terminate about the time the pronuclei begin to form. In this figure, only four Ca^{2+} oscillations are shown following the initial Ca^{2+} wave (L.M.M., unpublished observations).

2000). The Ca^{2+} increase causes ascidian and vertebrate eggs to enter anaphase and complete meiosis, and causes sea urchin eggs, which have already completed meiosis, to undergo DNA synthesis (see Jaffe *et al.*, 2001, for a discussion of the more complex case of starfish). These conclusions have been established by artificially elevating Ca^{2+} in eggs and, conversely, by inseminating eggs that have been injected with Ca^{2+} buffers to prevent the Ca^{2+} increase. The pathway connecting the Ca^{2+} increase to the occurrence of anaphase is only partly understood, but appears to involve a calmodulin-dependent protein kinase (Lorca *et al.*, 1993; Johnson *et al.*, 1998), which somehow leads to the proteolysis of securin and cyclin, which results in the proteolysis of the cohesin that holds the chromosomes together (Kawahara and Yokosawa, 1994; Aizawa *et al.*, 1996; Peter *et al.*, 2000; Stemmann *et al.*, 2001). The pathway connecting the Ca^{2+} increase to DNA synthesis in echinoderm eggs appears to involve the inactivation of MAP kinase (Tachibana *et al.*, 1997; Carroll *et al.*, 1999, 2000; see Jaffe *et al.*, 2001, for further discussion), but how Ca^{2+} causes this inactivation and how MAP kinase inactivation leads to DNA synthesis have not been determined.

Another incompletely understood issue is how far the embryonic cell cycle can proceed in response to artificially elevating Ca^{2+}. Rabbit eggs treated with a series of electric shocks to cause Ca^{2+} elevations proceed into mitosis and about a third of the way through embryonic development (Ozil and Huneau, 2001). However, the electrical stimuli could have consequences in addition to elevating Ca^{2+}. Evidence from studies of starfish and frogs has supported the conclusion that the continuation of embryonic cell cycles, beyond the initial events triggered by Ca^{2+}, requires the introduction of a centrosome, as a consequence of fertilization or experimental manipulation (Nomura and Nemoto, 1998; Tournier and Bornens, 2001).

In addition to reinitiating the cell cycle, the Ca^{2+} increase at fertilization causes other egg activation events; in particular, in many species, it causes cortical granule exocytosis, which establishes a mechanical block to polyspermy (Zucker and Steinhardt, 1978; Kline, 1988; Kline and Kline, 1992). However, some other events of fertilization are not caused by Ca^{2+}; for example, in sea urchin eggs, sperm entry and pronuclear formation (Carroll *et al.*, 1999), as well as the initial membrane conductance increase that contributes to polyspermy prevention (McCulloh *et al.*, 2000), occur independently of the Ca^{2+} rise.

TABLE 1
Signaling Components that Mediate Ca^{2+} Release in Eggs at Fertilization

	Echinoderms	Ascidians	Amphibians	Mammals
Inositol trisphosphate[a]	+	+	+	+
Phospholipase Cγ[b]	+	+	?	?
Tyrosine kinase[c]	+	+	+	?

[a] Echinoderms: Whitaker and Irvine, 1984; Ciapa and Whitaker, 1986; Chiba *et al.*, 1990; Mohri *et al.*, 1995; Carroll *et al.*, 1997, 1999; Lee and Shen, 1998; Shearer *et al.*, 1999; Iwasaki *et al.*, 2001. Ascidians: Toratani and Yokosawa, 1995; Albrieux *et al.*, 1997; Runft *et al.*, 2000. Amphibians: Busa *et al.*, 1985; Stith *et al.*, 1993; Nuccitelli *et al.*, 1993; Snow *et al.*, 1996; Runft and Jaffe, 1999; Yamamoto *et al.*, 2001. Mammals: Miyazaki, 1988; Miyazaki *et al.*, 1992, 1993; Kline and Kline, 1994.

[b] Echinoderms: Carroll *et al.*, 1997, 1999; Lee and Shen, 1998; Shearer *et al.*, 1999; Rongish *et al.*, 1999. Ascidians: Runft and Jaffe, 2000. Amphibians: Runft *et al.*, 1999; Sato *et al.*, 2000. Mammals: Dupont *et al.*, 1996; Mehlmann *et al.*, 1998, 2001; Jones *et al.*, 2000.

[c] Echinoderms: Ciapa and Epel, 1991; Shen *et al.*, 1999; Giusti *et al.*, 1999a,b, 2000; Abassi *et al.*, 2000; Kinsey and Shen, 2000. Ascidians: Ueki and Yokosawa, 1997; Runft and Jaffe, 2000. Amphibians: Glahn *et al.*, 1999; K. Sato *et al.*, 1996, 1999, 2000. Mammals: Dupont *et al.*, 1996; Talmor *et al.*, 1998.

In echinoderm, ascidian, and vertebrate eggs, the Ca^{2+} increase at fertilization (Fig. 1) is caused primarily by inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from the endoplasmic reticulum (ER) (see Table 1). Other small molecules such as cyclic GMP (cGMP) (Whalley *et al.*, 1992), cyclic ADP-ribose (cADP ribose) (Shen and Buck, 1993; Galione *et al.*, 1993; Lee *et al.*, 1993), nicotinic acid adenine dinucleotide phosphate (Perez-Terzic *et al.*, 1995; Galione *et al.*, 2000), and nitric oxide (Willmott *et al.*, 1996) can also cause Ca^{2+} release in sea urchin eggs, but current evidence (see below) indicates that IP_3 is the primary initiator of intracellular Ca^{2+} release at fertilization (see also Jaffe *et al.*, 2001). It is possible that these other small molecules act in concert with IP_3 to regulate the Ca^{2+} rise after it has been initiated.

This paper reviews studies of the signaling pathways that lead to IP_3 production and Ca^{2+} release at fertilization and how these pathways are initiated by sperm in eggs of echinoderms, ascidians, and vertebrates. Since IP_3 is generated by enzymes of the phospholipase C (PLC) family, which cleave the lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate IP_3 and diacylglycerol (Rhee, 2001), recent studies have examined whether PLCs and PLC regulatory pathways function in mediating egg activation during fertilization. Hypotheses as to how Ca^{2+} release at fertilization is initiated include sperm contact with a protein in the egg plasma membrane (Fig. 2A), or introduction of a factor(s) into the egg as a consequence of sperm–egg fusion. One possibility is that this factor is passed from the sperm membrane to the egg membrane (e.g., ion channels, membrane tyrosine kinases, or other membrane proteins or lipids) (Fig. 2B). Studies of mating in yeast provide an example of such a mechanism; as a consequence of fusion of the two yeast cells, a G-protein-linked receptor (Ste3p) from one cell membrane interacts with a transmembrane protein (Asg7p) from the other cell membrane, and this membrane protein interaction reinitiates the mitotic cycle in the diploid zygote, although it is unknown whether the process involves a Ca^{2+} rise (Roth *et al.*, 2000). Possibly, an analogous interaction of membrane proteins from the sperm and egg occurs at fertilization. Alternatively, the egg-activating factor could be passed from the sperm cytosol to the egg cytosol (e.g., Ca^{2+}, PLC enzymes, or cytoplasmic kinases) (Figs. 2C and 2D). Given the diversity of cell cycle control points and other events of egg activation among different species, how the initial gamete interaction starts the activation cascade may also vary among species.

