Potential role of a sperm-derived phospholipase C in triggering the egg-activating Ca^{2+} signal at fertilization

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An increase in intracellular Ca^{2+} at fertilization is the trigger for egg activation in all species that have been studied. Exactly how sperm–egg interaction leads to this Ca^{2+} increase has not been established. There is increasing support for the hypothesis that the spermatozoon introduces a Ca^{2+} -releasing protein into the egg cytoplasm after gamete membrane fusion. This review discusses the merits of this ‘sperm factor’ hypothesis and presents evidence indicating that the sperm factor, at least in mammals, consists of a phospholipase C with distinctive properties. This evidence leads us to propose that, after gamete fusion, a sperm-derived phospholipase C causes production of inositol 1,4,5-trisphosphate, which then generates Ca^{2+} waves from within the egg cytoplasm.

The initiation of development at fertilization in the plant or animal kingdom is triggered by an increase in the cytosolic free Ca^{2+} concentration of the egg (Stricker, 1999). This Ca^{2+} signal is both sufficient and necessary to trigger the events associated with egg activation and embryonic development (Whitaker and Swann, 1993; Schultz and Kopf, 1995; Stricker, 1999). In most species, the source of Ca^{2+} for activating the egg is intracellular, and is most probably the endoplasmic reticulum (Whitaker and Swann, 1993; Schultz and Kopf, 1995). In most species studied, the spermatozoon induces a Ca^{2+} increase that starts from the site of sperm–egg fusion (Whitaker and Swann, 1993; Jones *et al.*, 1998a; Deguchi *et al.*, 2000). This increase in Ca^{2+} propagates through the egg in a wave-like manner. In some species (for example, frogs, fish and sea urchins), only a single such Ca^{2+} wave is observed at fertilization, whereas in other species (for example, mammals and ascidians) Ca^{2+} oscillations occur that can last for either tens of minutes or for several hours (Swann and Ozil, 1994; Stricker, 1999). The initial Ca^{2+} waves propagate slowly from the site of sperm–egg fusion, but the subsequent Ca^{2+} waves originate from the endoplasmic reticulum-rich regions such as the ‘contraction pole’ in ascidians, or from the opposite side to the metaphase spindle in mammals (Speksnijder *et al.*, 1990; Kline *et al.*, 1999; Deguchi *et al.*, 2000).

Despite the importance of Ca^{2+} in egg activation, the general mechanism by which the spermatozoon causes Ca^{2+} release has not been resolved. In some eggs or oocytes, there is an increase in Ca^{2+} influx at fertilization and, in some marine invertebrates, this plays a role in activating development (Stricker, 1999). However, this review focuses upon the more commonly reported cases, in which the

spermatozoon triggers intracellular Ca^{2+} release and egg activation, as is most clearly the situation for mammalian eggs.

How do spermatozoa trigger Ca^{2+} release in eggs?

Three basic models have been proposed to explain how the spermatozoon achieves Ca^{2+} release. In the first model, Ca^{2+} is provided directly from the spermatozoon; in the second, an egg membrane receptor is activated by sperm binding and fusing; and in the third, a Ca^{2+} -releasing factor is released from the spermatozoon into the egg. These different models have been discussed extensively (Swann and Ozil, 1994; Schultz and Kopf, 1995; Jones 1998; Stricker, 1999). This review considers the first two models briefly, but concentrates on the ‘sperm factor’ hypothesis, focusing on numerous reports from the last few years, and citing previous reviews for some of the older original literature.

Ca^{2+} entry through the spermatozoon

It has been proposed that the spermatozoon triggers a Ca^{2+} increase by acting as a conduit for Ca^{2+} entry into the egg cytoplasm (Jaffe, 1991; Creton and Jaffe, 1995). Support for this mechanism comes from studies of frog and fish eggs, in which Ca^{2+} injection led to the generation of a Ca^{2+} wave (Nuccitelli, 1991). However, in mammalian eggs, Ca^{2+} influx does not generate Ca^{2+} oscillations (Swann and Ozil, 1994). Ca^{2+} concentrations at the site of sperm fusion in mice have been measured using two photon imaging techniques (Jones *et al.*, 1998a) and produced no evidence to indicate that Ca^{2+} influx plays a substantial role in mouse fertilization, as Ca^{2+} concentrations are the same in the

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spermatozoon and egg shortly after fusion in the period before the first Ca^{2+} increase. In addition, the Ca^{2+} increase at fertilization was not inhibited by removal of extracellular Ca^{2+} (Jones *et al.*, 1998a). These data support the conclusion that Ca^{2+} influx does not initiate signals in mammalian eggs. However, Ca^{2+} influx does appear to play a permissive role in maintaining oscillations, and capacitative Ca^{2+} entry into the egg can occur to replenish stores after each Ca^{2+} release event (McGuinness *et al.*, 1996).

