The soluble sperm factor that activates the egg: PLCzeta and beyond.

Karl Swann

School of Biosciences

Cardiff University.

The Sir Martin Evans Building

Museum Avenue

Cardiff CF10 3AX

Swannk1@cardiff.ac.uk

Short title: PLCζ and beyond.

Key words: sperm, egg, fertilization, calcium, PLCζ

Word count: 1739

Abstract

PLCzeta(ζ) initiates Ca^{2+} oscillations and egg activation at fertilization in mammals, but studies in mouse eggs fertilized by PLCζ knockout (KO) sperm imply that there is another slow acting factor causing Ca^{2+} release. Here, I propose a hypothesis for how this second sperm factor might cause Ca^{2+} oscillations in mouse eggs.

Egg activation is caused by increases in cytosolic Ca^{2+}, and in mammalian eggs (MII oocytes) the sperm triggers a prolonged series of repetitive transients, or oscillations, in the cytosolic free Ca^{2+} concentration (Swann & Lai 2016, Sanders & Swann 2016). These Ca^{2+} oscillations are driven by increased inositol 1,4,5-trisphosphate ($InsP_3$) production which causes cycles of Ca^{2+} release from the $InsP_3$-receptor (IP_3R). Since the 1990s we have known that mammalian sperm contain a soluble protein 'sperm factor' (or sperm-oocyte-activating-factor- SOAF), that can trigger Ca^{2+} oscillations after gamete fusion (Swann and Lai, 2016). Its existence inside the sperm can explain why **i**ntra**c**ytoplasmic **s**perm **i**njection (ICSI) mimics fertilization in causing Ca^{2+} oscillations in mouse and human eggs (Jones 2018, Kurokawa & Fissore 2003). It is now widely acknowledged that this sperm factor in mammals is the sperm-specific protein phospholipase PLCzeta(ζ) (Swann & Lai 2016, Jones 2018). Key evidence includes the finding that microinjection of PLCζ cRNA or protein causes prolonged sperm-like Ca^{2+} oscillations in all mammalian eggs studied (Swann & Lai 2016), and that functionally disruptive mutations in PLCζ alone lead to male factor infertility and egg activation failure in humans in IVF and ICSI (Escoffier *et al.* 2016).

Recently two groups have reported the phenotype of PLCζ knock out (KO) mice. They both found that injecting PLCζ KO mouse sperm into eggs (hence ICSI) fails to trigger any Ca^{2+} oscillations (Hachem *et al.* 2017, Nozawa *et al.* 2018). This shows that PLCζ accounts for the Ca^{2+} signals and egg activation after ICSI. However, during *in vitro* fertilization (IVF) and mating with PLCζ KO males some eggs are activated at fertilization and embryo development still occurs (Hachem *et al.* 2017, Nozawa *et al.* 2018). Success rates of IVF are lower and litter sizes are smaller with PLCζ KO males but the result contrasts with what happens with ICSI. The reason why IVF leads some

eggs to activate with PLCζ KO sperm is because there are ~3 large Ca^{2+} oscillations that occur about 40 mins later than expected when compared to wild type sperm (Nozawa *et al.* 2018). The existence of these delayed Ca^{2+} oscillations with PLCζ KO sperm has been reproduced in my lab (Fluks, Parrington and Swann unpublished). The late Ca^{2+} oscillations with PLCζ KO sperm lead to delayed egg activation, including cortical granule exocytosis which is required to block extra sperm entry (Nozawa *et al.* 2018). This means that many such zygotes fail to develop because they are polyspermic. Overall, the data suggest that PLCζ initiates the Ca^{2+} oscillations at fertilization, accounting for most of the Ca^{2+} spikes, but that during IVF the sperm has another mechanism for promoting later Ca^{2+} oscillations in the mouse (Jones 2018). Two characteristics of this secondary mechanism is that it is delayed after gamete fusion, and that it is active in IVF and not with ICSI.

In looking for PLCζ-independent mechanisms for Ca^{2+} oscillations we need to consider previous data gather from mammalian zygotes. First, all previous studies have shown that without sperm-egg membrane fusion in IVF there are no Ca^{2+} oscillations (Swann & Lai 2016). So, it's reasonable to assume that a second mechanism for Ca^{2+} release involves a sperm factor that is either soluble and enters the egg by cytosolic diffusion, or that it is introduced by the sperm membrane into the egg plasma membrane by two-dimensional diffusion. For either option I will describe it as a sperm factor. It has been suggested that the PLCζ-independent sperm factor may be 'cryptic' because it is only apparent when PLCζ is absent (Jones 2018). Whilst this is true from an observational point of view, it does not mean it is inactive during normal fertilization. In fact, it is difficult to see how a second factor could only arise when PLCζ was not present. As far as we know PLCζ is only active in eggs, so a lack of PLCζ would not be evident until after gametes have fused. Clearly, gene expression in spermatogenesis cannot compensate for future events, hence the second factor should operate in IVF with wild type sperm. In hindsight we can see evidence of a secondary mechanism because it was previously found that ICSI causes a shorter duration of Ca^{2+} oscillations than IVF in mouse zygotes[5]. If the secondary factor operates in normal IVF it also gives it a selective advantage for it to persist in the presence of PLCζ. One attractive idea is that this

factor is a ‘primitive’ factor from a role in egg activation in species earlier in the vertebrate lineage (Nozawa *et al.* 2018).

