Egg Activation at Fertilization by a Soluble Sperm Protein

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The most fundamental unresolved issue of fertilization is to define how the sperm activates the egg to begin embryo development. Egg activation at fertilization in all species thus far examined is caused by some form of transient increase in the cytoplasmic free Ca$^{2+}$ concentration. What has not been clear, however, is precisely how the sperm triggers the large changes in Ca$^{2+}$ observed within the egg cytoplasm. Here, we review the studies indicating that the fertilizing sperm stimulates a cytosolic Ca$^{2+}$ increase in the egg specifically by delivering a soluble factor that diffuses into the cytosolic space of the egg upon gamete membrane fusion. Evidence is primarily considered in species of eggs where the sperm has shown to elicit a cytosolic Ca$^{2+}$ increase by initiating Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores. We suggest that our best understanding of these signaling events is in mammals, where the sperm triggers a prolonged series of intracellular Ca$^{2+}$ oscillations. The strongest empirical studies to date suggest that mammalian sperm-triggered Ca$^{2+}$ oscillations are caused by the introduction of a sperm-specific protein, called phospholipase C-zeta (PLCζ) that generates inositol triphosphate within the egg. We will discuss the role and mechanism of action of PLCζ in detail at a molecular and cellular level. We will also consider some of the evidence that a soluble sperm protein might be involved in egg activation in nonmammalian species.

I. INTRODUCTION

A. The Central Problem of Egg Activation

One purpose of fertilization is that the egg is stimulated to begin development. In most cases that have been studied, this stimulus for development is provided in some way by the sperm. The question then arises as to how the relatively small sperm stimulates such a large cell as an egg. When a fertilized egg, or oocyte, is stimulated to develop there are many characteristic changes, some morphological others biochemical, which are all brought together under the collective term “activation.” The term egg activation usually covers “early” events of the zygote, where “early” means within the first mitotic cell cycle (130, 180, 202). If we want to know how the sperm stimulates the egg to begin development, we need to answer the question of how the sperm provides the signals for activation.

One of the major events of “egg activation” is the completion of meiosis. In most animals the egg, or more correctly the oocyte, is arrested before fertilization at a particular stage of the meiotic divisions (17). In some selected species, such as clams, the oocyte is arrested at the prophase stage of meiosis before fertilization. In most invertebrate species, the oocyte is arrested at metaphase of the first meiotic division (170). In many vertebrate species such as amphibians and mammals, oocyte arrest occurs at metaphase of the second meiotic division (MI stage) (17, 180). In some other species, notably the sea urchin, the oocyte completes meiosis before fertilization and is therefore correctly called an egg. The “eggs” of many other species are, technically speaking, oocytes, since they have not completed meiosis. However, throughout this article we will use the term that is most commonly used in the field of study. For example, mature frog and mouse eggs are really oocytes, since they are arrested at MII, but we will refer to them as eggs since this is used by most researchers who want to distinguish it from the immature oocyte which is arrested at prophase. The completion of meiosis is evident in oocytes and “eggs” by the formation of one or two polar bodies (180). Often the completion of meiosis and the end of activation is also signified by the formation of male and female pronuclei which may then undergo fusion. The completion of meiosis is then followed by immediate entry into the first embryonic cell cycle with the initiation of the first S phase as one of the final events of egg activation (32, 180, 202).

Another event of egg activation, common to many species, is the exocytosis of cortical granules which leads to the release of factors or the modification of cellular structures around the egg (108, 204). This is generally part of a safety mechanism...
blocking the entry of supernumerary sperm, which is inconsistent with normal development in most animal species. Such exocytosis is generally a rapid response that occurs within minutes of sperm-egg fusion. Other significant changes in the egg that mark activation include increased metabolism and protein synthesis. The sea urchin egg represents an extreme case where metabolism and protein synthesis are minimal before fertilization due in part to the low intracellular pH, and at fertilization there is a large increase in metabolism, oxygen consumption, and protein synthesis (204).

One of the most remarkable points about egg activation is that it is invariably stimulated by a large increase in the cytosolic free Ca$^{2+}$ level. The central role of Ca$^{2+}$ is true for egg activation in both plants and animals (5, 26, 72, 94, 180, 202). In many species it has been shown that there is a large increase in cytosolic free Ca$^{2+}$ shortly after sperm-egg interaction. The precise profile of the Ca$^{2+}$ increase varies from one species to another. There are also variations in the source of Ca$^{2+}$ since it can come from intracellular stores, or from Ca$^{2+}$ influx through the plasma membrane. Whatever the source of Ca$^{2+}$, the sperm-induced increases in Ca$^{2+}$ have been shown to be necessary for egg activation in a number of species, such as sea urchins, frogs, newts, medaka fish, hamster, and mouse. This has been demonstrated by introducing Ca$^{2+}$ ion-specific chelators, such as EGTA or BAPTA, into the cytoplasm which prevents any Ca$^{2+}$ increase and any sign of egg activation (86, 87, 230). In species where the source of the Ca$^{2+}$ increase is Ca$^{2+}$ influx through the plasma membrane, removal of extracellular Ca$^{2+}$ has also been shown to prevent egg activation (28, 29, 79). In addition to the Ca$^{2+}$ increase being necessary, it has also been shown that artificially increasing egg cytosolic Ca$^{2+}$, either by microinjection of Ca$^{2+}$, electroporation in the presence of Ca$^{2+}$, or by addition of Ca$^{2+}$ ionophores, can mimic the sperm in triggering all the major events of activation (32, 33, 202, 204). Hence, a Ca$^{2+}$ increase acts as a generic parthenogenetic stimulus in the unfertilized egg.

Some of the key processes stimulated by Ca$^{2+}$ in eggs have now been identified. Increases in Ca$^{2+}$ stimulate calmodulin and its dependent kinases such as calmodulin-dependent protein kinase II (CaMKII). The stimulation of CaMKII ultimately affects cell cycle proteins that lead to meiotic resumption (32). Other protein kinases such as protein kinase C and myosin light chain kinase are implicated in events such as cortical granule exocytosis (32). The Ca$^{2+}$-activated protein phosphatase calcineurin is also critical for egg activation and meiotic resumption in some species such as *Xenopus* and *Drosophila* (94).

The role of Ca$^{2+}$ in egg activation is also the same even when the sperm is not the immediate stimulus for development. In some species, such as the *Drosophila* or shrimp, oocyte activation occurs during ovulation and, hence, is a separate event from fertilization (78, 94, 111). Nevertheless, even in these cases, there is clear evidence that increased Ca$^{2+}$ is the stimulus for egg/oocyte activation. Hence, egg activation always involves an increase in cytosolic Ca$^{2+}$, and this feature appears to be conserved throughout metazoan reproduction. In this review, we shall only consider species where the sperm produces the Ca$^{2+}$ signal for activation. This is known to be the case for mammals, most vertebrates, and the majority of studied marine invertebrates. The phenomenon of gamete fusion-mediated Ca$^{2+}$ signaling is also unique to fertilization. We focus on species where the source of Ca$^{2+}$ is intracellular stores, since these cases provide the strongest evidence for a soluble sperm protein mediating egg activation. While we shall consider studies from a number of different species, we mostly discuss data on mammalian eggs in detail where we present the case that a sperm protein called phospholipase C-zeta (PLCζ) is the factor that generates the Ca$^{2+}$ signals at fertilization.

## II. THE MISSING MESSENGER

### A. Ca$^{2+}$ Release Dynamics in Eggs at Fertilization

The best clues to understanding how the sperm initiates the Ca$^{2+}$ signal in the egg have come from studies of the timing and pattern of Ca$^{2+}$ increases at fertilization. For example, in sea urchins and probably many other invertebrates, there is a small initial subcortical Ca$^{2+}$ increase in the egg at fertilization due to an action potential-mediated influx of extracellular Ca$^{2+}$. This is followed by a much larger increase in Ca$^{2+}$, caused by intracellular release from stores that originate from the point of sperm-egg interaction and travels as a wave across the egg (180, 202, 204). The voltage-gated Ca$^{2+}$ influx in echinoderm eggs does not start the Ca$^{2+}$ wave. Instead, the Ca$^{2+}$ wave is a distinct process uniquely triggered by the sperm. The wave of Ca$^{2+}$ increase causes a wave of exocytosis and, in species such as the sea urchin and frog, it has been shown to be a self-propagating wave that travels through all depths of the egg cytoplasm (204). In oocytes from polychete worms and limpets, there is an increase that also appears to be driven by voltage-gated Ca$^{2+}$ influx, and that occurs all around the egg without any obvious Ca$^{2+}$ wave (28, 177). In these species, the Ca$^{2+}$ influx leads to egg (oocyte) activation. In annelid worms, there is a combination of themes with an oocyte phase of Ca$^{2+}$ release with a later increase in Ca$^{2+}$ in the whole oocyte driven by Ca$^{2+}$ influx (37, 79, 132). Conversely, in mussel oocytes, there is an influx of Ca$^{2+}$ followed by a wave of internal release (29). In other oocyte species such newts, there is a single large and slow Ca$^{2+}$ increase caused by Ca$^{2+}$ release, but it does not appear to involve a propagating Ca$^{2+}$ wave (58, 69). In these species, multiple sperm enter the egg (physiological polyspermy), and with each sperm entry there is an incremental increase in Ca$^{2+}$ that occurs in a large proportion of the egg cyto-
Finally, in eggs of many species including ascidians and mammals, there are a series of repeated transient rises in Ca$^{2+}$, or so-called Ca$^{2+}$ oscillations, as a result of release from intracellular stores (26, 34, 41, 64, 124, 179, 191). These Ca$^{2+}$ oscillations also occur as waves, and the first one generally starts from near the point of sperm entry (30, 34, 124). These observations suggest that, depending on the species, the sperm can cause either Ca$^{2+}$ influx, Ca$^{2+}$ release, or some combination of both. The different sources of Ca$^{2+}$ that drive egg and oocyte activation are illustrated in the schematic of Figure 1.

