Focus on Fertilization

The cytosolic sperm factor that triggers \( \text{Ca}^{2+} \) oscillations and egg activation in mammals is a novel phospholipase C: PLC\( \varepsilon \)

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Abstract

When sperm activate eggs at fertilization the signal for activation involves increases in the intracellular free \( \text{Ca}^{2+} \) concentration. In mammals the \( \text{Ca}^{2+} \) changes at fertilization consist of intracellular \( \text{Ca}^{2+} \) oscillations that are driven by the generation of inositol 1,4,5-trisphosphate (InsP\( _3 \)). It is not established how sperm trigger the increases in InsP\( _3 \) and \( \text{Ca}^{2+} \) at fertilization. One theory suggests that sperm initiate signals to activate the egg by introducing a specific factor into the egg cytoplasm after membrane fusion. This theory has been mainly based upon the observation that injecting a cytosolic sperm protein factor into eggs can trigger the same pattern of \( \text{Ca}^{2+} \) oscillations induced by the sperm. We have recently shown that this soluble sperm factor protein is a novel form of phospholipase C (PLC), and it is referred to as PLC\( \varepsilon \)). We describe the evidence that led to the identification of PLC\( \varepsilon \) and discuss the issues relating to its potential role in fertilization.

Egg activation and the role of calcium

During fertilization the sperm fuses with the egg to introduce its paternal genes. In addition to the sperm delivering the genes there is also a need for a signal for the egg to activate and begin development. The delivery of paternal genes and the activation of development are the two most central features of what the sperm does at fertilization. These two roles can, in fact, be separated from each other. For example, in sea urchins, sperm can be microinjected into eggs without causing egg activation or development (Hiramoto 1962). On the other hand egg activation can be achieved in the absence of a sperm. This is seen as a natural means of reproduction for some animals, and can be achieved with certain chemical and physical treatments that cause so-called parthenogenetic development in the absence of fertilization (Mittwoch 1978, Swann & Ozil 1994). There are also situations in nature where a sperm fertilizes an egg but the activating role of the sperm is taken on by another stimulus. For example in eggs of the marine shrimp and zebrafish the sperm fuses and contributes its genetic material, but egg activation is triggered by the process of ovulation and subsequent contact with sea or pond water, rather than any factor from the sperm (Lindsay et al. 1992, Lee et al. 1999). However, in most animals studied to date, and certainly in mammals, the sperm provides its genome for development and it provides the essential stimulus for egg activation. Despite many advances in our understanding of events at fertilization, it is remarkable that the molecular nature of the trigger provided by the sperm to activate an egg has remained a mystery. In this review we describe our work that we believe takes significant steps towards identifying the factor in the sperm that activates development. Most of the data we discuss deal specifically with egg activation during mammalian fertilization. We anticipate that some of this story may turn out to apply to sperm-induced egg activation in non-mammalian species.

The most significant advance in our understanding of egg activation during fertilization is that an increase in intracellular \( \text{Ca}^{2+} \) in the egg cytoplasm stimulates all the events of activation. Jaffe and colleagues (Ridgway et al. 1977) first observed that a wave of intracellular \( \text{Ca}^{2+} \) increase occurred after sperm entry in fertilizing medaka fish eggs. Since then, \( \text{Ca}^{2+} \) increases in the form of waves or oscillations at fertilization have been detected in a variety of animals and plants (Stricker 1999). Even in species such as shrimp and zebrafish, where the sperm does not activate the egg, the activation process still involves a large increase in cytosolic free-\( \text{Ca}^{2+} \) ion concentration (Lindsay et al. 1992, Lee et al. 1999). This emphasizes the point that some form of \( \text{Ca}^{2+} \) increase at fertilization is
always observed during the initiation development. The importance of the Ca\(^{2+}\) increase is underlined by the finding that it is both necessary and sufficient for stimulating the early events of egg activation. If a Ca\(^{2+}\) increase is abolished at fertilization then, while sperm–egg membrane fusion still occurs, all other events of egg activation, such as meiotic resumption and exocytosis, are prevented (Kline & Kline 1992). The Ca\(^{2+}\) increase is known to be sufficient for development because causing an artificial Ca\(^{2+}\) rise in Ca\(^{2+}\) sufficient for development because causing an artificial Ca\(^{2+}\) rise in Ca\(^{2+}\) in the egg leads to stimulation of all of the early events of egg activation (Swann & Ozil 1994, Schultz & Kopf 1995). In many ways the problem of understanding how a sperm activates an egg at fertilization is centred on the issue of how the sperm triggers the Ca\(^{2+}\) changes in the egg cytosol. This single and critical issue has not been completely resolved in any species.

