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The characteristics of the Ca^{2+} signals that activate mammalian eggs at fertilization.

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Key Words

Calcium, egg, InsP_3 , ATP, sperm, phospholipase C

Abstract

Gamete membrane fusion in mammals brings the paternal genome into the cytoplasm of the egg. It also enables signals to pass from the sperm into the egg to trigger the completion of meiosis and the start of embryo development. The essential signal to activate development in all mammals studied, consists of a series of transient increases in the cytosolic Ca^{2+} concentration driven by cycles of InsP_3 production. This review focusses on the characteristics of these sperm-induced Ca^{2+} signals. I consider how some specific features of sperm-derived phospholipase C-zeta ($\text{PLC}\zeta$), along with the known properties of the type 1 InsP_3 receptor, provide a basis for understanding the mechanisms of the dynamic changes in Ca^{2+} observed in fertilizing eggs. I describe how the $\text{PLC}\zeta$ targeting of cytoplasmic vesicles in the egg cytoplasm, that contain $\text{PI}(4,5)\text{P}_2$, is necessary to explain the rapid waves associated with the rising phase of each Ca^{2+} transient. I also discuss the importance of the repetitive Ca^{2+} rises for egg activation and the way mitochondrial ATP production may modulate Ca^{2+} release in eggs. Finally, I consider the role that a sperm-induced ATP increase may play in the egg activation process.

The sperm-induced Ca^{2+} signals that cause egg activation.

The Ca^{2+} signals that are initiated by the sperm at fertilization in mammalian eggs consists of a series of oscillations in the cytosolic Ca^{2+} concentration (Miyazaki 2007). In all mammalian species studied, a series of repetitive increases in Ca^{2+} have been shown to persist for several hours after gamete fusion (Miyazaki and Igusa 1981; Cuthbertson and Cobbold 1985; Igusa and Miyazaki 1986; Kline and Kline 1992; Taylor et al. 1993). These Ca^{2+} oscillations are mediated by increased production of inositol (1,4,5)-trisphosphate (InsP_3) and Ca^{2+} release from the endoplasmic reticulum via the InsP_3 receptor (IP_3R), which also acts as the release channel (Miyazaki 1988; Miyazaki et al. 1992; Swann 1992; Kline and Kline 1994; Fissore et al. 1999; Malcuit et al. 2005). In mouse eggs the Ca^{2+} oscillations start within about a minute of gamete fusion and consist of 10-20 separate transient Ca^{2+} increases that each last for a few minutes (Cuthbertson and Cobbold 1985; Igusa and Miyazaki 1986; Miyazaki et al. 1986; Kline and Kline 1992; Mehlmann and Kline 1994; Lawrence et al. 1997). The spike-like increases in Ca^{2+} occur about 10-20 minutes apart. Figure 1 shows a typical series of Ca^{2+} oscillations that we measure using Oregon Green BAPTA dextran. This is a fluorescent Ca^{2+} sensitive dye that we find gives a very good signal to noise. We typically observe 10 to 20 large Ca^{2+} spikes, that occur from a baseline Ca^{2+} level between spikes that is slightly lower than that in unfertilized eggs. These Ca^{2+} oscillations occur as the egg completes meiosis, forming a second polar body and two

pronuclei (Marangos et al. 2003; Larman et al. 2004). The list of species where sperm-induced Ca^{2+} oscillations in eggs have been demonstrated includes mice, rats, hamsters, humans, pigs, cows and goats (Cuthbertson and Cobbald 1985; Igusa and Miyazaki 1986; Sun et al. 1992; Taylor et al. 1993; Sun et al. 1994; Ben-Yosef et al. 1995; Jellerette et al. 2006). A series of cytosolic Ca^{2+} oscillations are also seen in mouse and human eggs after intracytoplasmic sperm injection (ICSI) that is widely used as part of IVF treatment (Tesarik et al. 1994; Nakano et al. 1997; Yanagida et al. 2001).

In mice and hamsters, the pattern of Ca^{2+} increases in fertilizing eggs have been measured with temporal resolution of seconds and spatial resolution of a few microns. The initial Ca^{2+} increase spreads in a wave-like manner from the region of sperm egg fusion to the antipode in about 5-10 seconds (Miyazaki et al. 1986; Deguchi et al. 2000). Once it has spread across the egg the Ca^{2+} levels stay high for several minutes after which Ca^{2+} decreases over a few seconds in all regions of the egg at the same time. Over a period of 10-20 minutes following gamete fusion, the Ca^{2+} waves associated with each transient increase in speed and eventually (~30 min) they cross the egg much more rapidly, in about 1 second (Miyazaki et al. 1986; Deguchi et al. 2000). This change in wave speed is also manifest as an increase in the rate of rise of each Ca^{2+} transient (Swann and Lai 2013). The series of oscillations then stops just before the time of pronuclear formation. Studies in other species, including human eggs, also suggest that fertilization induced Ca^{2+} oscillations can last for several hours (Taylor et al. 1993). In mouse, hamster, and pig eggs it has been shown that Ca^{2+} oscillations are dependent upon Ca^{2+} influx (Igusa and Miyazaki 1983; Cheek et al. 1993; Zhang et al. 2018). The Ca^{2+} channels have been identified in mouse eggs and their roles in fertilization is discussed elsewhere (Wakai et al. 2019; Stein et al. 2020). I will concentrate here on other specific aspects of Ca^{2+} release via IP3Rs.

There are numerous publications showing measurements of Ca^{2+} oscillations in fertilizing mammalian eggs, but the amplitude of the Ca^{2+} transients is typically not calibrated. This is also the case in Figure 1 where the y-axis is plotted as the ratio of fluorescence divided by the fluorescence before the start of Ca^{2+} oscillations (F/F_0). The absolute concentration of the peak of each Ca^{2+} transient is worth considering because it is significant for understanding the mechanisms of oscillations that will be considered later. The most common dye used to measure Ca^{2+} in eggs is fura2. Some studies using Ca^{2+} sensitive fluorescent dyes like fura2 have suggested that the Ca^{2+} peaks are above $1\mu\text{M}$ (Mehlmann and Kline 1994), whilst other studies have suggested they might be in the range of 300 to 500nM (Miyazaki et al. 1992; Cheek et al. 1993). These differences reflect the difficulties in calibrating Ca^{2+} sensitive fluorescence dyes in the cytosol of egg. Attempts to calibrate fluorescent dyes *in vitro* is also compromised by the fact that many dyes have a different

affinity for Ca^{2+} in cytosol compared with calibration solutions, and this may require correction factors. Fura2 dye is also problematic because it has a high affinity for Ca^{2+} and is poor at resolving the amplitude of Ca^{2+} transients above $1\mu\text{M}$. Another issue is that many researchers use acetoxymethyl ester versions of dyes for loading them into eggs. This leads to dye compartmentalizing into organelles in eggs, so that much of the fluorescent signal is not emanating from the cytosol (Carroll et al. 1994). We microinject dextran-linked Ca^{2+} dyes into eggs which provides a prolonged signal that is entirely derived from the cytosolic compartment, but even with these dextran-linked dyes it is difficult to obtain a fluorescence maximum and minimum which are needed for the *in situ* calibration. Hence, despite their widespread use, fluorescent dyes have not provided an unequivocal determination of either the resting or the peak Ca^{2+} concentrations in fertilizing eggs. It is worth noting that if we assume the resting Ca^{2+} concentration in eggs is 100nM , which is typical of mammalian cells, then the peak Ca^{2+} transients in mouse eggs in the experiment shown in Figure 1 are generally $> 2\mu\text{M}$. This is consistent with some older studies on Ca^{2+} oscillations in mammalian eggs.

The first studies that measured and calibrated Ca^{2+} oscillations in fertilizing mouse and human eggs used aequorin to measure cytosolic Ca^{2+} (Cuthbertson and Cobbold 1985; Taylor et al. 1993). The luminescence from aequorin can be calibrated in drops of Ca^{2+} EGTA buffers and is less prone to problems in extrapolating from such buffers to conditions in the cytosol because aequorin is a protein. Furthermore, the calibration involves egg lysis at the end of an experiment which simply involves integrating all the light emitted. Aequorin is not ideal for measuring resting Ca^{2+} , but it is effective at higher Ca^{2+} concentrations and this study showed that the peak of each sperm-induced Ca^{2+} transient is close to $2\mu\text{M}$. This is consistent with another study in mouse eggs that used a low affinity Ca^{2+} dye (MagFura2) to measure the peaks of initial Ca^{2+} transients at fertilization (Halet et al. 2004). MagFura2 is not effective for measuring resting Ca^{2+} , but it can faithfully record the peak of each Ca^{2+} transient. The data with MagFura2 suggests that sperm induced Ca^{2+} transients are of an amplitude of between 1 and $3\mu\text{M}$, and this lasts for at least 1 minute (Halet et al. 2004). Increases in cytosolic free Ca^{2+} around $2\mu\text{M}$ will allow for the maximum stimulation of calmodulin which transduces the signal downstream. The calmodulin dependent protein kinase II (CAMKII) has been shown to be stimulated at fertilization by each rise in Ca^{2+} (Markoulaki et al. 2003; Markoulaki et al. 2004), and fluorescent calmodulin indicators also show full stimulation of CAM kinases in response to each Ca^{2+} rise (Ardestani et al. 2019). The most important processes that are targets for Ca^{2+} and calmodulin dependent kinases are meiotic resumption and cortical granule exocytosis. The mechanism of exit from meiotic metaphase arrest is mediated by CAMKII (Knott et al. 2006). The exocytosis of cortical

granules plays a key role in modifying the zona pellucida and blocking polyspermy (Evans 2020). This is triggered by myosin light chain kinase that is also stimulated by Ca^{2+} binding to calmodulin (Matson et al. 2006).

We know that Ca^{2+} is the physiological stimulus for the calmodulin dependent kinases because it has been shown that a rise in cytosolic Ca^{2+} is essential for mouse egg activation at fertilization. Loading mouse eggs with the Ca^{2+} chelator BAPTA can be done in a way to prevent all Ca^{2+} increases at fertilization (Kline and Kline 1992). BAPTA loading does not prevent sperm-egg fusion, but it blocks the two key markers of egg activation namely meiotic resumption and cortical granule exocytosis. Hence, if there are no Ca^{2+} increases there is no egg activation or development. Lower concentrations of BAPTA-AM can be used to stop most oscillations, but a single small Ca^{2+} transient can still occur (Kline and Kline 1992). In this case pronuclear formation can still be prevented. However, the use of BAPTA-AM to block Ca^{2+} oscillations can also inhibit protein synthesis, and the inhibition of protein synthesis can in itself promote egg activation because it leads to loss of cyclin B (Moses and Kline 1995; Lawrence et al. 1998). Hence, interpreting the effects of adding BAPTA-AM to oscillating eggs can be problematic. BAPTA loading of eggs can show that a Ca^{2+} change is necessary for initial meiotic resumption and for exocytosis, but the more subtle question of whether mammalian eggs require multiple Ca^{2+} transients to undergo full activation and development is more difficult to address.

The importance of multiple Ca^{2+} transients in egg activation.

The question of how many Ca^{2+} transients, of whatever amplitude or duration, are needed to activate mammalian eggs was first considered over 20 years ago. It has returned as a point of discussion because of the clinical use of Ca^{2+} ionophores in oocyte activation after fertilization failure that occasionally occurs in ICSI. To investigate the role of multiple Ca^{2+} transients in egg activation, one can stimulate eggs with different patterns of Ca^{2+} oscillations. A single large Ca^{2+} increase can be triggered in mouse eggs by incubation in 7% ethanol, and this method was commonly used to induce parthenogenetic activation in mouse eggs in the 1980s (Cuthbertson et al. 1981). This causes a large Ca^{2+} rise that persists for as long as ethanol is present, which is typically ~7 minutes (Swann and Ozil 1994). Incubation in Ca^{2+} ionophores, such as A23187 or ionomycin, also causes one Ca^{2+} transient and can activate mouse or human eggs. These data imply that a single Ca^{2+} increase can activate eggs. However, both ethanol and ionophore generally cause a larger and longer lasting Ca^{2+} increase than the initial one seen at fertilization (Swann and Ozil 1994; Rinaudo et al. 1997). In mouse eggs treated with Ca^{2+} ionophore the activation rates, as judged by

pronuclear formation, are often low (Moses et al. 1995). Furthermore, mouse eggs are generally only activated when the eggs are aged *in vitro* before stimulation, and freshly ovulated mouse eggs generally do not activate in response to Ca^{2+} ionophore treatment. In human eggs, the first study using Ca^{2+} ionophores reported rates of ~60% egg activation (Winston et al. 1991), but then two subsequent studies found that either no eggs, or only 16% of eggs, activated using Ca^{2+} ionophores (Winston et al. 1991; Balakier and Casper 1993; Rinaudo et al. 1997). There are numerous clinical studies suggesting that A23187 can be effective in activating eggs after ICSI, when previous cycles of treatment had been ineffective (Murugesu et al. 2017). In most cases where ionophores are found to be effective, the eggs from the previous ICSI cycle were used as the control group. More recent studies on human eggs, using a parallel control group, reported that a single application of A23187 was ineffective in activating unfertilized (aged) human eggs (Esbert et al. 2022). A single large Ca^{2+} rise is probably insufficient to induce human egg activation in most conditions.