In eggs that show Ca$^{2+}$ oscillations at fertilization, the multiple waves of Ca$^{2+}$ release change their characteristics with time. This is most clearly seen in the fertilizing ascidian oocyte. Here the sperm first causes a large Ca$^{2+}$ wave followed by a short series of secondary oscillations that cross the egg from the point of sperm entry. After a delay of ∼10 min, there is a secondary series of Ca$^{2+}$ waves that are initiated from a specific region called the contraction pole which is situated on the vegetal side of the oocyte (opposite to the spindle). Mammalian eggs also display multiple Ca$^{2+}$ waves, although they are less obviously polarized than in ascidian oocytes. An example of the Ca$^{2+}$ oscillations seen at fertilization in a mouse egg is shown in Figure 2. The Ca$^{2+}$ oscillations in mouse eggs are typical of mammalian eggs in that they are of relatively low frequency, large amplitude, and persist for several hours after sperm-egg interaction. The first Ca$^{2+}$ wave in mouse or hamster eggs occurs near the region of sperm-egg fusion which then spreads across to the antipode in 5-10 s. In hamster eggs, there follow two successive waves within 5 min, and these originate from a more diffuse location away from the point of sperm-egg contact (123). The successive oscillations occur another 5 min later which is around the time of the second Ca$^{2+}$ wave in the mouse egg. These subsequent waves originate from different parts of the egg cytoplasm, with some bias towards the cortical region, and consist of very rapid waves that cross the egg in less than a second (30, 123). The pattern of waves suggests that the sperm induces a Ca$^{2+}$ wave source in the cytoplasm that starts from the sperm-egg fusion region and then spreads across the egg in 5–10 min. Once this wave-eliciting region has spread throughout the egg, the Ca$^{2+}$ wave is initiated from random cortical locations and spread 10 times more rapidly throughout the egg.

The reason for the different patterns of Ca$^{2+}$ increases, whether single or multiple Ca$^{2+}$ increase, or the reasons why increases are driven by Ca$^{2+}$ release or by Ca$^{2+}$ influx is not known. It should also be noted that mammalian egg Ca$^{2+}$ oscillations are in a rather special class of their own because they are low in frequency and persist for many hours. This is probably related to the fact that meiotic completion in mammals takes many hours. Whatever the Ca$^{2+}$ signal profile, its initiation occurs within seconds to minutes after sperm-egg interaction. One of the key issues to resolve is the sequence of events that precede the initial Ca$^{2+}$ release in the egg at fertilization.

### B. Sperm-Egg Fusion and the Signal Transduction Step

The precise timing between sperm-egg fusion and the start of Ca$^{2+}$ release has been most accurately timed by electrical recordings of the plasma membrane currents and capacitance (56, 202). This is possible in cases where the initial sperm-egg interaction leads to an inward current that triggers an action potential and the cortical “flash of Ca$^{2+}$.”
When the sea urchin sperm fuses with the egg membrane, McCulloh and Chambers found that they could measure a jump in capacitance caused by the fusion of the sperm and egg membrane. In an elegant series of experiments, McCulloh and Chambers showed that the initial sperm-induced inward current is a depolarization or cortical flash. However, there are also electrical capacitance measurements in mouse eggs supporting the idea that fusion occurs before Ca\(^{2+}\) release. When membrane capacitance and Ca\(^{2+}\) are monitored during fertilization in mouse eggs, there is a small step-like increase in capacitance that precedes the Ca\(^{2+}\) rise by ~1–2 min (107). Since it is reasonable to assume that the small capacitance jump is due to sperm-egg fusion, the data suggest a delay between gamete fusion and the start of the first Ca\(^{2+}\) wave is at least 1 min.

The measurements of electrical changes clearly indicate that some continuity is established at the very start of the fertilization process, at least with regards to ions and other small molecules. However, there are other lines of evidence that sperm-egg membrane fusion precedes the initial Ca\(^{2+}\) increase and involves the exchange of much larger molecules such as proteins. For example, the timing of sperm-egg fusion and Ca\(^{2+}\) release comes from confocal imaging with fluorescent Ca\(^{2+}\)-sensitive dyes. If dextran-linked Ca\(^{2+}\) dyes (~10 kDa) are injected into the egg before fertilization, the dye stays in the cytoplasmic space (103). However, when a sperm fertilizes such dextran-loaded eggs, the fluorescence of the dye can be seen to enter the sperm before a Ca\(^{2+}\) increase. The uptake of dye can only occur as a result of cytoplasmic continuity; hence, this observation denotes sperm-egg fusion. Further studies of this type showed that much larger molecules of up to 240 kDa could diffuse into the sperm before Ca\(^{2+}\) release was initiated (77). Consequently, these data demonstrate the sperm and egg have effective cytosolic continuity well before the first Ca\(^{2+}\) increase occurs.

If the sperm and egg are fused together before the start of the Ca\(^{2+}\) release in sea urchins and mice, then one possible idea to explain how the sperm causes Ca\(^{2+}\) release is that the sperm contains a soluble sperm factor that diffuses into the egg. This idea receives support from a clinical procedure, intracytoplasmic sperm injection (ICSI), designed to overcome infertility due to low sperm counts or low sperm quality. During ICSI, a sperm is injected into the egg cytoplasm, and this method is effective in activating human and mouse eggs and leads to normal development to term (149). Significantly, ICSI in human and mouse eggs has been shown to lead to the generation of Ca\(^{2+}\) oscillations similar to those seen at fertilization (134, 147, 193). These data show that sperm do not need to make contact with the outer face of the egg plasma membrane to trigger Ca\(^{2+}\) oscillations. The important agents in causing Ca\(^{2+}\) release appear to be located inside the sperm head because it has been shown damage sustained by the sperm before injection plays an important role in determining how soon after injection that the Ca\(^{2+}\) oscillations start (217). Interestingly, the ICSI procedure is able to cause Ca\(^{2+}\) release between different species (e.g., human sperm injected into mouse eggs); hence, the putative presence of a sperm factor causing
Ca\textsuperscript{2+} oscillations seems to be conserved, at least within mammals (219).

C. Signaling Molecules

One idea for signal transduction at fertilization is that the sperm acts rather like a giant hormone molecule and stimulates plasma membrane receptors that then generate second messengers to cause intracellular Ca\textsuperscript{2+} release (36, 45, 162). There are some cases where surface receptors are probably involved in triggering receptor-based Ca\textsuperscript{2+} release. For example, in the annelid worm Pseudopotamilla occelata, the sperm causes an initial Ca\textsuperscript{2+} increase in the oocyte when it contacts the vitelline envelope (133). There is evidence that sperm contain an extracellularly active soluble peptide that can cause an initial Ca\textsuperscript{2+} increase that is required for a cytoplasmic projection that leads to sperm-egg fusion (133). This, therefore, is a soluble sperm factor that causes Ca\textsuperscript{2+} increase, but this initial increase does not activate the oocyte. There is a second phase of Ca\textsuperscript{2+} influx following sperm-egg fusion that actually activates the oocyte (133). Consequently, this is not a soluble egg-activating factor. We shall not discuss the idea of a receptor mechanism here in any more detail since it has been critically appraised many times elsewhere (79, 190, 202). It suffices to say that no physiological molecules (as opposed to synthetic peptides) have been identified on sperm that can trigger Ca\textsuperscript{2+} release in mammalian or sea urchin eggs. The egg plasma membrane receptors for sperm in mammals that have otherwise been identified seem to be linked to sperm-egg fusion rather than signal transduction leading to Ca\textsuperscript{2+} release (148). Furthermore, the G proteins or tyrosine kinases that are supposed to link membrane receptors to InsP\textsubscript{3} production in the egg are not involved in mouse egg fertilization (120, 207).

The way that most cells generate Ca\textsuperscript{2+} increases in response to hormones is via increased production of inositol 1,4,5-trisphosphate (InsP\textsubscript{3}), which is made after the enzymatic breakdown of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) (8). Studies in the sea urchin egg first demonstrated that an increased PIP\textsubscript{2} turnover occurred at fertilization and that injection of InsP\textsubscript{3} into sea urchin eggs causes a regenerative Ca\textsuperscript{2+} wave (19, 158, 189, 195). In the frog egg, InsP\textsubscript{3} was shown to increase at fertilization and that InsP\textsubscript{3} injection also triggers a Ca\textsuperscript{2+} wave, just like fertilization, that takes ~5 min to cross the egg (12, 145, 176). Additionally, InsP\textsubscript{3} injection was found to trigger a Ca\textsuperscript{2+} increase in hamster eggs and that sustained injection of InsP\textsubscript{3} causes Ca\textsuperscript{2+} oscillations in hamster and mouse eggs (50, 122, 185, 187). InsP\textsubscript{3} injection has also been shown to trigger a Ca\textsuperscript{2+} wave or oscillations in oocytes or eggs from a number of other mammalian and marine invertebrate species (29, 43, 34, 79, 106, 180, 196). In addition to InsP\textsubscript{3}, other molecules that might act as second messengers, such as cADPR and NAADP, have also been shown to cause Ca\textsuperscript{2+} release in sea urchin eggs and starfish oocytes (48, 49). It is not clear how these might be linked to sperm-egg interaction, even in a receptor-based theory, since there is currently no established pathway for generating these Ca\textsuperscript{2+} releasing agents in the cytosolic compartment of cells (48).