**Sperm factors and calcium release**

There have been several different proposals to explain how sperm cause Ca\(^{2+}\) release in eggs at fertilization. The relative merits of these ideas for different species have been reviewed extensively elsewhere (Nuccitelli 1991, Swann & Ozil 1994, Schultz & Kopf 1995, Evans & Kopf 1998, Stricker 1999, Jaffe et al. 2001, Runft et al. 2002). It is not our purpose to debate these theories here since they involve a discussion of data from a variety of species. In this review we are mainly concerned with events in mammals.

The idea that has gained most support in mammals over the last few years is referred to as the sperm factor hypothesis (Swann & Ozil 1994, Stricker 1999, Runft et al. 2002). This hypothesis proposes that fusion of sperm and egg membranes leads to the introduction of a factor into the egg cytoplasm. This factor could then initiate the Ca\(^{2+}\) release that leads to the appropriate pattern of waves or oscillations of Ca\(^{2+}\). This proposal appears to be a plausible mechanism in some species since in the mouse and sea urchin sperm–egg membrane fusion has been shown to precede the initial Ca\(^{2+}\) release in the egg (McCulloh & Chambers 1992, Lawrence et al. 1997). While it is now a predominant hypothesis for mammals, the first direct evidence for this hypothesis was presented in sea urchins. It was shown that microinjecting cytosolic sperm extracts from sea urchin sperm could trigger fertilization envelope elevation in unfertilized sea urchin eggs (Dale et al. 1985). This event of envelope elevation is normally associated with fertilization and is a good indication that a Ca\(^{2+}\) increase has occurred in the egg. Despite its potential significance, there have been no reports confirming this observation in sea urchins. It remains unclear whether the sea urchin sperm contain a soluble egg-activating factor. The same kind of observations were, however, made some years later when egg activation (pronuclear formation) was observed in rabbit and mouse eggs injected with rabbit sperm extracts (Stice & Robl 1990). This experiment in mammals has been confirmed many times, but the substantial evidence for a soluble sperm factor mechanism in mammals comes from a different type of experiment.

One of the problems with trying to demonstrate a sperm factor in these kinds of experiments is that many signs of egg activation, such as exocytosis, or even development to the blastocyst stage in mammals, can be mimicked to some extent by injecting Ca\(^{2+}\) into the egg (Swann & Ozil 1994, Schultz & Kopf 1995). Consequently, if there is any Ca\(^{2+}\) contamination of the injection solution, or the injection procedure itself leads to some Ca\(^{2+}\) influx into the egg, then it is a possibility that the activation is due to an experimental artefact rather than because of the injection of a sperm-borne activating agent. The situation is made more difficult in mammalian eggs since, depending upon the handling conditions, some degree of parthenogenetic activation can occur spontaneously. The only way to avoid this is to use an assay of activation that is not prone to this problem. An assay is needed that has a unique signature of the physiological events at fertilization.

While many eggs show a single Ca\(^{2+}\) increase at fertilization, the Ca\(^{2+}\) oscillations that occur during mammalian fertilization are highly stereotypic. The oscillations consist of a series of spike-like increases in Ca\(^{2+}\) that last about one minute and that occur at intervals of several minutes apart (Miyazaki et al. 1993, Swann & Ozil 1994). The typical pattern of oscillations in fertilizing mouse eggs is shown in Fig. 1. The precise shape of the Ca\(^{2+}\) spikes, as well as the frequency of Ca\(^{2+}\) spikes, seems to be a feature of the species of egg. The frequency of Ca\(^{2+}\) oscillations can also depend on the degree of polyspermy; multiple sperm–egg fusions give rise to higher-frequency oscillations (Faure et al. 1999). The special nature of these oscillations is illustrated by the fact that nearly all parthenogenetic activation agents work via a Ca\(^{2+}\) increase, and yet nearly all of them fail to cause oscillations. The classic parthenogenetic agents such as 7% ethanol, Ca\(^{2+}\) ionophore and electrical field stimulation cause just a single, long rise in Ca\(^{2+}\) (Swann & Ozil 1994). The only parthenogenetic agents that cause sustained Ca\(^{2+}\) oscillations are thimerosal and strontium ions.
(Swann 1991, Kline & Kline 1992). Despite causing oscillations, the exact pattern of Ca\(^{2+}\) changes triggered by these agents is distinguishable from those at fertilization. The Ca\(^{2+}\) spikes induced by strontium, for example, are of longer duration and smaller amplitude than those triggered by the sperm. Consequently, the Ca\(^{2+}\) oscillations at fertilization provide a signature for the sperm’s mechanism of egg activation, which allows us to assess whether a sperm-derived factor is physiologically relevant.