Previous studies restrict the options for how any factor can trigger Ca^{2+} oscillations in the absence of PLCζ. For example, one could propose that the second factor promotes Ca^{2+} influx into the egg, perhaps by the insertion of sperm derived Ca^{2+} channels into the egg membrane. However, there are many ways to increase Ca^{2+} influx into unfertilized mammalian eggs and none of them trigger Ca^{2+} oscillations without PLCζ. An updated version of the ‘Ca^{2+} conduit’ idea remains implausible (Swann & Lai 2016). The second factor cannot work via messengers such as NAADP, or cADPR, since these also fail to trigger Ca^{2+} oscillations in mouse eggs (Swann & Lai 2016). A sperm protein called PAWP has been suggested to trigger Ca^{2+} oscillations in eggs, but the key data on PAWP is not reproducible (Sanders and Swann, 2016). Furthermore, PAWP is supposed to cause Ca^{2+} oscillations during ICSI, but we now know that PLCζ accounts for these Ca^{2+} oscillations. Another study has suggested that extramitochondrial citrate synthase is the second sperm factor in mammals (Kang *et al.* 2020). However, the phenotype of extramitochondrial citrate synthase KO sperm at fertilization is apparently the same as PLCζ KO sperm, with delayed Ca^{2+} oscillations (Kang *et al.* 2020). This result is difficult to rationalize because these citrate synthase KO sperm still contained PLCζ and the initial Ca^{2+} oscillations should not be delayed. In addition, we have found that citrate synthase protein injection into mouse eggs does not trigger Ca^{2+} release (Sanders and Swann, unpublished observations). From what we know about how to cause Ca^{2+} oscillations in mouse eggs, we can conclude that the second factor is either making $InsP_3$, or else directly stimulating the IP_3Rs.

If the second sperm factor generates $InsP_3$ this implicates another PLC. There are many other PLC isoforms in mammalian sperm (Parrington *et al.* 2002). However, the other PLCs are about two or three orders of magnitude less active in causing Ca^{2+} release than PLCζ in eggs (Swann and Lai 2016; Mehlmann *et al.* 2001). To be active in eggs they would have to be expressed at >2pg per sperm, and yet there is only 40pg of total protein in a mouse sperm (Mehlmann *et al.* 2001). The

second sperm factor could stimulate an egg membrane derived PLC, but this is not consistent with some previous findings. For example, if eggs are imaged using GFP tagged C1 domains, there is no measurable diacylglycerol increase in the plasma membrane for at least two hours of sperm induced Ca^{2+} oscillations in mouse eggs, despite the ability of this probe to respond to other stimuli (Yu *et al.* 2008). Hence, it appears that a plasma membrane derived PLC activity is not stimulated in fertilizing eggs. This is not an issue for PLCζ which is the only mammalian PLC without a PH domain and it binds to PIP_2 in intracellular vesicles (Fig 1) and not the plasma membrane (Swann & Lai 2016). However, conventional PLCs (β, γ or δ1 class) locate to the plasma membrane with a PH domain, and one would expect some diacylglycerol increase to occur if they were active at fertilization. If the second sperm factor makes $InsP_3$ then it probably needs to stimulate another unconventional PLC that is localized on vesicles in the egg. It is not clear whether any other PLCs would match the unusual localization pattern of PLCζ, but it might be worth investigating the localization of the epsilon or eta class of PLCs in eggs.

In the absence of data on other PLCs I can suggest an alternative idea, that the second sperm factor acts to sensitize the IP_3R. Strontium ions or thimerosal both stimulate Ca^{2+} oscillations in mouse eggs, and they both appear to act directly to sensitize the IP_3R to release Ca^{2+} (Swann & Lai 2016). The schematic in Fig.1 shows the second factor affecting IP_3R induced Ca^{2+} release following PLCζ entry. If the target is the IP_3R, or vesicular PIP_2, the protein factor is likely to be soluble and diffuse into the cytosol. To explain why PLCζ independent Ca^{2+} release is not evident with ICSI, it is possible that the second factor is released from the sperm during their preparation when the sperm is damaged, or when the head is removed. Damaging the sperm membrane is standard practice before ICSI. Plasma membrane damage during cryopreservation may also lead to the loss of the second factor from sperm, which could explain why there was a lack of Ca^{2+} oscillations with most cryopreserved PLCζ KO sperm in IVF (Hachem *et al.* 2017). The other feature of the second sperm factor is a delayed action. It could be that the synthesis of a second sperm factor protein from RNA in the sperm could account for the >40 min delay before Ca^{2+} transients (Jones 2018). However, the total amount of RNA in a single mouse sperm (0.1pg) is