ECHINODERMS

Ca^{2+} Release Requires Activation of PLCγ and a Src Family Kinase

Considerable progress has been made in understanding the role played by PLCs in mediating the Ca^{2+} rise at fertilization in echinoderm eggs. In sea urchin eggs, the PLC inhibitor, U73122, can completely block the Ca^{2+} rise at fertilization without preventing sperm entry (Lee and Shen, 1998). This finding supports the hypothesis that Ca^{2+} release requires PLC activity, although this inhibitor can also have nonspecific effects, such as inhibiting the ER Ca^{2+} pump (De Moel *et al.*, 1995), inhibiting phospholipase D (Bosch *et al.*, 1998), and impairing the ability of IP_3 to mobilize Ca^{2+} (Hellberg *et al.*, 1996).

Recent work has focused on identifying the particular PLC(s) that may be required for Ca^{2+} release at fertilization. The PLC family of enzymes includes four subtypes: β, γ, δ, and ϵ. The mechanisms regulating PLCβ and -γ are better understood than those regulating PLCδ and -ϵ (reviewed by Rhee, 2001). PLCβ is activated by heterotrimeric G-proteins (α subunits of the G_q family or $\beta\gamma$ subunits of any G-protein family), and PLCγ can be activated by tyrosine phosphorylation. Translocation of PLCγ to the plasma membrane, where the PIP_2 substrate is located, can also contribute to PLCγ activation.

A functional G-protein–PLCβ-mediated Ca^{2+} release pathway is present in echinoderm eggs (Shilling *et al.*, 1990,

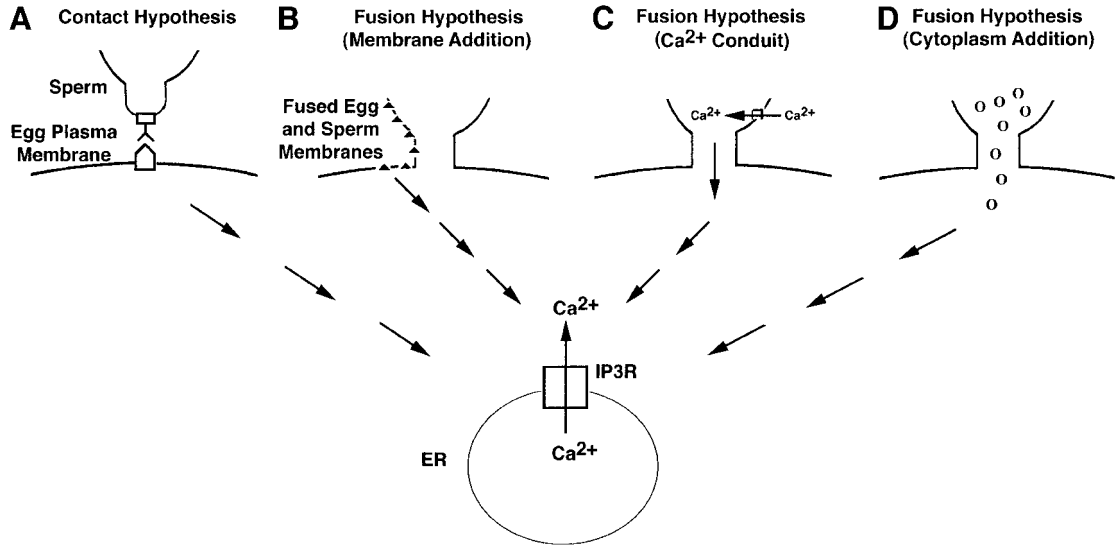

FIG. 2. Hypotheses for how the sperm might initiate Ca^{2+} release from the egg's ER at fertilization. (A) Contact hypothesis. A protein on the sperm surface contacts and activates a protein in the egg plasma membrane. (B) Membrane addition during fusion. An activating factor in the sperm membrane is incorporated into the egg membrane. (C) The sperm acts as a Ca^{2+} conduit during fusion. Extracellular Ca^{2+} enters the sperm via Ca^{2+} channels located on the sperm plasma membrane and diffuses into the egg. (D) Cytoplasm addition during fusion. An activating factor in the sperm cytoplasm diffuses into the egg cytoplasm. The arrows represent the multiple steps that could occur between the initial interaction of egg and sperm and the release of Ca^{2+} from the ER.

1994), and early studies in which sea urchin eggs were injected with GDP-β-S (a general inhibitor of GTP-dependent events) pointed to a role for this G-protein–PLCβ pathway in stimulating Ca^{2+} release at fertilization (Turner *et al.*, 1986). Subsequent work, however, has indicated that GDP-β-S is not sufficiently specific to be used as a meaningful inhibitor of G-proteins (Jaffe, 1990; Crossley *et al.*, 1991). Current evidence supports an alternative pathway mediated by PLCγ that leads to the Ca^{2+} rise at fertilization in echinoderm eggs (Carroll *et al.*, 1997, 1999; Shearer *et al.*, 1999; Ciapa and Chiri, 2000; Jaffe *et al.*, 2001).

PLCγ frequently interacts with other proteins (e.g., tyrosine kinases or linker proteins) via a specific interaction of its two tandem Src homology 2 (SH2) domains with a phosphotyrosine-containing sequence (Rhee, 2001). SH2 domains are sequences of ~100 amino acids that are found in many signaling proteins (Ottinger *et al.*, 1998); the SH2 domains of different proteins each recognize a specific phosphotyrosine containing sequence (Songyang and Cantley, 1995). Introducing recombinant PLCγ SH2 domains into cells can inhibit Ca^{2+} release, presumably by specifically blocking association of the full-length, endogenous PLCγ with an interacting protein required for activation of PLCγ (Roche *et al.*, 1996; Bae *et al.*, 1998; Mehlmann *et al.*, 1998; Runft *et al.*, 1999). Likewise, injecting the two tandem SH2 domains from mammalian PLCγ into echinoderm eggs can completely inhibit the fertilization-induced Ca^{2+} rise (Carroll *et al.*, 1997, 1999; Shearer *et al.*, 1999). Control SH2 domains from other proteins, or a point-mutated form of the PLCγ SH2 domains that has a reduced affinity for phosphorylated tyrosines, have no effect on Ca^{2+} release at fertilization (Carroll *et al.*, 1997, 1999; Shearer *et al.*, 1999; see Jaffe *et al.*, 2001). The PLCγ SH2 domains do not inhibit sperm entry, have no effect on the ability of IP_3 to stimulate Ca^{2+} release (Carroll *et al.*, 1997, 1999; Shearer *et al.*, 1999), and do not prevent PLCβ-mediated Ca^{2+} release in starfish eggs (Carroll *et al.*, 1997). These results indicate that the PLCγ SH2 domains prevent Ca^{2+} release at fertilization by specifically inhibiting SH2-mediated activation of PLCγ.

Additional studies have shown that IP_3 levels increase in echinoderm eggs at fertilization (Ciapa and Whitaker, 1986; Lee and Shen, 1998; Kuroda *et al.*, 2001), and that injection of heparin (a nonspecific inhibitor of the IP_3 receptor) can completely block the Ca^{2+} rise at fertilization (Mohri *et al.*, 1995). Furthermore, injection of the IP_3-binding region of the mouse IP_3 receptor, which sequesters IP_3, greatly reduces Ca^{2+} release at fertilization in starfish eggs (Iwasaki *et al.*, 2001). Taken together with the SH2 domain experiments, these findings argue that Ca^{2+} release at fertilization in echinoderm eggs is mediated mainly by IP_3 produced by PLCγ. cGMP and cADP ribose have also been considered as possible mediators of Ca^{2+} release at fertilization in sea urchin eggs, since injection of these compounds into eggs can stimulate Ca^{2+} release (Whalley *et al.*, 1992; Galione *et al.*, 1993; Lee *et al.*, 1993, 1996), and their levels rise in eggs at fertilization before Ca^{2+} release (Ciapa and Epel, 1996; Kuroda *et al.*, 2001). However, the ability of cGMP and

cADP ribose to induce a Ca^{2+} rise is not inhibited by PLCγ SH2 domains, unlike the Ca^{2+} rise at fertilization (Carroll *et al.*, 1999). Also, a cGMP analog, Rp-8-pCPT-cGMPS, inhibits cGMP-induced Ca^{2+} release, but not Ca^{2+} release at fertilization (Lee *et al.*, 1996). These findings indicate that the Ca^{2+} rise at fertilization is probably not initiated by cGMP or cADP ribose.