Since the pattern of Ca\(^{2+}\) oscillations provides the fingerprint, it is of some significance that the only real match for this fingerprint comes from an agent that is inside the sperm. Injecting cytosolic extracts from sperm, of the same species, has been shown to trigger Ca\(^{2+}\) oscillations in eggs from hamsters, mice, pigs and humans (Swann 1990, Homa & Swann 1994, Machaty et al. 2000, Tang et al. 2000). In each case injecting sperm extracts triggers Ca\(^{2+}\) oscillations that are a close mimic of the pattern of oscillations seen at fertilization. The factor responsible for this activity has been shown to be protein based and specific to sperm cytosol (Swann 1990, Fissore et al. 1998). These data clearly suggest that the sperm contain a protein factor that has the potential to pass into the egg at fertilization and initiate Ca\(^{2+}\) oscillations. Experiments along similar lines to those in mammals have now been carried out in other types of animals. In nemertean worms, ascidians and in newts, the injection of a cytosolic sperm factor has also been shown to trigger Ca\(^{2+}\) oscillations or waves (Stricker 1997, Kyozuka et al. 1998, Yamamoto et al. 2001). The sperm factor involved in all these cases is clearly some form of sperm-specific protein since it is heat and protease sensitive. The sperm factor protein is apparently not species specific because sperm extracts from pigs, for example, have been used to trigger Ca\(^{2+}\) oscillations in mouse, hamster, cow, human and nemertean worm eggs (Swann 1990, Homa & Swann 1994, Wu et al. 1997, Stricker et al. 2000). The sperm factor protein that is effective in mammals may be widespread since there are reports that cytosolic extracts made from frog, chicken or fish sperm can cause Ca\(^{2+}\) oscillations when injected into mouse eggs (Dong et al. 2000, Coward et al. 2003). Although it is not the most discerning form of assay for a sperm, it is important to note that the injection of these cytosolic sperm extracts does lead to egg activation and development up to the blastocyst stage in mammals (Fissore et al. 1998).

As well as data using soluble extracts, the injection of whole mammalian sperm has also been shown to activate development in mouse eggs. This is, in fact, essentially the procedure referred to as intracytoplasmic sperm injection (ICSI). This is increasingly used as a method for overcoming male factor infertility associated with in vitro fertilization (IVF). Not only does ICSI lead to egg activation, but in human, mouse, pig and horse eggs it has been shown to trigger a fertilization-like series of Ca\(^{2+}\) oscillations (Tesarik & Sousa 1994, Nakano et al. 1997). These data, therefore, offer another line of support for the hypothesis that the sperm activates eggs by introducing a factor after fusion. However, the solubility of the sperm factor that is active in ICSI is less clear. Injection of sperm heads, that have lost most of their soluble proteins, can cause Ca\(^{2+}\) oscillations and egg activation in the mouse (Perry et al. 2000). Extensive extraction of proteins from the sperm head is required to remove Ca\(^{2+}\)-releasing activity (Perry et al. 2000). The active factor appears to be a protein localized in the perinuclear theca which is a dense protein matrix between the sperm nucleus and plasma membrane (Kimura et al. 1998). Experiments that examined Ca\(^{2+}\) oscillations after ICSI in the mouse have also suggested that the sperm does not release all of its factor until 30 minutes after sperm injection into the cytoplasm (Knot et al. 2003). None of these data exclude the idea that the sperm protein that is effective in ICSI is the same one that has been previously characterized as being the soluble sperm factor, but they do suggest that the sperm factor protein may take some time to be completely released from the perinuclear theca in mouse sperm. It is of interest to note that during normal ICSI it is common to immobilize the sperm by mechanical damage to the sperm tail just before injection into the egg (Yanagida et al. 2001). The damage induced by this procedure could well help the sperm factor to be released once it is inside the egg since extensive mechanical disruption of the sperm membrane leads to a more rapid onset of Ca\(^{2+}\) oscillations after ICSI (Yanagida et al. 2001).

Since it may well represent the physiological activator of development it is clearly important to identify the soluble sperm factor in mammals and other species. The identification of the soluble sperm factor should also help resolve other issues regarding its solubility, localization and role in ICSI. Several years ago we made a sustained effort to identify the mammalian sperm factor protein by the direct approach of protein purification. We identified a 33 kDa protein that correlated with the ability of sperm extracts to cause Ca\(^{2+}\) oscillations (Parrington et al. 1996). However, this protein turned out to be a metabolic enzyme that was not responsible for Ca\(^{2+}\) release (Wolosker et al. 1998, Parrington et al. 1999). Other candidate sperm factors are the truncated form of the c-kit receptor, tr-kit (Sette et al. 1997), or a perinuclear theca protein (Sutovsky et al. 2003). Such proteins are sperm specific and have been shown to activate mammalian eggs after microinjection. However, as already discussed the activation assay alone is insufficient, and there are no reports of whether these proteins can cause Ca\(^{2+}\) oscillations in eggs. This makes them both rather premature candidates since a demonstration of the appropriate Ca\(^{2+}\)-releasing activity is the most important test for a sperm-derived molecule to be considered as an egg-activating factor.
The biochemical mechanism of the mammalian sperm factor

