Title

Sperm factors and oocyte activation: current controversies and considerations

Running title

Sperm factors and oocyte activation

Summary sentence

Current theories on the identity of sperm oocyte activating factor (SOAF) candidates

Key words: Oocyte activation; intracellular calcium (Ca^{2+}); sperm oocyte activating factor (SOAF); perinuclear theca (PT); PLCζ; PAWP

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ABSTRACT

The beginning of embryogenesis is preceded by a sequence of events mediated by the release of intracellular calcium in the ooplasm, a multi-faceted process known as 'oocyte activation'. It is now well established that a sperm protein factor introduced into the oocyte at the time of gamete fusion is responsible for initiating the cascade of signalling events involved. Several sperm proteins have been hypothesized as the sperm oocyte activating factor (SOAF) over the years, with phospholipase C zeta (PLCζ) emerging as the strongest candidate. A large body of consistent and reproducible evidence, from both biochemical and clinical settings, has accumulated in support of PLCζ, and data clearly demonstrates that oocyte activation ability can be rescued in PLCζ-deficient sperm by either PLCζ cRNA or recombinant PLCζ protein. However, a series of recent publications has challenged the dominant role of PLCζ and proposed an alternative candidate protein, post-acrosomal WW-domain binding protein (PAWP). These events have led to significant debate, fuelled by the opposing views of two independent laboratories, each defending their own respective SOAF candidate. This raises important questions with regards to the relative importance of these two proteins in diagnostic and therapeutic medicine, and invites urgent research attention from independent research groups. Here, it is our intention to provide an independent and unbiased review of this now very controversial area in order to engage the scientific and clinical communities in addressing the true importance of these two sperm proteins.

INTRODUCTION

Fusion of a sperm with an oocyte initiates an intricate cascade of signalling pathways in the ooplasm, commencing with oocyte activation and eventually leading to early embryo development. Key events transpiring in the ooplasm during this period include cortical granule exocytosis, pronuclear formation, maternal mRNA recruitment, release of meiotic arrest and the initiation of embryonic gene expression [1, 2, 3, 4]. A necessary component that triggers oocyte activation and these key events is the release of intracellular calcium (Ca^{2+}) stored in the endoplasmic reticulum (ER) [2-5]. The exact manner in which Ca^{2+} is released within the oocyte at fertilization has been debated for many years. While consensus of opinion supports the involvement of a sperm oocyte activating factor (SOAF) that is released into the oocyte upon gamete fusion, the identity of the precise sperm protein involved has been the source of much contention, with several candidates put forward for consideration [6-15]. The present review aims to provide an overview of the calcium-signalling pathway initiated within the oocyte upon gamete fusion, highlight the characteristics required of a SOAF, discuss past and present SOAF candidates, and critically consider recent controversial claims over two particular candidates.

CALCIUM SIGNALLING UPON FERTILIZATION

The oocyte undergoes a major change upon fertilization as Ca^{2+} stored in the ER is mobilized to the ooplasm in a carefully orchestrated temporal and spatial pattern, which ultimately initiates the cascade of molecular events that lead to oocyte activation [16, 17]. The frequency and amplitude of the evoked Ca^{2+} release pattern, which can manifest as either a single transient in non-mammals or a series of periodic oscillations in mammalian species, are crucial in activating the oocyte successfully and ensuring that the early stages of embryogenesis proceed normally [4, 5, 18-20].

Miyazaki et al. [17] provided distinct evidence that in mammalian oocytes, Ca^{2+} is released via the inositol-1,4,5-triphosphate receptors (IP_3R) in the ER membrane following binding of inositol-1,4,5-triphosphate (IP_3). The findings of this study corresponded with earlier data suggesting that upon

fertilization, a protein factor in the sperm was incorporated into the oocyte and subsequently led to the generation of IP_3 [16]. This line of evidence led to a concerted effort to identify the specific protein responsible, as clearly such a protein might hold significant diagnostic and/or therapeutic potential. The role played by this sperm protein is fundamental to the initiation of new life, and over time, the downstream cellular signalling system involved was elucidated in great detail. It is now well established that protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and mitogen activated protein kinase (MAPK), are all critical aspects of the downstream signalling cascade operating in an oocyte activated by Ca^{2+} release [16, 17, 20, 21]. These signalling molecules, and the events they orchestrate, lead to the release of cortical granules, the formation of a pronucleus, and allows the oocyte to alleviate metaphase II (MII) arrest and begin embryogenesis (reviewed by Amdani et al. [22]). Furthermore, we now know that a stored-operated calcium entry (SOCE) mechanism exists within the oocyte and plays a vital role in calcium homeostasis. The SOCE mechanism involves a group of molecules (STIM1, ORAI1, SERCA) and collectively permits the ER to refill with free Ca^{2+} from the ooplasm in order to generate new Ca^{2+} oscillations [23]. It follows that deficiency or abnormality in any of these signalling molecules or pathways may impart serious deleterious effects on the oocytes ability to activate, and thus have adverse effects upon embryonic viability or fertility.