Further characterization of how PLCγ functions in echinoderm fertilization has shown that PLCγ protein is present in sea urchin eggs, that PLCγ activity increases by 30 s after insemination, and that the PLCγ protein translocates from cytosolic to membrane fractions by 60 s after insemination (De Nadai *et al.*, 1998; Rongish *et al.*, 1999). In the PLCγ activity assays, the amount of sperm protein in the sample was calculated to be too small to contribute significantly to the assay, indicating that activation of an egg PLCγ accounts for the activity increase (Rongish *et al.*, 1999). Although increased tyrosine phosphorylation of PLCγ has not been detected in sea urchin eggs after insemination, the pool of PLCγ undergoing an increase in tyrosine phosphorylation at fertilization may be very small and difficult to detect (Rongish *et al.*, 1999). Alternatively, PLCγ may be activated by an SH2-mediated mechanism, such as translocation to the plasma membrane (Matsuda *et al.*, 2001; Wang *et al.*, 2001), that does not necessarily involve tyrosine phosphorylation of PLCγ itself.

Since cytoplasmic tyrosine kinases of the Src family often function in pathways leading to activation of PLCγ, by phosphorylating both PLCγ and/or linker proteins that associate with PLCγ (Rhee, 2001), a role for a Src family kinase (SFK) in initiating Ca^{2+} release at fertilization in echinoderm eggs has been examined. If the activity of a SFK mediates Ca^{2+} release at fertilization via a pathway involving PLCγ, several criteria should be fulfilled. A SFK should associate (directly or indirectly) with PLCγ in a fertilization-responsive manner, SFK activity should increase at fertilization, inhibition of SFK activity should prevent the Ca^{2+} rise at fertilization, and artificially raising SFK activity in the egg should lead to Ca^{2+} release. If the SFK operates upstream of PLCγ in the Ca^{2+} release pathway, then PLCγ SH2 domains should inhibit the Ca^{2+} rise in response to artificially raising SFK activity in the egg.

In starfish egg extracts, a 58-kDa protein that is recognized by a vertebrate SFK antibody associates with mammalian PLCγ SH2 domains by 15 s postinsemination (Giusti *et al.*, 1999a). Tyrosine kinase activity also associates with the PLCγ SH2 domains by 15 s postinsemination. In extracts of fertilized sea urchin eggs, active PLCγ protein binds to domains of the SFK Fyn (Kinsey and Shen, 2000). These results indicate that a SFK and PLCγ directly or indirectly interact with one another at fertilization.

An increase in protein tyrosine kinase activity at fertilization of sea urchin eggs was first reported almost 20 years ago (Dasgupta and Garbers, 1983). In this initial study, the earliest time point examined was at 1 h after insemination. Subsequent studies have detected an increase in tyrosine kinase activity at earlier and earlier time points, up to as early as 15 s after insemination (Ciapa and Epel, 1991; Kinsey, 1997). More specifically, immunoprecipitation studies using an antibody raised against a peptide present in mammalian Src have shown that SFK activity increases by 30 s after insemination in sea urchin eggs (Abassi *et al.*, 2000). Controls showed that this increase could not be accounted for by the addition of SFK present in nonfertilizing sperm in the sperm–egg mixture.

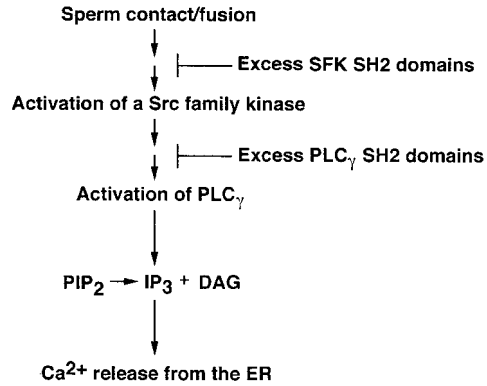

FIG. 3. Model of the signaling pathway that leads to Ca^{2+} release in echinoderm and ascidian eggs at fertilization. In this model, the initial interaction between egg and sperm leads to SH2 domain-mediated activation of a SFK, which then directly or indirectly leads to SH2 domain-mediated activation of PLCγ. Activation of PLCγ results in IP_3 production and Ca^{2+} release from the ER. Experiments using SH2 domains have provided evidence to support this model. See text for further details. Modified from Jaffe *et al.* (2001).

The general tyrosine kinase inhibitor genistein (Shen *et al.*, 1999), and the SFK inhibitor PP1 (Abassi *et al.*, 2000), both significantly delay Ca^{2+} release in sea urchin eggs at fertilization. Though genistein and PP1 can have nonspecific effects (Moore and Kinsey, 1995; Glahn *et al.*, 1999; Sato *et al.*, 2000; see Abram and Courtneidge, 2000), these findings hint that SFK activity may be required for the Ca^{2+} rise at fertilization. Src family SH2 domains have also been used as inhibitors of processes mediated by SFK activity (Roche *et al.*, 1995). Injecting echinoderm eggs with Src family SH2 domains can completely inhibit the Ca^{2+} rise at fertilization, while control SH2 domains from non-Src family cytoplasmic tyrosine kinases have no effect (Giusti *et al.*, 1999b; Abassi *et al.*, 2000; Kinsey and Shen, 2000). This indicates that SFK activity is required for the Ca^{2+} rise at fertilization.

Injecting active mammalian SFK protein into starfish eggs stimulates intracellular Ca^{2+} release, and this release is inhibited by PLCγ SH2 domains, supporting the hypothesis that a SFK operates upstream of PLCγ at fertilization (Giusti *et al.*, 2000). Related experiments have examined how the SFK may be activated. Though SFK SH2 domains can block the Ca^{2+} rise at fertilization, they do not inhibit Ca^{2+} release in response to injecting starfish eggs with an

TABLE 2

Experiments Examining the Initiation of Ca^{2+} Release in Eggs at Fertilization

	Echinoderms	Ascidians	Amphibians	Mammals
Contact with a protease-, lectin-, or integrin-binding peptide can cause Ca^{2+} release[a]	+	+	+	+
Extracellular Ca^{2+} influx is required for Ca^{2+} release at fertilization[b]	−	−	−	−
NO is required for Ca^{2+} release at fertilization[c]	?	−	?	−
Sperm extract injection can cause Ca^{2+} release/egg activation[d]	+	+	+	+
Sperm extract causes Ca^{2+} release via the same pathway as fertilization[e]	?	+	?	+

[a] Echinoderms: Steinhardt *et al.,* 1971; Carroll and Jaffe, 1995. Ascidians: Zalokar, 1980; Speksnijder *et al.,* 1990; Flannery and Epel, 1998. Amphibians: Iwao and Fujimura, 1996; Shilling *et al.,* 1997, 1998; Mizote *et al.,* 1999; K. Sato *et al.,* 1999. Mammals: Campbell *et al.,* 2000.

