

Flipping the Switch: How a Sperm Activates the Egg at Fertilization

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Sperm interaction with an egg in animals was first documented 160 years ago in sea urchins by Alphonse Derbès (1847) when he noted the formation of an “envelope” following the sperm’s “approach” to the egg. The “envelope” in sea urchins is an obvious phenotype of fertilization in this animal and over the past 35 years has served to indicate a presence of calcium released from cytoplasmic stores essential to activate the egg. The mechanism of calcium release has been intensely studied because it is a universal regulator of cellular activity, and recently several intersecting pathways of calcium release have been defined. Here we examine these various mechanisms with special emphasis on recent work in eggs of both sea urchins and mice. *Developmental Dynamics* 236:2027–2038, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

A new cellular potential is formed by the union of an egg and a sperm. The sperm supplies the paternal genome, and in most species the centriole, and the stimulus that activates the fertilized egg to develop into an embryo. Egg activation is a multi-faceted process that includes the initiation of the block to polyspermy (Wong and Wessel, 2006), resumption of meiosis and entry into the embryonic cell cycle, and changes in the expression of genes and their protein products within the zygote and early embryo (Whittingham, 1980; Whitaker and Steinhardt,

1982; Jaffe, 1983; Yanagimachi, 1994). The mechanism whereby the sperm activates the egg at fertilization has been a subject of interest for over a century, ever since pioneers such as Jacques Loeb and Frank Lillie began studying the process at the Marine Biological Laboratory at Woods Hole. The sea urchin has played a central role as a model organism for studying egg activation from those first studies to the present day, and is a key emphasis of this review (Fig. 1).

It was Loeb who first proposed, in 1898, that egg activation was a chemical process involving changes in the

concentration of ions within the egg (see Loeb, 1899), a bold idea given that many people at that time were still talking of embryo development in terms of “vital forces” (Pauly, 1987). Loeb’s proposal was based upon his discovery that sea urchin eggs could be induced to begin developing parthenogenetically, without the need for a sperm, simply by altering the proportion of ions in the solution bathing the eggs. Later, in 1902, Loeb suggested that the sperm initiated such a change in ionic composition in the egg by means of a “sperm factor” that was introduced into the egg during gamete

ABBREVIATIONS C2 conserved region 2 of PKC cADPR cyclic ADP ribose DAG diacyl glycerol DAG diacyl glycerol IP₃ inositol trisphosphate NAADP nicotinic acid adenine dinucleotide phosphate NO nitric oxide NOS nitric oxide synthase PH pleckstrin homology PIP₂ phosphatidylinositol (4,5)-bisphosphate PLC phospholipase C SFK Src family kinase SH2 Src homology domain 2.

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fusion. A different model of egg activation was put forward by Frank Lillie. He suggested that the process was triggered by an interaction between a sperm ligand and a receptor on the egg surface (Lillie, 1913). The importance of these pioneering scientists is demonstrated by the fact that these two alternative models are still the dominant frameworks for our understanding of the process of egg activation today.

Despite Loeb's pioneering suggestion that ionic changes were a key component of egg activation, it was not until much later that one ion in particular, calcium (Ca^{2+}), was shown to play the initiating role. The importance of Ca^{2+} was suggested during the 1930s by several investigators, most notably Daniel Mazia (Mazia, 1937), Lewis Victor Heilbrunn (see, e.g., Heilbrunn, 1952), and Floyd Moser (Moser, 1939). However, definitive evidence that a change in intracellular egg Ca^{2+} was the trigger for egg activation only emerged in the 1970s. A key step forward came with studies in which eggs were treated with the Ca^{2+} ionophore A23187 (Steinhardt and Epel, 1974; Steinhardt et al., 1974). This triggered egg activation, demonstrating that artificially raising intracellular egg Ca^{2+} induces events associated with egg activation (Steinhardt et al., 1974; Fulton and Whittingham, 1978). It was subsequently shown that a rise in Ca^{2+} within the egg accompanies fertilization (Ridgway et al., 1977; see, e.g., Stricker, 1999; Whitaker, 2006), and that blocking this rise in Ca^{2+} with chelating agents inhibited egg activation (Whitaker and Steinhardt, 1982; Kline, 1988; Kline and Kline, 1992). While many of these studies were first carried out on sea urchin eggs, it is now well established that Ca^{2+} acts as the trigger for egg activation in practically all species (Stricker, 1999; Whitaker, 2006). Despite being universally employed, the exact form of the Ca^{2+} signal at fertilization varies between animal groups: whereas a single wave of Ca^{2+} crosses the egg in sea urchins, frogs, and fish (Fig. 2), the sperm triggers a series of periodic increases that have been termed Ca^{2+} oscillations in mammals, nemertean worms, and ascidians (Stricker, 1999; Whitaker, 2006).

CALCIUM CONDUIT, SOLUBLE SPERM FACTOR, OR SURFACE INTERACTION?

Even though Ca^{2+} has been known to be the trigger of egg activation for several decades, the mechanism whereby the sperm induces a change in the concentration of Ca^{2+} within the egg remains to be fully resolved. Three major hypotheses dominate understanding of this process: (1) the Ca^{2+} bomb or conduit model; (2) the soluble sperm factor model; and (3) the membrane receptor model (Fig. 3; Whitaker and Swann, 1993; Evans and Kopf, 1998; Parrington, 2001). The first model was based around the idea that the sperm introduced a bolus of Ca^{2+} during gamete fusion (Jaffe, 1983, 1991), later modified to the suggestion that channels in the surface membrane of the sperm allowed it to act as a conduit for Ca^{2+} entry (Creton and Jaffe, 2001). Ca^{2+} introduced in this way would then act to trigger Ca^{2+} induced Ca^{2+} release (CICR) within the egg (Jaffe, 1991). Although attractive in its straightforwardness, and consistent with the finding that sperm-egg fusion in both the sea urchin and the mouse always precedes egg Ca^{2+} release (McCulloh and Chambers, 1992; Lawrence et al., 1997), a number of studies have now cast doubt upon the validity of this model. Counterevidence includes demonstration that injection of Ca^{2+} into sea urchin, Ascidian, or mammalian eggs fails to induce further Ca^{2+} release (Whitaker and Swann, 1993; Swann and Ozil, 1994), and that in the mouse, no elevation on the local cytoplasmic Ca^{2+} concentration occurs at gamete fusion, which would be expected if the sperm acted as a Ca^{2+} conduit (Jones et al., 1998).