In many cases in domesticated animal studies, egg activation protocols involve incubation in a Ca^{2+} ionophore, in combination with agents that cause a decrease in MPF activity such as protein kinase inhibitors, or protein synthesis inhibitors (Prather et al. 1999; Chung et al. 2000; Ross et al. 2008). This double treatment is used because the single Ca^{2+} increase with ionophore does not activate eggs effectively. A single Ca^{2+} increase can cause a decrease in MPF activity which may initiate meiotic resumption, but then a later return of MPF activity leads to the recondensation of chromosomes in a state referred to as MIII (Moses et al. 1995; Ducibella et al. 2002). In both mouse and bovine eggs, it was found that multiple Ca^{2+} increases can cause a decrease in MPF activity that is sustained and allows the complete exit from meiosis II and the formation of pronuclei (Collas et al. 1993a; Collas et al. 1993b; Ozil and Swann 1995; Ducibella et al. 2002). In mouse eggs the standard activation protocol involves incubation in Sr^{2+} containing medium. Medium containing millimolar Sr^{2+} , but no added Ca^{2+} , can cause long lasting Ca^{2+} oscillations in mouse and rat eggs (Kline and Kline 1992; Tomashov-Matar et al. 2005). These oscillations often involve long duration Ca^{2+} increases but are otherwise similar in amplitude to those seen at fertilization. Unlike A23187, Sr^{2+} medium can activate freshly ovulated mouse eggs and is the most effective artificial stimulus for parthenogenetic activation up to the blastocyst stage (Bos-Mikich et al. 1995; Ferrer-Buitrago et al. 2020). Sr^{2+} medium is very effective as a stimulus after ICSI in mouse eggs when they are injected with sperm that are either heat treated or lacking PLC ζ (Sanusi et al. 2015; Ferrer-Buitrago et al. 2020). Sr^{2+} medium is also routinely used to activate mouse eggs after nuclear transfer protocols designed for reproductive cloning. However, Sr^{2+} medium is not generally used in egg activation protocols for other species such as in

humans, because it does not cause Ca^{2+} oscillations in human eggs (Lu et al. 2018; Storey et al. 2021). The reasons for this lack of effect of Sr^{2+} in some species is discussed later. Regardless, the data obtained with Ca^{2+} ionophores and Sr^{2+} suggest that multiple Ca^{2+} transients are the most effective way to trigger egg activation. The most informative studies on Ca^{2+} and egg activation come from ways of mimicking Ca^{2+} transients in a more controllable manner.

The most effective way of artificially causing Ca^{2+} transients in eggs in a controlled manner is to use electroporation in medium containing Ca^{2+} . This causes Ca^{2+} influx and a large cytosolic Ca^{2+} increase (Ozil and Swann 1995). Electroporation is applied to mammalian eggs in medium of low ionic strength with low concentrations of Ca^{2+} . The formation of pores due to the electrical field leads to an influx of Ca^{2+} from the extracellular medium. If appropriate culture medium is very rapidly perfused onto eggs after electroporation (within 200msec), the pores close and the eggs recover (Ozil and Swann 1995). By using repeated perfusions and pulses, multiple Ca^{2+} transients can be generated with a variety of amplitudes and durations that depend on controllable variables such as the applied voltage. Using this approach Ozil and colleagues found that mouse eggs can be activated by a single large Ca^{2+} rise, but when a single increase is used, the amplitude and duration of such a rise needs to be higher for longer periods than that seen at fertilization (Swann and Ozil 1994; Ducibella et al. 2002; Ozil et al. 2005; Tóth et al. 2006). When physiologically sized Ca^{2+} transients are applied to mouse eggs, then 4 to 8 Ca^{2+} transients are only sufficient to initiate exocytosis and trigger metaphase to anaphase transition. To obtain full completion of meiosis and pronuclear formation, 24 Ca^{2+} transients were required (Ducibella et al. 2002). In addition, the number or frequency of Ca^{2+} transients can determine the timing of pronuclear formation, with higher frequency, or more total Ca^{2+} transients, resulting in more rapid pronuclear formation. These data show that in some cases a single large and rather prolonged Ca^{2+} increase can activate mouse eggs, but that a single physiologically sized Ca^{2+} increase is ineffective. Multiple physiological Ca^{2+} transients generally stimulate the greatest percentage of eggs to activate. Unfortunately, the repetitive electroporation technique has not been applied to human eggs, probably because the equipment required for the rapid perfusion is very complex and not commercially available.

PLC ζ induced Ca^{2+} oscillations in eggs.

In mammals the sperm stimulates Ca^{2+} release several seconds or minutes after sperm-egg membrane fusion, and it has been shown that fusion is necessary for the initiation of Ca^{2+} oscillations (Lawrence et al. 1997). The fusion allows the introduction of a soluble factor into the egg that can initiate Ca^{2+} release. PLC ζ is the only sperm derived soluble protein that

has been reproducibly shown to trigger Ca^{2+} oscillations in mammalian eggs. Microinjecting PLC ζ RNA, or recombinant protein, has been found to cause Ca^{2+} oscillations in mouse, pig, cow, horse, and human eggs (Saunders et al. 2002; Rogers et al. 2004; Malcuit et al. 2005; Yoneda et al. 2006; Bedford-Guaus et al. 2008). PLC ζ induced Ca^{2+} oscillations require a catalytically active site, and they cause downregulation of InsP_3 receptors (IP3Rs) (Saunders et al. 2002; Malcuit et al. 2005). The downregulation of IP3Rs only occurs in response to InsP_3 increases, so we know that PLC ζ is mediating its effects via hydrolysis of phosphatidyl-inositol (4,5)-trisphosphate (PI(4,5)P2), and increased InsP_3 production, then leading to the opening of the InsP_3 receptor (IP3R). The amount of PLC ζ in single sperm is sufficient to cause a series of Ca^{2+} oscillations similar to those seen at fertilization (Saunders et al. 2002). PLC ζ is remarkably effective in causing Ca^{2+} release in eggs and can trigger oscillations when present in eggs at $\sim 10\text{nM}$. Given that mammalian eggs have considerable autofluorescence, this concentration is well below the detection limit for fluorescent proteins, and the only effective way to monitor the expression of PLC ζ after injection of RNA is to use a luciferase tag. We have used this technique for many years to quantify the threshold for PLC ζ induced Ca^{2+} release (Nomikos et al. 2005; Nomikos et al. 2011). PLC ζ consists of four EF hand domains, X and Y catalytic domains and a C2 domain, and each domain is essential for its ability to cause Ca^{2+} oscillations in eggs (Kouchi et al. 2005; Nomikos et al. 2005). These findings and other aspects of the structure and function of PLC ζ activity in eggs is covered in several other reviews (Miyazaki and Ito 2006; Thanassoulas et al. 2022). My interest here is to concentrate on specific aspects of PLC ζ that have been linked to the characteristics of Ca^{2+} signals in eggs.

One of the key aspects of PLC ζ enzyme activity studied *in vitro* is that it is very sensitive to Ca^{2+} (Kouchi et al. 2004; Nomikos et al. 2005). All mammalian PLC enzymes are sensitive to Ca^{2+} but PLC ζ is unusual in that it is stimulated by resting Ca^{2+} concentrations in the eggs. As with peak Ca^{2+} signals the resting Ca^{2+} concentration in mammalian eggs is not entirely clear, but it is likely to be around 100nM . The resting level of Ca^{2+} will result in PLC ζ being as much as 50% active. Furthermore, when Ca^{2+} starts to increase during InsP_3 induced Ca^{2+} release, the production of InsP_3 will be enhanced by the rise in Ca^{2+} leading to a positive feedback loop of InsP_3 production. Its sensitivity to the physiological Ca^{2+} concentration is particularly significant when it is combined with another feature of PLC ζ that is perhaps the most unusual compared to other mammalian PLC subtypes.

One of the most unusual features of PLC ζ is that it does not localize to the plasma membrane (Larman et al. 2004; Yoda et al. 2004; Yu et al. 2012). Even, overexpressed GFP tagged versions of PLC ζ show no localization in the plasma membrane (Yoda et al. 2004; Yu

et al. 2012). Interestingly the fluorescently tagged versions PLC ζ do localize to pronuclei after they form, and this localization appears to play a key role in the termination of Ca²⁺ oscillations during fertilization in the mouse (Yoda et al. 2004). However, as noted above, once one can detect the expression of PLC ζ with a fluorescently tagged protein, then the expression level is an order of magnitude above the physiological concentrations. Luciferase tagged probes do not help with localization because they emit too little light, and they do not provide confocal images needed for subcellular resolution. The localization is best visualized with techniques that contain some signal amplification step, such as indirect immunofluorescence. Using a myc-tagged version of PLC ζ , that was expressed at physiological levels, the main localization of PLC ζ has been shown to be on small vesicles that are distributed throughout the egg cytoplasm (Yu et al. 2012). The identity of these vesicles is unclear, but they are not the endoplasmic reticulum or mitochondria. Significantly, a similar staining pattern on small cytoplasmic vesicles, was also seen with antibodies against PI(4,5)P₂, and with a fluorescently tagged PI(4,5)P₂ binding peptide called PBP10 (Yu et al. 2012; Sanders et al. 2018). The data suggests that the majority of the PI(4,5)P₂ in mature eggs is found in cytoplasmic vesicles and that PLC ζ binds to, and presumably hydrolyses the PI(4,5)P₂, in these vesicles. The importance of these vesicles in fertilization is suggested by experiments using a simple drug intervention. We found that the drug propranolol can block sperm and PLC ζ induced Ca²⁺ oscillations in mouse eggs (Sanders et al. 2018). This effect requires high concentrations and is probably related to the ability of propranolol to inhibit Golgi recycling to the endoplasmic reticulum. Whilst there may be concerns about the high concentrations used in these experiments it is important to note that propranolol does not block Sr²⁺-induced, or InsP₃-induced Ca²⁺ oscillations (Sanders et al. 2018). The effects of propranolol are also fully reversible. We found that propranolol completely abolished the staining of vesicles with PBP10, which implies that the PI(4,5)P₂ in vesicles is significant in generating PLC ζ -induced Ca²⁺ oscillations. In addition, it suggests that the vesicles might be Golgi derived because propranolol acts upon recycling Golgi vesicles. It is worth noting that the Golgi is broken up into small vesicles in MII eggs, in a manner that resembles the Golgi 'haze' of somatic cells during mitosis. It is not clear how PLC ζ might bind to the Golgi vesicles, nor how the PI(4,5)P₂ is generated in the Golgi which is otherwise rich in PI(4)P.

Whilst most studies assay the effects of PLC ζ induced production of InsP₃ and Ca²⁺ release, PLC ζ it will also generate diacylglycerol (DAG) which will be expected to stimulate protein kinase C (PKC). There is evidence that PKC is stimulated at fertilization in rat eggs (Eliyahu and Shalgi 2002). It has been proposed to play a role in exocytosis, but as discussed above, myosin light chain kinase may well be the main target for Ca²⁺ stimulated exocytosis (Matson

et al. 2006). As noted above, PLC ζ is localized on cytoplasmic vesicles and the DAG generated by PLC ζ should also be confined to the membrane phase of these vesicles. The consequences of this are unclear. What is known is that if one measures the production of DAG in mouse eggs using a GFP probe tagged to a C2 domain, then there is no indication of DAG production in the plasma membrane, either at fertilization or after PLC ζ injection (Yu et al. 2008a). This result is not due to a lack of sensitivity of the GFP-C2 domain indicator, because this probe shows clear plasma membrane localization in response to Ca $^{2+}$ ionophores or PLC δ 1 injection (Yu et al. 2008a). Instead, the data imply that DAG is not generated in the plasma membrane of eggs during fertilization. Studies that have monitored PKC translocation, or measured PKC activity at fertilization in the plasma membrane, suggest that the conventional PKCs are stimulated by a rise in Ca $^{2+}$ rather than by DAG (Halet 2004).

The mechanism of PLC ζ induced Ca $^{2+}$ oscillations

The identification of PLC ζ as the sperm factor that initiates Ca $^{2+}$ oscillations enable an understanding and explanation of some of the characteristic features of the Ca $^{2+}$ signals at fertilization. The two distinctive features of PLC ζ that matter most are that PLC ζ is stimulated by Ca $^{2+}$ and that PLC ζ is localized in vesicles throughout the cytoplasm. These two features come together in explaining the fast Ca $^{2+}$ waves seen in fertilizing mouse and hamster eggs.