In mammalian eggs, and some invertebrate eggs, it is very difficult to obtain enough material to accurately measure InsP\textsubscript{3} increases biochemically. However, the generation of InsP\textsubscript{3} in mammalian eggs can be assessed indirectly by monitoring the downregulation of InsP\textsubscript{3} receptors (10, 73). After fertilization in mouse and bovine eggs, the number of InsP\textsubscript{3} receptors significantly decreases due to proteolysis. This decrease in receptor level is detectable in groups of eggs and occurs a few hours after the start of Ca\textsuperscript{2+} oscillations (113, 114). Such “downregulation” has been shown to be strictly dependent on the generation of InsP\textsubscript{3} and binding of InsP\textsubscript{3} to its receptor since it does not, for instance, occur in eggs undergoing Ca\textsuperscript{2+} oscillations in response to Sr\textsuperscript{2+} which directly stimulates opening of InsP\textsubscript{3} receptor (10, 73, 113). However, decreased InsP\textsubscript{3} receptors can be readily detected after injecting adenophosphin, which binds with high affinity to the InsP\textsubscript{3} receptor. Hence, the observed downregulation of InsP\textsubscript{3} receptors in itself illustrates that an increase in InsP\textsubscript{3} occurs at fertilization. Furthermore, InsP\textsubscript{3} receptor downregulation can be artificially achieved by microinjecting adenophosphin into immature mouse oocytes that are subsequently allowed to undergo maturation in vitro. This generates mature eggs that are lacking InsP\textsubscript{3} receptors. When these eggs are fertilized, there are no Ca\textsuperscript{2+} oscillations, so it is clear that InsP\textsubscript{3} receptors are essential for generating Ca\textsuperscript{2+} oscillations at fertilization in mammalian eggs (10). It is also notable that in mouse and hamster eggs, a monoclonal antibody against the InsP\textsubscript{3} receptor has been shown to block both InsP\textsubscript{3}- and sperm-induced Ca\textsuperscript{2+} oscillations, so there is a strong case for InsP\textsubscript{3} being the only signaling molecule necessary to cause Ca\textsuperscript{2+} oscillations in fertilizing mammalian eggs (124).

D. Soluble Sperm Factors

The simplest idea to explain why Ca\textsuperscript{2+} release follows shortly after sperm-egg membrane fusion is that the sperm contains some soluble factor that triggers Ca\textsuperscript{2+} increases in the egg (180, 184). The factor is proposed to be soluble in the sense that it diffuses into the egg cytosol after gamete fusion. There could be a number of molecules in sperm that represent the factor that triggers Ca\textsuperscript{2+} release in eggs. One idea was that Ca\textsuperscript{2+} itself was effectively the “sperm factor,” or at least that Ca\textsuperscript{2+} influx across the sperm membrane might provide a conduit for the entry of extracellular Ca\textsuperscript{2+} which could trigger further Ca\textsuperscript{2+} release from intracellular stores (72, 218). However, there is direct evidence against this sperm conduit proposal in mammals. The moments leading up to and during the first Ca\textsuperscript{2+} increase have been measured in mouse eggs. It was shown that the Ca\textsuperscript{2+} in-
crease is first initiated within the egg cytoplasm and that the sperm has a low Ca\(^{2+}\) level when it fuses with the egg (77). Moreover, the initial Ca\(^{2+}\) release can also occur in the absence of extracellular Ca\(^{2+}\) (77). In mammals, it had also proved impossible to mimic the oscillations at fertilization by simply injecting Ca\(^{2+}\) into the egg in various ways (64, 185). Microinjecting Ca\(^{2+}\) is ineffective at triggering the Ca\(^{2+}\) wave in sea urchin eggs (189) and does not mimic the sperm effectively in frog eggs (11). Finally, it has also been shown that voltage clamping sea urchin eggs at very negative potentials, which should enhance Ca\(^{2+}\) influx, does not lead to reduction in the latent period, but instead causes the sperm to “unfuse” with the egg membrane (118). Hence, influx of Ca\(^{2+}\) into the egg via the sperm has to be minimized to maintain successful gamete fusion.

Another possible candidate for a soluble sperm egg-activating factor was InsP\(_3\) itself, which can trigger a regenerative Ca\(^{2+}\) wave in frog and sea urchin eggs (11, 198). Since the discovery of InsP\(_3\), there have been a number of other small molecules identified that can cause Ca\(^{2+}\) release in the sea urchin egg and that could be a candidate soluble sperm factor that enters the egg after sperm-egg fusion, such as cyclic ADP ribose (cADPR), NAADP, and cGMP (18, 49, 201). It has also been specifically suggested that nitric oxide (NO) could be the diffusible factor produced by the sperm to trigger Ca\(^{2+}\) release at fertilization in sea urchin eggs (95). However, NO does not cause Ca\(^{2+}\) release in mouse or ascidian eggs (63). In addition, while cADPR can trigger a Ca\(^{2+}\) wave in medaka fish eggs (44), novel Ca\(^{2+}\) releasing agents such as cADPR or NAADP do not appear to be effective in causing Ca\(^{2+}\) release in mammalian eggs, frog eggs, or ascidian oocytes (88, 215, 225). The only Ca\(^{2+}\)-mobilizing messenger molecule that causes Ca\(^{2+}\) release in every type of egg studied is InsP\(_3\).

In hamster and mouse eggs, injecting physiological amounts of InsP\(_3\) causes a single large Ca\(^{2+}\) increase within a second (88, 122). However, since the sperm causes prolonged, low-frequency oscillations, it is difficult to envisage how a single bolus of InsP\(_3\) alone could explain the characteristic Ca\(^{2+}\) oscillations at fertilization. Instead, the relevant mechanism involving InsP\(_3\) could involve some form of sustained or periodic production of InsP\(_3\). Prolonged injection of InsP\(_3\) or photo-activated release of InsP\(_3\) over a period of time does lead to a series of Ca\(^{2+}\) oscillations (76, 187). However, even in these cases, and particularly for hamster eggs, the oscillations again tend to be smaller and of higher frequency than those at fertilization (187). Injecting the InsP\(_3\) mimetic adenophostin into ascidian oocytes can cause a sustained series of Ca\(^{2+}\) oscillations, again suggesting that if InsP\(_3\) were to be produced in the egg it could account for the Ca\(^{2+}\) oscillations. However, as with mammals, the pattern of these oscillations is not exactly like fertilization, where there are two distinct series of cell cycle-dependent Ca\(^{2+}\) oscillations (119, 225). These data suggest that if a sperm factor could generate InsP\(_3\), it may need to make InsP\(_3\) in a way that is modulated in space and/or time to correlate with the specific pattern of oscillations at fertilization.

The first reproducible data that established the nature of a sperm-derived factor came from studies of sperm extracts injected into mammalian eggs. It was found that the microinjection of sperm extracts prepared from the cytosolic fraction of hamster or boar sperm could trigger a prolonged series of Ca\(^{2+}\) oscillations in hamster, human, or mouse eggs (184). These oscillations are remarkably similar to those seen at fertilization and do not diminish in amplitude with time. In the same year, the injection of soluble sperm extracts was also shown to cause egg activation events, such as second polar body emission and pronuclear formation (178). These data suggest that mammalian sperm contains some soluble protein(s) that can trigger the Ca\(^{2+}\) oscillations seen at fertilization. Many of these experiments in mammalian eggs were repeated and extended to other mammalian and nonmammalian species by independent groups (42, 62, 110, 112, 146, 210, 211, 212). They were also repeated in nemertean worms, whose oocytes also displayed Ca\(^{2+}\) oscillations characteristic of fertilization after sperm extract injection (179). Injection of soluble ascidian sperm extracts also causes Ca\(^{2+}\) oscillations in ascidian oocytes, and the sperm extract-induced oscillations have two distinct phases, just like those at fertilization (100, 161). In all of these cases, with different species, the relevant factor causing the Ca\(^{2+}\) oscillation was shown to be protein-based, since it was of high molecular weight, and also heat- and protease-sensitive. Such Ca\(^{2+}\) oscillation-inducing activity is only found in sperm extracts, and not in extracts made from a range of other tissues (179, 184).