A key step in identifying the sperm factor is to characterize its basic mechanism of Ca\(^{2+}\) release. In order to understand how the sperm factor mediates its effects, we employed a cell-free egg-based system to study Ca\(^{2+}\) release. The sea urchin egg homogenate has been used to study Ca\(^{2+}\) release by a number of different laboratories (Lee 1997). It can release Ca\(^{2+}\) in response to inositol 1,4,5-trisphosphate (InsP\(_3\)), cyclic ADP-ribose (ADPR) and NAADP. In fact, the novel messengers cADPR and NAADP were both discovered by using the sea urchin egg homogenate (Lee 1997). We found that, as well as responding to the above range of Ca\(^{2+}\)-releasing agents, the sea urchin egg homogenate also shows a distinctive phase of Ca\(^{2+}\) release after addition of mammalian sperm extracts (Galion et al. 1997, Jones et al. 1998). Figure 2a shows a sperm extract-induced Ca\(^{2+}\)-release response in the sea urchin egg homogenate. The factor in sperm extracts that is effective at triggering this Ca\(^{2+}\) release appears to be the same factor that causes Ca\(^{2+}\) oscillations in mouse eggs (Parrington et al. 1999). This means that, regardless of whether sea urchins have an active sperm factor, the sea urchin egg homogenate system is a simple and valid assay for examining the mechanism of action of the mammalian sperm factor.

The sperm factor causes Ca\(^{2+}\) release in the homogenate system with a distinctive delay and gradual upstroke, with the rate of rise of Ca\(^{2+}\) increase being a function of the amount of sperm factor added (Jones et al. 2000). This makes the egg homogenate a more quantitative assay for the sperm factor than microinjection into mouse eggs. There are also some biochemical experiments that can be performed with the sperm factor in the homogenate system that are not practical in intact mammalian eggs. For example, we found that addition of mammalian sperm extracts to the sea urchin egg homogenate causes a large increase in InsP\(_3\) levels (Jones et al. 1998, Rice et al. 2000). This increase plays a causal role in Ca\(^{2+}\) release because the prior desensitization of InsP\(_3\)-induced Ca\(^{2+}\) release blocks the sperm factor response (Jones et al. 1998). In contrast, desensitization of cADPR or NAADP signalling does not affect the sperm factor-induced Ca\(^{2+}\) release. Consequently, the homogenate study clearly shows that the mammalian sperm factor works via the generation of InsP\(_3\). These data are consistent with experiments on intact frog eggs where mammalian sperm extract injection is also accompanied by an increase in InsP\(_3\) levels (Wu et al. 2001).

Since the sperm factor leads to a rapid increase in InsP\(_3\), then it is likely that a phospholipase C (PLC) is involved. One possibility is that the sperm factor stimulates a PLC in the egg. In sea urchin, starfish and ascidian eggs it has been suggested that egg-derived Src-like tyrosine kinases, and PLC\(_{\gamma}\), are both critical for Ca\(^{2+}\) release at fertilization (Jaffe et al. 2001). This could explain how the sperm extracts cause Ca\(^{2+}\) release in the sea urchin egg-based system that we used. We initially carried out some studies to see if protein kinases may be important for mediating Ca\(^{2+}\) release via the sperm factor. Figure 2 shows sperm extract-induced Ca\(^{2+}\) release in sea urchin egg homogenates after addition of the tyrosine kinase inhibitor genistein, or a more general phosphorylation inhibitor staurosporine. High concentrations of either of these inhibitors, or other protein kinase inhibitors, failed to inhibit Ca\(^{2+}\) release induced by the sperm factor. We have also found that sperm extracts could cause Ca\(^{2+}\) release in homogenates after depletion of ATP with apyrase (data not shown). While these are negative data they still suggest that the idea that the sperm factor causes Ca\(^{2+}\) release via protein phosphorylation is not an easy hypothesis to sustain (see Kurokawa et al. and Talmor-Cohen et al. (this issue) for data on the role of Src kinases in egg activation).

It turns out that there is a much simpler hypothesis to explain the origin of InsP\(_3\) generation triggered by the sperm factor. We found that the sperm extracts themselves contain a PLC activity, and that this enzymatic activity is correlated with the Ca\(^{2+}\)-releasing activity (Jones et al. 1998). This PLC activity is very high in the sperm extracts that are effective in causing Ca\(^{2+}\) oscillations in eggs. In fact the PLC activity is sufficiently high that it could theoretically account for a significant generation of InsP\(_3\) when a single sperm equivalent is introduced into the egg cytoplasm (Rice et al. 2000). The sperm extract PLC is also unusual in that it is active at very low Ca\(^{2+}\) levels and that it can readily hydrolyse phosphatidylinositol 4,5-diacylglycerol.
bisphosphate (PIP$_2$) in free solution (Jones et al. 2000, Rice et al. 2000). The sperm PLC activity offers an obvious explanation of how sperm extracts cause InsP$_3$ production since the sperm factor could itself be a PLC enzyme. In this case the sperm is envisaged to act by introducing an exogenous PLC activity into the egg that leads to the InsP$_3$ production at fertilization.