The second model, which proposes that egg activation is triggered by a soluble factor released during gamete fusion from the sperm into the egg, was very controversial for many years due to the failure to identify such a factor at the molecular level. In mammals, this situation has greatly changed in the last few years with the discovery that the mammalian sperm factor appears to be a novel type of sperm-specific phospholipase C, PLC ζ (Saunders et al., 2002). While it has

now become generally accepted that PLC ζ is likely to be the physiological agent of egg activation in mammals (Swann et al., 2006), many questions remain about both its mechanism of action and whether such a sperm PLC plays a role during egg activation in other animals.

The third hypothesis, the membrane receptor model, was the dominant hypothesis for many years. Proposing that egg activation is triggered by an interaction between a ligand upon the sperm and a receptor on the egg (Jaffe, 1990; Schultz and Kopf, 1995; Evans and Kopf, 1998), this model had the virtue that such surface-mediated interactions are a normal feature of cell signaling in somatic cells. From the start, this model was centered around the idea that the signaling cascade triggered by such an interaction would involve activation of an egg PLC (Jaffe, 1990; Schultz and Kopf, 1995; Evans and Kopf, 1998). While studies in echinoderms and some other animals have pointed to a central role in egg activation for PLC γ (Townley et al., 2006), what remains far less clear is whether this is the consequence of a surface-mediated interaction between a sperm ligand and an egg receptor.

THE PHOSPHOINOSITIDE SIGNALLING PATHWAY

The PLC family of proteins are cytoplasmic enzymes that cleave phosphatidylinositol (4,5)-bisphosphate (PIP $_2$) at the plasma membrane to yield inositol triphosphate (IP $_3$) and diacyl glycerol (DAG), each of which regulate a variety of cell processes (Rhee, 2001). IP $_3$ may then bind its receptor, the IP $_3$ receptor, in the endoplasmic reticulum to release Ca^{2+} into the cytoplasm (see Fig. 5). At least six different subfamilies of PLC have been identified, including β , γ , δ , ϵ , and more recently also ζ and η (Rhee, 2001; Saunders et al., 2002; Nakahara et al., 2005). All PLC isoforms contain separate X and Y domains that come together to form the active site responsible for PIP $_2$ cleavage (Fig. 4). In addition to the X and Y domains, they also contain various combinations of regulatory domains, including PH domains (pleckstrin homology), SH2 domains (Src homology 2), and C2 do-

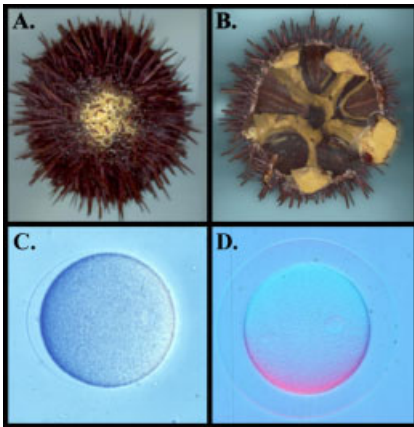


Fig. 1. **A:** Top view of the sea urchin *Strongylocentrotus purpuratus*, induced to shed eggs (orange mass in center). **B:** An adult opened to reveal the five lobes of ovary. **C:** An egg at the moment of activation showing the fusing sperm on the left, along with the rising fertilization envelop. **D:** The same egg 45 sec following insemination showing the fully formed fertilization envelope, and the two pronuclei within the egg.

mains (constant or conserved region 2 of PKC; Fig. 4).

The regulatory domains of PLC target different isoforms to their respective activators or substrates via protein–protein or protein–lipid interactions. The mechanism of PLC activation varies according to subfamily, and is dependent on the specific combination of regulatory domains present in each isoforms (see, e.g., Roux et al., 2006). PH domains bind polyphosphoinositides such as PIP_2 and PIP_3 , and thus serve to target the protein to the plasma membrane (Harlan et al., 1994; Rameh and Cantley, 1999). PH domains can also mediate interaction with and activation by the $\beta\gamma$ subunits of heterotrimeric G proteins (Camps et al., 1992; Smrcka and Sternweis, 1993; Lee et al., 1994). SH2 domains interact with receptor protein tyrosine kinases as well as with non-receptor tyrosine kinases like Src (Weiss, 1993; Noh et al., 1995). Finally, C2 domains bind Ca^{2+} and confer Ca^{2+} -mediated phospholipid-binding properties to the proteins that contain them (see below). Isoforms of $\text{PLC}\beta$, $\text{PLC}\gamma$, and $\text{PLC}\delta$ have been identified in eggs, while isoforms of $\text{PLC}\beta$, γ , δ , and most recently ζ , have been described in sperm (Dupont et al., 1996; Mehlmann et al., 1998; Wang et al., 1998; Wu et al., 2001; Parrington et al., 2002; Saunders et al., 2002).