Activation of an egg receptor by a sperm ligand

The second model proposes that the spermatozoon initiates Ca^{2+} release as a result of binding of sperm ligand to an egg plasma membrane receptor (Foltz and Shilling, 1993; Evans and Kopf, 1998). The receptor then activates an egg-derived phospholipase C (PLC), which generates the Ca^{2+} -releasing messenger inositol 1,4,5-trisphosphate (InsP_3) by hydrolysis of phosphatidylinositol 4-,5-bisphosphate (PIP_2). This theory is supported by the finding that InsP_3 -induced Ca^{2+} release occurs in eggs of many different species (Miyazaki *et al.*, 1993; Whitaker and Swann, 1993; Schultz and Kopf, 1995). In addition, sustained injection of InsP_3 or photolysis of eggs loaded with caged InsP_3 can cause Ca^{2+} oscillations in mammalian eggs (Swann and Ozil, 1994; Jones and Nixon, 2000). An increase in InsP_3 concentrations has also been demonstrated in both sea urchin and frog eggs at fertilization (Nuccitelli, 1991; Ciapa *et al.*, 1992; Snow *et al.*, 1996; Lee and Shen, 1998). A functional role for InsP_3 in fertilization is supported by the finding that injecting an inhibitory InsP_3 -receptor antibody blocks Ca^{2+} release at fertilization and thus egg activation (Miyazaki *et al.*, 1993). Finally, if the InsP_3 receptor is degraded in eggs, Ca^{2+} oscillations after egg insemination are inhibited (Brind *et al.*, 2000). These data show that InsP_3 -receptor mediated Ca^{2+} release is critical at fertilization. However, what is the evidence that receptor-mediated InsP_3 production is involved?

In somatic cells, receptor–ligand interaction stimulates InsP_3 production through PLC of the β or γ classes (Rhee *et al.*, 1997). Mammalian eggs contain both $\text{PLC}\beta$ and $\text{PLC}\gamma$ isoforms (Dupont *et al.*, 1996). Several lines of evidence also show that stimulating $\text{PLC}\beta$ s or $\text{PLC}\gamma$ s artificially can lead to Ca^{2+} release and activation in eggs (Schultz and Kopf, 1995; Mehlmann *et al.*, 1998). However, it remains to be shown whether a receptor-linked InsP_3 generative mechanism operates at fertilization. In the marine worm *Urechis caupo*, extracellular application of a sperm protein causes egg activation (Stephano and Gould, 1997). In frog and bovine eggs, extracellular addition of a peptide from a disintegrin domain, or peptides containing the RGD sequence, also cause Ca^{2+} release and egg activation (Shilling *et al.*, 1998; Campbell *et al.*, 2000). These proteins and peptides may play a role in Ca^{2+} release in eggs, but it is not known whether physiological amounts of these proteins are exposed on the surface of a single spermatozoon, or whether the receptors they stimulate are linked to PLC. The

best candidates for sperm receptors on the egg membrane in mammals are transmembrane proteins such as CD9 and integrins (Kaji *et al.*, 2000; Wasserman *et al.*, 2001). Despite the evidence supporting a role for CD9 and integrin ligands in gamete membrane fusion, appropriate knockout experiments indicate that these proteins are not involved in stimulating Ca^{2+} release (Cho *et al.*, 1998; Kaji *et al.*, 2000).