[b] Echinoderms: Schmidt *et al.,* 1982. Ascidians: Speksnijder *et al.,* 1989, 1990; Sensui and Morisawa, 1996. Amphibians: Wilkinson *et al.,* 1998. Mammals: Jones *et al.,* 1998a.

[c] Echinoderms: Willmott *et al.,* 1996; Lee *et al.,* 1996; Kuo *et al.,* 2000. Ascidians: Hyslop *et al.,* 2001. Mammals: Hyslop *et al.,* 2001.

[d] Echinoderms: Dale *et al.,* 1985. Ascidians: Dale, 1988; Wilding and Dale, 1998; Kyozuka *et al.,* 1998; McDougall *et al.,* 2000; Runft and Jaffe, 2000. Amphibians: Yamamoto *et al.,* 2001. Mammals: Swann, 1990, 1994; Homa and Swann, 1994; Wu *et al.,* 1997, 1998; Palermo *et al.,* 1997; Sakurai *et al.,* 1999; Perry *et al.,* 1999, 2000; Gordo *et al.,* 2000.

[e] Ascidians: Runft and Jaffe, 2000. Mammals: Oda *et al.,* 1999.

active SFK (Giusti *et al.,* 2000). This indicates that the step in the fertilization process that is interfered with by the SFK SH2 domains is upstream of Src activation.

In summary, evidence from echinoderm studies indicates that fertilization stimulates the SH2-mediated activation of a SFK, which either directly or indirectly activates PLCγ by a SH2-mediated mechanism (Table 1, Fig. 3). PLCγ activation then leads to IP_3 production and Ca^{2+} release. It should be noted that the SFK SH2 and PLCγ SH2 domains used in the echinoderm fertilization studies described above were derived from vertebrate proteins. Further studies using echinoderm-specific SH2 domains would be valuable.

Initiation of the Ca^{2+} Release Pathway in Echinoderm Eggs: An Unanswered Question

Although several components of the signal transduction pathway leading to intracellular Ca^{2+} release at fertilization have been identified, it is unknown whether sperm–egg contact or sperm–egg fusion initiates the signaling pathway (Table 2; Fig. 2). In support of the contact hypothesis (Fig. 2A) is the observation that application of proteases to echinoderm eggs can stimulate early events of egg activation, including Ca^{2+} release (Steinhardt *et al.,* 1971; Carroll and Jaffe, 1995). In support of the fusion hypothesis (Fig. 2D), injection of an extract of sea urchin sperm into sea urchin eggs leads to egg activation (Dale *et al.,* 1985). However, it is unknown whether proteases or sperm extract cause Ca^{2+} release by the same pathway used at fertilization. Since PLCs can be activated by 1–10 μM Ca^{2+} (Wahl *et al.,* 1992; Jhon *et al.,* 1993; Hwang *et al.,* 1996; Rhee, 2001), another possibility is that egg activation is due to an influx of extracellular Ca^{2+} into the egg after sperm–egg fusion through Ca^{2+} channels located in the sperm membrane (Fig. 2C; see Créton and Jaffe, 1995). This idea, however, is not consistent with the observation that echinoderm eggs can be activated by fertilization in a low-Ca^{2+} medium (44 nM Ca^{2+}) (Schmidt *et al.,* 1982).

Experiments examining the timing of fertilization events in sea urchin eggs have indicated that both contact and fusion precede the release of intracellular Ca^{2+}, such that either of these events could be the cause of Ca^{2+} release. The time of sperm–egg fusion has been determined by measurements of membrane capacitance, which is proportional to membrane surface area, and these measurements have indicated that sperm–egg fusion occurs at the same time as a membrane conductance increase (McCulloh and Chambers, 1992). Within milliseconds, the conductance increase causes a Ca^{2+} action potential (Chambers and de Armendi, 1979), which is seen as a small transient Ca^{2+} rise in the egg (see Fig. 1A). Thus, the conductance increase or the action potential provides a marker of the time of sperm–egg fusion, which can be compared with the time of Ca^{2+} release from the ER (see Fig. 1A). Based on such Ca^{2+} records, it has been concluded that sperm–egg fusion precedes Ca^{2+} release in echinoderm eggs by about 4–12 s (Mohri *et al.,* 1995; Carroll *et al.,* 1997, 1999; Giusti *et al.,* 1999b, 2000).

One observation that favors the fusion hypothesis is that clamping the sea urchin egg's membrane potential at a positive level prevents both sperm–egg fusion, as indicated by sperm entry into the egg cytoplasm, and Ca^{2+} release, as indicated by cortical granule exocytosis, without having an obvious effect on sperm–egg binding (Jaffe, 1976). Voltage clamp studies over a range of membrane potentials have shown that Ca^{2+} release (cortical granule exocytosis) is only seen when there is at least a transient sperm–egg fusion event (as indicated by a conductance increase) (Lynn and Chambers, 1984; Lynn *et al.,* 1988; McCulloh and Chambers, 1992), supporting the idea that sperm–egg fusion is a necessary step in the events leading to Ca^{2+} release in echinoderm eggs.

Recently, Kuo *et al.* (2000) presented evidence that the sperm-induced Ca^{2+} rise in sea urchin eggs at fertilization may be mediated by nitric oxide (NO), produced in the egg at fertilization due to the introduction of NO synthase from the sperm. NO is believed to stimulate Ca^{2+} release in sea urchin eggs via a cGMP/protein kinase G-mediated pathway that leads to cADP ribose production; cADP ribose may stimulate Ca^{2+} release from ryanodine receptors (a type of Ca^{2+} channel found in the ER of some cells) (Galione *et al.*, 1993; Lee *et al.*, 1993; Willmott *et al.*, 1996). Kuo *et al.* (2000) showed that, based on an increase in fluorescence of diaminofluorescein, NO rises in the egg at fertilization, that NO donors stimulate egg activation, and that oxyhemoglobin (an NO scavenger) reduces the amplitude of the Ca^{2+} rise at fertilization (Kuo *et al.*, 2000). However, other findings are not consistent with the conclusion that NO mediates the Ca^{2+} rise at fertilization. The protein kinase G inhibitor, Rp-8-pCPT-cGMPS, blocks Ca^{2+} release in sea urchin eggs in response to NO (Willmott *et al.*, 1996), but has no effect on the Ca^{2+} rise at fertilization (Lee *et al.*, 1996).

ASCIDIANS

Ca^{2+} Release in Ascidian Eggs Occurs by a Pathway Similar to That in Echinoderms

Ascidians are marine invertebrates that are grouped with vertebrates in the phylum Chordata. Experiments like those described above for echinoderm eggs have indicated that the pathway leading to Ca^{2+} release at fertilization in ascidian eggs also involves a SFK, PLCγ, and IP_3 (Table 1; Fig. 3). In particular, IP_3 is produced at fertilization (Toratani and Yokosawa, 1995), introducing IP_3 causes Ca^{2+} release (McDougall and Sardet, 1995; Albrieux *et al.*, 1997; Yoshida *et al.*, 1998), and injecting PLCγ SH2 or Src family SH2 domains completely and specifically inhibits Ca^{2+} release at fertilization (Runft and Jaffe, 2000). In addition, ruthenium red, an inhibitor of the ryanodine receptor, does not inhibit Ca^{2+} release at fertilization in ascidian eggs (Wilding and Dale, 1998; Yoshida *et al.*, 1998), consistent with an IP_3-mediated pathway. The requirement for tyrosine kinase activity is further supported by the inhibition of egg activation events at fertilization by the tyrosine kinase inhibitor erbstatin (Ueki and Yokosawa, 1997).