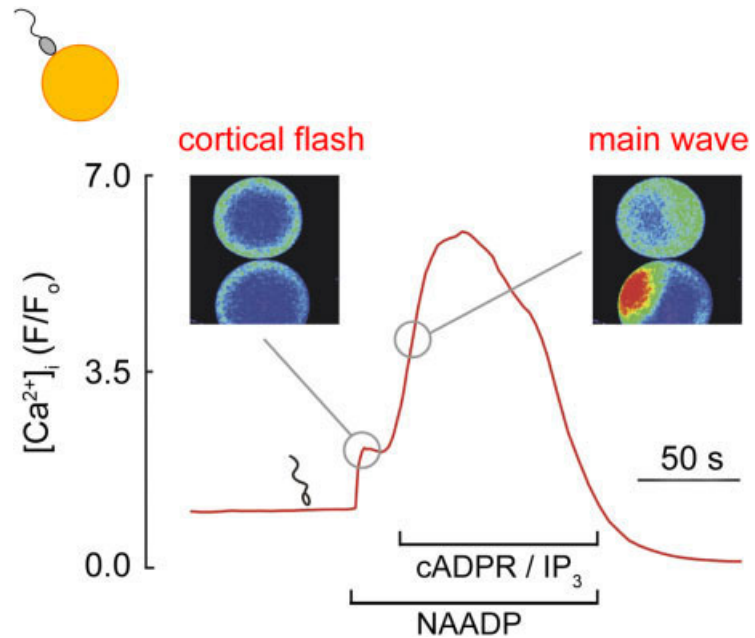


Fig. 2. Two phases of Ca^{2+} increase occur at fertilization, reflecting two spatially and temporally different events. The first increase is the cortical flash due to a rapid influx of Ca^{2+} across the plasma membrane. The second phase is due to Ca^{2+} release from intracellular stores, which propagates as a wave across the egg from the sperm entry point. These different phases of response rely on different Ca^{2+} mobilizing messengers. The cortical flash is uniquely reliant upon NAADP, whereas IP_3 and cADPR activate and prolong the Ca^{2+} spike, respectively. cADPR is mostly produced after the spike upstroke; however, this is not entirely clear because of messenger redundancy.

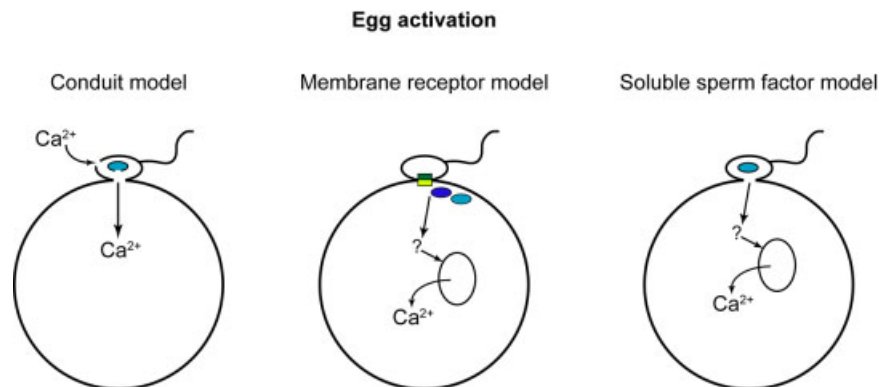


Fig. 3. The three major models of egg activation. The conduit model suggests that Ca^{2+} enters the egg via a Ca^{2+} channel opening in the sperm plasma membrane; the membrane receptor model suggests that a receptor activated by sperm binding relays a signal to initiate the Ca^{2+} release mechanism, and the soluble sperm factor model suggests that sperm deliver a molecule that initiates the Ca^{2+} release mechanism. See text for additional details.

THE ROLE OF THE PHOSPHOINOSITIDE PATHWAY DURING EGG ACTIVATION

The idea that the phosphoinositide pathway (PI) plays a central role during egg activation has been supported by a number of studies over the last two decades. Biochemical measurements in sea urchin and frog eggs

showed that there is an increased turnover of phospholipids and an increase in inositol trisphosphate (IP_3) levels at fertilization (Turner et al., 1986; Stith et al., 1994). Further evidence of a role for the PI pathway is the fact that the PLC inhibitor U73122 inhibits both Ca^{2+} release and egg activation in sea urchins and mice (Dupont et al., 1996; Lee et al., 1998) while egg activation in mice is

blocked by treatment with an antibody raised against the IP₃ receptor (Miyazaki et al., 1992). Additional evidence also shows that an egg PLC plays a central role during egg activation in echinoderms (Townley et al., 2006), and that a sperm PLC ζ is the physiological agent of egg activation in mammals (Swann et al., 2006).

THE IDENTIFICATION OF THE MAMMALIAN SPERM FACTOR AS PLC

The first evidence that a soluble sperm factor might be the trigger of egg activation came from a study in sea urchins, in which injection of a sea urchin sperm extract induced egg activation (Dale et al., 1985). A later study identified a similar putative egg activation factor in Ascidian sperm (Dale, 1988). It was not clear from these studies whether the activating stimulus was a protein or a small molecular weight Ca²⁺ mobilizing messenger. However, evidence for the existence of a soluble sperm protein that could mimic the ability to trigger a pattern of Ca²⁺ release similar to that seen at fertilization and induce egg activation emerged from a series of studies in which sperm extracts were injected into eggs of a variety of mammals, marine worms, and ascidians (Swann, 1990; Stricker, 1999; Kyozuka et al., 1998; Parrington, 2001). More recently, sperm extracts from frogs, chickens, and tilapia fish have also been shown to trigger Ca²⁺ oscillations in mouse eggs (Dong et al., 2000; Coward et al., 2003, 2005).

Many years of effort were spent trying to identify the sperm factor at the molecular level (Parrington, 2001). A breakthrough came with the discovery that the mammalian sperm factor was a sperm-specific PLC that had very distinctive enzymatic properties compared to other known PLCs (Jones et al., 1998, 2000; Parrington et al., 1999, 2002). This discovery was made possible by the use of a cell-free egg-based Ca²⁺ release assay, the sea urchin egg homogenate, which enabled a biochemical dissection of the sperm factor's mechanism of action in a way that would have been much more difficult using an intact egg. The discovery of an apparent sperm-specific factor initiated a search for a novel type

of PLC by analysis of cDNA databases, leading to the identification of PLC ζ (Saunders et al., 2002). Confirmation that PLC ζ was the long-sought-after mouse sperm factor came with the finding that recombinant PLC ζ RNA and protein trigger Ca²⁺ oscillations when injected into a mouse egg (Saunders et al., 2002; Kouchi et al., 2005). In addition, immuno-depletion of PLC ζ from sperm extracts abolished their ability to cause Ca²⁺ release (Saunders et al., 2002). Further studies have shown that the presence of PLC ζ in fractionated sperm extracts correlates with their ability to induce Ca²⁺ oscillations in the egg (Fujimoto et al., 2004; Kurokawa et al., 2005).