Other experiments to investigate the receptor theory of egg activation involve the use of specific inhibitors. Many transmembrane receptors are coupled to GTP-binding proteins (G proteins). The finding that functionally inhibitory antibodies to G proteins do not affect Ca^{2+} release at fertilization in mammals or frogs indicates that G proteins are not essential for signalling (Williams *et al.*, 1998; Runft *et al.*, 1999). An alternative transmembrane signalling system involves tyrosine phosphorylation of $\text{PLC}\gamma$, which can occur after binding of the $\text{PLC}\gamma$ SH2 domain to a phosphorylated tyrosine on the cytoplasmic tail of a membrane receptor. Support for this mechanism at fertilization comes from the finding that exogenously introduced SH2 constructs from a bovine $\text{PLC}\gamma$ block sperm-induced Ca^{2+} release in starfish (Carroll *et al.*, 1997), sea urchin and ascidian (Carroll *et al.*, 1999; Shearer *et al.*, 1999; Jaffe *et al.*, 2001) eggs. Such SH2 constructs probably act by competing with endogenous $\text{PLC}\gamma$ for phosphorylated tyrosine residues on appropriate target proteins that may be members of the src kinase family (Jaffe *et al.*, 2001). However, a mechanistic explanation involving receptors and $\text{PLC}\gamma$ is lacking because tyrosine kinase inhibitors do not block sperm-induced Ca^{2+} release in eggs of species in which a src kinase– $\text{PLC}\gamma$ pathway has been proposed to operate (for example, Moore and Kinsey, 1995). In addition, the relevance of these findings for egg activation in vertebrates is unclear because SH2 domain-containing proteins do not inhibit fertilization and Ca^{2+} release in mouse or frog eggs (Mehlmann *et al.*, 1998; Runft *et al.*, 1999).

Egg activation by a sperm factor

The 'sperm factor' hypothesis proposes that the spermatozoon contains an activating factor released into the egg after gamete fusion. This model requires that Ca^{2+} release is initiated only after sperm–egg fusion. In sea urchins, sperm–egg membrane fusion occurs several seconds before Ca^{2+} release (Whitaker and Swann, 1993). In mice, fluorescent dextrans, or globular proteins of up to 250 kDa, can diffuse between egg and spermatozoon before Ca^{2+} release (Lawrence *et al.*, 1997; Jones *et al.*, 1998a). In fact, the time between fusion and Ca^{2+} release in mouse fertilization is probably at least 1 min (Lawrence *et al.*, 1997; Lee *et al.*, 2001).

The main support for the sperm factor theory is the finding that injection of a soluble cytosolic sperm extract into eggs generates Ca^{2+} oscillations very similar to those seen at fertilization (Fig. 1). The ability of soluble sperm extracts to cause Ca^{2+} release in eggs has been observed in many mammalian species (Swann, 1990; Homa and

Swann, 1994; Wu *et al.*, 1997; Fissore *et al.*, 1998), in newts and ascidians (Kyojuka *et al.*, 1998; Yamamoto *et al.*, 2001), as well as in a protostome nemertean worm (Stricker, 1997). Sperm extract injection in mammals also triggers development up to at least the blastocyst stage (Fissore *et al.*, 1998). The key feature of sperm extract injection into eggs is that the pattern of Ca²⁺ oscillations triggered matches closely the pattern normally observed at fertilization. In ascidian eggs, for example, fertilization is associated with two phases of Ca²⁺ oscillations, separated by an interval during which the first polar body is extruded (Fig. 1). The same temporal response can be mimicked by sperm extracts, but not by other Ca²⁺-releasing agents (Kyojuka *et al.*, 1998; McDougall *et al.*, 2000). In addition, the sperm factor-induced Ca²⁺ oscillations in ascidians are affected in the same manner as fertilization by the injection of SH2 constructs and by manipulations in the activity of maturation-promoting factor (McDougall *et al.*, 2000; Runft and Jaffe, 2000).

The sperm factor does not appear to be species-specific within mammals (Homa and Swann, 1994; Wu *et al.*, 1997). A soluble factor from sperm extracts taken from non-mammalian species, such as frogs or chickens, induces Ca²⁺ oscillations after injection into mouse eggs (Dong *et al.*, 2000). Injection of human spermatozoa extracts causes oscillations in ascidian oocytes (Wilding *et al.*, 1997), and injection of pig sperm extracts causes Ca²⁺ oscillations in nemertean worm oocytes (Stricker *et al.*, 2000). These data indicate a highly conserved evolutionary mechanism of egg activation, although the same factor may not be active in every species, as there are no reports that ascidian or nemertean worm sperm factor cause Ca²⁺ oscillations in mammalian eggs.