We made an attempt to identify the sperm extract PLC by the use of a panel of antibodies against the known isoforms. When we started this line of enquiry there were a variety of known mammalian PLCs classified as the $\beta$, $\gamma$ and $\delta$ forms (Katan 1998). There are also subtypes of the $\beta$, $\gamma$ and $\delta$ forms. Screening sperm extracts with a variety of these antibodies failed to show evidence for high concentrations of any of the known PLC isoforms in sperm extracts (Parrington et al. 2002). Even where low levels of a known PLC could be detected, for example PLC$_{G2}$, there was no correlation between this PLC isoform and Ca$^{2+}$-releasing activity (Parrington et al. 2002). The only PLC that we did not have antibodies to was the recently described PLC$_{Gz}$, but this is unlikely to be the sperm factor PLC since PLC$_{Gz}$ has a molecular mass of $>$200 kDa (Kelley et al. 2001), and the molecular size of the sperm factor is somewhere between 30 and 70 kDa (Wu et al. 1998, Parrington et al. 2002). The molecular size data alone suggest that the PLC would most likely to be of a PLC$8$ class, but the PLC$8$s are not concentrated in active sperm extracts (Parrington et al. 2002). Furthermore, although PLC$84$ is found in whole sperm, it is evidently not a sperm factor since sperm from PLC$84$ knockout mice still cause Ca$^{2+}$ oscillations during fertilization, or ICSI (Fukami et al. 2001).

Another way of investigating the role of a PLC in mediating the effects of the sperm factor was to investigate if any of the known PLCs could trigger Ca$^{2+}$ release in eggs. We used purified recombinant PLCs of $\gamma$, $\beta$ and $\delta$ classes and found no evidence for Ca$^{2+}$ release associated with any isoform after they were injected into intact mouse eggs, or added to the sea urchin egg homogenate (Jones et al. 2000). The specific activity of these PLCs was higher than the PLC activity of sperm extracts that were effective in causing Ca$^{2+}$ release, so these data clearly suggest that the common isoforms of PLCs cannot account for the sperm factor’s ability to generate InsP$_3$. Although we found no Ca$^{2+}$-releasing activity of PLC$_{G1}$ and PLC$_{G1}$ in mouse eggs, there are reports that show that injection of recombinant PLC$_{G1}$, or PLC$_{G1}$, can cause a short-lasting series of Ca$^{2+}$ oscillations (Mehlmann et al. 2001, Kouchi et al. 2004). We do not know why these results differ from ours. However, we did find that one of the problems of using apparently pure preparations of PLC proteins is that they can be contaminated with InsP$_3$, which by itself can cause short-lasting Ca$^{2+}$ oscillations (Jones et al. 2000). Regardless of the difference in results with other PLCs, there is no doubt that the level of PLC$_{G1}$ activity that was reported as being effective at causing Ca$^{2+}$ oscillations in mouse eggs was more than 500 times higher that the total PLC activity in a single mouse sperm. Consequently, it was argued by Jaffe’s group that the sperm factor is unlikely to be a PLC (Mehlmann et al. 2001, Runft et al. 2002). However, this suggestion discounts the possibility that an unidentified PLC could be responsible for InsP$_3$ production by the sperm factor. If the sperm factor is another type of PLC, then it would have to have some quite distinct features compared with PLC$_{G1}$, or probably any of the other known PLC isoforms.

The discovery of a novel phospholipase C: PLC$_{Gz}$

Following a search of a mouse testis expressed sequence tag (EST) database a novel PLC was identified (Saunders et al. 2002). This PLC was smaller than other mammalian PLCs ($\sim$70 kDa) and, having some distinctive domain structures, it was termed PLC$z$ (zeta). Northern blot analysis revealed that PLC$_{Gz}$ expression could only be detected in testis. Further analysis using PCR suggests that expression of PLC$_{Gz}$ occurs in spermatids and not in the meiotic steps of spermiogenesis (Saunders et al. 2002). Protein blots revealed that PLC$_{Gz}$ protein is specifically present in whole sperm and in sperm extracts from mouse, hamster, pig and humans (Cox et al. 2002, Saunders et al. 2002). Furthermore, when sperm extracts were fractionated and assayed for the ability to cause Ca$^{2+}$ release in sea urchin egg homogenates, the Ca$^{2+}$-releasing activity correlated with the presence of PLC$_{Gz}$ protein (Saunders et al. 2002). All these data suggested that PLC$_{Gz}$ might be a key component of the mammalian sperm factor protein. However, the critical experiment is to examine if PLC$_{Gz}$ alone can trigger Ca$^{2+}$ oscillations in eggs and, therefore, whether PLC$_{Gz}$ is sufficient to explain the cytosolic sperm factor activity in eggs.