Current questions of interest about PLC ζ include its exact mode of action once within the fertilized egg. Recent studies have shown that PLC ζ appears to become sequestered in the pronuclei of the fertilized egg (Larman et al., 2004; Yoda et al., 2004), suggesting a possible role in mediating the cell-cycle dependency of Ca²⁺ oscillations in the early embryo, but the exact functional significance of this pattern of localization remains to be determined. Of great interest is how PLC ζ is stored and packaged in the sperm, and whether it plays a role during egg activation in other animal groups besides mammals. This latter point has support, as demonstrated in a recent study that identified a chicken orthologue of PLC ζ with similar sperm specificity and functional properties as mammalian PLC ζ (Coward et al., 2005), suggesting perhaps a conserved, widespread mechanism for egg activation in a number of vertebrates.

The definitive test remains as to whether PLC ζ is both necessary and sufficient for physiological egg activation in mammals. A recent study used RNA interference to create mouse sperm partially deficient in PLC ζ . These sperm had partial deficiencies in their ability to trigger Ca²⁺ oscillations in mouse eggs and to cause egg activation (Knott et al., 2005), suggesting that PLC ζ is necessary. However, a concrete answer will require the creation of a mouse knockout in PLC ζ , or the identification of a human infertility patient with no functional PLC ζ . The fact that some infertile men appear to produce sperm defi-

cient in the ability to trigger egg activation (Flaherty et al., 1998; Mahutte and Arici, 2003; Heindryckx et al., 2004; Yanagida, 2004) provides the possibility to further study the link between PLC ζ structure and function in mammals.

A PLC ζ homologue has not been identified in echinoderms or ascidians, so the controversy over the universality of a sperm-delivered PLC ζ as a master regulator of Ca²⁺ release and egg activation remains to be resolved. Instead, evidence suggests that egg activation in echinoderms and in some vertebrates, like amphibians, is mediated by a distinct mechanism, with an egg PLC γ playing a central role.

THE CENTRAL ROLE OF EGG PLC γ DURING EGG ACTIVATION IN ECHINODERMS

In species where a PLC ζ has not been identified, other members of the PLC family of proteins, mainly PLC γ , are essential mediators of Ca²⁺ release (Runft and Jaffe, 2000; Runft et al., 2002, 2004; Giusti et al., 2003). Fertilization in both echinoderms and frogs, for example, is associated with an increase in the activity of PLC γ (Fig. 5) (Rongish et al., 1999; Sato et al., 2000). Most of the functional evidence implicating PLC γ in the release of Ca²⁺ in invertebrate eggs has relied on the use of dominant-negative SH2 domains, of which PLC γ contains two, bind Src family kinases (SFKs), a group of tyrosine kinases that are well-known activators of PLCs. Injection of PLC γ SH2 domains into Ascidian and echinoderm eggs competes away Src binding and inhibits Ca²⁺ release at fertilization, suggesting that PLC γ is an important activator of the IP₃ pathway in invertebrates (Carroll et al., 1997; Giusti et al., 1999; Runft et al., 2002). In frogs, PLC γ associates with an SFK known as Ytk and becomes tyrosine phosphorylated shortly after fertilization (Sato et al., 2000). While injection of PLC γ SH2 domains into vertebrate eggs, including frogs and mammals, has no inhibitory effect on Ca²⁺ release pathways (Mehlmann et al., 1998; Runft et al., 1999, 2002), injection of recombinant PLC γ 1 can induce Ca²⁺ release in mouse oocytes (Mehl-

mann et al., 2001). The amounts of recombinant PLC γ 1 protein required to trigger these Ca $^{2+}$ oscillations are orders of magnitude higher than those contained in a single sperm head (Mehlmann et al., 2001), so while it clearly supports a role for the PLC enzymes during egg activation, it remains unclear what significance this finding has for the physiological mechanism of activation in mammals.

Much effort has focused on the elucidation of the mechanism(s) that lead to PLC γ activation. One possibility is that it is recruited to the plasma membrane and activated by a sperm-delivered factor (Runft et al., 2002). Alternatively, it is possible that PLC γ is activated by signaling mechanisms intrinsic to the egg, including tyrosine kinases and/or G proteins, both of which are known to regulate the activity of PLC γ isoforms. Both signaling pathways are present in the eggs of vertebrates and invertebrates (Runft et al., 2002), and although the two have usually been presented as mutually exclusive signaling pathways, such a premise is unnecessary. In fact, growing evidence suggests there is a high degree of interaction between G protein signaling and SFK pathways (Luttrell and Luttrell, 2004). SFK activation occurs in the context of large protein complexes in which SFKs can interact with other proteins, including membrane receptors and scaffolding proteins (Luttrell and Luttrell, 2004). In vertebrates for example, G proteins can directly stimulate SFK activity (Hall et al., 1999; Ma et al., 2000; Luttrell and Lefkowitz, 2002), and, in turn, SFKs can modulate the activity of G proteins by tyrosine phosphorylation (Bushman et al., 1990; Luttrell et al., 1992). An increase in tyrosine kinase activity has been reported following fertilization in sea urchins (Ciapa and Epel, 1991; Ciapa and Chiri, 2000) and, recently, the first sea urchin upstream activating Src family kinase, named SFK1, was reported (Giusti et al., 2003).