The sperm factor has been generally referred to as a “soluble” factor because of its presence in the cytosolic fraction of sperm extracts. However, studies of ICSI with mouse sperm suggested that the factor might not be soluble. Injection of mouse sperm into mouse eggs causes egg activation, but the active factor was found to be associated with the sperm head (83, 153, 183). The active factor could only be extracted from mouse sperm heads by extensive incubation in reducing conditions. It was suggested that this factor (referred to as SOAF, for sperm-borne oocyte activating
The primary action for an egg-activating sperm factor is that it can cause Ca\(^{2+}\) oscillations in eggs. So the first attempts to identify the sperm factor protein involved protein fractionation using column chromatography and the subsequent assay of discrete fractions by microinjection into mouse eggs and measuring Ca\(^{2+}\) (152). A 33-kDa protein with sequence homology to glucosamine deaminase correlated with activity and was proposed to be part of the active factor (152). However, subsequent studies showed that the recombinant version of this protein did not cause Ca\(^{2+}\) oscillations in mouse eggs and that it did not always correlate with Ca\(^{2+}\) mobilizing activity (150, 208). Other proteins were also suggested to be a potential sperm factor. A truncated and cytosolic form of c-kit receptor, tr-kit, was proposed to be the sperm factor since its injection into mouse eggs was shown to cause egg activation (169). Subsequent studies suggested that tr-kit activated mouse eggs via activation of Src-like kinases, since its ability to activate receptors derived from a testis cDNA library revealed the identification of a novel isoform of PLC. Analysis of expressed sequence tag (EST) database sequences derived from a testis cDNA library revealed the existence of a novel PLC isoform that, when fully sequenced, was distinctive in having no PH domain (168). This novel testis PLC was smaller than all other mammalian PLCs and was named PLC-\(\zeta\) (PLC\(\zeta\)). Initial studies showed that PLC\(\zeta\) was a \(\sim 70\) kDa protein that was present as RNA in spermatids and only found as a protein in mature sperm (168). To test the effect of this novel PLC, the cRNA encoding PLC\(\zeta\) was microinjected into mouse eggs. After a short delay for protein synthesis in the egg, PLC\(\zeta\) was found to cause a prolonged series of Ca\(^{2+}\) oscillations very similar to those at fertilization, and to those seen after injecting cytosolic sperm extracts (168). Importantly, recombinant PLC\(\zeta\) is readily expressed in eggs by injection of cRNA, and protein expression can be monitored by fluorescent or luminescent protein tags on either the NH\(_2\) or COOH terminus of PLC\(\zeta\) (136, 220, 228). Microinjecting PLC\(\zeta\) cRNA was then shown to cause Ca\(^{2+}\) oscillations and to efficiently activate development of mouse, human, pig, bovine, and horse eggs, up to the embryonic blastocyst stage (6, 7, 157, 159, 160, 166, 188, 221, 222). An example of a mouse egg undergoing Ca\(^{2+}\) oscillations after microinjection of PLC\(\zeta\) cRNA is shown in Figure 3. Furthermore, the PLC\(\zeta\) in sperm extracts can be specifically depleted using antibodies, and this leads to a loss of Ca\(^{2+}\)-mobilizing activity of extracts either in intact mouse eggs, or in the sea urchin egg homogenate (163). Hence, the previously described soluble sperm factor in mammals correlates with all the observed properties of sperm PLC\(\zeta\). Table 1 shows the different stud-
ies that have been carried out to show that PLCζ is able to trigger Ca^{2+} increases in homologous or heterologous species of eggs.

Following the identification of PLCζ, the nature of the “insoluble” factor (SOAF) that causes egg activation in mouse ICSI was investigated. For these experiments, sperm were treated with Triton to remove membrane proteins and readily soluble intracellular proteins. The active component of sperm was then extracted using reducing agents and separated using column chromatography (47). Analysis of the active fractions found that mouse sperm PLCζ alone correlated with the ability to activate mouse eggs (47). These data resolve previous issues by showing that the poorly soluble mouse PLCζ also has the properties of a sperm factor that causes egg activation and Ca^{2+} oscillations after mouse ICSI. The data suggest there are differences in solubility between species, because hamster sperm has a soluble PLCζ, while others such as mouse have a less soluble sperm PLCζ that is bound to the sperm head. Boar sperm may represent an intermediate case since the PLCζ in boar sperm can be partially extracted by simply breaking the sperm by sonication, for example (98, 99). However, the remaining PLCζ remains bound to the sperm head and can only be extracted with high pH treatment, suggesting that a fraction of PLCζ in the sperm may be insoluble in standard salt buffers.

As expected for any PLC isoform, the sperm PLCζ does cause Ca^{2+} release via the generation of InsP_{3} because microinjection of PLCζ with a point mutation in its catalytic domain that is predicted to abolish enzyme activity, does not cause any Ca^{2+} oscillations in eggs (38, 168). In addition, microinjection of recombinant PLCζ protein that is enzymatically active also causes Ca^{2+} oscillations similar to fertilization and to oscillations triggered by PLCζ cRNA injection (92, 144). Furthermore, injection of PLCζ has been shown to lead to the downregulation of InsP_{3} receptors, which is a direct indication that InsP_{3} is being generated in the mouse egg (106). PLCζ injection into eggs in which Ca^{2+} rises are prevented by the Ca^{2+} chelator BAPTA (106) also leads to InsP_{3} receptor downregulation. Hence, PLCζ can generate InsP_{3} in eggs even in the absence of any Ca^{2+} rise.

B. Evidence for the Role of PLCζ in Fertilization

There are several lines of evidence that PLCζ plays a direct role in fertilization in mammalian eggs. The first comes from a quantitative analysis of the PLCζ in sperm. The amount of PLCζ expression required to cause a fertilization-like series of Ca^{2+} oscillations in mouse eggs, estimated using Myc-tagged PLCζ, is 30–50 fg (168, 136). Independent studies using different antibodies to native PLCζ have concurred that the level of PLCζ in a single mouse sperm is estimated at 20–50 fg (47, 168). The considerable overlap for these two estimates shows that the amount of PLCζ in a single mouse sperm is within the same range as the amount that produces normal Ca^{2+} os-

<table>
<thead>
<tr>
<th>Species of PLCζ</th>
<th>Egg/Oocyte Species</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Mouse, cow, horse</td>
<td>7, 20, 92, 159, 168</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse</td>
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<td>Human</td>
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<td>Cow</td>
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<td>Pig</td>
<td>Pig, mouse</td>
<td>188, 221, 222</td>
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<tr>
<td>Horse</td>
<td>Mouse</td>
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<tr>
<td>Monkey</td>
<td>Mouse</td>
<td>25</td>
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<td>125</td>
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<td>Chicken</td>
<td>Mouse</td>
<td>23</td>
</tr>
<tr>
<td>Medaka (fish)</td>
<td>Mouse</td>
<td>68</td>
</tr>
</tbody>
</table>
cillations (168). The precise frequency of oscillations is sensitive to the amount of PLCζ, which fits with the observations that when more than one sperm fuses with the egg, there is a small but measurable increase in frequency (40). Other studies suggest that there is around 130 fg of PLCζ in bull sperm and at least 350 fg PLCζ in pig sperm (20, 99). These higher values appear reasonable since these species’ eggs have a greater volume, and hence, a soluble protein like PLCζ will be diluted into a greater volume after fertilization. Consequently, the presence of PLCζ in sperm can be said to provide a sufficient explanation for the generation of Ca2+ oscillations at fertilization in mammalian eggs.

The way the Ca2+ waves at fertilization start can also be explained by the character of PLCζ and its diffusion into the egg after sperm-egg fusion. As mentioned above in hamster eggs, the source of the Ca2+ waves, essentially a trigger zone, spreads across the egg in 5–10 min (123). This spread is consistent with the diffusion of a molecule with a mass such as PLCζ (~70 kDa), which would take ~400 s to diffuse 70 μm in cytoplasm. We assume here that hamster sperm PLCζ starts to diffuse away from the sperm as soon as gamete fusion occurs. This seems reasonable since the hamster sperm factor is very soluble, and the estimates of the time between fusion and the first Ca2+ transient is ~10 s (64, 65). In the mouse, sperm-egg fusion is followed by a delay of a few minutes before the initial Ca2+ increase (103). This delay is determined by the species of sperm, since fusion of mouse sperm with hamster eggs is characterized by a similar delay of several minutes between sperm-egg fusion and the first Ca2+ transient (65). The delay may be due to the lower solubility of mouse PLCζ which may require some time of exposure to reducing conditions in the egg for it to start to be released by the sperm (47). By carrying out ICSI and then removing the sperm at later time points, it has been shown that up to 90 min is required for all the PLCζ to be removed from the mouse sperm head (223). This period matches that for the release of Ca2+ oscillation-inducing activity previously described for mouse sperm (89).

Another key feature of Ca2+ oscillations, in mouse eggs at least, is that they stop just before the formation of the pronuclei (116). Studies of fertilizing mouse eggs show that pronuclear formation is necessary for the sudden cessation of Ca2+ oscillations (116). There are then a further short series of Ca2+ oscillations during the first mitosis in the mouse zygote that are triggered shortly after nuclear envelope breakdown. This subsequent pattern is unique to fertilization because the mitotic Ca2+ transients, for example, are not seen in artificially activated mouse eggs (91, 116). It had already been suggested previously that the sperm factor might localize to the pronuclei in activated mouse eggs and that its release during the first mitosis could explain the occurrence of Ca2+ oscillations in mitotic zygotes (91). Observations of PLCζ seem to fulfill this type of behavior. Localization studies of PLCζ have found that it enters the pronuclei when they form after successful egg activation (66, 102, 220). Furthermore, within the amino acid sequence of mouse PLCζ there is a KKRKRK polybasic region that is a predicted nuclear localization sequence. Changing specific positive residues in this sequence blocks the pronuclear localization of PLCζ (66, 102). This disruption of PLCζ’s nuclear localization also leads to additional Ca2+ oscillations persisting for some hours after pronuclear formation. These data support the idea that the PLCζ undergoes nuclear localization, and this may be the immediate cause of the cessation of Ca2+ oscillations. They also illustrate how a subtle feature of the pattern of Ca2+ oscillations in mouse eggs is replicated and explained by the properties of sperm PLCζ. However, it is worth noting that whilst the nuclear localization of PLCζ occurs in mouse, it is not always so evident with PLCζ from other species (147). Bovine, horse, and human PLCζ do not undergo nuclear localization in mouse eggs or cell lines (68, 166). It is not yet clear what might determine the cessation of Ca2+ oscillations in these species.