To test whether PLC$_{Gz}$ can cause Ca$^{2+}$ changes in eggs we chose to inject the cRNA encoding for PLC$_{Gz}$. Injecting cRNA has previously been used as an effective means of expressing specific proteins in mouse eggs. In fact we have previously shown that injecting mouse eggs with mRNA from spermatogenic cells can cause fertilization-like Ca$^{2+}$ oscillations, suggesting that the sperm factor should be capable of being translated into an active state in egg cytoplasm (Parrington et al. 2000). The method of injecting RNA also avoids problems of generating and stabilizing the activity of a protein made in another expression system. In addition, the injection of artificially made cRNA avoids the possibility of injecting eggs with contaminating molecules derived from the cell system used to make a recombinant PLC$_{Gz}$. We found that when we injected PLC$_{Gz}$ cRNA into mouse eggs, it triggered a prolonged series of Ca$^{2+}$ oscillations (see Fig. 3). The initial Ca$^{2+}$ transient at fertilization in mouse has a characteristic form with smaller oscillations on top of a larger increase, and this pattern is mimicked by injection of PLC$_{Gz}$ cRNA. The frequency and number of subsequent oscillations depended upon the amount of PLC$_{Gz}$ cRNA injected (Saunders et al. 2002). The amount
of PLCζ protein expressed in these experiments was measured by injecting a Myc-tagged version of PLCζ that could be assayed by immunoblots. This method showed that 10–75 fg PLCζ was effective in causing Ca2+ oscillations in mouse eggs at a frequency that mimicked the response at fertilization. Since it was estimated that a single mouse sperm contains about 20–50 fg PLCζ, these data clearly suggest that the sperm-derived PLCζ can offer an explanation of the ability of a single mouse sperm to cause Ca2+ oscillations in eggs during fertilization. It also shows that the presence of PLCζ in sperm extracts offers a sufficient explanation of why they can cause Ca2+ oscillations when injected into eggs.

In order to establish if PLCζ is necessary to explain the ability of sperm extracts to cause Ca2+ release, we used specific antibodies to PLCζ to deplete selectively the PLCζ protein from sperm extracts. We found that sperm extracts depleted of PLCζ did not cause Ca2+ oscillations in mouse eggs under conditions where control antibody-depleted extracts did cause oscillations (Saunders et al. 2002). The same immunodepletion experiments also showed that PLCζ was essential for triggering Ca2+ release in sea urchin egg homogenates. These data, therefore, indicate that PLCζ is necessary to explain the effect of the sperm factor on Ca2+ release. Although it remains a formal possibility that some other accessory protein could be bound to PLCζ it must be either insignificant for biological activity, or else be present in the egg, because the introduction of PLCζ alone into eggs is sufficient to cause the complete series of oscillations triggered at fertilization. It is also worth noting that two previous estimates of the molecular mass of the sperm factor are within the range of 30–70 kDa (Wu et al. 1998, Parrington et al. 2002), and since PLCζ is ~70 kDa there is little scope for incorporating other proteins into an active complex. Consequently, we are confident that a monomeric PLCζ molecule is synonymous with the previously described cytosolic sperm factor in soluble sperm extracts.

Although we injected PLCζ cRNA, a recent report has now shown that a recombinant PLCζ can also cause Ca2+ oscillations when injected into mouse eggs (Kouchi et al. 2004). Slightly more recombinant PLCζ protein was required to cause Ca2+ oscillations than we found as being effective after expression in vitro (Kouchi et al. 2004). This probably reflects the problems inherent in stabilizing the activity of PLCζ. Nevertheless, it is significant that the recombinant PLCζ protein was also shown to be active in eggs and to have enzymatic activity in vitro. The PLCζ activity in vitro is unusual in that it is active at much lower free-Ca2+ concentrations than other PLCs (Kouchi et al. 2004). This result is consistent with the high sensitivity to Ca2+ that was previously shown with boar sperm extracts (Rice et al. 2000). These data suggest that PLCζ is able to cause substantial amounts of InsP3 production at resting levels of Ca2+. The increase in PLC activity with increasing Ca2+ also offers a mechanism for positive feedback on InsP3 production that may have a role in generating the pattern of Ca2+ oscillations.

As well as being highly effective in causing Ca2+ oscillations it is also important that PLCζ is an effective parthenogenetic activating agent for mouse eggs. Injecting cRNA to express physiological amounts of PLCζ in mouse eggs leads to activation and development up to the blastocyst stage (Saunders et al. 2002). This suggests that the Ca2+ oscillations induced by PLCζ are a sufficient explanation of how the sperm stimulates preimplantation development in mammals. In accordance with previous studies of the sperm factor, we have also found that PLCζ is not species specific. We have identified a PLCζ gene in mice, humans and cynomolgous monkeys. The human and monkey PLCζ are effective at causing Ca2+ oscillations in mouse eggs and at triggering development up to the blastocyst stage (Cox et al. 2002). The main difference that is apparent between these different forms of PLCζ is with regards to their activities. In terms of the amount of injected cRNA, the human form of PLCζ is more than one order of magnitude more effective at causing Ca2+ oscillations and egg activation, in mouse eggs, than either the mouse or monkey forms. The reasons for this difference in activity of PLCζ from different species are not yet clear.