A ROLE FOR G PROTEINS IN EGG ACTIVATION

Heterotrimeric G proteins are conserved and ubiquitously expressed GTPases that mediate a variety of signaling events (Sprang, 1997). Each complex consists of three subunits, known as G α , G β , and G γ , with dis-

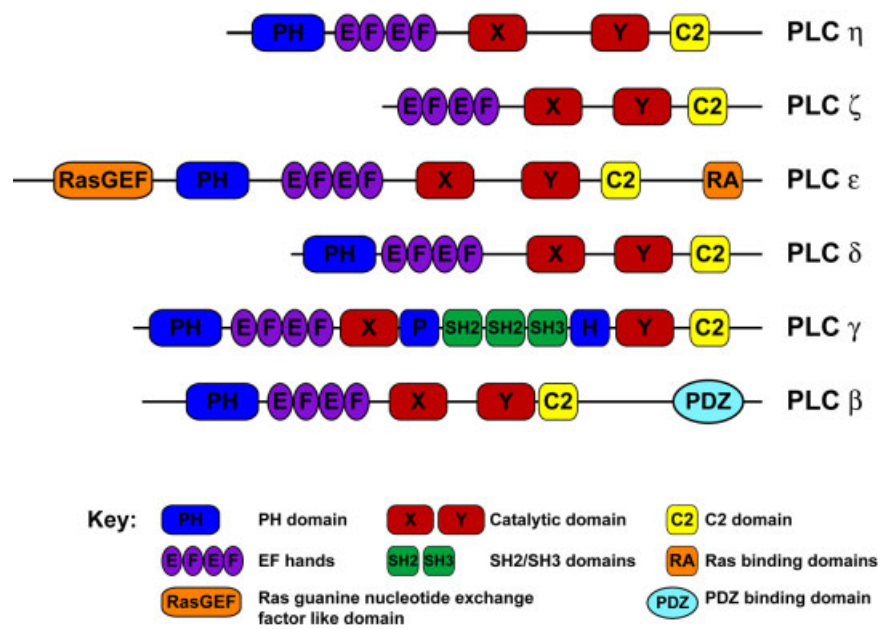


Fig. 4. Major structural features of various PLCs. At least six different subfamilies of PLC have been identified: β , γ , δ , ϵ , ζ , and η . All contain the X and Y catalytic domain; the C2 domain, which is involved in conferring Ca $^{2+}$ -mediated phospholipid-binding properties and interactions with G protein subunits; and EF hand domains, which are involved in binding Ca $^{2+}$. Other domains confer specific properties on the PLC isoforms that contain them, for instance SH2 domains in PLC γ allows it to interact with tyrosine kinases. PLC ζ is distinguished from other isoforms by its absence of a PH domain, the motif that mediates binding to phospholipids.

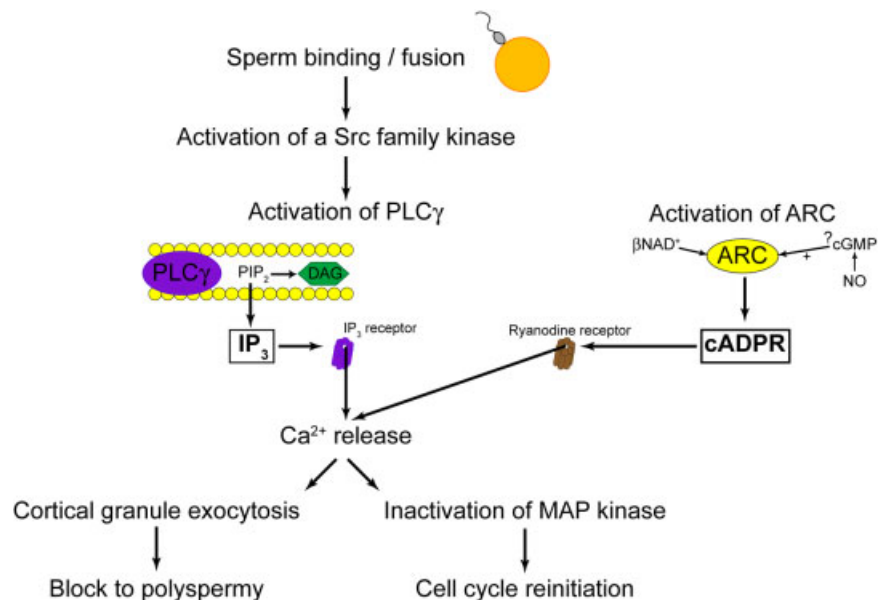


Fig. 5. A convergence of calcium release pathways resulting in a vast array of egg activation pathways. One of the central mediators of Ca $^{2+}$ release and egg activation in sea urchins is thought to be an egg PLC γ , which is activated by an upstream tyrosine kinase of the Src family. However, another Ca $^{2+}$ mobilizing messenger, cADPR, has also been shown to play a role during egg activation. It is generated by ADP-ribosyl cyclase enzymes, which can be activated by cGMP following activation by the NO signalling pathway.

tinct isoforms present in various tissues, with specific intracellular targets. The GTPase activity is present within the G α subunit: in its inactive

state, the α subunit of the heterotrimeric complex is bound by GDP. Upon exchange of GDP for GTP, the α subunit dissociates from the $\beta\gamma$ het-

erodimer, and each component is free to interact with and regulate the activity of distinct downstream targets. GTP hydrolysis results in the inactivation of the α subunit, which then re-associates with the $\beta\gamma$ heterodimer to reconstitute the inactive heterotrimeric complex.

The involvement of G proteins in Ca^{2+} release and egg activation events at fertilization has been suspected since PLC was shown to induce Ca^{2+} release via the IP_3 pathway (Fig. 5). Various data support a role for G protein signaling in the induction of Ca^{2+} release and egg activation, but the exact mechanism of action, or the contribution of each G protein, remains poorly understood. Early studies showed that sperm activates G proteins that stimulate the production of IP_3 , leading to Ca^{2+} release and cortical granule exocytosis in sea urchins (Turner et al., 1986). Activation of G proteins via the non-hydrolysable GTP analog GTP- γ S was also shown to cause Ca^{2+} release and cortical granule exocytosis in mammalian oocytes and in frog and echinoderm eggs (Cran et al., 1988; Jaffe et al., 1988; Miyazaki, 1988; Crossley et al., 1991; Fissore and Robl, 1994). In contrast, injection of the G protein antagonist GDP- β S inhibited Ca^{2+} transients in hamster and rabbit oocytes (Miyazaki 1988; Fissore and Robl, 1994). Later on, the G protein activator AlF_4 was shown to induce cortical granule exocytosis in mouse oocytes (Tahara et al., 1996).