Initiation of the Ca^{2+} Release Pathway in Ascidian Eggs: Evidence for an Activator Protein from the Sperm Cytosol

In ascidians, current evidence favors the fusion hypothesis for how the sperm initiates Ca^{2+} release at fertilization (Table 2; Fig. 2). Although externally applied lectins can stimulate Ca^{2+} release and other early events of egg activation (Zalokar, 1980; Speksnijder *et al.*, 1990; Flannery and Epel, 1998), there is no evidence as to whether such a contact-mediated mechanism operates at fertilization. Extracellular Ca^{2+} influx has also been considered as a possible initiator of Ca^{2+} release, but is not likely since ascidian eggs can be activated by fertilization in very low Ca^{2+}-containing media (Speksnijder *et al.*, 1989, 1990; Sensui and Morisawa, 1996).

In support of the fusion hypothesis, injecting an extract of ascidian sperm into ascidian eggs stimulates Ca^{2+} release that is temporally and spatially similar to the Ca^{2+} rise that occurs at fertilization (Dale, 1988; Wilding and Dale, 1998; Kyozuka *et al.*, 1998; McDougall *et al.*, 2000; Nixon *et al.*, 2000; Runft and Jaffe, 2000). Extract from approximately one to three sperm is sufficient to stimulate egg activation (Kyozuka *et al.*, 1998; Runft and Jaffe, 2000). Injecting PLCγ SH2 domains or Src family SH2 domains into ascidian eggs blocks Ca^{2+} release in response to sperm extract, indicating that fertilization and sperm extract injection use the same signaling molecules to initiate Ca^{2+} release (Table 2). These findings support the hypothesis that ascidian egg activation at fertilization is initiated by a cytosolic factor introduced from the sperm into the egg following sperm–egg fusion (Fig. 2D).

The activating factor(s) from ascidian sperm has been partially characterized. It is heat- and trypsin-sensitive and is therefore most likely a protein (Kyozuka *et al.*, 1998; Runft and Jaffe, 2000; McDougall *et al.*, 2000). It has a molecular weight between 30 and 100 kDa, and its activity is not reduced by high-speed centrifugation of the sperm extract or by protease inhibitors in the extraction buffer, indicating that it is soluble and probably not a protease (Wilding and Dale, 1998; Runft and Jaffe, 2000; Hyslop *et al.*, 2001). The activating factor from the sperm extract appears to operate via a cytoplasmic mechanism only, as applying it to the surface of ascidian eggs does not cause Ca^{2+} release (Runft and Jaffe, 2000). It also appears to be sperm-specific, because injecting ascidian eggs with an extract of ascidian ovary does not stimulate Ca^{2+} release (Runft and Jaffe, 2000). However, the activating factor may not be species-specific, as injecting human sperm extract into ascidian eggs also stimulates egg activation (Wilding *et al.*, 1997).

Since NO synthase has been proposed as an activating factor from sperm in sea urchin fertilization (Kuo *et al.*, 2000), the role of NO in ascidian fertilization has recently been examined. Though NO can stimulate Ca^{2+} release in ascidian eggs, a rise in NO has not been detected at fertilization (Hyslop *et al.*, 2001). Furthermore, treating ascidian eggs with an inhibitor of the NO-mediated Ca^{2+} release signaling pathway (N^G-nitro-L-arginine methyl ester) has no effect on Ca^{2+} release at fertilization (Hyslop *et al.*, 2001). These results indicate that Ca^{2+} release at fertilization in ascidian eggs is probably not mediated by NO.

Because Src family SH2 domains can inhibit Ca^{2+} release induced by sperm extract (Runft and Jaffe, 2000), the activating factor in the extract may be a regulator, directly or indirectly, of an egg SFK. Thus, SFK regulators known from studies of somatic cells, including kinases and phosphatases, and molecules that bind the SH2 and SH3 domains of

SFKs (see Abram and Courtneidge, 2000) are possible candidates for the activating factor in ascidian sperm.

VERTEBRATES

The Pathway Leading to Ca^{2+} Release at Fertilization in Vertebrate Eggs Requires IP_3 but Not SH2-Mediated Activation of PLCγ

As in echinoderms and ascidians, intracellular Ca^{2+} release at fertilization in vertebrate eggs is mediated by IP_3 (Table 1). IP_3 levels rise in *Xenopus* eggs at fertilization (Stith *et al.*, 1993; Snow *et al.*, 1996; Sato *et al.*, 2000), and injecting IP_3 into frog, fish, and mammalian eggs can stimulate a rise in intracellular Ca^{2+} (Busa *et al.*, 1985; Iwamatsu *et al.*, 1988; Miyazaki, 1988). A monoclonal antibody directed against the C-terminal domain of the mouse type I IP_3 receptor can completely block Ca^{2+} release and other events of egg activation at fertilization of hamster and mouse eggs (Miyazaki *et al.*, 1992, 1993; Xu *et al.*, 1994). A similar mammalian IP_3 receptor antibody largely inhibits the Ca^{2+} rise at fertilization of *Xenopus* eggs, though small, localized rises in Ca^{2+} do occur (Fontanilla and Nuccitelli, 1998; Runft *et al.*, 1999). It is unclear whether these local Ca^{2+} rises result from an IP_3-independent pathway or because the antibody is not a complete inhibitor; the latter is suggested by the finding that the antibody only partially inhibits Ca^{2+} release in response to injected IP_3 (Runft *et al.*, 1999). The importance of IP_3 in mediating Ca^{2+} release at fertilization in vertebrate eggs indicates that PLCs function in this pathway, and consistently, the PLC inhibitor U73122 has an inhibitory effect on Ca^{2+} release at fertilization in mouse and frog eggs (Dupont *et al.*, 1996; Sato *et al.*, 2000). However, U73122 can have nonspecific effects as discussed above (see Echinoderms).

Signaling pathways for both G-protein-PLCβ-mediated and tyrosine kinase-PLCγ-mediated intracellular Ca^{2+} release are present in vertebrate eggs, as indicated by the ability of frog and mouse eggs to activate after the stimulation of exogenously expressed receptors that function through these pathways (Kline *et al.*, 1988; Williams *et al.*, 1992; Moore *et al.*, 1993; Yim *et al.*, 1994; Mehlmann *et al.*, 1998; Runft *et al.*, 1999). To examine whether a G-protein is required for Ca^{2+} release at fertilization, a general inhibitor of GTP-dependent events, GDP-β-S, was used initially. GDP-β-S prevents Ca^{2+} release and other early events of egg activation in hamster and mouse eggs (Miyazaki, 1988; Moore *et al.*, 1994), but whether this is due to inhibition of a heterotrimeric G-protein or some other GTP-dependent process is uncertain. In *Xenopus*, attempts to test the requirement for a G-protein using GDP-β-S were inconclusive due to nonspecific effects (Kline *et al.*, 1990). More recently, mouse and *Xenopus* eggs have been treated with more specific inhibitors of G-proteins, including pertussis toxin (which inhibits G_i family members G_i and G_o), and an antibody that blocks G_q activation by 7-transmembrane receptors. Although pertussis toxin and the G_q antibody were shown to be effective inhibitors of the corresponding G-proteins in the egg, neither has an effect on Ca^{2+} release at fertilization (Kline *et al.*, 1991; Moore *et al.*, 1994; Williams *et al.*, 1998; Runft *et al.*, 1999). Likewise, using phosducin or excess G-protein α subunits in the GDP-bound form to sequester G-protein $\beta\gamma$ subunits does not inhibit early events of egg activation, including the ZP2-to-$ZP2_f$ transition in mouse (Moore *et al.*, 1994) and Ca^{2+} release in frog (Runft *et al.*, 1999). These results argue against a function for a heterotrimeric G-protein and PLCβ in mediating Ca^{2+} release at fertilization in mouse and frog eggs.