Future directions

There are still many basic questions to be addressed regarding the mechanism of action of PLCζ, and its full role at fertilization. One of the most important issues to address is the unequivocal demonstration of the role of PLCζ in normal fertilization. We have shown that PLCζ is the soluble sperm factor. Nevertheless, the demonstration of the full role of PLCζ in Ca2+-signalling during normal in vitro fertilization will only be absolutely clear when a specific means is used to stop sperm-derived PLCζ from causing InsP3 production. The precise localization of PLCζ in the sperm before fertilization also needs to be established. The PLCζ gene does not have a signal sequence that would target the protein product into an
organelle, so it is reasonable to suggest that it is within the cytosolic compartment of the sperm. This is consistent with the cytosolic, or sub-plasma membrane, localization of most of the known mammalian PLCs (Katan 1998). However, exactly where and how PLCζ is packaged within the rather limited space of a sperm cytoplasm is unclear.

It will be important to understand how PLCζ is regulated in the sperm and egg. For example, in the sperm it is known that an increase in phosphoinositide hydrolysis is involved in the acrosome reaction (Breitbart 2002). The sperm does not undergo an acrosome reaction until capacitated, and since it appears to contain substantial amounts of PLCζ it is remarkable how it avoids undergoing continuous unstimulated phosphoinositide turnover. There must clearly be some very effective switch in signaling capacity of PLCζ during fertilization. The sperm contains a potent PLCζ that is apparently inactive inside the sperm, and yet when introduced into the egg PLCζ is extremely active under conditions where other PLC iso-enzymes are either inactive, or much less effective.

It is likely that the structure of PLCζ will in some way shed light on a number of issues surrounding the actions of PLCζ. The domain structure of PLCζ is different from other animal PLCs. Most mammalian PLC isoforms contain a distinct series of domains. These include so-called X and Y domains that are responsible for catalytic activity. In addition there are EF hand domains that may bind Ca2+, a C2 domain that can bind Ca2+ or phospholipids, and a PH domain that binds to polyphosphoinositides such as PIP2, or other proteins (Katan 1998). The closest relatives of PLCζ in animals are PLCs of the ε class. The PLCεs are made from all of the above protein domains. PLCζ1 in particular is known to have a PH domain that targets it to PIP2 in the plasma membrane. PLCζ has X and Y domains, two EF hands, and a C2 domain, but it does not have a PH domain. This domain structure of PLCζ is preserved in all three species in which it has been identified. The X and Y catalytic domains appear to be functional in hydrolysing PIP2 because the mutation of a specific aspartate residue, that is essential for the catalytic activity on PLCζ, is able to abolish the ability of PCLζ to cause Ca2+ oscillations (Saunders et al. 2002). The most unusual feature of PLCζ, however, is that it does not contain a PH domain. This makes it unclear how it binds to membranes where PIP2 is located. The lack of a PH domain is not unprecedented since the plant forms for PIP2-specific PLCs also lack a PH domain. It is worth noting that the lack of a PH domain does not, by itself, confer the specific features on PLCζ because injecting cRNA for a PLCζ that lacks a PH domain does not cause Ca2+ oscillations in eggs (Saunders et al. 2002). We can only assume that some specific characteristics of the remaining domains of PLCζ allow it to bind to an appropriate source of PIP2 in the egg. The appropriate source of PIP2 for PLCζ is also unclear. Most cells have PIP2 predominantly localized in the plasma membrane, but experiments in the sea urchin egg homogenate suggest that the sperm factor PLC activity is acting upon a substrate that is localized on acid vesicles (Rice et al. 2000).