The function of G proteins was addressed in a more specific manner using pertussis and cholera toxins, which inhibit $\text{G}_{\alpha i}$ and activate $\text{G}_{\alpha s}$, respectively. In mammals, neither pertussis nor cholera toxin had an effect on Ca^{2+} release (Miyazaki, 1988; Williams et al., 1992; Moore et al., 1994). This suggested that if G_{α} proteins were involved, they must belong to a pertussis and cholera toxin insensitive subfamily, which includes $\text{G}_{\alpha q}$ and $\text{G}_{\alpha 11}$. In frogs, neither $\text{G}_{\alpha q}$ nor $\text{G}_{\alpha 11}$ are essential for egg activation (Runft et al., 2002), but in sea urchins, $\text{G}_{\alpha q}$ and $\text{G}_{\alpha s}$ contribute to Ca^{2+} release pathways (Voronina and Wessel, 2004). Furthermore, it has been established that the effects of $\text{G}_{\alpha q}$ and $\text{G}_{\alpha s}$ signaling are transduced by the release of $\text{G}\beta\gamma$ subunits rather than by

activation of specific G_{α} subunits (Voronina and Wessel, 2004).

THE ROLE OF NITRIC OXIDE AND CYCLIC ADP RIBOSE DURING FERTILIZATION

Despite the prominence of the PI signaling pathway in models of egg activation, increasing evidence suggests that other intracellular Ca^{2+} signaling pathways also play important roles. The important Ca^{2+} mobilizing messenger cyclic ADP-ribose (cADPR) was first identified using the sea urchin egg homogenate assay (Clapper et al., 1987). Heparin, an IP_3 receptor antagonist, does not prevent the Ca^{2+} wave during sea urchin fertilization (Rakow and Shen, 1990; Crossley et al., 1991), an event that is shaped by cADPR (Fig. 2) (Galione et al., 1993; Lee et al., 1993). Further studies in sea urchin egg homogenates revealed that cADPR mediates Ca^{2+} release via the ryanodine receptor (Fig. 5) (Galione et al., 1991; Lee, 1993; Lee et al., 1995) (Fig. 5). The timing of cADPR production suggested that it might have a role during the later phase of the Ca^{2+} transient (Leckie et al., 2003). In line with this result, microinjection of an antagonist of cADPR, 8-amino-cADPR, decreased the duration of the fertilization Ca^{2+} transient, suggesting that cADPR plays a major part in sustaining the Ca^{2+} signal. This is in contrast to IP_3 production, which precedes, and coincides with, the fertilization Ca^{2+} wave, but which is short lived (Ciapa and Whitaker, 1986; Carroll et al., 1999; Thaler et al., 2004).

Precisely how sperm-induced egg activation leads to an increase in egg cADPR levels is at present unknown. cADPR is believed to be synthesized from βNAD^+ by ADP ribosyl cyclase. Most studies have focused on a role for cGMP and nitric oxide (NO) in mediating the increase in cADPR levels at fertilization via the activation of ADP-ribose cyclase. Injection of cGMP into sea urchin eggs resulted in a Ca^{2+} transient most closely resembling that of both fertilization (Whalley et al., 1992) and cADPR-induced prolongation of the Ca^{2+} signal (Galione et al., 1993; Lee et al., 1993; Leckie et al., 2003). NO, by virtue of its activation

of soluble guanylyl cyclase and hence cGMP production, could also evoke Ca^{2+} signals in sea urchin eggs via the cADPR signaling pathway (Willmott et al., 1996). Furthermore, inhibitors of ADP-ribose cyclase such as nicotinamide, and inhibitors of cGMP-dependent protein kinases inhibited cGMP-induced Ca^{2+} release (Sethi et al., 1996). These results indicate that cGMP mobilizes Ca^{2+} in sea urchin eggs by initiating a signaling pathway promoting cADPR synthesis (Fig. 5).

An important question still to be answered is where NO functions in the hierarchy of messengers involved in activation of the egg. While activation of Ca^{2+} release via the NO pathway and the ryanodine receptor does not lead to full egg activation in mammalian eggs, one report did suggest that NO may be the initiator of Ca^{2+} release in sea urchins (Kuo et al., 2000). This was based on the observation that microinjection of NO donors or of nitric oxide synthase (NOS), the enzyme responsible for NO production, was able to activate the egg, whereas microinjection of NO scavengers inhibited egg activation after fertilization. A more recent study, where levels of Ca^{2+} and NO were measured simultaneously, shows that NO-mediated Ca^{2+} release through the ryanodine receptor is a late event in the fertilization response in sea urchins (Leckie et al., 2003), suggesting that NO is not responsible for the initiation of the Ca^{2+} wave. Instead, these authors propose that activation of the NO pathway is needed to maintain the duration of the Ca^{2+} transient initially elicited via the IP_3 pathway. Nevertheless, the overall results clearly show that, at least in invertebrates, NO-mediated signaling is an important component of normal Ca^{2+} dynamics at fertilization.

Whether the NO pathway has any role in Ca^{2+} release in vertebrates remains to be conclusively established. One study using an antibody that recognizes type 2 and 3 ryanodine receptors, both of which are present in the mouse egg, concluded that Ca^{2+} release via these two receptor subtypes was not necessary for Ca^{2+} release in this species (Ayabe et al., 1995). Whether the type 1 ryanodine receptor could have an overlapping function though, it is not clear. A different

study that again simultaneously measured Ca^{2+} and NO levels in the egg showed that NO has no role at least in the initiation of Ca^{2+} release (Hyslop et al., 2001). Whether or not NO plays a role in the propagation of the Ca^{2+} wave, as it seems to do in invertebrates (Leckie et al., 2003), is still unknown.