One issue to resolve for PLCζ is whether it can be shown to be solely responsible and essential for egg activation at fertilization. The standard way to address this problem is to make a PLCζ “knockout” mouse. So far there is just one preliminary report of a PLCζ-null mouse (67). The PLCζ-null males were sterile, but this was due to a lack of spermatids resulting from mouse spermatogenesis failure. Hence, absence of mouse PLCζ appears to prevent the formation of morphologically mature sperm. It is not clear why this might be the case. Since mouse spermatids normally lack egg-activating ability, it has not proved possible for a PLCζ-null mouse to establish the role of PLCζ at fertilization in a definitive manner. A different approach has been successfully used to knock-down the level of PLCζ in sperm (90). Transgenic mice have been generated that express interfering RNA that reduced the expression of PLCζ protein in sperm. During in vitro fertilization, the sperm from these transgenic mice caused a reduced number of Ca2+ oscillations in eggs compared with control sperm (90). The male mice used in this study would have displayed a high degree of germline mosaicism, which could result in different levels of PLCζ in different sperm (90). This could account for the variable number of Ca2+ transients seen at fertilization in the transgenic mice. However, it was noted that while the founder males were able to breed to produce some offspring, the RNAi-expressing transgene was never passed onto the next generation. So the sperm which were most likely to have reduced PLCζ levels did not appear to be able to fertilize eggs in a manner that gave rise to full-term development. While not conclusive, the data imply that PLCζ plays an important role in initiating embryogenesis.
C. Is PAWP an Egg-Activating Sperm Factor in Mammals?

Despite evidence supporting the role of PLCζ in mediating sperm-induced Ca²⁺ release in mammals, another sperm protein has more recently been suggested as an egg-activating sperm factor. PAWP is a postacrosomal sheath WW domain binding protein found in mammalian sperm (209). Microinjection of recombinant PAWP protein has been shown to cause egg activation in pig, cow, and Xenopus eggs (209). The amount of PAWP in the sperm is around 80 fg, and this is within the range of recombinant PAWP protein required for egg activation (209). The proposed mechanism of action for PAWP is through binding to a Yes-associated protein (YAP) which may then interact with and stimulate PLCγ (209). This could involve a noncanonical pathway that acts via SH3 domains (1). The interaction of PAWP with YAP can be blocked by a PAWP peptide containing the PPGY conserved sequence (209). Interestingly, peptides containing this PPGY sequence were found to block PAWP-induced egg activation in pig eggs (209). They were also shown to block egg activation after sperm injection during ICSI. These data imply that PAWP protein plays an important role in egg activation at fertilization. This idea is supported by data showing the levels of PAWP in human sperm correlate with clinical success rates after ICSI in humans (2).

The key experiment to substantiate the role of PAWP in fertilization is to show that it can cause a Ca²⁺ increase in eggs that mimic the response at fertilization. The first experiments to assess this were carried out on frog eggs. Microinjection of PAWP protein was shown to cause a Ca²⁺ increase in frog (Xenopus) eggs (2). It was found that a Ca²⁺ increase occurred throughout the frog egg within 2 min of PAWP injection (2). While suggesting that PAWP can cause Ca²⁺ release, these data are difficult to interpret because at fertilization in frog eggs the sperm triggers a traveling distinctive Ca²⁺ wave that takes 5–6 min to cross the egg (13, 46). This distinctive wave is seen with every stimulus that triggers Ca²⁺ release in frog eggs, including the injection of InsP₃ or Ca²⁺ itself (12, 198). In contrast PAWP is not causing the distinctive Ca²⁺ wave that is seen specifically at fertilization or egg activation in frog eggs.

More recently, it was reported that injection of either human recombinant PAWP or PAWP cRNA causes Ca²⁺ oscillations in mouse and human eggs (2). These PAWP-induced Ca²⁺ oscillations resemble those seen after ICSI in these eggs (2). This provides the strongest evidence that PAWP is involved in generating Ca²⁺ oscillations at fertilization. It raises a scenario where either, or both, PLCζ and PAWP could be involved in Ca²⁺ signaling in eggs. To investigate the Ca²⁺ mobilizing role of PAWP, we have microinjected human and mouse PAWP recombinant protein into mouse and human eggs and found that it did not cause any Ca²⁺ increase (141, 142). We have expressed tagged versions of PAWP protein and used cRNA injection, but failed to see any sign of Ca²⁺ release over a range of concentrations (141, 142). It is possible that PAWP is not active with exogenous fusion-protein tags, so we have also injected untagged versions of PAWP cRNA over a range of concentrations and failed to detect any Ca²⁺ oscillations (142). In such experiments with untagged PAWP cRNA, we also verified expression of recombinant full-length PAWP protein in cRNA-injected eggs by immunoblot analysis using a PAWP-specific antibody (142). In these experiments, we carried out parallel runs with PLCζ protein or cRNA, and in all cases the PLCζ-injected eggs displayed sustained Ca²⁺ oscillations. It was also reported by Aarabi et al. (1) that peptides that matched sequences from PAWP were able to block PAWP- and ICSI-induced Ca²⁺ oscillations or egg activation (1, 3, 209). We injected the same PPGY motif-containing peptide used by Aarabi et al. (142), at the same, or higher, concentrations and found no inhibition of Ca²⁺ oscillations at fertilization or after ICSI (142). More recently, another group has made a PAWP null mouse, and it was found that males produce normal sperm lacking PAWP protein (167). Those mouse sperm, devoid of PAWP, are still able to fertilize eggs and trigger a normal pattern of Ca²⁺ oscillations and egg activation (167). These data suggest that PAWP is not a suitable candidate for the egg-activating sperm factor.

IV. PLCζ STRUCTURE AND FUNCTION

A. The Structural Domains of PLCζ

PLCζ is the smallest mammalian PLC and consists of just four discrete domains (168). These are the X and Y catalytic domains that are common to all PI-specific PLCs (55, 155), which are flanked by four EF-hand domains at the NH₂-terminal end and a C2 domain at the COOH-terminal end. The domain structure of PLCζ is shown schematically in FIGURE 4. The function of the main domains has been ex-
PLCζ has more basic residues and higher positive charge density than in mouse PLCζ (25). This is correlated with a greater affinity of human PLCζ for PIP2 and may account for its greater relative potency in causing Ca2+ oscillations in mouse eggs (25, 143, 228). All these data are consistent with a role for the X-Y linker in targeting PIP2 in eggs.

B. The Phylogeny of PLCζ

There is now evidence for a sperm PLCζ that is active in causing Ca2+ oscillations in the eggs of several different mammalian species. In the case of sperm PLCζ from pigs, humans, and cows, the same species of egg has been used. However, in many cases mouse eggs are used as a more available model system, since they readily express the injected RNA and show robust cytoplasmic Ca2+ oscillations in response to PLCζ derived from many different mammalian species. Homologous PLCζ protein sequences have now been identified in nonmammalian species such as chickens and medaka fish (23, 68). In each case, the injection of cRNA was able to cause Ca2+ oscillations in mouse eggs. These data suggest that PLCζ may play a role in fertilization of many different vertebrate species as well as the evidence for a vital role already observed in mammals. In pufferfish, a PLCζ homolog has been identified, but its expression is enriched in the ovary and brain (24). However, in these cases the PLCζ was not active in causing Ca2+ oscillations in mouse eggs, possibly due to important differences in their sequences (24). The canine PLCζ may be different since it is predicted from genomic sequence data to have an extended NH2 terminus, but this has yet to be verified and investigated for functional significance. Figure 5 shows a phylogenetic analysis of the different PLCζ isoforms that have been identified to date. The clustering of the primate PLCζ sequences is evident from the analysis of sequence phylogeny and indicates that the most recent divergence occurs for the human PLCζ. The shorter amino acid sequence of human PLCζ relative to most mammalian PLCζ sequences is particularly interesting and is due to the reduced length of the X-Y linker region (25). This is due to the absence from the human protein of a 123-bp exon corresponding to a 41-amino acid sequence that is retained in the otherwise near-identical primate sequences. The shorter human sequence produces a higher positive charge density in the human X-Y linker region and may correlate with a higher PIP2 binding and functional potency of the human PLCζ in generating Ca2+ oscillations in mouse eggs (143).

C. PLCζ Localization and Regulation in the Eggs and Sperm

One expectation of an egg-activating factor is that it should be localized on the sperm in an appropriate region to gain entry into the egg for it to trigger the initial Ca2+ release. This has been studied using immunocytochemistry with an-
tibodies raised to PLCζ, which has indicated localization to the equatorial region of the sperm head, although other regions of sperm such as the acrosome and mid-piece have also been stained with such antibodies (47, 80, 223, 144). The equatorial region is of particular interest since this is the part of sperm underlying the membrane that first fuses with the egg (218). This pattern of localization has now been supported by immunogold labeling studies providing higher resolution using electron microscopy (39). Hence, PLCζ is present within the region in the sperm that should facilitate it to initiate Ca\(^{2+}\) release in the egg. The localization of PLCζ in sperm may vary between species. In horse, sperm immunocytochemistry studies found that PLCζ was localized in the sperm midpiece as well as the sperm head (6). This localization may be genuine since the injection of the midpiece, as well the sperm head, of horse sperm into mouse eggs causes egg activation. It should be noted that one study has suggested that PLCζ is not localized within the sperm after the acrosome reaction (3). However, this study used PLCζ antibodies where their Western blots indicated that the antibody reactivity was to four distinct protein bands, only one of which has the correct molecular mass to be PLCζ, so it unclear whether the protein being analyzed in the immunocytochemistry studies is another protein(s). The suggestion by these authors that PLCζ is not located within the sperm is also directly contradicted by studies showing the extraction of PLCζ, which was unambiguously identified by tandem mass spectrometry, from within isolated perinuclear matrix material in mouse sperm (47).