One of the characteristics of sperm-induced Ca $^{2+}$ oscillations in mouse and hamster eggs is that, whilst the first few increases occur as slow waves of $\sim 10\mu\text{m}/\text{sec}$ from the point of sperm-egg fusion, the later Ca $^{2+}$ waves originate from different parts of the egg cortex and are much more rapid at over $50\mu\text{m}/\text{s}$ (Miyazaki et al. 1986; Deguchi et al. 2000). The time of ~ 6 secs for the initial slow Ca $^{2+}$ wave to cross the mouse egg is consistent with the time for a molecule the size of InsP $_3$ to diffuse across an egg of $\sim 70\mu\text{m}$ (Swann 2023). However, the later fast waves that cross the egg in less than 1 sec are not easily explained when one considers the specific mechanism of Ca $^{2+}$ oscillations.

There are two classes of models for explaining cytosolic based Ca $^{2+}$ oscillations in cells that involve IP3Rs (Politi et al. 2006; Sneyd et al. 2006). The first class is based upon the behaviour of IP3Rs and was evidenced by experiments measuring single channel activity, either with isolated IP3Rs or with IP3Rs within the endoplasmic reticulum membrane from cells such as *Xenopus* oocytes. It was shown that Ca $^{2+}$ has a biphasic effect on the IP3R; it opens the channel at low Ca $^{2+}$ and then closes the channel at higher Ca $^{2+}$ concentrations

(Bezprozvanny et al. 1991; Finch et al. 1991). The stimulation, and then inhibition of IP3R is mediated by two different binding sites on the IP3R channel (see Figure.2) (Foskett et al. 2007). The biphasic effect of Ca^{2+} provides a basis for a class of models of Ca^{2+} oscillations that involve stimulation and then inhibition of IP3R induced Ca^{2+} release (Atri et al. 1993). These are referred to as IP3R based models since the IP3R provides the key element of the positive and negative feedback needed for oscillations. These models suggest that at fertilization the sperm causes a prolonged and constant increase in InsP_3 concentration. This model is supported by the finding that sustained microinjection of InsP_3 into hamster or mouse eggs causes Ca^{2+} oscillations (Swann et al. 1989; Swann 1992; Galione et al. 1994). Also, microinjecting the potent IP3R agonist, adenophostin, causes Ca^{2+} oscillations in mouse and bovine eggs (Sato et al. 1998; Malcuit et al. 2005). Despite these observations, the effects of InsP_3 in these studies is not compelling because the oscillations do not effectively mimic the Ca^{2+} response at fertilization. For example, the InsP_3 -induced oscillations in hamster eggs, are of higher frequency and lower amplitude than those seen at fertilization. Regardless, this model of Ca^{2+} oscillations is still used many schematics of InsP_3 signalling at fertilization.

The second class of models involves a different positive feedback loop in which the Ca^{2+} released via IP3Rs stimulates $\text{PLC}\zeta$ to make more InsP_3 , and hence increase Ca^{2+} release (Politi et al. 2006; Sneyd et al. 2006). These can be referred to as PLC based models since they rely on Ca^{2+} stimulated PLC activity. There is very good evidence for Ca^{2+} stimulated $\text{PLC}\zeta$ as discussed above. Furthermore, measurements of InsP_3 in mouse eggs using fluorescent indicators show that there are oscillations in InsP_3 that accompany the Ca^{2+} oscillations, as expected by with PLC positive feedback loop models (Shirakawa et al. 2006; Matsu-Ura et al. 2019). The detection of InsP_3 oscillations does not, however, prove that such oscillations themselves are essential for Ca^{2+} oscillations. There is, however, a way one can determine which model of oscillations is operating in eggs.

The two different mechanisms of Ca^{2+} oscillations can be distinguished by examining the effects of sudden pulses of InsP_3 , that can be delivered into the egg cytoplasm by uncaging InsP_3 (Sneyd et al. 2006). It has been shown that in all mathematical models based upon IP3Rs, a sudden pulse of InsP_3 temporarily increases the frequency of oscillations (Sneyd et al. 2006). In contrast, with all models based upon Ca^{2+} stimulated InsP_3 production, a sudden pulse of InsP_3 causes an immediate Ca^{2+} transient but this does not increase the frequency of oscillations. Instead, there is a resetting of the phase of Ca^{2+} oscillations. We have shown that when a pulse of InsP_3 is triggered in mouse eggs, that are oscillating in response to $\text{PLC}\zeta$, there is always a resetting of the phase of Ca^{2+} oscillations (Sanders et al. 2018). This is only consistent with the Ca^{2+} stimulated PLC models. This result is

unequivocal and implies that during each Ca^{2+} rise there is an increase in InsP_3 that plays an active role in a positive feedback loop. Therefore, InsP_3 acts as a dynamic variable and InsP_3 oscillations are absolutely necessary for $\text{PLC}\zeta$ induced Ca^{2+} oscillations.

The Ca^{2+} waves that are generated by Ca^{2+} and InsP_3 production are not only rapid, but they travel through the centre of the egg (Deguchi et al. 2000). It is very difficult to explain this type of Ca^{2+} wave if the InsP_3 is being produced at the plasma membrane, because the diffusion of InsP_3 from the membrane to the centre of the egg will take several seconds (Swann 2023). The only way that a Ca^{2+} wavefront can propagate through the egg, at the speeds that are observed, is if InsP_3 is generated within the cytoplasm (Sanders et al. 2018). This idea has been suggested from previous models of Ca^{2+} waves in fertilizing ascidian oocytes (Dupont and Dumollard 2004). In mouse eggs the vesicles that we found to contain $\text{PI}(4,5)\text{P}_2$ appear to be about $2\text{-}3\mu\text{m}$ apart, which is sufficiently small to allow for the diffusion of InsP_3 to play its role in propagating the Ca^{2+} waves (Sanders et al. 2018). Hence, the mechanism of the Ca^{2+} oscillations requires a cytoplasmic source of InsP_3 , and the vesicular distribution of $\text{PI}(4,5)\text{P}_2$ is not an odd epiphonema, but an absolutely necessary feature of a mature fertilizable egg.

There are two specific new issues to resolve with the PLC based InsP_3 positive feedback model of Ca^{2+} oscillations in eggs. First, there is the one of how the Ca^{2+} reaches $>1\mu\text{M}$. InsP_3 opens all IP_3Rs , and the Ca^{2+} plays a role in both opening and closing IP_3Rs (see Figure 2). It is notable that studies of IP_3Rs imply that Ca^{2+} cannot rise particularly high because the IP_3R will be mostly closed above $2\mu\text{M}$ Ca^{2+} , in the same way that they are mostly closed at 100nM Ca^{2+} (Bezprozvanny et al. 1991). This is problematic for eggs because we know the Ca^{2+} increase in fertilizing eggs is around $2\mu\text{M}$ and this state lasts for about 1 minute. Mathematical models of Ca^{2+} oscillations in somatic cells, circumnavigate this problem by introducing a time delay so that the IP_3R does not close in response to Ca^{2+} until after Ca^{2+} release is initiated (Atri et al. 1993; Politi et al. 2006; Sneyd et al. 2006). This delayed IP_3R closing mechanism allows the models to generate Ca^{2+} oscillations. However, the evidence for this mechanism for IP_3Rs undergoing delayed closure suggests a time scale of about 1 second (Finch et al. 1991), which does not allow Ca^{2+} to stay elevated for a minute or more as seen in mammalian eggs.

An alternative view of the problem of high Ca^{2+} for all IP_3R models is to recognize that the concentration at which Ca^{2+} inhibits IP_3Rs can be very dependent upon the concentration of InsP_3 . The predominant form of IP_3R in mammalian eggs is the type 1 receptor ($\text{IP}_3\text{R1}$) and a version of this type is found in *Xenopus* oocytes (Mak et al. 1998). In studies of *Xenopus* oocytes, Mak and Fosket showed, that if the InsP_3 concentration is increased above 100nM ,

then the IP3R1 is no longer inhibited by Ca^{2+} in the physiological range, and the Ca^{2+} inhibitory site (Fig.2) is unable to close the IP3R1 (Mak et al. 1998). Hence, if there is a substantial increase in both Ca^{2+} and InsP_3 at the same time, then the IP3R1s do not close until Ca^{2+} reaches levels well above $10\mu\text{M}$. A large and simultaneous increase in InsP_3 and Ca^{2+} is exactly what occurs with $\text{PLC}\zeta$ induced Ca^{2+} transients. Hence, the positive feedback of Ca^{2+} and InsP_3 production means that InsP_3 overrides the inhibitory effect of Ca^{2+} on the IP3Rs. This can explain why sperm and $\text{PLC}\zeta$ cause larger Ca^{2+} transients than seen after InsP_3 injection. However, the release system now becomes locked into a state of high Ca^{2+} , high InsP_3 , and open IP3R1s. This brings in the second problem of how each Ca^{2+} rise is terminated. To resolve this type of problem, some models of Ca^{2+} oscillations invoke the emptying of Ca^{2+} stores to terminate a cytosolic increase (Dupont and Goldbeter 1993). However, it is known that Ca^{2+} stores do not run out of Ca^{2+} during each Ca^{2+} peak (Wakai et al. 2013). Instead, I suggest it is possible that the IP3R1s desensitize to InsP_3 itself, and hence Ca^{2+} release decreases. This is plausible because IP3R mediated Ca^{2+} release shows clear and specific desensitization to InsP_3 in sea urchin egg homogenates (Jones et al. 1998). This idea has not been fully investigated in mathematical models of Ca^{2+} oscillations.

Other soluble sperm factors and Ca^{2+} release in eggs.

The key experiments to establish a role of $\text{PLC}\zeta$ in fertilization have involved generating male mice that produce sperm with no functional $\text{PLC}\zeta$. Several groups have now made $\text{PLC}\zeta$ knockout mouse lines in which sperm are produced that do not express $\text{PLC}\zeta$, or else contain highly truncated and non-functional version of the enzyme (Hachem et al. 2017; Nozawa et al. 2018; Kashir et al. 2024). Sperm that lack functional $\text{PLC}\zeta$ fail to cause Ca^{2+} oscillations or egg activation after ICSI (Hachem et al. 2017; Nozawa et al. 2018). However, after normal *in vitro* fertilization, with the sperm fusing with the egg, there are 3 to 4 Ca^{2+} spikes that appear to be delayed by ~40 mins from the time of sperm egg fusion (Nozawa et al. 2018). These delayed Ca^{2+} spikes can activate eggs and can account for a small number of live young being born after mating female mice with $\text{PLC}\zeta$ deficient males (Hachem et al. 2017; Nozawa et al. 2018). The delayed nature of the Ca^{2+} spikes means that many $\text{PLC}\zeta$ knockout sperm fertilized eggs do not develop an effective block to extra sperm entry and most eggs become polyspermic (Nozawa et al. 2018). These data clearly prove that $\text{PLC}\zeta$ accounts for Ca^{2+} oscillations and egg activation after ICSI, but the results during normal fertilization are more complex to interpret. The data suggest that $\text{PLC}\zeta$ initiates Ca^{2+} release and causes the majority of Ca^{2+} oscillations, but that some other factor, or signalling system from the sperm also causes Ca^{2+} release in eggs. It is unclear why this other mechanism, or sperm factor, is not active in ICSI. Nor why it should work with a distinct delay. It is also

unclear how much this applies to human fertilization. There are now cases of men, attending IVF clinics, who have been identified as having homologous mutations in PLC ζ that lead to a loss of function (Escoffier et al. 2016; Abdulsamad et al. 2023). These men and their female partners came to attention of researchers because of repeated total fertilization failure during ICSI treatment, and presumably failed conception following 2 or more years of intercourse. So, in humans a lack of functional PLC ζ appears to lead to a complete loss of fertility. It is not clear if there is a genuine difference between mice and men in these findings, and if so, why this would arise.

The fact that PLC ζ deficient sperm lead to delayed Ca²⁺ oscillations and cause some mouse eggs to activate, implies that another sperm factor could be involved in Ca²⁺ release. This factor has been variously referred to as the primitive, cryptic, or secondary sperm factor (Jones 2018; Nozawa et al. 2018; Swann 2020). It seems likely that this secondary factor would also be active at fertilization. This is based upon the observations that spermatogenesis and sperm motility seem to be completely unaffected by the lack of a functional PLC ζ (Hachem et al. 2017). This implies that PLC ζ cannot affect spermatogenesis and that the first time that a lack of PLC ζ becomes evident is after the sperm fuses with the egg. In such case it is difficult to see how any secondary factor could be 'upregulated' to compensate for a lack of PLC ζ . Hence the secondary factor is expected to enter the egg in normal fertilization. In a sense it is only a 'secondary' sperm factor by virtue of the fact that its effects are delayed about an hour after PLC ζ initiates Ca²⁺ oscillations. Any secondary factor is constrained in how it might act to cause Ca²⁺ oscillations, because we have a clear idea of what factors can cause Ca²⁺ oscillations in mouse eggs. For example, increasing Ca²⁺ influx by itself does not cause Ca²⁺ oscillations in mouse eggs so that is not a viable mechanism to use (Swann and Ozil 1994). All the evidence suggests that fertilization induced Ca²⁺ oscillations are mediated by the IP3R, which means that a secondary factor needs to stimulate the IP3R.