An important unresolved question is what signaling mechanisms lie upstream of NO. NO is produced by the family of nitric oxide synthases (NOS). Upon activation, these enzymes catalyze the conversion of the amino acid L-arginine into NO and citrulline. Three distinct isoforms of NOS have been identified in mammalian tissues, including neuronal NOS (nNOS), which functions in the nervous system, endothelial NOS (eNOS), which functions in the cardiovascular system, and inducible NOS (iNOS), which functions in the immune system (Moncada et al., 1991; Dinerman et al., 1993; Kerwin et al., 1995). The relative contribution of each isoform to Ca^{2+} release pathways at fertilization remains unclear. Two distinct NOS cDNAs that are expressed in testes and ovary have been identified in sea urchins (Cox et al., 2001; Sodergren, 2006).

One proposed mechanism of activation for NOS proteins is via PKC-mediated phosphorylation (Matsubara et al., 2003), which places them downstream of PLC activation. Recall that cleavage of PIP_2 by PLC not only produces IP_3 , which induces Ca^{2+} release via the IP_3 receptor, but also DAG, which activates PKC (Fig. 5) (Liu and Heckman, 1998; Rhee, 2001). Thus, upon activation by DAG, PKC can phosphorylate NOS, which in turn produces NO and stimulates Ca^{2+} release via the ryanodine receptor (Fig. 5). The fact that PLCs produce both IP_3 and DAG upon cleavage of PIP_2 again places them at center stage because it suggests that PLCs are regulatory nodes that can induce Ca^{2+} release via both the IP_3 and NO pathways. Though it is tempting to speculate that a single protein may initiate all egg activation events, it is also likely that signal transduction pathways are complex networks of interactions that integrate and transduce diverse cellular signals. One

point of convergence for diverse cellular signals is NOS.

NOS can be activated not only by PKC-mediated phosphorylation, but also by Ca^{2+} and calmodulin (Matsubara et al., 2003). In fact, binding to calmodulin via the highly conserved calmodulin-binding domains is essential for NOS function (Cox et al., 2001; Li and Poulos, 2005) and different NOS isoforms bind calmodulin with different affinities. While nNOS and eNOS, which are expressed in a constitutive manner, bind calmodulin in a Ca^{2+} -dependent manner, iNOS, which is expressed upon induction of the immune response, binds calmodulin constitutively at physiological Ca^{2+} levels of about 100 nM (Li and Poulos, 2005).

Indirect evidence suggests that the NOS isoforms regulating NO production in eggs are nNOS or eNOS, or both. First, nNOS and eNOS are inhibited by the specific NOS antagonist N^G -nitro-L-arginine (L-NAME), but iNOS is not (Li and Poulos, 2005). In sea urchin eggs, NOS activity can be suppressed using L-NAME (Kuo et al., 2000). Second, given that egg activation is a Ca^{2+} -dependent process, it is likely that NO production in the egg is modulated by enzymes that are responsive to Ca^{2+} levels. Both nNOS and eNOS, for example, are sensitive to Ca^{2+} levels in a calmodulin-dependent manner (Michel and Feron, 1997). Calmodulin can regulate the activity of many other proteins at fertilization, including calmodulin-dependent kinase II (CaM kinase II). CaM kinase II activity undergoes oscillations following fertilization in mouse eggs (Markoulaki et al., 2003, 2004), and this activity is important for cortical granule exocytosis and progression through the cell cycle, based on evidence that shows inhibitors of calmodulin or CaM kinase II block both latter processes (Abbott and Ducibella, 2001; Tatone et al., 2002). Notably, CaM kinase II has the ability to regulate NOS function (Michel and Feron, 1997; Schneider et al., 2003). NOS proteins can also be phosphorylated by tyrosine kinases like Src (Li et al., 2004), suggesting possible cross-talk between various putative regulators of the Ca^{2+} release response in invertebrate eggs. Little is known about NOS function in

eggs, so a thorough understanding of the role of the NO pathway at fertilization will only be achieved once we identify the various mechanisms by which NOS can be activated in this cell.

A sea urchin homolog of the seven trans-membrane G protein-coupled receptor for histamine (suH₁R) was recently identified on the cell surface of sea urchin eggs that leads to NO production (Leguia and Wessel, 2006). Treatment of eggs with histamine causes fluctuations in the resting levels of NO in the egg, while antagonists or antibodies of H₁R inhibit the rise of NO normally observed at fertilization. Thus, inhibition of suH₁R function decreases the maintenance, but not the amplitude, of the Ca^{2+} transient and suggests that it is an integral part of the overall pathway leading to egg activation at fertilization in sea urchins.

The suH₁R of the egg impinges on multiple different signaling pathways, including heterotrimeric G-protein cascades, NOS activation, and Ca^{2+} release. It is unlikely that the phenotypes resulting from modifying suH₁R activity affected only one of its target signaling pathways. For example, although Ca^{2+} is clearly a target of suH₁R receptor activation, a resultant phenotype must be interpreted in the context of, at least, altered NO dynamics, and heterotrimeric G-protein activities, the vast majority of which are currently unknown. This is further complicated by the fact that each animal egg has unique requirements for each of these pathways, and minor alterations in the amplitude, timing, duration, and frequency of even one pathway such as Ca^{2+} , can have major consequences on fertilization and early development (Ozil et al., 2005). Thus, is it not possible to conclude that the decreased exocytosis of cortical granules observed when suH₁R is inhibited is solely the result of the decreased Ca^{2+} levels. This issue is further complicated in light of recent evidence showing that the G $\beta\gamma$ subunits of heterotrimeric G proteins can inhibit the Ca^{2+} -induced exocytotic machinery that mediates membrane fusion (Blackmer et al., 2005; Gerachshenko et al., 2005) perhaps by competing with the Ca^{2+} sensor synaptotagmin for binding of the C-terminal tail of SNAP-25, and/or by

regulating fusion pore dynamics (Schneider, 2001). Added to this equation are the effects of NO, which includes nitrosylation of other receptor and signaling proteins, so that inappropriate histamine receptor activation or repression could lead to the misregulation of multiple cellular targets, including Ca^{2+} -sensing proteins (Tao and English, 2003), ryanodine receptors (Xu et al., 1998), Ca^{2+} channels (Poteser et al., 2001), and fusion pore dynamics (Schneider, 2001; Thorn et al., 2004).