As well as providing a potential explanation for how the sperm initiates the series of Ca2+ oscillations at fertilization, the molecular properties of PLCζ may provide a greater understanding of how the oscillations can be stopped in mammalian eggs. The Ca2+ oscillations at fertilization in mammals last for several hours. Exactly when the oscillations are seen to stop can vary depending upon the quality of the oocyte, and probably the methods of measuring Ca2+ (Cheung et al. 2000). However, using minimal concentrations of dextran-conjugated fluorescent indicators to reduce Ca2+ buffering, it has been shown that Ca2+ oscillations eventually stop around the time of pronuclei formation (Marangos et al. 2003). The formation of pronuclei is inhibitory for generating Ca2+ release and no Ca2+ transients are observed in interphase. However, in mouse zygotes Ca2+ oscillations are seen again just after the breakdown of pronuclei (Marangos et al. 2003). A number of lines of evidence have led to the suggestion that this cell cycle dependency of Ca2+ oscillations at fertilization is due to nuclear sequestration of a sperm-derived factor (Carroll 2001). The proposal is that the localization of a sperm-derived factor in the nucleus stops it from causing Ca2+ oscillations, and that the release of the factor during the first mitosis causes the extra Ca2+ oscillations that are specifically seen in zygotes. We have recently shown that PLCζ becomes localized to pronuclei in PLCζ-activated mouse eggs (Larman et al. 2004). The nuclear localization plays a causal role in terminating the PLCζ-induced Ca2+ oscillations because blocking nuclear import of PLCζ leads to prolonged Ca2+ oscillations in eggs (Larman et al. 2004). We also found that the nuclear localization of PLCζ is due to a specific sequence of basic amino acids localized within a region of the protein between the X and Y catalytic domains (Larman et al. 2004). These data suggest that the sequestration of PLCζ in the pronuclei of embryos can explain why zygotes specifically show Ca2+ oscillations during the meiotic and mitotic phase of the first cell cycle, and not during interphase. They also suggest that PLCζ may be regulated by novel mechanisms compared with other somatic tissue-derived PLCs.

We consider that PLCζ offers the molecular basis of an explanation of how Ca2+ release is triggered during mammalian fertilization (see Fig. 4). If this is the case then there are implications for explaining certain cases of male factor infertility. For example 40% of failed fertilization after ICSI are reported to be due to the failure of egg activation (Rawe et al. 2000). In these cases the sperm is within the egg cytoplasm but a stimulus for activation is apparently missing. Our knowledge of PLCζ may also have wider implications for our understanding of fertilization in general. There is clearly evidence for the existence of a Ca2+-releasing sperm factor in some non-mammalian species. It is possible that molecules similar to PLCζ can
also offer the basis for explanation of sperm-induced Ca\textsuperscript{2+} release in a wide range of animal species.

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References

Carroll J 2001 The initiation and regulation of Ca\textsuperscript{2+} signalling at fertilization in mammals. Seminars in Cell and Developmental Biology 12 37–43.


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


Faure JE, Myles DG & Primakoff P 1999 The frequency of calcium oscillations in mouse eggs at fertilization is modulated by the number of fused sperm. Developmental Biology 213 370–377.

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


Gallone A, Jones KT, Lai FA & Swann K 1997 A cytosolic sperm factor mobilizes Ca\textsuperscript{2+} from intracellular stores by activating multiple Ca\textsuperscript{2+} release mechanisms independently of low molecular weight messengers. Journal of Biological Chemistry 272 28901–28905.


Kline D & Kline JT 1992 Repetitive calcium transients and the role of calcium in oocytosol and cell cycle activation in the mouse egg. Developmental Biology 149 80–89.


Kouchi Z, Fukami K, Shikano T, Oda S, Nakamura Y, Takenawa T & Miyazaki S 2004 Recombinant phospholipase C-zeta has high Ca\textsuperscript{2+} sensitivity and induces Ca\textsuperscript{2+} oscillations in mouse eggs. Journal of Biological Chemistry (In Press).


Larman MG, Saunders CM, Carroll J, Lai FA & Swann K Cell cycle-dependent Ca\textsuperscript{2+} oscillations in mouse embryos are regulated by nuclear targeting of PLC\textsubscript{\zeta}, Journal of Cell Science (In Press).

Lawrence Y, Whitaker M & Swann K 1997 Sperm-oocyte fusion is the prelude to the initial Ca\textsuperscript{2+} increase at fertilization in the mouse. Development 124 223–241.


McCulloh DH & Chambers EL 1992 Fusion of membranes during fertilization: increases of sea urchin egg’s membrane capacitance and membrane conductance at the site of contact with the sperm. Journal of General Physiology 99 137–175.


Marangos P, FitzHarris G & Carroll J 2003 Ca^{2+} oscillations at fertilization in mammals are regulated by the formation of pronuclei. Development 130 1461–1472.

Mehlmann LM, Chattopadhyay A, Carpenter G & Jaffe LA 2001 Evidence that phospholipase C from the sperm is not responsible for initiating Ca^{2+} release at fertilization in mouse eggs. Developmental Biology 236 492–501.


Miyazaki S, Shirakawa H, Nakada K & Honda Y 1993 Essential role of the inositol 1,4,5-trisphosphate/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. Developmental Biology 58 62–78.


Parrington J, Jones KT, Lai FA & Swann K 1999 The soluble sperm factor that causes Ca^{2+} release from sea urchin egg homogenates also triggers Ca^{2+} oscillations after injection into mouse eggs. Biochemical Journal 341 1–4.


Stricker SA 1999 Comparative biology of calcium signaling during fertilization and egg activation in animals. Developmental Biology 211 157–176.


Tang TS, Dong JB, Huang XY & Sun FZ 2000 Ca^{2+} oscillations induced by a cytosolic sperm factor are mediated by a maternal machinery that functions only once in mammalian eggs. Development 127 1141–1150.