When considering other non-PLC ζ sperm derived factors, it is notable that there have already been other proteins that have been suggested to cause Ca²⁺ oscillations in mammalian eggs. These proteins include tr-kit, PAWP and citrate synthase (Sette et al. 1997; Wu et al. 2007; Aarabi et al. 2014; Kang et al. 2020). One of the general issues with these candidate sperm factors is the reproducibility of the original findings. The original paper suggesting the tr-kit could activate mouse eggs was published in 1997 and yet no one has replicated the data, nor has anyone ever shown that tr-kit causes Ca²⁺ oscillations. The paper suggesting PAWP protein could cause Ca²⁺ oscillations in mouse and human eggs was published in 2012. We tried to replicate the results in this study but could not find any

evidence that PAWP causes Ca^{2+} release in eggs (Nomikos et al. 2014; Nomikos et al. 2015). More recently it was suggested that citrate synthase might be a Ca^{2+} releasing sperm factor in mouse eggs (Kang et al. 2020). Citrate synthase is commercially available, and it is simple to test if it activates eggs but there have been no positive reports from other labs so far. We have injected citrate synthase into mouse eggs and failed to detect any Ca^{2+} oscillations. In contrast with the lack of replications in such studies, it took just two years for two independent laboratories to reproduce our key finding that $\text{PLC}\zeta$ causes Ca^{2+} oscillations and egg activation in the mouse. This was followed by numerous other replications in other species.

The sensitivity of Ca^{2+} release to different stimuli.

Whilst all mammalian eggs tested show Ca^{2+} release at fertilization, and in response to the microinjection of InsP_3 or $\text{PLC}\zeta$, there are differences in the sensitivity of eggs to various stimuli. These differences were evident in studies from the 1980s on hamster eggs. A remarkable feature of hamster eggs is that, when the zona pellucida is removed, they can be fertilized by sperm from other mammalian species and even become polyspermic, with multiple sperm entering the egg. When mouse sperm fertilize hamster eggs, they trigger Ca^{2+} oscillations and egg activation. However, it requires between 5 and 10 mouse sperm to fuse before the hamster egg generates any Ca^{2+} transients and becomes activated (Igusa et al. 1983). Obviously one mouse sperm will cause a series of Ca^{2+} oscillations in a mouse egg, so we can deduce that the hamster egg is somehow much less sensitive than the mouse egg to the mouse factor - now known to be $\text{PLC}\zeta$. This difference in sensitivity of eggs from different species is also seen with experiments injecting InsP_3 . InsP_3 can be injected continuously into eggs by inserting a sharp micropipette containing a high concentration of InsP_3 (Galione et al. 1994; Swann 1994). In mouse eggs this causes sustained Ca^{2+} oscillations with pipette concentrations as low as $10\mu\text{M}$ InsP_3 (Swann 1994). In hamster eggs the same technique causes Ca^{2+} oscillations but one needs to use 1mM InsP_3 in the micropipette, and the oscillations are damped and generally do not persist beyond 10 mins (Galione et al. 1994). This suggests that mouse eggs are more sensitive to InsP_3 than hamster eggs, and that this can explain their greater sensitivity to a fertilizing sperm.

A significant difference in sensitivity is also seen between mouse eggs and human eggs. Human $\text{PLC}\zeta$ is very potent at causing Ca^{2+} oscillations in mouse eggs. It requires as little as 30 times less human $\text{PLC}\zeta$ to trigger Ca^{2+} oscillations in mouse eggs than mouse $\text{PLC}\zeta$ (Yu et al. 2008b). Since human $\text{PLC}\zeta$ appears to cause a similar series of Ca^{2+} oscillations in human eggs to what is seen in fertilizing mouse eggs then we can deduce that the greater

potency of human PLC ζ must be because the human egg requires a more potent stimulus. Some of this requirement may be because the human egg is larger than the mouse egg, but the volume difference is only about 5-fold, so it appears that the human egg is somehow less sensitive to PLC ζ induced signalling. As with the hamster egg, this difference is potentially explained by IP3R sensitivity. We used UV pulses to un-cage and release InsP₃ to stimulate IP3R mediated Ca²⁺ transients in mouse eggs and human eggs (Storey et al. 2021). The amount of UV light needed to stimulate Ca²⁺ release in human eggs is about 10 times greater than that required to cause Ca²⁺ release in a mouse egg, implying that human eggs are many times less sensitive to InsP₃ than mouse eggs (Storey et al. 2021).

The differences in the sensitivity of Ca²⁺ release between different species of mammalian eggs is also evident from the way they respond to Sr²⁺ medium. As noted above both mouse and rat eggs can be stimulated to undergo prolonged Ca²⁺ oscillations in Sr²⁺ medium (Kline and Kline 1992; Tomashov-Matar et al. 2005; Lu et al. 2018; Storey et al. 2021). This is consistent with the findings that Sr²⁺ stimulates Ca²⁺ release via the IP3R (Marshall and Taylor 1994; Sanders et al. 2018). However, human eggs do not undergo Ca²⁺ oscillations in Sr²⁺ medium (Lu et al. 2018; Storey et al. 2021). Sr²⁺ probably enters eggs via TRPV3 channels, but both human eggs and mouse eggs have TRPV3 channels so the inability of human eggs to respond cannot be explained by a lack of Sr²⁺ influx (Lu et al. 2018). It's notable that there are no reports of pig, cow, or hamster eggs undergoing Ca²⁺ oscillations in Sr²⁺ medium, despite many studies of Ca²⁺ release in these types of eggs. The implication is that mouse and rat eggs have a much more sensitive Ca²⁺ release system than eggs from other species such as humans and hamsters. It's not obvious why there is such a marked difference in sensitivity because mammalian eggs appear to predominantly have the same type 1 IP3R (Fissore et al. 1999; Mann et al. 2010). We recently found evidence that the difference is due to the modulation of IP3R1.

Single channel recordings of IP3R1s have identified three ligands that are likely to be physiological: Ca²⁺, InsP₃ and ATP (Foskett et al. 2007). The interacting roles of Ca²⁺ and InsP₃ are widely acknowledged, but the potential role of ATP is generally overlooked. When referring to 'ATP', it should be understood that I am referring to the total ATP in a cell or in the cytosol. However, in all cells ATP exists in two forms: MgATP²⁻ and ATP⁴⁻. Most of the cells ATP is bound to Mg²⁺, but some of the ATP is Mg²⁺ free in the form of ATP⁴⁻ (Grubbs 2002). The studies of IP3R1s in *Xenopus* oocytes show that the Mg²⁺ free form ATP⁴⁻ can open the IP3R1 channel, and in fact that ATP⁴⁻ acts as an allosteric stimulator of the IP3R1 at the Ca²⁺ stimulatory site (see Figure 2) (Mak et al. 1999). The half maximal concentration of ATP⁴⁻ to open IP3R1s is around 270 μ M which is physiologically relevant (Mak et al. 1999). Most mammalian cells, including eggs, maintain a concentration of 1 to 4 mM total ATP. With

this level of total ATP there will be about 100-200 μ M of unbound ATP⁴⁻. Hence ATP⁴⁻ is normally within the range where it could modulate IP3R1 activity and increases in ATP⁴⁻ could potentiate, or else help trigger, Ca²⁺ release. Its notable that ATP⁴⁻ is the immediate product of mitochondrial ATP production because it is exported from mitochondria via the adenine nucleotide exchanger (Foskett et al. 2007). This is important because mammalian eggs are unusual in that they generate nearly all their ATP from mitochondria (Dumollard et al. 2008). The type 1 InsP₃ receptor is one of three main subtypes in mammalian cells and is the one most sensitive to ATP⁴⁻, and most likely to respond in the physiological range (Tu et al. 2005). This makes eggs particularly likely to be sensitive to an effect of ATP on Ca²⁺ release.

We recently showed that the concentration of ATP in mouse eggs affects their ability to generate Ca²⁺ oscillations in response to Sr²⁺. Mouse eggs invariably generate Ca²⁺ oscillations when incubated in Sr²⁺ medium, but if all the carbohydrate metabolites are removed from the incubation medium, mouse eggs fail to respond to Sr²⁺ (Storey et al. 2021). The lack of metabolites causes the egg to have a low ATP level. When pyruvate is added back to such mouse eggs the ATP concentration rises back to normal levels as mitochondrial activity increases. At the same time as ATP increases, Ca²⁺ oscillations are generated. These data suggest that the concentration of ATP in mouse eggs critically affects their ability to respond to Sr²⁺, and that with low ATP levels, Ca²⁺ oscillations are not generated. This is potentially significant because we also found that the concentration of ATP (as total ATP) in human eggs is consistently lower than that in mouse eggs, and in fact the level of ATP in a human egg is similar to that of a pyruvate deprived mouse egg (Storey et al. 2021). This implies that the relatively low ATP concentration in human eggs can explain why they do not activate in response to Sr²⁺. This may also be the case for eggs from domestic animals. It certainly appears to be the case in hamster eggs. As discussed above, hamster eggs appear to be significantly less sensitive to InsP₃-induced Ca²⁺ release than mouse eggs. One study that involved measuring ATP in both mouse and hamster eggs (to look at the effects of ageing) found that the mouse egg ATP level was about six times higher than that in hamster eggs (Simsek-Duran et al. 2013). This is consistent with the hypothesis that ATP levels set the sensitivity of Ca²⁺ release in eggs. It should be noted that these measurements of ATP, with cross-species comparisons, involve egg lysis and use kits that measure the total ATP. However, if we assume that intracellular free Mg²⁺ concentrations are similar in eggs from different species, then the concentration of ATP⁴⁻ will be proportional to the total ATP. Hence, ATP⁴⁻ is expected to be significantly higher in mouse eggs compared to human or hamster eggs. Unfortunately, there are no probes to measure ATP⁴⁻ since firefly

luciferase, and all the available fluorescent ATP probes, respond to MgATP^{2-} , which in most cases, is almost the same as the total ATP.

Changes in ATP concentration at fertilization

The oscillatory changes in cytosolic Ca^{2+} at fertilization cause an increase in mitochondrial activity. In mouse eggs it was found that each Ca^{2+} transient leads to a distinct increase in NADH and FADH levels (Dumollard et al. 2004). The increases in NADH and FADH outlast each Ca^{2+} transient. Measurements using Ca^{2+} probes inside the mitochondrial matrix show that matrix Ca^{2+} increases occur with each cytosolic Ca^{2+} increase (Dumollard et al. 2008). This suggests that each NADH or FADH change is a result of Ca^{2+} influx into mitochondria which can then stimulate mitochondrial dehydrogenases. Hence Ca^{2+} oscillations stimulate mitochondria during the process of fertilization. Stimulating mitochondrial activity may be a way of avoiding a decrease in ATP levels, because the pumping of Ca^{2+} into the endoplasmic reticulum is likely to consume a significant fraction of the egg's ATP. We know that mitochondrial ATP production is critical in maintaining Ca^{2+} oscillations because if mouse eggs are fertilized in the absence of pyruvate, to reduce ATP production, then Ca^{2+} oscillations are interrupted by a persistent high level of Ca^{2+} (Liu et al. 2001; Dumollard et al. 2008). Hence, the Ca^{2+} oscillations appear to act to simultaneously increase the egg's supply and demand of ATP.

The idea of Ca^{2+} mediated increased ATP supply and demand at fertilization, is however, more complex. The changes in ATP in mouse eggs can be measured by microinjecting firefly luciferase and imaging the luminescence of luciferase alongside the fluorescence from Ca^{2+} sensitive dyes. During the sperm-induced Ca^{2+} oscillations there is a substantial increase in the concentration of ATP in eggs (Campbell and Swann 2006; Ikie-Eshalomi et al. 2023). Figure 3 shows the increase in ATP alongside Ca^{2+} oscillations in a fertilizing mouse egg. The ATP increase occurs in two phases, the first starts during the initial Ca^{2+} transient, and then a secondary ATP increase occurs about 1 hour later into the Ca^{2+} oscillations. The two-step change in ATP can also be seen when luciferase is targeted to the egg's mitochondria (Dumollard et al. 2008). These data imply that a substantial 'overproduction' of ATP occurs in mitochondria during fertilization.

