Focus on Fertilization

The cytosolic sperm factor that triggers Ca^{2+} oscillations and egg activation in mammals is a novel phospholipase C: PLC ζ K Swann¹, M G Larman^{1,2}, C M Saunders² and F A Lai²¹ Department of Anatomy and Developmental Biology, University College London, London WC1E 6BT, UK and² Cell Signalling Laboratory, Wales Heart Research Institute, University of Wales College of Medicine, Cardiff CF14 4XN, UKCorrespondence should be addressed to K Swann; Email: ucgakas@ucl.ac.uk**Abstract**

When sperm activate eggs at fertilization the signal for activation involves increases in the intracellular free Ca^{2+} concentration. In mammals the Ca^{2+} changes at fertilization consist of intracellular 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 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 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 ζ (zeta). We describe the evidence that led to the identification of PLC ζ and discuss the issues relating to its potential role in fertilization.

Reproduction (2004) **127** 431–439**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 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 Ca^{2+} increase occurred after sperm entry in fertilizing medaka fish eggs. Since then, 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- Ca^{2+} ion concentration (Lindsay *et al.* 1992, Lee *et al.* 1999). This emphasizes the point that some form of 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 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+} itself 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

Figure 1 Ca^{2+} oscillations in mouse eggs during *in vitro* fertilization. Zona-free mouse eggs were fertilized as described previously (Saunders *et al.* 2002). Intracellular Ca^{2+} was measured with fura red. The y-axis represents increasing Ca^{2+} concentrations in units of the fluorescence excitation ratio (RU) for fura red (420/490 nm). Sperm were added to eggs at the start of the trace and the subsequent increases in Ca^{2+} are represented as spike-like upward deflections. The oscillations in Ca^{2+} last for several hours after sperm–egg interaction.

(Swann 1991, Kline & Kline 1992). Despite causing oscillations, the exact pattern of Ca²⁺ changes triggered by these agents is distinguishable from those at fertilization. The Ca²⁺ spikes induced by strontium, for example, are of longer duration and smaller amplitude than those triggered by the sperm. Consequently, the Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ oscillations or waves (Stricker 1997, Kyojuka *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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺

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 Ca²⁺ oscillations and egg activation in the mouse (Perry *et al.* 2000). Extensive extraction of proteins from the sperm head is required to remove Ca²⁺-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 Ca²⁺ 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 (Knott *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 Ca²⁺ 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 Ca²⁺ oscillations (Parrington *et al.* 1996). However, this protein turned out to be a metabolic enzyme that was not responsible for Ca²⁺ 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 Ca²⁺ oscillations in eggs. This makes them both rather premature candidates since a demonstration of the appropriate Ca²⁺-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 (Galione *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

Figure 2 Ca^{2+} release in the sea urchin egg homogenate. Ca^{2+} concentrations were monitored in sea urchin egg homogenates with Fluo3 fluorescence units (RFUs). See Jones *et al.* (1998) for full experimental details. (a) Ca^{2+} release in the homogenate system is triggered by addition of boar sperm extract. (b, c) The addition of staurosporine, or genistein, did not inhibit Ca^{2+} release caused by subsequent addition of sperm extracts as in (a) (genistein by itself caused a small rise in Ca^{2+}). Similar results were seen with other protein kinase inhibitors such as 6-dimethylaminopurine (6-DMAP) or tyrphostin B42 (data not shown).

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 $\text{PLC}\gamma$, 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 genestein, 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

bisphosphate (PIP₂) in free solution (Jones *et al.* 2000, Rice *et al.* 2000). The sperm PLC activity offers an obvious explanation of how sperm extracts cause InsP₃ 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 InsP₃ 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 β , γ and δ forms (Katan 1998). There are also subtypes of the β , γ and δ 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 δ 2, there was no correlation between this PLC isoform and Ca²⁺-releasing activity (Parrington *et al.* 2002). The only PLC that we did not have antibodies to was the recently described PLC ϵ , but this is unlikely to be the sperm factor PLC since PLC ϵ 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 δ class, but the PLC δ s are not concentrated in active sperm extracts (Parrington *et al.* 2002). Furthermore, although PLC δ 4 is found in whole sperm, it is evidently not a sperm factor since sperm from PLC δ 4 knockout mice still cause Ca²⁺ 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 Ca²⁺ release in eggs. We used purified recombinant PLCs of γ , β and δ classes and found no evidence for Ca²⁺ 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 Ca²⁺ release, so these data clearly suggest that the common isoforms of PLCs cannot account for the sperm factor's ability to generate InsP₃. Although we found no Ca²⁺-releasing activity of PLC γ 1 and PLC δ 1 in mouse eggs, there are reports that show that injection of recombinant PLC γ 1, or PLC δ 1, can cause a short-lasting series of Ca²⁺ 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 InsP₃, which by itself can cause short-lasting Ca²⁺ oscillations (Jones *et al.* 2000). Regardless of the difference in results with other PLCs, there is no doubt that the level of PLC γ activity that was reported as being effective at causing Ca²⁺ oscillations in mouse eggs was more than 500 times higher than 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 InsP₃ 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 γ 1, or probably any of the other known PLC isoforms.