similar to the amount of PLCζ RNA injected into an egg to cause Ca^{2+} oscillations, and yet sperm RNA is made up of several hundred varieties. Any protein synthesized from sperm RNA would have to be >100 times more potent than PLCζ which is active at concentrations of less than 10nM. A more realistic idea is that secondary factor is another protein delivered by the sperm. The delay could be because this protein needs to first diffuse and equilibrate throughout the egg and then act indirectly to sensitize IP_3Rs. The second factor may not be active in human fertilization since human eggs are less sensitive to Ca^{2+} release, and for example do not oscillate in response to strontium medium (Lu *et al.* 2018). This could explain why inactivating mutations in human PLCζ lead to male factor infertility with both normal conception and ICSI (Escoffier *et al.* 2016). The second factor may only be evident in mouse and rat eggs, or possibly in some non-mammalian species that do not appear to use PLCζ to activate the egg (Swann & Lai 2016).

Declaration of Interests

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this article.

Funding.

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author Contribution.

KS conceived the ideas and wrote the paper.

Acknowledgements.

I am grateful to Jessica Sanders and Monika Fluks who helped generate the unpublished data I refer to, and to John Parrington for sending me the PLCζ KO male mice.

References

Escoffier J, Lee HC, Yassine S, Zougari R, Martinez G, Karaouzene T, Coutton C, Kherraf ZE, Halouni L, Triki C, Nef S, Thierry-Mieg N, Savinov SN, Fissore R, Ray PF & Arnoult C (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** 878-891. (https://doi.org/10.1093/hmg/ddv617)

Hachem A, Godwin J, Ruas M, Lee HC, Ferrer-Buitrago M, Ardestani G, Bassett A, Fox A, Naverrete F, De Sutter P, Heindryckx B, Fissore RA & Parrington J (2017) PLCζ is the physiological trigger of the Ca^{2+} oscillations that induced embryogeneis in mammals but conception can occur in its absence, Development. **144** 2914-2924. (https://doi.org/10.1242/dev.150227)

Jones KT (2018) Mammalian sperm contain two factors for calcium release and egg activation: Phospholipase C zeta and a cryptic activating factor. Molecular Human Reproduction. **24** 465-468. (https://doi.org/10.1093/molehr/gay038)

Kang W, Harada Y, Yamatoya K, Kawano N, Kanai S, Miyamoto Y, Nakamura A, Miyado M, Hayashi Y, Kuoki Y, Saito H, Iwao Y, Umezawa A & Miyado K (2020) Extra-mitochondrial citrate synthase initiates calcium oscillation and supresses age-dependent sperm dysfunction. Laboratory Investigation. **100,** 583-595 (https://doi.org/10.1038/s41374-019-0353-3)

Kurokawa M & Fissore RA (2003) ICSI-generated mouse zygotes exhibit altered calcium oscillations, inositol 1,4,5-trisphohate receptor 1 downregulation, and embryo development. Molecular Human Reproduction **9,** 523-533. (https://doi.org/10.1093/molehr/gag072)

Lu Y, Reffy R, Ferrer Buitrago M, Vander Jeught M, Neupane J, De Vos WH, Van der Abbeel E, Lierman S, De Sutter P & Heindryckx B (2018) Strontium fails to trigger Ca^{2+} release and activation in human oocytes despite the presence of functional TRPV3 channels. Human Reproduction Open **3,** hoy005. (https://doi.org/10.1093/hropen/hoy005)

Mehlmann LM, Chattopadhyay A, Carpenter G and Jaffe LA (2001) Evidence that phospholipase C from the sperm is not responsible for initiating Ca^{2+} release at fertilization in mouse eggs. Dev. Biol. **236,** 492-501. (https://doi.org/10.1006/dbio.2001.0329)

Nozawa K, Satouh Y, Fujimoto T, Oji A, & Ikawa M (2018) Sperm borne phospholipase C zeta1 ensures monospermic fertilization in mice. Sci. Rep. **8,** 1315. (https://doi.org/10.1038/s41598-018-19497-6)

Parrington J, Jones ML, Tunwell R, Devader C, Katan M & Swann K (2002) Phospholipase C isoforms in mammalian spermatozoa: potential components of the sperm factor that causes Ca^{2+} release in eggs. Reproduction **123,** 31-39.

(https://doi.org/10.1530/rep.0.1230031)

Sanders J & Swann K (2016) Molecular triggers of egg activation at fertilization in mammals, Reproduction **152,** R41-R50. (http://dx.doi.org/10.1530/REP-16-0123)

Swann K & Lai FA (2016) Egg activation by a soluble sperm protein. Physiological Reviews **96**, 127-149. (https://doi.org/10.1152/physrev.00012.2015)

Yu Y, Halet G, Lai FA & Swann K (2008) Regulation of diacylglycerol production and protein kinase C stimulation during sperm- and PLCζ-mediated mouse egg activation. Biol. Cell **100,** 633-643. (https://doi.org/10.1042/BC20080033)

Figure Legend.

Fig.1. A schematic representation of the hypothesis for PLCζ and a second factor may act to cause Ca^{2+} oscillations in fertilizing mouse eggs.