The active sperm factor in all these 'sperm factor' experiments is protein-based, as it is both trypsin- and heat-sensitive. Ultrafiltration membrane and gel filtration chromatography studies have shown that the factor has a molecular mass > 30 kDa (Swann, 1990; Parrington *et al.*, 1996; Stricker, 1997; Kyojuka, *et al.*, 1998; Wu *et al.*, 1998). The ability of extracts to cause Ca²⁺ oscillations appears to be sperm-specific, as soluble extracts prepared from other tissues do not cause Ca²⁺ oscillations when microinjected into eggs (Swann, 1990; Stricker, 1997; Wu *et al.*, 1997). Microinjection of between one and eight sperm equivalents have been calculated to be capable of stimulating Ca²⁺ oscillations (Nixon *et al.*, 2000). This finding indicates that the amount of sperm extract used in these experiments is within the physiological range, although no account is made for these estimates of any possible loss of protein during the extraction procedure. All the protein would need to be extracted from a spermatozoon and there would need to be no loss of activity to achieve a value of exactly one sperm equivalent. Neither of these requirements is likely to be met in practice.

Other evidence that spermatozoa express a specific protein encoding the sperm factor comes from another type of injection experiment. Injection of mRNA isolated from

Fig. 1. The main evidence for the sperm factor hypothesis: Ca²⁺ changes at fertilization and after sperm extract injection. (a) Ca²⁺ oscillations at fertilization and after sperm extract injection in a mouse egg. (b) Ca²⁺ oscillations at fertilization and after sperm extract injection in an egg of an ascidian (*Ascidella aspersa*). In (a) and (b), the sperm extract injection mimics the response at fertilization. Intracellular Ca²⁺ was measured by taking fluorescence excitation ratios of the Ca²⁺-sensitive fluorescent dyes fura red (a) and fura 2 (b). (See Lawrence *et al.*, 1997 and McDougall *et al.*, 2000 for experimental details.)

spermatogenic cells triggers fertilization-like Ca²⁺ oscillations in mouse oocytes (Parrington *et al.*, 2000). This effect depends upon protein synthesis in the injected oocytes, which are effectively used as an expression and assay system for the injected mRNA. Since injecting mRNA from a range of other tissues does not cause Ca²⁺ oscillations, these data indicate that spermatogenic cells contain a specific mRNA encoding the sperm factor protein (Parrington *et al.*, 2000).

Support for the existence of an intracellular sperm factor also comes from experiments in which spermatozoa are injected directly into eggs during intracytoplasmic sperm injection (ICSI). After ICSI in both mouse and human eggs, a series of Ca²⁺ oscillations, similar to that seen at fertilization, is initiated (Tesarik and Sousa, 1994; Nakano *et al.*, 1997; Yanagida *et al.*, 2001). ICSI also leads to Ca²⁺ oscillations in nemertean worms (Stricker, 1996). Since ICSI in mammals involves injection of one spermatozoon, these data again indicate that the spermatozoon contains sufficient amounts of a sperm factor to cause Ca²⁺ oscillations.

How soluble is the soluble sperm factor?

As well as supporting the proposed existence of a sperm factor, the ICSI experiments indicate that the egg-activating

factor is present in the head of mouse spermatozoa and not in the tail (Kimura *et al.*, 1998; Perry *et al.*, 1999). However, results of ICSI studies in mice have also shown that Ca^{2+} oscillations and activation can be triggered with spermatozoa that have been demembrated before injection and consequently should have lost any soluble sperm factors (Perry *et al.*, 2000). The sperm factor that is active in these demembrated spermatozoa can then be removed from the sperm heads by incubation with reducing agents (Perry *et al.*, 1999). These data indicate that sperm heads contain an insoluble sperm factor, which may be different from the soluble sperm factor used in the above cytosolic extract experiments (Perry *et al.*, 1999). However, this interpretation may be premature because it is not known how much of the soluble sperm factor activity remains in a pellet fraction after preparation of the cytosolic extracts. The differences in interpretation about the solubility of a sperm factor may, in fact, be explained by differences in extraction of the sperm factor in different species. For example, soluble extracts from hamster, boar and human spermatozoa are very active in causing Ca^{2+} oscillations in eggs, but soluble extracts from mouse spermatozoa are not (Swann, 1990; K. Swann and J. Parrington, unpublished). A difference between mouse extracts and other species was also evident in a report on the effect of a soluble sperm factor (Stice and Robl, 1990). This indicates that, although a sperm factor is released during permeabilization in many spermatozoa, mouse spermatozoa retain much of the factor in the sperm head. Consistent with this contention is the finding that human spermatozoa that have been frozen and thawed are unlike mouse spermatozoa in that they lose their ability to activate eggs when introduced by ICSI (Rybouchkin *et al.*, 1996). Therefore, it is possible that there is only one sperm factor involved in causing Ca^{2+} oscillations, but that there are differences among species in the solubility of such a factor.