The discovery of a novel phospholipase C: PLC ζ

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 (~70 kDa) and, having some distinctive domain structures, it was termed PLC ζ (zeta). Northern blot analysis revealed that PLC ζ expression could only be detected in testis. Further analysis using PCR suggests that expression of PLC ζ occurs in spermatids and not in the meiotic steps of spermiogenesis (Saunders *et al.* 2002). Protein blots revealed that PLC ζ 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 Ca²⁺ release in sea urchin egg homogenates, the Ca²⁺-releasing activity correlated with the presence of PLC ζ protein (Saunders *et al.* 2002). All these data suggested that PLC ζ might be a key component of the mammalian sperm factor protein. However, the critical experiment is to examine if PLC ζ alone can trigger Ca²⁺ oscillations in eggs and, therefore, whether PLC ζ is sufficient to explain the cytosolic sperm factor activity in eggs.

To test whether PLC ζ can cause Ca²⁺ changes in eggs we chose to inject the cRNA encoding for PLC ζ . 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 Ca²⁺ 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 ζ . We found that when we injected PLC ζ cRNA into mouse eggs, it triggered a prolonged series of Ca²⁺ oscillations (see Fig. 3). The initial Ca²⁺ 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 ζ cRNA. The frequency and number of subsequent oscillations depended upon the amount of PLC ζ cRNA injected (Saunders *et al.* 2002). The amount

Figure 3 Ca^{2+} oscillations in a mouse egg after injection of 0.02 mg/ml PLC ζ cRNA (see Saunders *et al.* 2002 for details). Conditions are the same as in Fig. 1 except that fura2-dextran was used to measure intracellular Ca^{2+} and the ratio units are for fura2-dextran excitation ratio (350/380 nm). The egg was injected ~30 min before the start of the recording. It can be seen that PLC ζ expression leads to a prolonged series of repetitive Ca^{2+} rises similar to those seen at fertilization.

of PLC ζ protein expressed in these experiments was measured by injecting a Myc-tagged version of PLC ζ that could be assayed by immunoblots. This method showed that 10–75 fg PLC ζ was effective in causing Ca^{2+} 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 PLC ζ these data clearly suggest that the sperm-derived PLC ζ can offer an explanation of the ability of a single mouse sperm to cause Ca^{2+} oscillations in eggs during fertilization. It also shows that the presence of PLC ζ in sperm extracts offers a sufficient explanation of why they can cause Ca^{2+} oscillations when injected into eggs.

In order to establish if PLC ζ is necessary to explain the ability of sperm extracts to cause Ca^{2+} release, we used specific antibodies to PLC ζ to deplete selectively the PLC ζ protein from sperm extracts. We found that sperm extracts depleted of PLC ζ did not cause Ca^{2+} 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 PLC ζ was essential for triggering Ca^{2+} release in sea urchin egg homogenates. These data, therefore, indicate that PLC ζ is necessary to explain the effect of the sperm factor on Ca^{2+} release. Although it remains a formal possibility that some other accessory protein could be bound to PLC ζ it must be either insignificant for biological activity, or else be present in the egg, because the introduction of PLC ζ 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 PLC ζ is ~70 kDa there is little scope for incorporating other proteins into an active complex. Consequently, we are confident that a monomeric PLC ζ molecule is synonymous with the previously described cytosolic sperm factor in soluble sperm extracts.