In frog eggs, PLCγ activity increases significantly within 2 min after insemination and PLCγ tyrosine phosphorylation also appears to increase (Sato *et al.*, 2000), but one uncertainty is the possible contribution of nonfertilizing sperm in the sperm–egg mixture to these assays (see Rongish *et al.*, 1999). Whether this increase in PLCγ activity is required for Ca^{2+} release at fertilization in frog eggs is unknown. Although PLCγ SH2 domains block PLCγ-mediated Ca^{2+} release in response to platelet-derived growth factor (PDGF) in mouse and frog eggs expressing PDGF receptors, the PLCγ SH2 domains do not inhibit Ca^{2+} release at fertilization in these eggs, even at a 10× higher concentration than that used to inhibit PDGF-induced Ca^{2+} release (Mehlmann *et al.*, 1998; Runft *et al.*, 1999). These findings demonstrate that the mechanism of Ca^{2+} release at fertilization in vertebrate eggs differs from that in echinoderms and ascidians in that it does not require SH2-mediated activation of PLCγ (Table 1). Coinjecting frog eggs with both PLCγ SH2 domains and the G_q antibody does not inhibit the Ca^{2+} rise at fertilization either, arguing against the possibility that G_q-mediated activation of PLCβ and SH2-mediated activation of PLCγ operate redundantly at fertilization (Runft *et al.*, 1999). PLCγ activity can also be stimulated by its translocation to the plasma membrane by a pathway requiring phosphatidylinositol-3-kinase (PI3 kinase) (Rhee, 2001). However, in mouse eggs, fertilization-induced Ca^{2+} release is not inhibited by the PI3 kinase inhibitor wortmannin (Mehlmann *et al.*, 2001).

In summary, though IP_3 is required for Ca^{2+} release at fertilization in vertebrate eggs and studies using the general PLC inhibitor U73122 suggest a role for PLC activity in the Ca^{2+} release pathway, which PLC subtype is involved has not been conclusively determined.

Evidence That a Tyrosine Kinase Functions in Egg Activation at Fertilization in Vertebrates

In zebrafish eggs, a SFK is activated at fertilization, as demonstrated by increased tyrosine kinase activity in immunoprecipitates of the SFK Fyn from inseminated eggs (Wu and Kinsey, 2000). This increase occurs within 30 s postinsemination, and cannot be accounted for by Fyn activity present in nonfertilizing sperm in the insemination mixture. Likewise, studies of *Xenopus* eggs indicate that

the kinase activity of a partially purified SFK called Xyk increases by 10 min postinsemination (K. Sato *et al.,* 1996, 1999). One approach to examine whether Xyk functions in causing Ca^{2+} release at fertilization in frog eggs has been to immunoprecipitate PLCγ from frog egg lysates at various times after insemination, and to examine whether active Xyk coimmunoprecipitates (Sato *et al.,* 2000). These experiments showed that tyrosine kinase activity was present in PLCγ immunoprecipitates made from eggs at 2 min postinsemination, and suggested that the activity was due to Xyk.

Studies using tyrosine kinase inhibitors have provided further evidence that tyrosine kinase activity is required for the Ca^{2+} rise at fertilization in *Xenopus* eggs. The tyrosine kinase inhibitor lavendustin inhibits Ca^{2+} release at fertilization in frog eggs (Glahn *et al.,* 1999), as do two inhibitors that are considered to be relatively specific for Src family kinases, the peptide A7 and the pharmacological inhibitor PP1 (K. Sato *et al.,* 1999, 2000). However, A7 and PP1 also inhibit sperm–egg fusion, raising the question of whether the action of these inhibitors is on egg activation or on an earlier sperm function. Furthermore, it remains uncertain how a tyrosine kinase might lead to Ca^{2+} release at fertilization in frog eggs, since PLCγ SH2 domains do not inhibit Ca^{2+} release at fertilization in this species (Runft *et al.,* 1999).

In mouse eggs, several tyrosine kinase inhibitors delay Ca^{2+} release at fertilization and reduce the number of Ca^{2+} oscillations (Dupont *et al.,* 1996). However, since these inhibitors were applied extracellularly, the delay could result from an effect on sperm function prior to egg activation. A further indication that a tyrosine kinase, and in particular a SFK, may function in mammalian fertilization comes from a study showing that Fyn is localized to the region of sperm entry in rat eggs observed at 1 h after fertilization (Talmor *et al.,* 1998). Although these findings provide hints that a SFK may function in mammalian fertilization, further studies are needed to determine whether and how this might occur.

What Initiates Ca^{2+} Release at Fertilization in Amphibian Eggs?

Both sperm-contact and soluble sperm factor hypotheses (Table 2; Fig. 2) have been examined in amphibian eggs. Although there is, as yet, no answer as to which of these mechanisms operates at fertilization, results supporting each are summarized below. In support of the contact hypothesis (Fig. 2A), the protease cathepsin B causes an increase in intracellular Ca^{2+} when applied to *Xenopus* eggs (Mizote *et al.,* 1999). A protease from sperm of the newt *Cynops* also induces early events of egg activation when applied to *Xenopus* eggs (Mizote *et al.,* 1999). A similar protease is present in *Xenopus* sperm extract (Mizote *et al.,* 1999; see Iwao, 2000), but has not been applied to *Xenopus* eggs. Fertilization in *Xenopus* can be blocked by incubating eggs with protease inhibitors or with substrate peptides for the *Cynops* sperm protease (Mizote *et al.,* 1999). These inhibitors may prevent the sperm protease from acting on egg molecules involved in activation; it is also possible, however, that these inhibitors block molecules involved in the acrosome reaction and/or other sperm functions prior to egg activation.

Other evidence suggests that Ca^{2+} release is initiated in frog eggs when a disintegrin molecule on the sperm surface contacts an integrin on the egg surface (see Shilling *et al.,* 1998). Applying peptides containing the integrin-binding amino acid sequence RGDS to *Xenopus* eggs can induce Ca^{2+} release, cortical contraction, and resumption of meiosis (Iwao and Fujimura, 1996; K. Sato *et al.,* 1999). A cDNA encoding a metalloprotease/disintegrin termed xMDC16 has been isolated from *Xenopus* testis (Shilling *et al.,* 1997), and applying a peptide derived from the disintegrin region of xMDC16 to *Xenopus* eggs can either induce intracellular Ca^{2+} release or block fertilization, depending on the peptide concentration used (Shilling *et al.,* 1997; 1998). Though the protease, RGDS, and xMDC16 studies show that *Xenopus* eggs are capable of being activated by a contact-stimulated mechanism, the egg molecules with which the examined proteases and disintegrins interact have not been identified, and whether a protease or disintegrin on the sperm surface operates at fertilization is unknown.

Xenopus eggs can activate when fertilized in a Ca^{2+}-free medium (Wilkinson *et al.,* 1998), arguing against egg activation by means of extracellular Ca^{2+} influx through sperm Ca^{2+} channels after sperm–egg fusion (Table 2; Fig. 2C). In support of the idea that sperm–egg fusion introduces an activating factor from the sperm cytoplasm into the egg (Fig. 2D), is a study showing that injection of newt sperm extract into newt eggs stimulates a Ca^{2+} rise similar both temporally and spatially to that at fertilization (Yamamoto *et al.,* 2001). Although an extract of *Xenopus* sperm can stimulate Ca^{2+} release when injected into mouse eggs, this extract has not been injected into *Xenopus* eggs (Dong *et al.,* 2000). The identity of the factor in the newt or *Xenopus* sperm extract that causes a Ca^{2+} rise in eggs, and the necessity of this factor for fertilization, have yet to be investigated.