Some of this increase in ATP concentration in fertilizing eggs can be explained by Ca^{2+} entering mitochondria and stimulating matrix dehydrogenases, as discussed above. This is shown by the fact that $\text{PLC}\zeta$ can also cause an increase in ATP (Ikie-Eshalomi et al. 2023). However, the ATP increase with $\text{PLC}\zeta$ is only about half of that seen at fertilization (Ikie-Eshalomi et al. 2023). Furthermore, with $\text{PLC}\zeta$ there is no secondary increase in ATP. Interestingly, the secondary increase in ATP still occurs at around 1 hour after sperm egg

fusion even when eggs are loaded with BAPTA to prevent all but the initial Ca^{2+} transient (Ikke-Eshalomi et al. 2023). This shows that the secondary increase in ATP is sperm mediated, and not driven by $\text{PLC}\zeta$, or by Ca^{2+} . The simplest way to interpret these data is to propose that the sperm provides a stimulus, separate to $\text{PLC}\zeta$, that increases mitochondrial activity after sperm-egg fusion. It is not clear what this factor might be, but it is possible that a sperm protein, or set of proteins, targets egg mitochondria. This idea is similar to one previously suggested by Sakkas and colleagues for glucose metabolism at fertilization (Urner and Sakkas 1999). It was found that glucose metabolism increases through both glycolysis and the pentose phosphate pathway during fertilization in mouse eggs (Urner and Sakkas 1999). The increase in glucose metabolism is not mimicked by parthenogenetic activation and so it appears to be sperm specific. Together with our observations, the data imply that sperm stimulates increases in both glycolysis and oxidative phosphorylation in eggs in a way that does not involve $\text{PLC}\zeta$.

It is not clear what role, if any, the increase in ATP plays at fertilization in mouse eggs. It is possible that an increase in ATP concentration just reflects an imbalance between supply and demand. However, I consider this unlikely because adding excess pyruvate to eggs, does not increase the ATP concentration which suggests that ATP levels are specifically regulated (Dumollard et al. 2009). Overproduction of ATP is also unlikely because the increase in ATP at fertilization lasts for more than 2 hours, and yet the turnover time for ATP in eggs is likely to be about 1 minute (Leese 1991). Hence, the homeostatic system controlling ATP levels has ample time to readjust. Overproduction is also literally a waste of energy for the cell. Furthermore, as judged by the changes in luciferase signal, the increase in ATP at fertilization in mouse eggs appears to be larger than the increase in ATP detected in pancreatic beta cells responding to glucose (Kennedy et al. 1999; Campbell and Swann 2006; Ikke-Eshalomi et al. 2023). The ATP increase in beta cells plays a vital role in insulin secretion and depends upon the specific rewiring of metabolism (Rutter et al. 2020). It seems unusual to suppose that an even larger change of ATP in eggs during fertilization were just an accident, a type of epiphenomenon. I suggest it is more likely that the sperm induced ATP increase has a consequence, and a role to play in the process of egg activation.

One possible function of the increase is that the extra ATP promotes energy using steps in fertilization. The energy requiring usage of ATP in cells is dominated by protein turnover, and Na^+ and Ca^{2+} pumping (Rolfe and Brown 1997). Protein synthesis rates increase after fertilization in mouse eggs by about 40% and it is possible that increases in ATP drives some of this change (Schultz et al. 1979). Another major use of ATP, as mentioned above, will be associated with pumping Ca^{2+} back into stores, or out of the egg. A doubling of ATP levels in

a cell will make little difference to the free energy available for pumping Ca^{2+} . This is because the free energy available depends upon the logarithm of the ATP concentration, and the reaction $\text{ATP} \leftrightarrow \text{ADP} + \text{P}_i$ is already ~ 10 orders of magnitude away from equilibrium (Wilson 2015). However, an increase in the concentration of ATP will speed up the kinetics of Ca^{2+} pumping and this could increase the rate of Ca^{2+} uptake into stores (Verjovski-Almeida and Inesi 1979). Increases in the activity of Ca^{2+} ATPases will lower cytosolic Ca^{2+} and hence the increase in ATP could explain why the 'inter-spike' Ca^{2+} concentration at fertilization is lower than the resting Ca^{2+} concentration before fertilization (see Figure 1).

Another possible effect of the ATP increase could be to promote Ca^{2+} release in eggs. This is related to the stimulation of the IP3R by ATP^{4-} discussed above. It is notable that the secondary increase in ATP starts about an hour after the initial Ca^{2+} spike at fertilization. This secondary ATP increase occurs about 10 minutes before a small increase in the frequency of Ca^{2+} oscillations, that is seen in many eggs. It is notable that the transient increase in frequency of Ca^{2+} oscillations occur during the rising phase of ATP (technically MgATP^{2-}). This is the time when mitochondrial ATP^{4-} production is likely to be at a maximum because the increase in total ATP is driven by mitochondria. The mitochondrially derived ATP^{4-} may act as a co-agonist with InsP_3 in promoting Ca^{2+} release and increasing the number of Ca^{2+} oscillations at fertilization. Experiments to investigate these potential effects of ATP^{4-} are, however, non-trivial because any interference in mitochondrial ATP production, for example by removing pyruvate or adding mitochondrial poisons, causes a rapid rise in Ca^{2+} due to the inhibition of Ca^{2+} pumps (Dumollard et al. 2004; Dumollard et al. 2008). It is difficult to separate out the role of ATP^{4-} , as an allosteric activator of the IP3R1, from the role of MgATP^{2-} as an energy source for Ca^{2+} pumping.

Unresolved problems.

There are various issues to resolve about sperm induced Ca^{2+} signals in mammalian eggs. We still do not know how $\text{PLC}\zeta$ binds to vesicles in eggs and whether this involves a specific egg protein. It's also unclear how the vesicles containing $\text{PI}(4,5)\text{P}_2$ generate and recycle their inositol containing lipids. There is also the problem of the nature of the putative secondary sperm factor that can trigger Ca^{2+} oscillations in the absence of $\text{PLC}\zeta$. This issue is clearly important to understand mouse egg activation, but it is important to establish the nature of the factor in order to know whether it has implications for egg activation in humans, or even non mammalian eggs. I would add to this specific problem another one of establishing the nature of potential factors that increase metabolism in eggs at fertilization, and specifically how the sperm causes the secondary increase in ATP.

Another issue that would benefit from further investigation is the mechanism by which Ca^{2+} signals during egg activation could influence events much later in development. The connection has been difficult to address because the main phase of embryonic genome expression occurs days after the Ca^{2+} oscillations at fertilization have stopped. However, recent studies suggest that mammalian embryonic gene expression may be initiated during the first cell cycle and a possible link to the pattern of Ca^{2+} spikes may need to be revisited (Perry et al. 2023) .

Finally, it is worth noting that there is a significant clinical problem with regards to Ca^{2+} signals in eggs. ICSI is used in most IVF treatment cycles around the world (Haddad et al. 2021). Total fertilization failure, where all ovulated eggs fail to fertilize, occurs in about 1-4% of these ICSI cases and a significant cause is failed egg activation (Ferrer-Buitrago et al. 2018; Bonte et al. 2019; Ferrer-Buitrago et al. 2019). However, low fertilization rates occur in about one third of all ICSI cycles, so the room for improving the success rates of ICSI treatment could probably have a much larger potential impact (Scaravelli et al. 2021). In many cases of failed fertilization, it is now becoming common to offer artificial egg activation as a therapy. The main treatment approved for clinical use to induce artificial egg activation is Ca^{2+} ionophore. The Ca^{2+} ionophore A23187 was first shown to be capable of causing egg activation in the 1970s and it can only generate a single large Ca^{2+} increase. There is evidence that multiple Ca^{2+} transients would be a more effective stimulus for egg activation but it's not clear how this can be achieved in clinical practice. The obvious solution would be to microinject $\text{PLC}\zeta$, but it is a very unstable enzyme, and it has been difficult to develop a commercially available form of the recombinant protein. It would be invaluable to find a way to improve $\text{PLC}\zeta$'s stability or else provide an alternative way of activating human eggs that invokes multiple Ca^{2+} transients.

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References

- Aarabi, M., Balakier, H., Bashar, S., Moskovtsev, S. I., Sutovsky, P., Librach, C. L. and Oko, R. 2014. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *Faseb j* 28(10), pp. 4434-4440. doi: 10.1096/fj.14-256495
- Abdulsamad, H. M. R. et al. 2023. The Therapeutic and Diagnostic Potential of Phospholipase C Zeta, Oocyte Activation, and Calcium in Treating Human Infertility. *Pharmaceuticals (Basel)* 16(3). doi: 10.3390/ph16030441
- Ardestani, G., West, M. C., Maresca, T. J., Fissore, R. A. and Stratton, M. M. 2019. FRET-based sensor for CaMKII activity (FRESCA): A useful tool for assessing CaMKII activity in response to Ca(2+) oscillations in live cells. *J Biol Chem* 294(31), pp. 11876-11891. doi: 10.1074/jbc.RA119.009235
- Atri, A., Amundson, J., Clapham, D. and Sneyd, J. 1993. A single-pool model for intracellular calcium oscillations and waves in the *Xenopus laevis* oocyte. *Biophys J* 65(4), pp. 1727-1739. doi: 10.1016/s0006-3495(93)81191-3
- Balakier, H. and Casper, R. F. 1993. Experimentally induced parthenogenetic activation of human oocytes. *Hum Reprod* 8(5), pp. 740-743. doi: 10.1093/oxfordjournals.humrep.a138132
- Bedford-Guaus, S. J., Yoon, S. Y., Fissore, R. A., Choi, Y. H. and Hinrichs, K. 2008. Microinjection of mouse phospholipase C zeta complementary RNA into mare oocytes induces long-lasting intracellular calcium oscillations and embryonic development. *Reprod Fertil Dev* 20(8), pp. 875-883. doi: 10.1071/rd08115
- Ben-Yosef, D., Oron, Y. and Shalgi, R. 1995. Low temperature and fertilization-induced Ca²⁺ changes in rat eggs. *Mol Reprod Dev* 42(1), pp. 122-129. doi: 10.1002/mrd.1080420116
- Bezprozvanny, I., Watras, J. and Ehrlich, B. E. 1991. Bell-shaped calcium-response curves of Ins(1,4,5)P₃- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* 351(6329), pp. 751-754. doi: 10.1038/351751a0
- Bonte, D. et al. 2019. Assisted oocyte activation significantly increases fertilization and pregnancy outcome in patients with low and total failed fertilization after

intracytoplasmic sperm injection: a 17-year retrospective study. *Fertil Steril* 112(2), pp. 266-274. doi: 10.1016/j.fertnstert.2019.04.006

Bos-Mikich, A., Swann, K. and Whittingham, D. G. 1995. Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. *Mol Reprod Dev* 41(1), pp. 84-90. doi: 10.1002/mrd.1080410113

Campbell, K. and Swann, K. 2006. Ca²⁺ oscillations stimulate an ATP increase during fertilization of mouse eggs. *Dev Biol* 298(1), pp. 225-233. doi: 10.1016/j.ydbio.2006.06.032

Carroll, J., Swann, K., Whittingham, D. and Whitaker, M. 1994. Spatiotemporal dynamics of intracellular [Ca²⁺]_i oscillations during the growth and meiotic maturation of mouse oocytes. *Development* 120(12), pp. 3507-3517. doi: 10.1242/dev.120.12.3507

Cheek, T. R., McGuinness, O. M., Vincent, C., Moreton, R. B., Berridge, M. J. and Johnson, M. H. 1993. Fertilisation and thimerosal stimulate similar calcium spiking patterns in mouse oocytes but by separate mechanisms. *Development* 119(1), pp. 179-189. doi: 10.1242/dev.119.1.179

Chung, J. T., Keefer, C. L. and Downey, B. R. 2000. Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI). *Theriogenology* 53(6), pp. 1273-1284. doi: 10.1016/s0093-691x(00)00271-5

Collas, P., Fissore, R., Robl, J. M., Sullivan, E. J. and Barnes, F. L. 1993a. Electrically induced calcium elevation, activation, and parthenogenetic development of bovine oocytes. *Mol Reprod Dev* 34(2), pp. 212-223. doi: 10.1002/mrd.1080340214

Collas, P., Sullivan, E. J. and Barnes, F. L. 1993b. Histone H1 kinase activity in bovine oocytes following calcium stimulation. *Mol Reprod Dev* 34(2), pp. 224-231. doi: 10.1002/mrd.1080340215

Cuthbertson, K. S. and Cobbold, P. H. 1985. Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca²⁺. *Nature* 316(6028), pp. 541-542. doi: 10.1038/316541a0

Cuthbertson, K. S., Whittingham, D. G. and Cobbold, P. H. 1981. Free Ca²⁺ increases in exponential phases during mouse oocyte activation. *Nature* 294(5843), pp. 754-757. doi: 10.1038/294754a0

Deguchi, R., Shirakawa, H., Oda, S., Mohri, T. and Miyazaki, S. 2000. Spatiotemporal analysis of Ca(2+) waves in relation to the sperm entry site and animal-vegetal axis

during Ca^{2+} oscillations in fertilized mouse eggs. *Dev Biol* 218(2), pp. 299-313. doi: 10.1006/dbio.1999.9573

Ducibella, T. et al. 2002. Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Dev Biol* 250(2), pp. 280-291.