What is the sperm factor protein?

A major unsolved problem is the identity of the sperm factor protein. Attempts have been made to identify the sperm factor by column chromatography and correlating the ability of extracts to cause Ca^{2+} oscillations with a 33 kDa protein (Parrington *et al.*, 1996). This protein was located in the head of the spermatozoon in the region that is first to contact the egg cytoplasm during fusion. However, further experiments showed that this protein does not produce Ca^{2+} oscillations by itself, and does not always correlate with Ca^{2+} releasing ability in eggs (Shevchenko *et al.* 1998; Wolosker *et al.*, 1998; Wu *et al.*, 1998; Parrington *et al.*, 1999). Evidently, the active Ca^{2+} oscillation-inducing protein is not the 33 kDa protein and must be another protein present in the extracts.

Another candidate sperm factor is a truncated form of the c-kit receptor named tr-kit. When either the tr-kit protein or tr-kit mRNA is microinjected into mouse eggs it causes egg activation (Sette *et al.*, 1997). Such activation is Ca^{2+} -

dependent, and this has led to the suggestion that tr-kit is the sperm factor. However, a number of significant caveats cast doubt on the validity of tr-kit. Firstly, tr-kit does not appear to be in the appropriate part of the spermatozoon as it is located predominantly in the sperm midpiece (Sette *et al.*, 1997) rather than the sperm head which activates eggs in ICSI. Secondly, tr-kit-induced egg activation is blocked by an SH3 domain-containing construct (Sette *et al.*, 1998), yet the same construct does not block egg activation at fertilization (Mehlmann *et al.*, 1998). In addition, there are no reports that show whether tr-kit can actually induce Ca^{2+} oscillations in eggs, the definitive assay for the sperm factor.

Another suggestion for the identity of the sperm factor has come from recent studies in sea urchins in which nitric oxide (NO) has been shown to cause Ca^{2+} release (Kuo *et al.*, 2000). The same study also found that NO scavengers inhibit Ca^{2+} release at fertilization in sea urchin eggs (Kuo *et al.*, 2000). Kuo *et al.* (2000) suggest that the sperm factor in echinoderms may be a NO synthase (NOS), although NO does not appear to be a universal trigger for egg activation. Fertilization in mouse and ascidian eggs does not appear to be associated with any change in NO, and an NOS inhibitor that blocked endogenous NOS failed to block Ca^{2+} release at fertilization (Hyslop *et al.*, 2001).

How does the sperm factor cause Ca^{2+} release?

Owing to the lack of success in identifying the sperm factor through direct protein purification methods, we have used a different strategy and have examined the ability of sperm extracts to release Ca^{2+} in homogenates prepared from sea urchin eggs. These cell-free homogenates are well characterized and have been used extensively to identify the novel Ca^{2+} -releasing agents cyclic ADP ribose (cADPR) and nicotinic acid dinucleotide phosphate (NAADP) (Lee, 1997). Initial experiments showed that mammalian sperm extracts cause a Ca^{2+} increase in these egg homogenates (Jones *et al.* 1998b) and so a series of experiments was performed to determine how this Ca^{2+} increase was achieved.