While there is general consensus on PLCζ localization within the sperm, it is less clear where PLCζ is localized in the egg after gamete fusion. Fluorescently tagged fusion proteins of PLCζ do not show any distinct localization (220, 226, 227). PLCζ can be seen to enter pronuclei when they form after activation, but it is apparently distributed uniformly throughout the egg cytoplasm during the preceding phase of Ca\(^{2+}\) oscillations. There is no obvious binding to the plasma membrane which is expected to be a major factor in PLCζ localization. However, in these cases, it is possible that the egg cytoskeleton or other cytoplasmic structures may play a role in the localization of PLCζ.

**FIGURE 5.** Cladogram illustrating the phylogeny of aligned PLCζ sequences derived from various animal species. For simplicity, the tree branch lengths are omitted. The phylogenetic tree demonstrates the close similarity of the primate sequences (human, chimpanzee, gibbon, macaque, rhesus monkey), as well as the rodent (rat, mouse, hamster) and bird (chicken, quail, zebra finch) sequences. Numbers after species name denote sequence length. The most recent evolutionary divergence occurs between the human and chimpanzee. **TABLE 1** shows the species where Ca\(^{2+}\) releasing activity has been clearly demonstrated.
source of PIP2 in the egg, as it is in other cells (57). However, recent studies using Myc-tagged PLCζ have shown that it is mostly localized in vesicles that occur throughout the egg cytoplasm (229). Furthermore, immunostaining has also been used to show that PIP2 is also contained in vesicles throughout the cytoplasm (229). So it remains a possibility that PLCζ causes hydrolysis of an intracellular membrane source of PIP2. This could explain why no DAG production is detectable in the plasma membrane at fertilization, or after PLCζ injection in mouse eggs (227). The idea that PLCζ interacts with vesicles in the cytoplasm is illustrated schematically in Figure 6. Interestingly, previous studies on sea urchin and frog eggs had shown that a significant proportion of PIP2 is found in yolk vesicles (156, 176). It therefore is possible that PLCζ is distinct from most other PLCs in acting upon a discrete intravesicular source of PIP2.

An issue that may be correlated with an unusual localization of PLCζ in eggs is the control of its activity. One of the remarkable features of PLCζ is the way it is apparently fully active as soon as it enters the egg cytoplasm. The mammalian sperm is thousands of times smaller in volume than the egg, so it may contain thousands of times more PLC than the egg. If PLCζ is autonomously active in the sperm, it would be expected to rapidly consume all the available phosphoinositide lipids. Hence, we can only assume that PLCζ is not functionally active in the sperm. Remarkably this situation can be mimicked in a PLCζ-expressing stable cell line. PLCζ has been expressed in mammalian cell lines at levels that are 100-1,000 times higher than that found in eggs (154). Despite this level of expression, there is no alteration in resting Ca2+ or major effect upon agonist-stimulated Ca2+ increases (154). However, when extracts from these PLCζ-transfected cells are injected into mouse eggs, Ca2+ oscillations are stimulated (154). Injection of whole CHO cells expressing PLCζ also triggers Ca2+ oscillations (154). These cell lines, therefore, mimic the situation in sperm in that PLCζ is apparently inactive in their cytoplasm, yet can initiate Ca2+ oscillations as soon as it is placed within the egg cytoplasm. Interestingly, when sperm extracts containing PLCζ are injected into CHO cells, it is ineffective in triggering Ca2+ oscillations (154). This suggests that PLCζ cannot hydrolyze PIP2 in CHO cells. It was noted that CHO cells did not show any evidence for intracellular vesicles containing PIP2. Hence, these cell lines are distinct from eggs. It is possible that PLCζ acts exclusively on PIP2 in intracellular vesicles, and this could provide the basis for why it is active in eggs and not cell lines. The idea that PLCζ might only be active in eggs is consistent with transgenic mice studies where it was expressed ectopically in all tissues (226). In such mice, there was a distinct lack of effect in nearly all tissues other than the egg, which showed “spontaneous” Ca2+ oscillations associated with egg activation (226).

D. PLCζ and the Mechanism of Ca2+ Oscillations

One of the key characteristics of PLCζ is its high sensitivity to Ca2+. The sensitivity is such that PLCζ is ~50% active at resting Ca2+ levels of ~100 nM (92, 136). PLCζ also shows a steep rise in activity as Ca2+ levels increase from resting levels up to around 1 μM (92, 136). Hence, InsP3 production will increase greatly during each Ca2+ rise, and it may be expected that oscillations in InsP3 will accompany oscillations in Ca2+. The possibility that the InsP3 concentration oscillates is significant because it can provide a mechanism for generating Ca2+ oscillations. There are two classes of mathematical models for InsP3-induced Ca2+ oscillations in cells. There are those that have a strong positive feedback role for Ca2+ on the InsP3 receptor, and then a negative feedback of Ca2+ at higher Ca2+ concentration (8, 175). These are the so-called “InsP3 receptor” based models that predict that Ca2+ oscillations occur with constant InsP3. Then there are other models that involve a positive feedback effect of Ca2+ on a PLC that leads to oscillations in InsP3 to
accompany the Ca²⁺ oscillations. There is experimental evidence that either one of these mechanisms can operate in somatic cells depending on the cell type studied and the type of agonist stimulation used (175).

In some somatic cells, the models of Ca²⁺ oscillations have been resolved using fluorescent probes to monitor InsP₃ concentrations in real time. The GFP-tagged PH domain from PLCδ1 has been used as an indirect probe for InsP₃ since it binds plasma membrane PIP₂, but when InsP₃ concentrations increase, there is a translocation of GFP-PH away from the plasma membrane (57). When this probe is introduced into mouse eggs there is a familiar plasma membrane staining (57). However, at fertilization there is an increase in GFP-PH staining and not a decrease (57). The increase is driven by increased synthesis of PIP₂, which is in turn due to exocytosis (57). A decrease in plasma membrane staining can only be seen with very high concentrations of InsP₃. Hence, this probe cannot be used to detect physiological InsP₃ changes in mouse eggs.

A further attempt has been made to measure InsP₃ in mouse eggs using a different type of fluorescent indicator called “fretino.” This indicator consists of the InsP₃ binding domain of the InsP₃ receptor placed between CFP and YFP fluorescent proteins (174). There is a change in the efficiency of fluorescence resonance energy transfer (FRET) between the CFP and YFP upon InsP₃ binding. This probe did not show any FRET signal changes at fertilization, but oscillations in InsP₃ were detected alongside Ca²⁺ oscillations when PLCζ was injected and allowed to overexpress (174). From these data it is clear that InsP₃ can oscillate in synchrony with Ca²⁺ oscillations when sufficient amounts of PLCζ are present. The reason why there was no signal change at fertilization could be because InsP₃ does not oscillate significantly under physiological levels of PLCζ, or because the probe was not sensitive enough to detect the changes in InsP₃.

Despite the attempt to measure InsP₃ in eggs, the different models of InsP₃-induced Ca²⁺ in cells can be distinguished by a simple experiment. A theoretical analysis of the two types of Ca²⁺ oscillators revealed they respond differently to a sudden increase in InsP₃ (175). Models based on the kinetics of the InsP₃ receptor alone show an increase in Ca²⁺ oscillation frequency if there is a sudden increase in InsP₃ concentration. This is because in this type of model, InsP₃ is acting as a control parameter that modulates the frequency of oscillations. In contrast, models based on Ca²⁺-dependent InsP₃ production show an immediate Ca²⁺ transient in response to a sudden increase in InsP₃, but this transient then leads to a resetting of the oscillations in phase from the immediate Ca²⁺ transient (175). Oscillations then continue to occur at a similar frequency to before. This type of phase resetting behavior is seen because in the Ca²⁺-dependent InsP₃ models, InsP₃ is acting as a dynamic variable rather than a control parameter. The difference in behavior has been shown with 13 different models of Ca²⁺ oscillations (175). A sudden increase in InsP₃ can be generated in mouse eggs by photorelease of caged InsP₃. When tested in fertilizing mouse eggs, it was found the photorelease of InsP₃ leads to an immediate Ca²⁺ transient that then resets the phase of Ca²⁺ oscillations (190). The same phase resetting was seen with PLCζ-induced Ca²⁺ oscillations in mouse eggs (190). These data demonstrate that PLCζ and sperm cause oscillations through a positive feedback cycle involving Ca²⁺-dependent InsP₃ generation and InsP₃-induced Ca²⁺ release. The schematic in Figure 6 illustrates the idea that there is a positive feedback cycle of Ca²⁺ release and InsP₃ production that underlies the Ca²⁺ waves and oscillations induced by PLCζ. The implication of this scheme is that InsP₃ and Ca²⁺ together play a key role in determining the timing of each Ca²⁺ transient. This is probably more significant than, for example, the refilling of Ca²⁺ stores, which otherwise appears to occur passively during each interspike interval (199).