Dumollard, R., Campbell, K., Halet, G., Carroll, J. and Swann, K. 2008. Regulation of cytosolic and mitochondrial ATP levels in mouse eggs and zygotes. *Dev Biol* 316(2), pp. 431-440. doi: 10.1016/j.ydbio.2008.02.004

Dumollard, R., Carroll, J., Duchen, M. R., Campbell, K. and Swann, K. 2009. Mitochondrial function and redox state in mammalian embryos. *Semin Cell Dev Biol* 20(3), pp. 346-353. doi: 10.1016/j.semcdb.2008.12.013

Dumollard, R., Marangos, P., Fitzharris, G., Swann, K., Duchen, M. and Carroll, J. 2004. Sperm-triggered $[\text{Ca}^{2+}]$ oscillations and Ca^{2+} homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. *Development* 131(13), pp. 3057-3067. doi: 10.1242/dev.01181

Dupont, G. and Dumollard, R. 2004. Simulation of calcium waves in ascidian eggs: insights into the origin of the pacemaker sites and the possible nature of the sperm factor. *J Cell Sci* 117(Pt 18), pp. 4313-4323. doi: 10.1242/jcs.01278

Dupont, G. and Goldbeter, A. 1993. One-pool model for Ca^{2+} oscillations involving Ca^{2+} and inositol 1,4,5-trisphosphate as co-agonists for Ca^{2+} release. *Cell Calcium* 14(4), pp. 311-322. doi: 10.1016/0143-4160(93)90052-8

Eliyahu, E. and Shalgi, R. 2002. A role for protein kinase C during rat egg activation. *Biol Reprod* 67(1), pp. 189-195. doi: 10.1095/biolreprod67.1.189

Esbert, M., Carmody, A., Ballesteros, A., Seli, E. and Scott, R. T. 2022. Calcium ionophore A23187 treatment to rescue unfertilized oocytes: a prospective randomized analysis of sibling oocytes. *Reprod Biomed Online* 45(5), pp. 878-883. doi: 10.1016/j.rbmo.2022.06.021

Escoffier, J. et al. 2016. Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. *Hum Mol Genet* 25(5), pp. 878-891. doi: 10.1093/hmg/ddv617

Evans, J. P. 2020. Preventing polyspermy in mammalian eggs-Contributions of the membrane block and other mechanisms. *Mol Reprod Dev* 87(3), pp. 341-349. doi: 10.1002/mrd.23331

Ferrer-Buitrago, M., Bonte, D., Dhaenens, L., Vermorgen, S., Lu, Y., De Sutter, P. and Heindryckx, B. 2019. Assessment of the calcium releasing machinery in oocytes that failed to fertilize after conventional ICSI and assisted oocyte activation. *Reprod Biomed Online* 38(4), pp. 497-507. doi: 10.1016/j.rbmo.2018.12.035

Ferrer-Buitrago, M. et al. 2018. Human oocyte calcium analysis predicts the response to assisted oocyte activation in patients experiencing fertilization failure after ICSI. *Hum Reprod* 33(3), pp. 416-425. doi: 10.1093/humrep/dex376

Ferrer-Buitrago, M. et al. 2020. Comparative study of preimplantation development following distinct assisted oocyte activation protocols in a PLC-zeta knockout mouse model. *Mol Hum Reprod* 26(11), pp. 801-815. doi: 10.1093/molehr/gaaa060

Finch, E. A., Turner, T. J. and Goldin, S. M. 1991. Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science* 252(5004), pp. 443-446. doi: 10.1126/science.2017683

Fissore, R. A., Longo, F. J., Anderson, E., Parys, J. B. and Ducibella, T. 1999. Differential distribution of inositol trisphosphate receptor isoforms in mouse oocytes. *Biol Reprod* 60(1), pp. 49-57. doi: 10.1095/biolreprod60.1.49

Foskett, J. K., White, C., Cheung, K. H. and Mak, D. O. 2007. Inositol trisphosphate receptor Ca^{2+} release channels. *Physiol Rev* 87(2), pp. 593-658. doi: 10.1152/physrev.00035.2006

Galione, A., Swann, K., Georgiou, P. and Whitaker, M. 1994. Regenerative and non-regenerative calcium transients in hamster eggs triggered by inositol 1,4,5-trisphosphate. *J Physiol* 480 (Pt 3)(Pt 3), pp. 465-474. doi: 10.1113/jphysiol.1994.sp020375

Grubbs, R. D. 2002. Intracellular magnesium and magnesium buffering. *Biometals* 15(3), pp. 251-259. doi: 10.1023/a:1016026831789

Hachem, A. et al. 2017. PLC ζ is the physiological trigger of the Ca^{2+} oscillations that induce embryogenesis in mammals but conception can occur in its absence. *Development* 144(16), pp. 2914-2924. doi: 10.1242/dev.150227

Haddad, M. et al. 2021. Thoughts on the popularity of ICSI. *J Assist Reprod Genet* 38(1), pp. 101-123. doi: 10.1007/s10815-020-01987-0

- Halet, G. 2004. PKC signaling at fertilization in mammalian eggs. *Biochim Biophys Acta* 1742(1-3), pp. 185-189. doi: 10.1016/j.bbamcr.2004.09.012
- Halet, G., Tunwell, R., Parkinson, S. J. and Carroll, J. 2004. Conventional PKCs regulate the temporal pattern of Ca^{2+} oscillations at fertilization in mouse eggs. *J Cell Biol* 164(7), pp. 1033-1044. doi: 10.1083/jcb.200311023
- Igusa, Y. and Miyazaki, S. 1983. Effects of altered extracellular and intracellular calcium concentration on hyperpolarizing responses of the hamster egg. *J Physiol* 340, pp. 611-632. doi: 10.1113/jphysiol.1983.sp014783
- Igusa, Y. and Miyazaki, S. 1986. Periodic increase of cytoplasmic free calcium in fertilized hamster eggs measured with calcium-sensitive electrodes. *J Physiol* 377, pp. 193-205. doi: 10.1113/jphysiol.1986.sp016181
- Igusa, Y., Miyazaki, S. and Yamashita, N. 1983. Periodic hyperpolarizing responses in hamster and mouse eggs fertilized with mouse sperm. *J Physiol* 340, pp. 633-647. doi: 10.1113/jphysiol.1983.sp014784
- Ikie-Eshalomi, C., Aliyev, E., Hoehn, S., Jurkowski, T. P. and Swann, K. 2023. Sperm induce a secondary increase in ATP levels in mouse eggs that is independent of Ca^{2+} oscillations. *Biochem J* 480(24), pp. 2023-2035. doi: 10.1042/bcj20230065
- Jellerette, T., Melican, D., Butler, R., Nims, S., Ziomek, C., Fissore, R. and Gavin, W. 2006. Characterization of calcium oscillation patterns in caprine oocytes induced by IVF or an activation technique used in nuclear transfer. *Theriogenology* 65(8), pp. 1575-1586. doi: 10.1016/j.theriogenology.2005.08.025
- Jones, K. T. 2018. Mammalian sperm contain two factors for calcium release and egg activation: Phospholipase C zeta and a cryptic activating factor. *Mol Hum Reprod* 24(10), pp. 465-468. doi: 10.1093/molehr/gay038
- Jones, K. T., Cruttwell, C., Parrington, J. and Swann, K. 1998. A mammalian sperm cytosolic phospholipase C activity generates inositol trisphosphate and causes Ca^{2+} release in sea urchin egg homogenates. *FEBS Lett* 437(3), pp. 297-300. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/9824311> doi: 10.1016/s0014-5793(98)01254-x
- Kang, W. et al. 2020. Extra-mitochondrial citrate synthase initiates calcium oscillation and suppresses age-dependent sperm dysfunction. *Lab Invest* 100(4), pp. 583-595. doi: 10.1038/s41374-019-0353-3

Kashir, J. et al. 2024. The mammalian sperm factor phospholipase C zeta is critical for early embryo division and pregnancy in humans and mice. *Hum Reprod* 39(6), pp. 1256-1274. doi: 10.1093/humrep/deae078

Kennedy, H. J., Pouli, A. E., Ainscow, E. K., Jouaville, L. S., Rizzuto, R. and Rutter, G. A. 1999. Glucose generates sub-plasma membrane ATP microdomains in single islet beta-cells. Potential role for strategically located mitochondria. *J Biol Chem* 274(19), pp. 13281-13291. doi: 10.1074/jbc.274.19.13281

Kline, D. and Kline, J. T. 1992. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev Biol* 149(1), pp. 80-89. doi: 10.1016/0012-1606(92)90265-i

Kline, J. T. and Kline, D. 1994. Regulation of intracellular calcium in the mouse egg: evidence for inositol trisphosphate-induced calcium release, but not calcium-induced calcium release. *Biol Reprod* 50(1), pp. 193-203. doi: 10.1095/biolreprod50.1.193

Knott, J. G., Gardner, A. J., Madgwick, S., Jones, K. T., Williams, C. J. and Schultz, R. M. 2006. Calmodulin-dependent protein kinase II triggers mouse egg activation and embryo development in the absence of Ca²⁺ oscillations. *Dev Biol* 296(2), pp. 388-395. doi: 10.1016/j.ydbio.2006.06.004

Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T. and Miyazaki, S. 2004. Recombinant phospholipase C ζ has high Ca²⁺ sensitivity and induces Ca²⁺ oscillations in mouse eggs. *J Biol Chem* 279(11), pp. 10408-10412. doi: 10.1074/jbc.M313801200

Kouchi, Z., Shikano, T., Nakamura, Y., Shirakawa, H., Fukami, K. and Miyazaki, S. 2005. The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase C ζ . *J Biol Chem* 280(22), pp. 21015-21021. doi: 10.1074/jbc.M412123200

Larman, M. G., Saunders, C. M., Carroll, J., Lai, F. A. and Swann, K. 2004. Cell cycle-dependent Ca²⁺ oscillations in mouse embryos are regulated by nuclear targeting of PLC ζ . *J Cell Sci* 117(Pt 12), pp. 2513-2521. doi: 10.1242/jcs.01109

Lawrence, Y., Ozil, J. P. and Swann, K. 1998. The effects of a Ca²⁺ chelator and heavy-metal-ion chelators upon Ca²⁺ oscillations and activation at fertilization in mouse eggs suggest a role for repetitive Ca²⁺ increases. *Biochem J* 335 (Pt 2)(Pt 2), pp. 335-342. doi: 10.1042/bj3350335

Lawrence, Y., Whitaker, M. and Swann, K. 1997. Sperm-egg fusion is the prelude to the initial Ca^{2+} increase at fertilization in the mouse. *Development* 124(1), pp. 233-241. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/9006083>

Leese, H. J. 1991. Metabolism of the preimplantation mammalian embryo. *Oxf Rev Reprod Biol* 13, pp. 35-72.

Liu, L., Hammar, K., Smith, P. J., Inoue, S. and Keefe, D. L. 2001. Mitochondrial modulation of calcium signaling at the initiation of development. *Cell Calcium* 30(6), pp. 423-433. doi: 10.1054/ceca.2001.0251

Lu, Y. et al. 2018. Strontium fails to induce Ca^{2+} release and activation in human oocytes despite the presence of functional TRPV3 channels. *Hum Reprod Open* 2018(3), p. hoy005. doi: 10.1093/hropen/hoy005

Mak, D. O., McBride, S. and Foskett, J. K. 1998. Inositol 1,4,5-trisphosphate [correction of tris-phosphate] activation of inositol trisphosphate [correction of tris-phosphate] receptor Ca^{2+} channel by ligand tuning of Ca^{2+} inhibition. *Proc Natl Acad Sci U S A* 95(26), pp. 15821-15825. doi: 10.1073/pnas.95.26.15821

Mak, D. O., McBride, S. and Foskett, J. K. 1999. ATP regulation of type 1 inositol 1,4,5-trisphosphate receptor channel gating by allosteric tuning of Ca^{2+} activation. *J Biol Chem* 274(32), pp. 22231-22237. doi: 10.1074/jbc.274.32.22231

Malcuit, C., Knott, J. G., He, C., Wainwright, T., Parys, J. B., Robl, J. M. and Fissore, R. A. 2005. Fertilization and inositol 1,4,5-trisphosphate (IP3)-induced calcium release in type-1 inositol 1,4,5-trisphosphate receptor down-regulated bovine eggs. *Biol Reprod* 73(1), pp. 2-13. doi: 10.1095/biolreprod.104.037333

Mann, J. S., Lowther, K. M. and Mehlmann, L. M. 2010. Reorganization of the endoplasmic reticulum and development of Ca^{2+} release mechanisms during meiotic maturation of human oocytes. *Biol Reprod* 83(4), pp. 578-583. doi: 10.1095/biolreprod.110.085985

Marangos, P., FitzHarris, G. and Carroll, J. 2003. Ca^{2+} oscillations at fertilization in mammals are regulated by the formation of pronuclei. *Development* 130(7), pp. 1461-1472. doi: 10.1242/dev.00340