THE ROLE OF THE NAADP SIGNALING PATHWAY AT FERTILIZATION

A second important Ca^{2+} signalling messenger has been identified using the sea urchin egg homogenate assay, nicotinic acid adenine dinucleotide phosphate (NAADP) (Lee and Aarhus, 1995; Perez Terzic et al., 1995). Studies in the sea urchin egg first demonstrated that the NAADP-sensitive Ca^{2+} store has a distinct sub-cellular localization from that of the IP_3 or RyR/cADPR pathways (Lee and Aarhus, 1995; Genazzani et al., 1997) and that NAADP appears to target an acidic organelle such as the lysosome (Fig. 6). Stratification of egg organelles showed that the NAADP-sensitive store segregated to the opposite pole from the endoplasmic reticular stores (Lee and Aarhus, 2000). Furthermore, the use of a lysosome-disrupting agent (glycylphenylalanine 2-naphthylamide, GPN), inhibited NAADP from mobilising Ca^{2+} in both microsomes and intact eggs, without affecting the Ca^{2+} released by IP_3 or cADPR (Churchill et al., 2002). Additional evidence for a lysosome-like organelle NAADP-sensitive store was provided from fractionation of egg homogenates, which showed that the store co-distributed with lysosomal enzyme markers (Churchill et al., 2002). A key goal in the Ca^{2+} signaling field is now to identify the NAADP receptor.

NAADP also appears to play a role during fertilization in sea urchins. Microinjection of NAADP in intact sea urchin eggs induced a Ca^{2+} signal that, as previously found in sea urchin egg homogenates, was not blocked by heparin or by prior desensitization to

IP_3 or cADPR (Perez Terzic et al., 1995). Spatially resolved measurements of Ca^{2+} in intact sea urchin eggs (Churchill et al., 2003) and matured starfish oocytes (Santella et al., 2000; Lim et al., 2001; Moccia et al., 2003) revealed that NAADP-induced Ca^{2+} release is biphasic, consisting of an initial Ca^{2+} increase confined to the perimeter of the egg that is followed by a global Ca^{2+} increase, which extended through the egg cytoplasm. Removal of extracellular Ca^{2+} or addition of the Ca^{2+} channel blocker (Cd^{2+}) did not affect the second Ca^{2+} increase (consistent with Ca^{2+} release from intracellular stores), whereas the localized Ca^{2+} increase at the plasma membrane was abolished (Churchill et al., 2003). These data provide evidence that NAADP may stimulate Ca^{2+} entry across the plasma membrane, in addition to mobilizing intracellular Ca^{2+} stores (Patel, 2004).

Sperm, like NAADP, also stimulate a localized Ca^{2+} increase at the plasma membrane, termed the cortical flash (Fig. 2), which involves activation of voltage-sensitive Ca^{2+} channels (Shen and Buck, 1993) that may aid in establishing the so-called fast block to polyspermy (Jaffe, 1976; McCulloh and Chambers, 1992). Inactivation of NAADP receptors abolishes the cortical flash at fertilization, suggesting that NAADP and sperm-induced Ca^{2+} entry are mediated by similar means (Churchill et al., 2003). Supramaximal, micromolar NAADP levels were found in *Lytechinus pictus* sea urchin sperm (Billington et al., 2002) and importantly these levels markedly increase (~4-fold) upon contact with egg jelly (Churchill et al., 2003). These data raise the possibility that a concentrated source of NAADP is delivered into the egg as a bolus of messenger when the sperm fuses with the egg. Measurement of NAADP levels in fertilized eggs indicates that the increase in NAADP at fertilization occurs in two phases: an initial surge, which may be sperm-derived, and a second later increase peaking 4–5 min post-fertilization (Churchill et al., 2003), probably resulting from synthesis of NAADP within the egg.

THE SEA URCHIN EGG AS A MODEL FOR EGG ACTIVATION AND CALCIUM SIGNALING IN MAMMALS

Ultimately, the use of model organisms for studying processes such as fertilization and development assumes that findings made using such organisms will help further understanding of these processes in our own human bodies. Research into the mechanism of egg activation in sea urchins was of key importance in the light it shed on the central role of Ca^{2+} as the trigger of this process. At the same time, the last few years have led to a recognition that there may be critical differences in the signaling components employed to generate a Ca^{2+} signal in sea urchins compared to mammals. Thus, while it is now widely accepted that a key feature of echinoderm egg activation is the activation of an egg $\text{PLC}\gamma$ by a tyrosine kinase mechanism, such a mechanism does not appear to be conserved in mammalian eggs. Similarly, mammalian egg activation is thought to be triggered by a sperm phospholipase C, $\text{PLC}\zeta$, yet sequencing of the sea urchin genome has failed to identify an ortholog. These differences may reflect very real divergences in the demands made upon the egg activation signaling machinery in echinoderms and mammals.

It would be unwise, however, to judge the studies of signaling processes in the sea urchin egg purely in terms of their importance for our understanding of fertilization. The sea urchin egg has been essential for understanding intracellular Ca^{2+} signaling in general. Sea urchin egg homogenates, for example, have allowed the initial identification of the novel Ca^{2+} mobilizing messengers cADPR and NAADP, which play vital roles in the regulation of processes in the mammalian body as diverse as insulin secretion by the pancreas, cardiac function, and nerve growth and differentiation.

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