E. Technological and Clinical Implications

Human eggs display a series of Ca²⁺ oscillations at fertilization and after ICSI (191, 193). In in vitro fertilization clinics, the ICSI procedure now accounts for the majority of fertilizations. Whilst ICSI is generally successful, there are still 1–4% of cases where fertilization completely fails in all eggs from a patient (80, 216, 135). In addition, ~30% of eggs fail to fertilize after ICSI (135). In some of these cases, it was shown that the failure of fertilization was due to the inability of the sperm to activate eggs. There are now several reports showing that the lack of egg-activating ability in these cases is often associated with a lack of PLCζ protein in the sperm (105, 135, 186, 197, 224). Most interesting, however, are cases where a lack of egg-activating ability was associated with a mutant form of PLCζ (60). In a particular patient, there was a mutation of the PLCζ gene on both alleles (on chromosome 17), and both mutations involved single amino acid substitutions in the catalytic domains which lead to a complete loss of PLCζ activity (81, 82, 135). The only current option available in these cases where PLCζ is deficient, or the sperm is otherwise unable to activate the egg in ICSI, is to use Ca²⁺ ions to artificially activate the eggs (80, 197, 216). This treatment causes only a single Ca²⁺ increase and is not an optimal way to activate mammalian eggs (80). In domesticated animals, ICSI has also been used as a way of generating transgenic animals. However, unlike the mouse, ICSI success rates are low in pigs, cows, and horses (51). Again, one of the main problems is a low activation rate after sperm injection, and it appears that this is due to a lack of an effective Ca²⁺ signal (115).

Mouse eggs can be easily activated by incubation in Sr²⁺ ions as this provides a simple means of causing Ca²⁺ oscil-
lutions (87). However, Sr\(^{2+}\) media have never been shown to cause Ca\(^{2+}\) oscillations in eggs from humans or domestic animals (157). In contrast, injecting human PLC\(_\zeta\) cRNA has been shown to cause Ca\(^{2+}\) oscillations in human oocytes, and to activate development up to the blastocyst stage (157). Injecting bovine PLC\(_\zeta\) cRNA has also been shown to activate cow embryo development up to the blastocyst stage (159). Both of these studies revealed that PLC\(_\zeta\) might be useful as a parthenogenetic activating agent in reproductive technologies. While microinjecting PLC\(_\zeta\) cRNA is an effective means of causing Ca\(^{2+}\) oscillations in all eggs studied, it appears to be important to use it within a limited range. Studies in mouse and bovine embryos indicate that overexpression of PLC\(_\zeta\) can lead to high-frequency Ca\(^{2+}\) oscillations, which does cause egg activation, but also produces poor embryo development (159, 228). The expression level of PLC\(_\zeta\) due to injected cRNA will accumulate with time and is therefore difficult to control, particularly in human eggs, so it may be more effective and reliable to use recombinant PLC\(_\zeta\) protein. We have reported the successful expression of a stable human PLC\(_\zeta\) using a NusA-fusion tag that can trigger Ca\(^{2+}\) oscillations in mouse and human eggs (144). It is possible that such recombinant versions of PLC\(_\zeta\) could be used to overcome problems of low fertilization rates after ICSI as well as improve ICSI success rates in domesticated animal species. PLC\(_\zeta\) could also be used to enhance activation efficiency during somatic cell nuclear transfer (160).

V. UNRESOLVED SPERM FACTORS IN OTHER SPECIES

A. Amphibians and Birds

Cytosolic sperm extracts from *Xenopus laevis* sperm do not cause egg activation when injected in *Xenopus* eggs (69). However, injecting *Xenopus* sperm extracts have been shown to cause Ca\(^{2+}\) oscillations when injected into mouse eggs (31), and injecting sperm extracts from the frog *Bufo arenarum* sperm can activate frog eggs (9). So it is possible that a soluble sperm factor may exist in frogs, but the evidence is inconsistent. Otherwise, the receptor theory is favored by most studies on *Xenopus* eggs where the addition of disintegrin peptides, or peptides with an RGD motif that interacts with integrin receptors, have been shown to trigger a Ca\(^{2+}\) increase and egg activation (70, 173). The addition of such peptides may cause an increase in tyrosine kinase activity in the egg cortex and stimulate PLC\(_\gamma\) activity, but it is not clear how this occurs because tyrosine kinases are implicated and yet the injection of SH2 domains does not block fertilization (53, 163, 164, 165). The signaling scenarios are difficult to resolve in frog eggs because activation, and the Ca\(^{2+}\) wave itself, could be triggered by a number of stimuli that lead to an initial Ca\(^{2+}\) increase, and it is difficult to distinguish the sperm-induced response from artificial mimics. Precise analysis of the Ca\(^{2+}\) dynamics in frog eggs has shown that InP\(_3\) is the best mimic of fertilization for starting the Ca\(^{2+}\) wave (11), but this level of detailed analysis is rarely carried out in studies of frog eggs.

There is direct evidence for a soluble sperm factor in newt eggs, *Cynops pyrrhogaster*. The eggs of this species are naturally fertilized by multiple sperm, a situation referred to as physiological polyspermy (69). This occurs in urodeles (newts and salamanders) and results in many sperm entering the cytoplasm, followed by selective fusion of the female pronucleus with just one male pronucleus (69). Fertilization in these types of eggs is associated with a slow Ca\(^{2+}\) increase that moves over a limited region of cytoplasm (69, 196). Microinjection of cytlosic sperm extracts can cause a Ca\(^{2+}\) increase and egg activation in newt eggs (214). The factor in newt sperm extracts was purified and found to be correlated with a major 45 kDa protein which was identified as citrate synthase (59). Microinjection of mammalian recombinant citrate synthase can cause Ca\(^{2+}\) release in newt eggs, but the mechanism is unclear. It has been suggested that it could involve the conversion of citrate to acetyl CoA and oxaloacetate (58), but this has yet to be established.

More recently, studies of Ca\(^{2+}\) release in quail eggs have suggested that both PLC\(_\zeta\) and citrate synthase might be necessary for Ca\(^{2+}\) signals at fertilization. Quail sperm contain PLC\(_\zeta\), and injection of sperm extracts or PLC\(_\zeta\) cRNA into quail eggs caused a Ca\(^{2+}\) increase and egg activation (125, 126). The fractionation of sperm extracts also found that activity correlated with metabolic enzymes such as citrate synthase and aconitase (125). However, these enzymes are normally found in the mitochondrial matrix, so it is unclear whether they would be delivered to the egg cytosol at fertilization. Another issue with the proposal that citrate synthase, or other metabolic enzymes, mediate Ca\(^{2+}\) release in newts or birds is that the amount of enzyme being used may not be physiological. For example, while ~10 sperm enter the newt egg at fertilization, the Ca\(^{2+}\) increases in newt egg demonstrated so far have been with sperm extract or citrate synthase levels equivalent to ~300-2,000 sperm (59). With quail eggs there has been no quantification of the levels of either PLC\(_\zeta\) or metabolic enzymes required for causing Ca\(^{2+}\) release and how they relate to levels in the sperm.

B. Ascidians

As with mammals, ascidian cytlosic sperm extracts can also cause Ca\(^{2+}\) oscillations when injected into ascidian oocytes (100, 119, 161). It is again significant that the ascidian sperm extract mimics fertilization in causing two sets of Ca\(^{2+}\) oscillations that correlated with the first and second meiotic divisions (109, 119). The sperm factor is high molecular mass and heat sensitive, suggesting that the ascidian sperm factor is also a protein (100). The ascidian sperm factor activity can also be purified to some extent using...
column chromatography (McDougall, person communication). These data are similar to early observations on the mammalian sperm factor. However, the ascidian sperm factor may not be PLCζ. The sperm extract-induced Ca^{2+} oscillations in ascidians are inhibited by SH2 domains from PLCγ or Fyn kinase (161). Both these SH2 domains also block fertilization-induced Ca^{2+} oscillations in ascidian oocytes (161) but are ineffective at fertilization in mouse (120). The ascidian sperm extract is also affected by the activity of cdk1 in that the second series of Ca^{2+} oscillations are dependent on cdk1 activity (119). This appears to be due to an action of cdk1 on the ability of the sperm factor to generate InsP₃ (119). Again, this is different from PLCζ which continues to be active in causing oscillations for well over an hour after the decline in cdk1 activity in fertilizing mouse eggs (100). There is currently no indication for an ascidian PLCζ. However, the ascidian sperm factor might be a protein that stimulates an egg-derived PLC, or else a different type of PLC. As with mouse eggs, the PLC may act upon intracellular PIP₂ since the pattern of Ca^{2+} oscillations can only be readily explained by assuming InsP₃ is generated throughout the egg cytoplasm (35). Interestingly, there is always a distinct delay between injection of sperm extracts and the start of the first Ca^{2+} increase in ascidian eggs which could imply additional steps are required before PLC stimulation. Identifying the ascidian sperm factor will represent a significant step in our general understanding of egg activation in nonmammalian species.