Markoulaki, S., Matson, S., Abbott, A. L. and Ducibella, T. 2003. Oscillatory CaMKII activity in mouse egg activation. *Dev Biol* 258(2), pp. 464-474. doi: 10.1016/s0012-1606(03)00133-7

- Markoulaki, S., Matson, S. and Ducibella, T. 2004. Fertilization stimulates long-lasting oscillations of CaMKII activity in mouse eggs. *Dev Biol* 272(1), pp. 15-25. doi: 10.1016/j.ydbio.2004.04.008
- Marshall, I. C. and Taylor, C. W. 1994. Two calcium-binding sites mediate the interconversion of liver inositol 1,4,5-trisphosphate receptors between three conformational states. *Biochem J* 301 (Pt 2)(Pt 2), pp. 591-598. doi: 10.1042/bj3010591
- Matson, S., Markoulaki, S. and Ducibella, T. 2006. Antagonists of myosin light chain kinase and of myosin II inhibit specific events of egg activation in fertilized mouse eggs. *Biol Reprod* 74(1), pp. 169-176. doi: 10.1095/biolreprod.105.046409
- Matsu-Ura, T. et al. 2019. Dual-FRET imaging of IP(3) and Ca(2+) revealed Ca(2+)-induced IP(3) production maintains long lasting Ca(2+) oscillations in fertilized mouse eggs. *Sci Rep* 9(1), p. 4829. doi: 10.1038/s41598-019-40931-w
- Mehlmann, L. M. and Kline, D. 1994. Regulation of intracellular calcium in the mouse egg: calcium release in response to sperm or inositol trisphosphate is enhanced after meiotic maturation. *Biol Reprod* 51(6), pp. 1088-1098. doi: 10.1095/biolreprod51.6.1088
- Miyazaki, S. 1988. Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. *J Cell Biol* 106(2), pp. 345-353. doi: 10.1083/jcb.106.2.345
- Miyazaki, S. 2007. Intracellular calcium oscillations in mammalian eggs at fertilization. *J Physiol* 584(Pt 3), pp. 713-714. doi: 10.1113/jphysiol.2007.144238
- Miyazaki, S., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y. and Hiramoto, Y. 1986. Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. *Dev Biol* 118(1), pp. 259-267. doi: 10.1016/0012-1606(86)90093-x
- Miyazaki, S. and Igusa, Y. 1981. Fertilization potential in golden hamster eggs consists of recurring hyperpolarizations. *Nature* 290(5808), pp. 702-704. doi: 10.1038/290702a0
- Miyazaki, S. and Ito, M. 2006. Calcium signals for egg activation in mammals. *J Pharmacol Sci* 100(5), pp. 545-552. doi: 10.1254/jphs.cpj06003x
- Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S. and Mikoshiba, K. 1992. Block of Ca²⁺ wave and Ca²⁺ oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science* 257(5067), pp. 251-255. doi: 10.1126/science.1321497

Moses, R. M. and Kline, D. 1995. Release of mouse eggs from metaphase arrest by protein synthesis inhibition in the absence of a calcium signal or microtubule assembly. *Mol Reprod Dev* 41(2), pp. 264-273. doi: 10.1002/mrd.1080410218

Moses, R. M., Kline, D. and Masui, Y. 1995. Maintenance of metaphase in colcemid-treated mouse eggs by distinct calcium- and 6-dimethylaminopurine (6-DMAP)-sensitive mechanisms. *Dev Biol* 167(1), pp. 329-337. doi: 10.1006/dbio.1995.1027

Murugesu, S. et al. 2017. Does the use of calcium ionophore during artificial oocyte activation demonstrate an effect on pregnancy rate? A meta-analysis. *Fertil Steril* 108(3), pp. 468-482.e463. doi: 10.1016/j.fertnstert.2017.06.029

Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwabara, Y. and Miyazaki, S. 1997. Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. *Mol Hum Reprod* 3(12), pp. 1087-1093. doi: 10.1093/molehr/3.12.1087

Nomikos, M. et al. 2005. Role of phospholipase C-zeta domains in Ca^{2+} -dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca^{2+} oscillations. *J Biol Chem* 280(35), pp. 31011-31018. doi: 10.1074/jbc.M500629200

Nomikos, M. et al. 2011. Male infertility-linked point mutation disrupts the Ca^{2+} oscillation-inducing and PIP(2) hydrolysis activity of sperm PLC ζ . *Biochem J* 434(2), pp. 211-217. doi: 10.1042/bj20101772

Nomikos, M. et al. 2015. Functional disparity between human PAWP and PLC ζ in the generation of Ca^{2+} oscillations for oocyte activation. *Mol Hum Reprod* 21(9), pp. 702-710. doi: 10.1093/molehr/gav034

Nomikos, M. et al. 2014. Sperm-specific post-acrosomal WW-domain binding protein (PAWP) does not cause Ca^{2+} release in mouse oocytes. *Mol Hum Reprod* 20(10), pp. 938-947. doi: 10.1093/molehr/gau056

Nozawa, K., Satouh, Y., Fujimoto, T., Oji, A. and Ikawa, M. 2018. Sperm-borne phospholipase C zeta-1 ensures monospermic fertilization in mice. *Sci Rep* 8(1), p. 1315. doi: 10.1038/s41598-018-19497-6

Ozil, J. P. et al. 2005. Egg activation events are regulated by the duration of a sustained $[\text{Ca}^{2+}]_{\text{cyt}}$ signal in the mouse. *Dev Biol* 282(1), pp. 39-54. doi: 10.1016/j.ydbio.2005.02.035

- Ozil, J. P. and Swann, K. 1995. Stimulation of repetitive calcium transients in mouse eggs. *J Physiol* 483 (Pt 2)(Pt 2), pp. 331-346. doi: 10.1113/jphysiol.1995.sp020589
- Perry, A. C. F., Asami, M., Lam, B. Y. H. and Yeo, G. S. H. 2023. The initiation of mammalian embryonic transcription: to begin at the beginning. *Trends Cell Biol* 33(5), pp. 365-373. doi: 10.1016/j.tcb.2022.08.008
- Politi, A., Gaspers, L. D., Thomas, A. P. and Höfer, T. 2006. Models of IP₃ and Ca²⁺ oscillations: frequency encoding and identification of underlying feedbacks. *Biophys J* 90(9), pp. 3120-3133. doi: 10.1529/biophysj.105.072249
- Prather, R. S., Tao, T. and Macháty, Z. 1999. Development of the techniques for nuclear transfer in pigs. *Theriogenology* 51(2), pp. 487-498. doi: 10.1016/s0093-691x(98)00242-8
- Rinaudo, P., Pepperell, J. R., Buradgunta, S., Massobrio, M. and Keefe, D. L. 1997. Dissociation between intracellular calcium elevation and development of human oocytes treated with calcium ionophore. *Fertil Steril* 68(6), pp. 1086-1092. doi: 10.1016/s0015-0282(97)00406-8
- Rogers, N. T., Hobson, E., Pickering, S., Lai, F. A., Braude, P. and Swann, K. 2004. Phospholipase C ζ causes Ca²⁺ oscillations and parthenogenetic activation of human oocytes. *Reproduction* 128(6), pp. 697-702. doi: 10.1530/rep.1.00484
- Rolfe, D. F. and Brown, G. C. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol Rev* 77(3), pp. 731-758. doi: 10.1152/physrev.1997.77.3.731
- Ross, P. J. et al. 2008. Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C ζ . *BMC Dev Biol* 8, p. 16. doi: 10.1186/1471-213x-8-16
- Rutter, G. A., Georgiadou, E., Martinez-Sanchez, A. and Pullen, T. J. 2020. Metabolic and functional specialisations of the pancreatic beta cell: gene disallowance, mitochondrial metabolism and intercellular connectivity. *Diabetologia* 63(10), pp. 1990-1998. doi: 10.1007/s00125-020-05205-5
- Sanders, J. R., Ashley, B., Moon, A., Woolley, T. E. and Swann, K. 2018. PLC ζ Induced Ca²⁺ Oscillations in Mouse Eggs Involve a Positive Feedback Cycle of Ca²⁺ Induced InsP(3) Formation From Cytoplasmic PIP(2). *Front Cell Dev Biol* 6, p. 36. doi: 10.3389/fcell.2018.00036

Sanusi, R., Yu, Y., Nomikos, M., Lai, F. A. and Swann, K. 2015. Rescue of failed oocyte activation after ICSI in a mouse model of male factor infertility by recombinant phospholipase C ζ . *Mol Hum Reprod* 21(10), pp. 783-791. doi: 10.1093/molehr/gav042

Sato, Y., Miyazaki, S., Shikano, T., Mitsuhashi, N., Takeuchi, H., Mikoshiba, K. and Kuwabara, Y. 1998. Adenophostin, a potent agonist of the inositol 1,4,5-trisphosphate receptor, is useful for fertilization of mouse oocytes injected with round spermatids leading to normal offspring. *Biol Reprod* 58(3), pp. 867-873. doi: 10.1095/biolreprod58.3.867

Saunders, C. M. et al. 2002. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* 129(15), pp. 3533-3544. doi: 10.1242/dev.129.15.3533

Scaravelli, G. et al. 2021. Fertilization rate as a novel indicator for cumulative live birth rate: a multicenter retrospective cohort study of 9,394 complete in vitro fertilization cycles. *Fertil Steril* 116(3), pp. 766-773. doi: 10.1016/j.fertnstert.2021.04.006

Schultz, R. M., Letourneau, G. E. and Wassarman, P. M. 1979. Program of early development in the mammal: changes in patterns and absolute rates of tubulin and total protein synthesis during oogenesis and early embryogenesis in the mouse. *Dev Biol* 68(2), pp. 341-359. doi: 10.1016/0012-1606(79)90209-4

Sette, C., Bevilacqua, A., Bianchini, A., Mangia, F., Geremia, R. and Rossi, P. 1997. Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. *Development* 124(11), pp. 2267-2274. doi: 10.1242/dev.124.11.2267

Shirakawa, H., Ito, M., Sato, M., Umezawa, Y. and Miyazaki, S. 2006. Measurement of intracellular IP3 during Ca²⁺ oscillations in mouse eggs with GFP-based FRET probe. *Biochem Biophys Res Commun* 345(2), pp. 781-788. doi: 10.1016/j.bbrc.2006.04.133

Simsek-Duran, F., Li, F., Ford, W., Swanson, R. J., Jones, H. W., Jr. and Castora, F. J. 2013. Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes. *PLoS One* 8(5), p. e64955. doi: 10.1371/journal.pone.0064955

Sneyd, J., Tsaneva-Atanasova, K., Reznikov, V., Bai, Y., Sanderson, M. J. and Yule, D. I. 2006. A method for determining the dependence of calcium oscillations on inositol trisphosphate oscillations. *Proc Natl Acad Sci U S A* 103(6), pp. 1675-1680. doi: 10.1073/pnas.0506135103

- Stein, P., Savy, V., Williams, A. M. and Williams, C. J. 2020. Modulators of calcium signalling at fertilization. *Open Biol* 10(7), p. 200118. doi: 10.1098/rsob.200118
- Storey, A., Elgmati, K., Wang, Y., Knaggs, P. and Swann, K. 2021. The role of ATP in the differential ability of Sr^{2+} to trigger Ca^{2+} oscillations in mouse and human eggs. *Mol Hum Reprod* 27(1). doi: 10.1093/molehr/gaaa086
- Sun, F. Z., Bradshaw, J. P., Galli, C. and Moor, R. M. 1994. Changes in intracellular calcium concentration in bovine oocytes following penetration by spermatozoa. *J Reprod Fertil* 101(3), pp. 713-719. doi: 10.1530/jrf.0.1010713
- Sun, F. Z., Hoyland, J., Huang, X., Mason, W. and Moor, R. M. 1992. A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. *Development* 115(4), pp. 947-956. doi: 10.1242/dev.115.4.947
- Swann, K. 1992. Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store. *Biochem J* 287 (Pt 1)(Pt 1), pp. 79-84. doi: 10.1042/bj2870079
- Swann, K. 1994. Ca^{2+} oscillations and sensitization of Ca^{2+} release in unfertilized mouse eggs injected with a sperm factor. *Cell Calcium* 15(4), pp. 331-339. doi: 10.1016/0143-4160(94)90072-8
- Swann, K. 2020. The soluble sperm factor that activates the egg: PLC ζ and beyond. *Reproduction* 160(1), pp. V9-v11. doi: 10.1530/rep-20-0079
- Swann, K. 2023. Sperm-Induced Ca^{2+} Release in Mammalian Eggs: The Roles of PLC ζ , InsP(3), and ATP. *Cells* 12(24). doi: 10.3390/cells12242809
- Swann, K., Igusa, Y. and Miyazaki, S. 1989. Evidence for an inhibitory effect of protein kinase C on G-protein-mediated repetitive calcium transients in hamster eggs. *Embo j* 8(12), pp. 3711-3718. doi: 10.1002/j.1460-2075.1989.tb08546.x
- Swann, K. and Lai, F. A. 2013. PLC ζ and the initiation of Ca^{2+} oscillations in fertilizing mammalian eggs. *Cell Calcium* 53(1), pp. 55-62. doi: 10.1016/j.ceca.2012.11.001
- Swann, K. and Ozil, J. P. 1994. Dynamics of the calcium signal that triggers mammalian egg activation. *Int Rev Cytol* 152, pp. 183-222. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/8206704>doi: 10.1016/s0074-7696(08)62557-7