Sea urchin egg homogenates display the useful phenomenon of 'homologous desensitization'. For example, after a single large addition of InsP_3 , the homogenate becomes desensitized such that further additions of InsP_3 no longer cause Ca^{2+} release. However, such InsP_3 -insensitive homogenates still respond to other Ca^{2+} releasing agents that act independently of the InsP_3 receptor (NAADP and cADPR). Similarly, homogenates can be desensitized to cADPR, but still respond to InsP_3 and NAADP. When homogenates were desensitized to InsP_3 , they no longer responded to sperm extracts, and the converse was also true (Jones *et al.*, 1998b). However, homogenates still responded to sperm extract when it was desensitized to NAADP or cADPR. These data show that the sperm extracts trigger Ca^{2+} release through the InsP_3 receptor. Critically, a mass assay was used to show that InsP_3 concentrations increased in homogenates after application of sperm extracts. This increase in

Fig. 2. Schematic diagram to explain how the sperm factor initiates Ca²⁺ release and Ca²⁺ waves in mammalian eggs. After fusing with the egg, the sperm factor phospholipase C (PLC) (purple) diffuses into the egg cytoplasm. It then leads to production of inositol 1,4,5-trisphosphate (InsP₃), which causes Ca²⁺ release from intracellular stores (red). The Ca²⁺ then acts back upon the sperm-derived PLC to stimulate more InsP₃ production. The sperm PLC may hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP₂) from intracellular membrane organelles such as yolk or lysosomes (yellow), as well as from the plasma membrane.

InsP₃ and Ca²⁺ release is blocked by prior treatment of egg homogenates with a bacterial inositol-specific PLC, which depletes the membrane of endogenous inositol-containing lipids (Jones *et al.*, 1998b). These data show that InsP₃ generation is both necessary and sufficient for sperm extracts to trigger Ca²⁺ release in this system. It appears that other Ca²⁺ release mechanisms are not involved in explaining the actions of the sperm factor. These data are also consistent with the findings in intact mammalian eggs, in which sperm factor injection leads to downregulation of InsP₃ receptors. Such downregulation is strictly dependent upon InsP₃ production (Jellerette *et al.*, 2000).

As InsP₃ is produced from inositol phospholipids, the above data show that PLC activity is involved in the sperm extract response. The key questions are: (i) is the factor responsible for Ca²⁺ release in the egg homogenate also responsible for Ca²⁺ oscillations in mammalian eggs? and (ii) is the relevant PLC in the egg or in the spermatozoon? Fractionation experiments indicate that the sperm factor active in causing Ca²⁺ release in the homogenates is the same as the factor causing Ca²⁺ oscillations in intact eggs (Parrington *et al.*, 1999). Sperm extracts were fractionated

over three separate chromatographic columns. On all three columns, fractions that caused Ca²⁺ oscillations when microinjected into mouse eggs also caused Ca²⁺ release when added to sea urchin egg homogenate. If different factors are responsible for Ca²⁺ release in mouse eggs and sea urchin egg homogenates, then it is unfortunate that they co-purified in this way. The simplest interpretation is that the same factor causes Ca²⁺ release in both assay systems.

So, is the PLC in the egg or in the spermatozoon? If the PLC is in the egg, then the sperm extract would contain a factor that acts to switch on egg PLC activity. So far, there is no indication that the mechanisms used to switch on PLC activity in somatic cells operate after addition of sperm extract to egg homogenates. No inhibitory effect on sperm extract-induced Ca²⁺ release of agents that inhibit G-proteins or tyrosine kinases has been observed (K. Swann and J. Parrington, unpublished), which is consistent with findings in intact mouse eggs at fertilization (Mehlmann *et al.*, 1998). In contrast, when sperm extracts were examined for PLC activity, active sperm extracts readily hydrolysed added PIP₂ to InsP₃. Hence, the sperm extracts contain a PLC activity. This PLC activity in spermatozoa co-purified in

fractions that were able to release Ca^{2+} in sea urchin egg homogenate and cause Ca^{2+} oscillations in mouse eggs (Parrington *et al.*, 1999).

The contention that this sperm PLC activity is significant is supported by the finding that it is two orders of magnitude greater than that found in other mammalian tissue extracts prepared in the same way as spermatozoa (Rice *et al.*, 2000). The PLC activity in a single spermatozoon is such that a rough estimate indicates it may be sufficient to account for Ca^{2+} release in a mammalian egg (Rice *et al.*, 2000). It should be noted that mouse sperm extracts have PLC activity similar to that of other tissues (Mehlmann *et al.*, 2001). However, soluble sperm extracts from mice are a poor source of an active sperm factor and therefore it would have been interesting to determine the Ca^{2+} -releasing ability of the mouse sperm extracts used in the study of Mehlmann *et al.* (2001). The PLC activity reported by Mehlmann *et al.* (2001) for mouse sperm extracts is more than 100-fold lower than the PLC activity measured in boar sperm extracts that were active in causing Ca^{2+} release in eggs (Jones *et al.*, 2000).