What Initiates Ca^{2+} Release at Fertilization in Mammalian Eggs?

In the past several years, much progress has been made in identifying fertilization-related molecules in the plasma membranes of mammalian sperm and eggs. In particular, current evidence indicates that the binding of the sperm and egg membranes may involve the binding of an ADAM (a disintegrin and metalloprotease) family protein in the sperm (see Cho *et al.,* 1998; Nishimura *et al.,* 2001) to an unknown receptor in the egg (see Miller *et al.,* 2000). The subsequent fusion of the sperm and egg membranes depends on the presence in the egg membrane of CD9, a member of the tetraspanin family of proteins (Chen *et al.,* 1999; Le Naour *et al.,* 2000; Miyado *et al.,* 2000; Kaji *et al.,* 2000; Miller *et al.,* 2000). A glycosyl-phosphatidylinositol

anchored protein in the egg membrane also appears to be required for fusion (Coonrod *et al.*, 1999). However, there is no evidence that any of these proteins have a signaling function in initiating Ca^{2+} release in eggs.

Investigations of how fertilization activates Ca^{2+} release in mammalian eggs have pointed to a mechanism in which an activating protein from the sperm cytoplasm is introduced into the egg cytoplasm. One exception to this conclusion is a study in which application of a peptide containing the RGD sequence recognized by integrins caused two Ca^{2+} transients in bovine eggs (Campbell *et al.*, 2000). Although this finding supports the possibility that sperm–egg contact could cause Ca^{2+} release, evidence reviewed below argues more strongly for a fusion-mediated mechanism.

Insights from ICSI

The technique of intracytoplasmic sperm injection (ICSI) has become widely used in assisted reproduction and has provided some information about the basic science of fertilization. Mammalian eggs injected with whole mammalian sperm can exhibit normal events of egg activation, including Ca^{2+} release (Tesarik *et al.*, 1994; Nakano *et al.*, 1997; Meng and Wolf, 1997; M. S. Sato *et al.*, 1999), and can develop to term (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993; Kimura and Yanagimachi, 1995a; Kuretake *et al.*, 1996; Kimura *et al.*, 1998). This suggests that contact of sperm and egg plasma membranes is not a critical step for egg activation. Studies using ICSI have shown that only the sperm head is critical for egg activation and subsequent embryonic development (Kuretake *et al.*, 1996; Meng and Wolf, 1997; Kimura *et al.*, 1998). Injecting sperm at various stages of spermiogenesis has demonstrated that elongating spermatids can activate eggs, whereas round spermatids and secondary spermatocytes cannot (Ogura *et al.*, 1994; Kimura and Yanagimachi, 1995b,c; Sousa *et al.*, 1996; Tesarik, 1998; Sato *et al.*, 1998; Sakurai *et al.*, 1999). Egg activation and subsequent development following ICSI are sperm-specific and not the result of artifactual introduction of Ca^{2+} into the egg from the outside medium; while there is often an initial release of Ca^{2+} upon insertion of the injection pipet, neither this initial Ca^{2+} release nor injecting culture medium alone activates eggs (Tesarik *et al.*, 1994; Dozortsev *et al.*, 1995a; Laybaert *et al.*, 1996; Nakano *et al.*, 1997; M. S. Sato *et al.*, 1999).

ICSI initiates Ca^{2+} oscillations in eggs resembling those seen at fertilization, beginning 4–10 h after sperm injection into human eggs (Tesarik *et al.*, 1994) and 15–30 min after sperm injection into mouse eggs (Nakano *et al.*, 1997; M. S. Sato *et al.*, 1999). Interestingly, injected sperm are often more effective at causing resumption of meiosis if the sperm plasma membrane is first disrupted or removed, perhaps allowing an activating substance (see below) to diffuse from the sperm more easily (Tesarik and Sousa, 1995; Fishel *et al.*, 1995; Dozortsev *et al.*, 1995b; Kasai *et al.*, 1999). In summary, the ability of an injected sperm to cause egg activation is consistent with the hypothesis that a substance from the sperm is transferred to the egg following sperm–egg fusion. However, it is not certain that these mechanisms are the same as those that operate during natural fertilization.

Sperm–Egg Fusion and Activation of Mammalian Eggs

In *in vitro* fertilization, sperm–egg fusion precedes mammalian egg activation. Dye transfer studies that can determine the time of sperm–egg fusion, in parallel with monitoring intracellular Ca^{2+} levels, show that sperm–egg fusion occurs ~1–5 min before the initial rise in Ca^{2+} in mouse eggs (Lawrence *et al.*, 1997; Jones *et al.*, 1998a). The idea that the sperm contributes to the initial Ca^{2+} rise by acting as a conduit to allow an influx of Ca^{2+} into the egg through the sperm membrane at sperm–egg fusion (Fig. 2C) is ruled out by the observations that there is no localized increase of Ca^{2+} in the mouse egg near the site of sperm–egg fusion, and that normal Ca^{2+} release occurs in eggs fertilized at very low (13 nM) extracellular Ca^{2+} concentrations (Jones *et al.*, 1998a). Therefore, Ca^{2+} release following sperm–egg fusion does not appear to be the result of an increased plasma membrane permeability to Ca^{2+}. A more likely explanation for how the sperm causes Ca^{2+} release in mammalian eggs is that it transfers a Ca^{2+}-releasing substance(s) to the egg following sperm–egg fusion.

In mammalian eggs, the "fusion hypothesis"—specifically, the idea that the sperm introduces a soluble activating substance into the egg upon sperm–egg fusion (Fig. 2D)—has been studied considerably in the last decade. The first evidence in support of this hypothesis came from experiments demonstrating that injection of extracts prepared from hamster or boar sperm induced Ca^{2+} oscillations in hamster eggs similar to those seen at fertilization (Swann, 1990). Since then, many groups have confirmed that sperm extracts from a number of mammalian species cause Ca^{2+} release in eggs similar to those seen at fertilization (Swann, 1990, 1994; Homa and Swann, 1994; Sousa *et al.*, 1996; Wu *et al.*, 1997; Palermo *et al.*, 1997; Sakurai *et al.*, 1999). In addition to causing Ca^{2+} release, sperm extracts fully activate eggs, causing cortical granule exocytosis, second polar body formation, pronuclear formation, and cleavage (Meng and Wolf, 1997; Wu *et al.*, 1998; Sakurai *et al.*, 1999; Gordo *et al.*, 2000). The Ca^{2+}-releasing ability of sperm extracts is not species-specific and is abolished after heating and protease treatment, indicating that the active substance is a protein (Swann, 1990; Wu *et al.*, 1997).

Despite widespread agreement that sperm extracts contain a protein(s) that causes egg activation when injected into eggs, two major issues remain to be resolved. First, this protein(s) has not been identified; and second, it still remains to be shown whether this protein(s) is the physiological activator of eggs at fertilization. The finding that a function-blocking antibody against the IP_3 receptor blocks sperm extract-induced Ca^{2+} release in mouse eggs (Oda *et*

al., 1999) is consistent with the hypothesis that the sperm extract activates eggs by the same pathway used at fertilization (Table 2).