- Taylor, C. T., Lawrence, Y. M., Kingsland, C. R., Biljan, M. M. and Cuthbertson, K. S. 1993. Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. *Hum Reprod* 8(12), pp. 2174-2179. doi: 10.1093/oxfordjournals.humrep.a137999
- Tesarik, J., Sousa, M. and Testart, J. 1994. Human oocyte activation after intracytoplasmic sperm injection. *Hum Reprod* 9(3), pp. 511-518. doi: 10.1093/oxfordjournals.humrep.a138537
- Thanassoulas, A., Swann, K., Lai, F. A. and Nomikos, M. 2022. SPERM FACTORS AND EGG ACTIVATION: The structure and function relationship of sperm PLCZ1. *Reproduction* 164(1), pp. F1-f8. doi: 10.1530/rep-21-0477
- Tomashov-Matar, R., Tchetchik, D., Eldar, A., Kaplan-Kraicer, R., Oron, Y. and Shalgi, R. 2005. Strontium-induced rat egg activation. *Reproduction* 130(4), pp. 467-474. doi: 10.1530/rep.1.00746
- Tóth, S., Huneau, D., Banrezes, B. and Ozil, J. P. 2006. Egg activation is the result of calcium signal summation in the mouse. *Reproduction* 131(1), pp. 27-34. doi: 10.1530/rep.1.00764
- Tu, H., Wang, Z., Nosyreva, E., De Smedt, H. and Bezprozvanny, I. 2005. Functional characterization of mammalian inositol 1,4,5-trisphosphate receptor isoforms. *Biophys J* 88(2), pp. 1046-1055. doi: 10.1529/biophysj.104.049593
- Urner, F. and Sakkas, D. 1999. Characterization of glycolysis and pentose phosphate pathway activity during sperm entry into the mouse oocyte. *Biol Reprod* 60(4), pp. 973-978. doi: 10.1095/biolreprod60.4.973
- Verjovski-Almeida, S. and Inesi, G. 1979. Fast-kinetic evidence for an activating effect of ATP on the Ca^{2+} transport of sarcoplasmic reticulum ATPase. *J Biol Chem* 254(1), pp. 18-21.
- Wakai, T., Mehregan, A. and Fissore, R. A. 2019. Ca^{2+} Signaling and Homeostasis in Mammalian Oocytes and Eggs. *Cold Spring Harb Perspect Biol* 11(12). doi: 10.1101/cshperspect.a035162
- Wakai, T., Zhang, N., Vangheluwe, P. and Fissore, R. A. 2013. Regulation of endoplasmic reticulum Ca^{2+} oscillations in mammalian eggs. *J Cell Sci* 126(Pt 24), pp. 5714-5724. doi: 10.1242/jcs.136549

Wilson, D. F. 2015. Programming and regulation of metabolic homeostasis. *Am J Physiol Endocrinol Metab* 308(6), pp. E506-517. doi: 10.1152/ajpendo.00544.2014

Winston, N., Johnson, M., Pickering, S. and Braude, P. 1991. Parthenogenetic activation and development of fresh and aged human oocytes. *Fertil Steril* 56(5), pp. 904-912.

Wu, A. T. et al. 2007. PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem* 282(16), pp. 12164-12175. doi: 10.1074/jbc.M609132200

Yanagida, K., Katayose, H., Hirata, S., Yazawa, H., Hayashi, S. and Sato, A. 2001. Influence of sperm immobilization on onset of Ca^{2+} oscillations after ICSI. *Hum Reprod* 16(1), pp. 148-152. doi: 10.1093/humrep/16.1.148

Yoda, A. et al. 2004. Ca^{2+} oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation. *Dev Biol* 268(2), pp. 245-257. doi: 10.1016/j.ydbio.2003.12.028

Yoneda, A. et al. 2006. Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase Czeta. *Reproduction* 132(3), pp. 393-401. doi: 10.1530/rep.1.01018

Yu, Y., Halet, G., Lai, F. A. and Swann, K. 2008a. Regulation of diacylglycerol production and protein kinase C stimulation during sperm- and PLCzeta-mediated mouse egg activation. *Biol Cell* 100(11), pp. 633-643. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/18471090>doi: 10.1042/BC20080033

Yu, Y., Nomikos, M., Theodoridou, M., Nounesis, G., Lai, F. A. and Swann, K. 2012. PLC ζ causes Ca^{2+} oscillations in mouse eggs by targeting intracellular and not plasma membrane PI(4,5)P(2). *Mol Biol Cell* 23(2), pp. 371-380. doi: 10.1091/mbc.E11-08-0687

Yu, Y., Saunders, C. M., Lai, F. A. and Swann, K. 2008b. Preimplantation development of mouse oocytes activated by different levels of human phospholipase C zeta. *Hum Reprod* 23(2), pp. 365-373. doi: 10.1093/humrep/dem350

Zhang, L., Chao, C. H., Jaeger, L. A., Papp, A. B. and Machaty, Z. 2018. Calcium oscillations in fertilized pig oocytes are associated with repetitive interactions between STIM1 and ORAI1. *Biol Reprod* 98(4), pp. 510-519. doi: 10.1093/biolre/iroy016

Figure Legends

Fig.1. Ca^{2+} oscillations at fertilization in mouse eggs.

This trace shows a recording of cytosolic Ca^{2+} from a single mouse egg that was injected with the Ca^{2+} sensitive dye Oregon Green BAPTA dextran. The fluorescence level from the whole egg was measured every 10 seconds during *in vitro* fertilization. Full experimental details are given elsewhere (Ikie-Eshalomi et al. 2023). The fluorescence intensity is plotted as the fluorescence (F) divided by the initial fluorescence value (F0), hence F/F0. Ca^{2+} oscillations persisted for several hours.

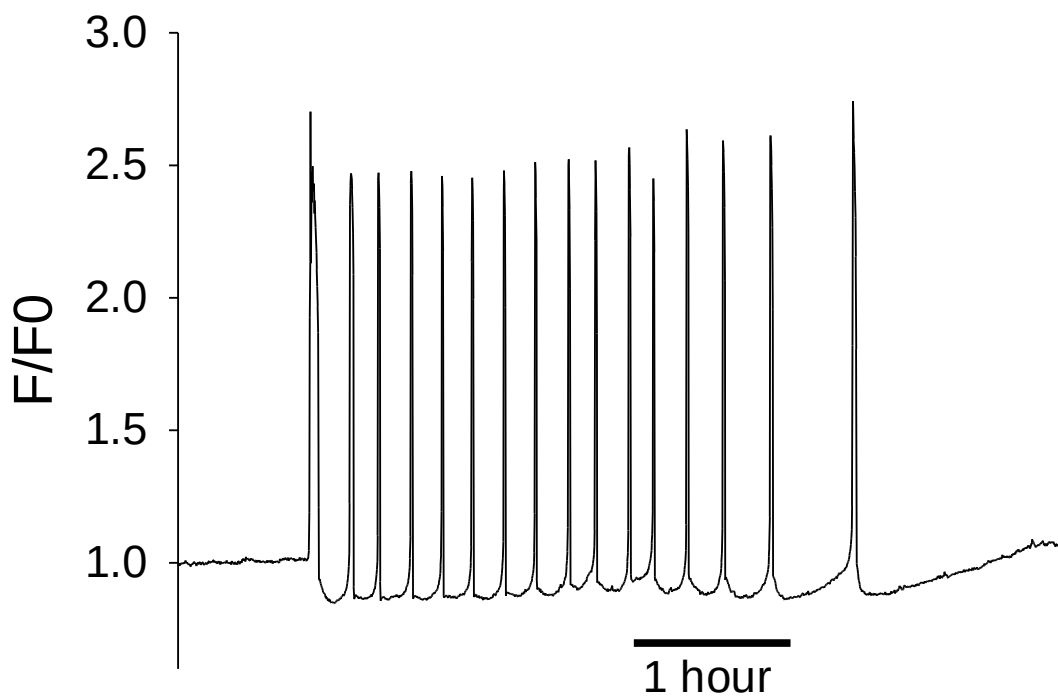


Fig.2. A model of how InsP_3 and Ca^{2+} modulate the IP3R in eggs during fertilization.

The IP3R1 is a Ca^{2+} channel and represented by the two blocks in the middle. IP3R1s have a Ca^{2+} binding site that opens the channel (+ve) at low Ca^{2+} ($< \mu\text{M}$) and one that closes the channel (-ve) at higher Ca^{2+} concentrations. Ca^{2+} released into the cytosol stimulates $\text{PLC}\zeta$ to make InsP_3 . The InsP_3 then opens the IP3R1 by inhibiting the inhibitory (-ve) Ca^{2+} binding site. ATP^{4-} promotes opening of the IP3R1 by enhancing the Ca^{2+} sensitivity of the stimulatory Ca^{2+} (+ve) binding site (Foskett et al. 2007).

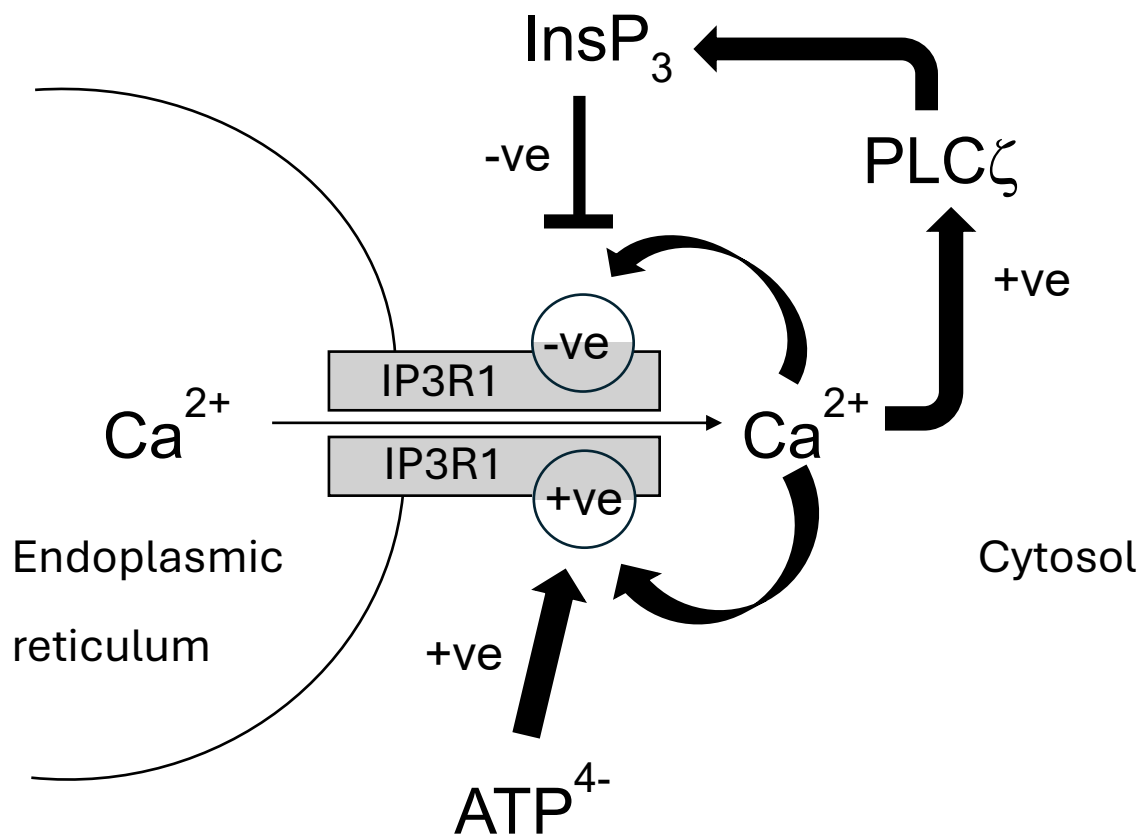


Fig.3. Changes in ATP and Ca^{2+} at fertilization in mouse eggs.

Mouse eggs were injected with firefly luciferase, to monitor ATP (solid trace), and with Oregon Green BAPTA dextran, to measure Ca^{2+} (dotted trace) as described previously (Ikke-Eshalomi et al. 2023). During fertilization in this mouse egg there were a series of Ca^{2+} oscillations and at the same time a two phased increase in ATP. The second phase of increase is only seen with sperm-induced Ca^{2+} oscillations and starts at the point indicated by the arrow. The fluorescence signals or luminescence signals (S) are plotted as intensity values divided by starting values (S_0).