As there is a highly active PLC in sperm extracts, and as this correlates with Ca^{2+} -releasing activity, the simplest interpretation of our data is that the sperm factor consists of a PLC isozyme. If the spermatozoon contains sufficient amounts of such a PLC, then this finding may offer a straightforward explanation of how the spermatozoon triggers InsP_3 formation at fertilization. A schematic diagram of our hypothesis to explain how the spermatozoon causes Ca^{2+} release in mammalian eggs is shown (Fig. 2). After fusing with the egg plasma membrane, the sperm structures decondense in the egg cytoplasm and release the sperm PLC that then initiates InsP_3 production in the egg.

There are two other features of the mammalian sperm PLC activity that can be incorporated into the schematic hypothesis (Fig. 2). Firstly, the PLC activity in spermatozoa is modulated by the physiological range of free Ca^{2+} concentrations (Rice *et al.*, 2000), so that an increase in Ca^{2+} concentration stimulates the PLC activity. Consequently, when InsP_3 causes some initial Ca^{2+} increase, this will act by positive feedback to generate more InsP_3 and hence the release of more Ca^{2+} (Fig. 2). This positive feedback loop provides a mechanism for generating the rapid Ca^{2+} waves that account for the sharp upstroke of each Ca^{2+} increase in eggs undergoing fertilization. Such a positive feedback loop involving Ca^{2+} -dependent InsP_3 production has been shown to be a key part of some mathematical models of cytosolic Ca^{2+} oscillations (Meyer and Stryer, 1988) and may also underlie the regenerative Ca^{2+} release described in hamster eggs undergoing fertilization (Miyazaki *et al.*, 1993; Swann and Ozil, 1994).

Another feature of the mammalian sperm PLC is that some of its substrate (PIP_2) may have an unusual location. In conventional transmembrane signalling, a receptor-stimulated PLC, such as those of the β or γ classes, will hydrolyse PIP_2 derived from the inner leaflet of the plasma membrane (Rhee *et al.*, 1997). However, when sperm

extracts are added to sea urchin egg homogenate, they appear to act mainly on another source of PIP_2 , as plasma membrane represents only a minor component of this cell-free extract. The subfraction of egg homogenates that generates most InsP_3 when treated with the sperm PLC is the subfraction rich in yolk platelets (Rice *et al.*, 2000). Yolk platelets are acid vesicles in eggs and the corresponding organelles in mammalian eggs are the lysosomes (Albertini, 1984). Therefore, it is possible that the sperm PLC can lead to InsP_3 generation from organelles within the cytoplasm of the egg, which would help explain why the sperm factor causes regenerative Ca^{2+} waves that are propagated throughout the egg cytoplasm (Oda *et al.*, 1999).

We envisage that the next major step forward in our investigations will be the identification of the PLC in sperm extracts that we consider to be responsible for Ca^{2+} release. Of the four subtypes of PLC (β , γ , δ , and ϵ) the roles of only $\text{PLC}\beta$ and $\text{PLC}\gamma$ at fertilization have been considered (Carroll *et al.*, 1997; Williams *et al.*, 1998). $\text{PLC}\beta$ and $\text{PLC}\gamma$ isoforms have been detected in mammalian spermatozoa (Mehlmann *et al.*, 1998; Parrington *et al.*, 1999). However, fractionation experiments have indicated that $\text{PLC}\beta$ and $\text{PLC}\gamma$ are not components of the boar sperm factor (Parrington and Swann, 1999). Recombinant or purified $\text{PLC}\beta 1$, $\delta 1$ and $\gamma 1$ are also unable to mimic the sperm factor in causing Ca^{2+} release in egg homogenates or intact eggs (Jones *et al.*, 2000). This finding indicates that the active component may instead be another PLC isoform that is either specific to spermatozoa or specifically regulated in spermatozoa. Our model (Fig. 2) indicates that such a sperm-derived PLC plays the sole role of egg activation trigger. Our model is simple and readily tested, since candidate sperm PLCs can be assessed to see whether they meet the properties of a trigger molecule for Ca^{2+} oscillations and egg activation.

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