In Search of the Elusive Sperm Factor

There have been several candidates suggested for the egg-activating protein from the sperm. The first was a ~33-kDa protein that had high sequence homology with the bacterial enzyme glucosamine-6-phosphate isomerase. This protein was partially purified from hamster sperm lysate and was termed "oscillin" (Parrington *et al.,* 1996). Oscillin is found in fractions of hamster sperm lysate that contain Ca^{2+}-releasing activity (Parrington *et al.,* 1996; Swann and Lai, 1997; Wolny *et al.,* 1999). Oscillin is also present in sperm of other species, including human (Wolny *et al.,* 1999; Montag *et al.,* 1998).

However, recombinant oscillin, though enzymatically active, does not cause Ca^{2+} release when injected into mouse eggs (Wolosker *et al.,* 1998; Shevchenko *et al.,* 1998; Wolny *et al.,* 1999). In addition, immunodepletion of oscillin from sperm extract fails to abolish the Ca^{2+}-releasing ability of the extract (Wolny *et al.,* 1999). Furthermore, oscillin is expressed in a wide variety of tissues (Nakamura *et al.,* 2000; Amireault and Dubé, 2000), whereas the protein from the sperm that causes Ca^{2+} release appears to be sperm-specific, as injection of tissue extracts from brain (Wu *et al.,* 1997; Jones *et al.,* 2000), kidney, and liver (Jones *et al.,* 2000) into eggs fails to cause Ca^{2+} release. Therefore, although oscillin is present in mammalian sperm, it is not likely to be the egg-activating protein.

Another protein proposed to be the activating factor in sperm extract is the truncated form of the c-kit tyrosine kinase receptor (tr-kit) (Sette *et al.,* 1997, 1998). Tr-kit protein accumulates in elongating spermatids during spermiogenesis (Albanesi *et al.,* 1996). Injecting extracts of COS cells expressing recombinant tr-kit into mouse eggs has been reported to cause egg activation events, such as cortical granule exocytosis, second polar body formation, and pronuclear formation. These events are inhibited by preincubating eggs in BAPTA-AM, suggesting that tr-kit is acting by causing Ca^{2+} release (Sette *et al.,* 1997). Tr-kit protein stimulates PIP_2 hydrolysis and tyrosine phosphorylation of PLCγ1 when transfected in COS cells (Sette *et al.,* 1998), adding to its attraction as a candidate for the sperm Ca^{2+}-releasing protein. When coinjected with the SH3 domain of PLCγ1, the ability of tr-kit to activate eggs is significantly reduced, suggesting that tr-kit activates PLCγ1 in some way that involves PLCγ1's SH3 domain. However, injecting the PLCγ1 SH3 domain into mouse eggs at a much higher concentration does not inhibit Ca^{2+} release at fertilization (Mehlmann *et al.,* 1998), indicating that tr-kit releases Ca^{2+} by a pathway differing from that operating at fertilization. Recombinant tr-kit protein has not yet been purified and shown to cause Ca^{2+} oscillations when injected into eggs; such experiments need to be done in order to test whether tr-kit has a role in initiating Ca^{2+} release at fertilization.

More recently, PLC, the enzyme that produces IP_3, has been implicated as the Ca^{2+}-releasing factor from sperm. In support of this hypothesis, injection of recombinant PLCγ1 protein into mouse eggs causes Ca^{2+} oscillations that closely resemble those seen at fertilization (Mehlmann *et al.,* 2001). PLCγ1, PLCγ2, and PLCδ4 are present in mammalian sperm (Dupont *et al.,* 1996; Tomes *et al.,* 1996; Mehlmann *et al.,* 1998; Wu *et al.,* 2001; Fukami *et al.,* 2001), and there is at least one report that PLCβ1 is present as well (Walensky and Snyder, 1995; but see Mehlmann *et al.,* 2001). Other PLC isoforms have not yet been identified in sperm. Sperm lysates have measurable PLC activity (Ribbes *et al.,* 1987; Tomes *et al.,* 1996; Jones *et al.,* 2000; Rice *et al.,* 2000; Mehlmann *et al.,* 2001), and boar sperm extracts cause the production of IP_3 when added to sea urchin egg homogenates (Jones *et al.,* 1998b; Rice *et al.,* 2000). The PLC inhibitor, U73122, inhibits Ca^{2+}-releasing activity in boar sperm lysates (Jones *et al.,* 2000; Wu *et al.,* 2001). However, these results are difficult to interpret because the concentration of U73122 used was higher than those concentrations shown to have nonspecific effects (see Echinoderms).

Of the PLCs present in sperm, PLCγ1, PLCγ2, and PLCδ4 are not present in fractions of sperm extracts containing Ca^{2+}-releasing ability (Heyers *et al.,* 2000; Wu *et al.,* 2001). In addition, sperm from mice lacking PLCδ4 cause Ca^{2+} release when injected into mouse eggs (Fukami *et al.,* 2001). It has been reported that the PLC activity in a single boar sperm is sufficient to generate enough IP_3 to activate an egg, based on incubation of sperm extract with PIP_2 and measurements of the IP_3 produced (Rice *et al.,* 2000). However, the concentration of PIP_2 present in the reaction mixture was not specified to be the same as that present in an egg, and may have been considerably higher. In another study, measurements of the PLC activity in a single mouse sperm were compared with the PLC activity in the minimum amount of PLCγ protein needed to cause Ca^{2+} release in mouse eggs. The PLC activity per sperm was found to be ~500–900 times less than the PLC activity in the amount of recombinant PLCγ required for Ca^{2+} release, indicating that sperm-derived PLC is not responsible for initiating Ca^{2+} release at fertilization (Mehlmann *et al.,* 2001).

Another potential candidate for the soluble sperm protein is NO synthase, which could cause production of NO in the egg and cause Ca^{2+} release through a cGMP pathway (Willmott *et al.,* 1996). One report suggests that NO might have a role in fertilization of sea urchin eggs (Kuo *et al.,* 2000; see Echinoderms). However, a subsequent study that examined a role for NO at fertilization of mammalian eggs indicates that NO is not the Ca^{2+}-releasing agent (Hyslop *et al.,* 2001).

One of the potential shortcomings of the aforementioned studies of a soluble egg-activating protein from the sperm is that the extracts are prepared from sperm that have not been incubated in conditions that promote the acrosome

reaction. Therefore, these extracts contain acrosomal contents that would not normally be present in the natural fertilization setting.

Recent experiments showing that injection of demembranated sperm heads activates mouse eggs have suggested that the sperm-derived egg-activating protein may be associated with the sperm perinuclear material (Kuretake *et al.*, 1996; Kimura *et al.*, 1998). Sperm heads treated with the detergent Triton X-100 are completely demembranated and lack the acrosomal vesicle and cytoplasmic contents, though they retain the perinuclear matrix that surrounds the nucleus. Injection of such sperm heads into mouse eggs causes polar body and pronuclear formation, followed by normal development; live offspring have been obtained in this way. Sperm heads treated with Triton X-100 followed by trypsin or SDS, which remove the perinuclear matrix (but might also inactivate a Ca^{2+}-releasing protein), are incapable of activating eggs (Kimura *et al.*, 1998). These results support the hypothesis that the factor from the sperm that activates eggs is associated with the sperm perinuclear material, but the active component in this material has not been identified (see Perry *et al.*, 1999, 2000). It is possible that the sperm contains both soluble proteins as well as perinuclear matrix-associated proteins that serve as redundant mechanisms to ensure that the sperm will be able to activate the egg. Alternatively, it is possible that the same egg-activating protein is present in both fractions.

In summary, current evidence favors the hypothesis that, during mammalian fertilization, a protein present in the sperm enters the egg cytoplasm as a consequence of sperm–egg fusion, and causes egg activation. However, the identification of this protein remains a problem for the next century of fertilization research.

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