Although we injected PLC ζ cRNA, a recent report has now shown that a recombinant PLC ζ can also

cause Ca^{2+} oscillations when injected into mouse eggs (Kouchi *et al.* 2004). Slightly more recombinant PLC ζ protein was required to cause Ca^{2+} 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 PLC ζ . Nevertheless, it is significant that the recombinant PLC ζ protein was also shown to be active in eggs and to have enzymatic activity *in vitro*. The PLC ζ activity *in vitro* is unusual in that it is active at much lower free- Ca^{2+} concentrations than other PLCs (Kouchi *et al.* 2004). This result is consistent with the high sensitivity to Ca^{2+} that was previously shown with boar sperm extracts (Rice *et al.* 2000). These data suggest that PLC ζ is able to cause substantial amounts of InsP_3 production at resting levels of Ca^{2+} . The increase in PLC activity with increasing Ca^{2+} also offers a mechanism for positive feedback on InsP_3 production that may have a role in generating the pattern of Ca^{2+} oscillations.

As well as being highly effective in causing Ca^{2+} oscillations it is also important that PLC ζ is an effective parthenogenetic activating agent for mouse eggs. Injecting cRNA to express physiological amounts of PLC ζ in mouse eggs leads to activation and development up to the blastocyst stage (Saunders *et al.* 2002). This suggests that the Ca^{2+} oscillations induced by PLC ζ 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 PLC ζ is not species specific. We have identified a PLC ζ gene in mice, humans and cynomolgous monkeys. The human and monkey PLC ζ are effective at causing Ca^{2+} 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 PLC ζ is with regards to their activities. In terms of the amount of injected cRNA, the human form of PLC ζ is more than one order of magnitude more effective at causing Ca^{2+} oscillations and egg activation, in mouse eggs, than either the mouse or monkey forms. The reasons for this difference in activity of PLC ζ from different species are not yet clear.

Future directions

There are still many basic questions to be addressed regarding the mechanism of action of PLC ζ , and its full role at fertilization. One of the most important issues to address is the unequivocal demonstration of the role of PLC ζ in normal fertilization. We have shown that PLC ζ is the soluble sperm factor. Nevertheless, the demonstration of the full role of PLC ζ in Ca^{2+} -signalling during normal *in vitro* fertilization will only be absolutely clear when a specific means is used to stop sperm-derived PLC ζ from causing InsP_3 production. The precise localization of PLC ζ in the sperm before fertilization also needs to be established. The PLC ζ 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 PLC ζ is packaged within the rather limited space of a sperm cytoplasm is unclear.

It will be important to understand how PLC ζ 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 PLC ζ it is remarkable how it avoids undergoing continuous unstimulated phosphoinositide turnover. There must clearly be some very effective switch in signalling capacity of PLC ζ during fertilization. The sperm contain a potent PLC ζ that is apparently inactive inside the sperm, and yet when introduced into the egg PLC ζ is extremely active under conditions where other PLC isozymes are either inactive, or much less effective.

It is likely that the structure of PLC ζ will in some way shed light on a number of issues surrounding the actions of PLC ζ . The domain structure of PLC ζ is different from other animal PLCs. Most mammalian PLC isozymes 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 PLC ζ in animals are PLCs of the δ class. The PLC δ s are made from all of the above protein domains. PLC δ 1 in particular is known to have a PH domain that targets it to PIP₂ in the plasma membrane. PLC ζ has X and Y domains, two EF hands, and a C2 domain, but it does not have a PH domain. This domain structure of PLC ζ 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 PLCs, is able to abolish the ability of PLC ζ to cause Ca²⁺ oscillations (Saunders *et al.* 2002). The most unusual feature of PLC ζ , 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 PLC ζ because injecting cRNA for a PLC δ 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 PLC ζ allow it to bind to an appropriate source of PIP₂ in the egg. The appropriate source of PIP₂ for PLC ζ 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 PLC ζ 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 PLC ζ becomes localized to pronuclei in PLC ζ -activated mouse eggs (Larman *et al.* 2004). The nuclear localization plays a causal role in terminating the PLC ζ -induced Ca²⁺ oscillations because blocking nuclear import of PLC ζ leads to prolonged Ca²⁺ oscillations in eggs (Larman *et al.* 2004). We also found that the nuclear localization of PLC ζ 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 PLC ζ 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 PLC ζ may be regulated by novel mechanisms compared with other somatic tissue-derived PLCs.

We consider that PLC ζ 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 PLC ζ 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 PLC ζ can

Figure 4 Schematic representation of the basic hypothesis for how PLC ζ initiates Ca^{2+} release in mammalian eggs. After fusion of the sperm and egg plasma membranes the sperm-derived PLC ζ protein diffuses into the egg cytoplasm. This hydrolyses PIP_2 , from an unknown source, to generate InsP_3 . It is possible that the subsequent rise in Ca^{2+} leads to the regulation of PLC ζ activity.

also offer the basis for explanation of sperm-induced Ca^{2+} release in a wide range of animal species.

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