### C. Echinoderms

Studies in sea urchin eggs show a multitude of Ca^{2+} release mechanisms, and there are many candidate molecules that increase at fertilization and that could mediate Ca^{2+} release (97). These molecules include InsP₃, cADPR, and NAADP. It appears that both heparin, which blocks InsP₃-induced release, and ruthenium red, which blocks cADPR-induced release, both need to be injected to block Ca^{2+} release at fertilization in sea urchin and starfish eggs (49). However, other studies found that a higher concentration of heparin alone can block Ca^{2+} release at fertilization in sea urchins (129). It is possible that both InsP₃ and cADPR are somehow involved in amplifying the Ca^{2+} increase in sea urchin eggs, but it is not clear which specific molecule would start the wave. It has also been suggested that NAADP is a sperm-derived messenger delivered to the egg after gamete fusion and is involved in generating the initial depolarization in the sea urchin and starfish eggs (18, 127, 128). The principal evidence for this is that NAADP can trigger Ca^{2+} influx and membrane depolarization, and that this inhibits the subsequent Ca^{2+} influx or depolarization at fertilization. However, these effects may not be relevant to the way the sperm depolarizes the egg. In sea urchin eggs at least, it is already known that the action potential is triggered by the flow of current into the egg through the sperm membrane after fusion (16, 117). In their experiments on sea urchin fertilization, McCulloh and Chambers (117) also noticed that, at negative membrane holding potentials, the sperm can “unfuse” with the egg membrane after several seconds (117). Such “unfusion” is coincident with a “switch off” of the sperm inward current. Hence, the initial sperm-induced depolarization of the sea urchin egg cannot be due to any diffusible factor transferred into the egg.

Other work has suggested that NO, or cGMP, could be a factor delivered to the egg to trigger Ca^{2+} release (95). These two molecules form part of the same signaling pathway in which NO activates guanyl cyclase to increase cGMP (104). This leads to the stimulation of G-kinase that activates the enzymatic elevation of cADPR. However, this pathway is independent of InsP₃ and too slow to account for the initial Ca^{2+} release in sea urchin eggs (104, 202).

The mechanism of egg activation in echinoderm egg fertilization seems to involve Src family kinases and PLCγ (71, 84, 85). This is based on the observation that injecting SH2 domains from PLCγ or Fyn kinases can block the main Ca^{2+} increase and egg activation in sea urchin and starfish fertilization (15, 52, 85 171). These data suggest that the sperm might mediate Ca^{2+} release via tyrosine phosphorylation and ultimately stimulation of an egg-derived PLCγ via a plasma membrane receptor (45). However, in the presence of SH2 domains, there are small localized Ca^{2+} increases that can be detected at the sites of sperm-egg fusion in sea urchin eggs (171). It is possible that these localized hotspots of Ca^{2+} are sites where the sperm provides a signal that initiates a Ca^{2+} wave. The Ca^{2+} increase may then require PLCγ to spread as a wave across the egg. Interestingly, PLCγ has been shown to be localized on intracellular vesicles in sea urchin eggs (14). Hence, PLCγ may be involved mainly in propagating the Ca^{2+} wave through the egg cytoplasm. Whether it initiates the Ca^{2+} wave is not yet certain.

Given that sperm-egg fusion occurs before the start of the Ca^{2+} wave in sea urchin eggs (117), it is still possible that the sea urchin sperm contains an egg-activating factor. There are rather indirect lines of evidence for this proposal. The first piece of evidence is from an experiment using sea urchin eggs and starfish sperm. Sea urchin eggs cannot be fertilized by starfish sperm. However, starfish sperm can be “engulfed” by sea urchin eggs, and when the fusogen polyethylene glycol is added to such eggs, the starfish sperm fuse with the sea urchin eggs and they are then activated (101). The eggs are not activated by polyethylene glycol alone, so these data imply that the starfish sperm contain an egg-activating factor (101). The second point is that it has been shown that injection of >10 sea urchin sperm into a mouse egg can lead to egg activation, which implies the existence of a sperm factor that can cause InsP₃ production (200). If there is a soluble egg-activating sperm factor in sea urchins, then we might expect it to be an enzyme and not a small
molecule that releases Ca\(^{2+}\). In sea urchin eggs, the time between the initial depolarization, and hence fusion, and the Ca\(^{2+}\) wave (the latent period) is typically 10–20 s (202, 205). This latent period is very temperature dependent and has a high \(Q_{10}\) that is characteristic of an enzymatic step. So, in sea urchin egg, it is possible that the sperm delivers an enzyme that somehow generates InsP\(_3\) after gamete fusion. There is no evidence for a PLC\(\zeta\) in the genome of sea urchins, even though PLCs of the \(\beta, \delta, \) and \(\gamma\) class have all been described in the egg (21, 22, 71).

### D. Other Species

After mammals, newts, and ascidians, there is one more example where a soluble sperm factor effect has been unequivocally demonstrated within the cognate species. Microinjection of a cytosolic sperm extract from the nemertean worm *Cerebratulus lacteus* into oocytes of the same species causes a series of Ca\(^{2+}\) oscillations (179). The characteristics of the Ca\(^{2+}\) oscillations caused by sperm extract injection are identical to those seen at fertilization (179, 181). Furthermore, the amount of sperm extract required to initiate these responses is around one sperm equivalent. As with mammals, the oscillations are not mimicked by injecting Ca\(^{2+}\) itself. The sperm factor involved is evidently a protein, since it is high molecular weight and its activity is abolished by protease treatment (179). The factor has not yet been identified and might be different from putative factors in ascidians, or sea urchins. It was found that the injection of boar sperm extracts could cause a similar series of Ca\(^{2+}\) oscillations in *Cerebratulus* oocytes (182). Since we now know that PLC\(\zeta\) is the active agent in boar sperm extracts, the data suggest that PLC\(\zeta\) can generate InsP\(_3\) in the cytoplasm of oocytes from highly divergent animals. Whether these nemertean worms possess a PLC\(\zeta\)-like gene remains to be seen.

### VI. CONCLUSIONS AND SUMMARY

We have presented the evidence that, where the sperm activates development of the egg, it probably involves a sperm protein that is introduced into the egg after gamete membrane fusion. One idea that has been raised is that there might be multiple messengers from the sperm to cause egg activation (206). There are multiple Ca\(^{2+}\) release molecules to choose from, particularly if one studies the sea urchin egg. However, with fertilization the target for the sperm in egg activation is the increase of Ca\(^{2+}\) ions in the cytoplasm. Hence, physiologically there is just one target output. If the source of Ca\(^{2+}\) is essentially intracellular stores, as it is in most vertebrates, it seems reasonable to propose that the task can be achieved with just one factor for a given species. Of course, the nature of this factor may vary between different classes of animals. In this way, we envisage fertilization as being analogous to neuromuscular transmission where just one factor (acetylcholine for vertebrates) carries the message.

The single largest problem in the field is the nature of the bioassays used to assess a sperm factor candidate. If egg activation is used as the assay for a “sperm factor,” then any number of molecules may become candidates, including Ca\(^{2+}\) itself. In eggs with a single Ca\(^{2+}\) rise, whether it is a wave or not, the assay would be problematic, since any agent, or pathway causing Ca\(^{2+}\) release could again mimic the sperm at fertilization. This difficulty has not helped yield progress in species such as the sea urchin. There may also be an issue with eggs activated by Ca\(^{2+}\) influx, since if the influx is voltage gated, then any molecule that can cause even the slightest depolarization of the plasma membrane could mimic the sperm and trigger Ca\(^{2+}\) influx and activate the egg. The most discerning assay for a sperm factor is to have a series of distinctive Ca\(^{2+}\) oscillations since, in general, injection of Ca\(^{2+}\) or many other agents does not mimic fertilization. Hence, we suggest that the best evidence for a soluble sperm factor being involved at fertilization is for eggs of mammals, ascidians, and nemertean worms, all of which require a unique signature of Ca\(^{2+}\) oscillations. If a frog egg is being used, then a careful analysis of the pattern of the Ca\(^{2+}\) wave is necessary (11).

In the face of a profusion of molecules and themes, we suggest a simple way of understanding how a sperm drives Ca\(^{2+}\) increase in the egg at fertilization. In many species, the sperm causes an action potential in the egg which leads to voltage-gated Ca\(^{2+}\) influx (FIGURE 1). In some cases this Ca\(^{2+}\) influx plays the key role in egg activation, and this could be due to a plasma membrane receptor. However, it should be noted that if sperm-egg fusion is the cause of depolarization, then there is no real need for a sperm-specific factor, and certainly no need for a diffusible messenger. To clarify the role of sperm-egg membrane fusion, it would be useful if there were more experiments similar to those of McCulloh and Chambers on sea urchin eggs to examine the precise timing of sperm-egg fusion in species where membrane depolarization is the initial event at fertilization.

In many more species, the source of Ca\(^{2+}\) increase that activates the egg is intracellular and the best candidate for how the sperm mediates this Ca\(^{2+}\) release is InsP\(_3\) (FIGURE 1). It is possible in many of these cases that the sperm uses a soluble factor to generate InsP\(_3\). In mammals, there is no depolarization event, so the fusion is merely the “silent” prelude to the introduction by diffusion of the soluble protein that generates InsP\(_3\). In mammals, the cumulative evidence points primarily towards just one agent, namely, PLC\(\zeta\), a sperm-derived protein that causes InsP\(_3\) generation and the release of Ca\(^{2+}\) within the egg. The known structural and functional properties of PLC\(\zeta\) alone can explain all the key features of sperm-induced Ca\(^{2+}\) release in mammalian eggs. The main issues for PLC\(\zeta\) are the need for...
experiments to prove that it is the only factor causing Ca$^{2+}$ release at fertilization, a clear definition of how its binds to the intracellular source of PIP$_2$ in the egg and how the distinctive Ca$^{2+}$ oscillation frequency and amplitude is maintained and then terminated.

**ACKNOWLEDGMENTS**

We thank Alex McDougall for helpful comments and allowing citation of his unpublished data.

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**GRANTS**

Our work on PLC$\zeta$ has been supported by the Biotechnology and Biological Sciences Research Council and Wellcome Trust.

**DISCLOSURES**

Cardiff University and the authors hold intellectual property rights for PLC$\zeta$.

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