THE SPERM OOCYTE ACTIVATING FACTOR (SOAF)

Functional and developmental characteristics of the SOAF

General consensus suggests that in order for a protein factor to be considered as a SOAF it must possess appropriate functional and developmental characteristics. Theory arose that the definitive sperm factor should elicit Ca^{2+} release from ooplasmic sources in a manner indistinguishable from that observed during mammalian fertilization [7, 11, 24]. This mechanism involves the increased production of IP_3 regulated by the phosphoinositide signalling pathway [17]. The phospholipase C (PLC) family are cytosolic enzymatic proteins that catalyze the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) to generate IP_3 and diacylglycerol (DAG). IP_3 causes the release of Ca^{2+} via IP_3R localized on the surface of

71 the ER, while DAG and Ca^{2+} activate the PKC pathway such that the Ca^{2+} signals are transformed, or
72 decoded, into cellular responses [5, 25, 26, 27]. There are currently thirteen PLC isozymes that differ by
73 structure and regulatory mechanisms, and while it has been suggested that some endogenous oocyte PLCs
74 play a role in activation, there has been significant interest in identifying sperm PLCs [7, 14, 27, 28], as
75 these would represent strong candidates for the SOAF.
76
77 The developmental features of the SOAF are strongly considered to involve the perinuclear theca
78 (PT) of the sperm head, a region that contains cytoskeletal proteins crucial for maintaining sperm head
79 architecture and coats the sperm nucleus [29]. Initially thought to be limited to cytoskeletal proteins,
80 cytosolic proteins associated with fertilization are also present in the PT, a structure that spans three
81 regions of the sperm head: a sub-acrosomal region (SAR-PT), the equatorial segment (ES-PT) and the
82 post-acrosomal sheath (PAS-PT) [29, 30]. Fusion between the fertilising sperm and oocyte begins in the
83 ES-PT and continues to the PAS-PT, the region that is first exposed to the ooplasm. It follows therefore,
84 that the SOAF must reside in the PAS-PT [29]. These characteristics played a key role in facilitating and
85 directing targeted research into identifying the SOAF. Historically, however, the primary concern
86 focussed upon the exact mechanism by which the sperm first introduces the SOAF upon gamete fusion.
87
88 *Historical perspectives: theories of the SOAF's mechanism of action*
89 'Sperm factor' and 'receptor-based' mechanisms were the two dominant theories and the focus of
90 much debate for over a century. While both seemed entirely feasible, the 'sperm factor' mechanism, in
91 which a soluble sperm factor thought to be released into the oocyte in order to trigger oocyte activation,
92 was rapidly accepted in favour of the opposing theory [16, 31, 32]. The combination of advanced
93 intracellular calcium imaging technology, the failure to identify an appropriate 'receptor-based'
94 mechanism, and the clinical use of intracytoplasmic sperm injection (ICSI) - a technique which is
95 successful but bypasses any sperm/oocyte membrane binding mechanism, led to the almost complete

dismissal of a sperm-ligand binding to an oocyte receptor in order for activation to commence, [7, 8, 16, 33, 34].

Given its fundamental importance to successful fertilization, the clinical relevance of the SOAF cannot be denied. Oocyte activation deficiency (OAD) and total fertilization failure (TFF) are known causes of infertility, and are common in cases of recurrent ICSI failure. Reported abnormalities in the SOAF, or the associated molecular machinery within the oocyte, are thus likely to be the predominant underlying causes of OAD and TFF [11, 22, 26, 35-37]. It follows that a certain sub-set of idiopathic cases of infertility may also be affected. Despite the advances in modern laboratory technology, and our ability to image Ca^{2+} in living cells, our diagnostic and therapeutic options for cases of OAD or TFF in the clinic remain highly restricted unless the specific proteins involved are identified and can be assayed accurately. Clearly such proteins, whether sperm- or oocyte-borne, represent valuable pharmaceutical targets.

Identification of SOAF candidates

Two proteins, oscillin and citrate synthase, were initially proposed as SOAF candidates in the past but were soon cast aside. Oscillin was the first sperm protein proposed as the putative soluble sperm factor capable of initiating Ca^{2+} oscillations in mammalian eggs [32, 38] and was supported clinically by the fact that the localization of oscillin in the equatorial segment of human sperm appeared to have a positive impact upon oocyte activation and fertilization rates. However, the potential SOAF candidature of oscillin ended abruptly when a recombinant form of human oscillin failed to induce Ca^{2+} oscillations upon injection into mouse oocytes [39, 40, 41]. Next, the potential of citrate synthase as a SOAF emerged when a purified 45 kDa protein, homologous to *Xenopus* citrate synthase, was injected into the unfertilised oocytes of the newt, *Cynops pyrrhogaster*, and induced oocyte activation [42]. While this protein initially appeared to be a promising candidate, at least in the newt, there has been no subsequent reports describing the possible involvement with mammalian fertilization. Attention, soon turned to

phospholipase C zeta (PLCζ), which gained significant support amongst the scientific and clinical community as the predominant SOAF candidate. Mounting biochemical and clinical evidence still points to PLCζ as being the dominant protein responsible for activating oocytes at fertilization [7, 8]. However, over recent years, a new candidate has emerged, post-acrosomal WW-domain binding protein (PAWP). PAWP localizes in an appropriate region of the sperm head, causes Ca^{2+} oscillations when injected into oocytes, and protein levels have been correlated with fertilization outcome [9]. However, some of the emergent data relating to PAWP have been refuted amongst the scientific community, leading to significant confusion, particularly amongst the clinical arena. It is thus the intention of this review to focus particularly upon PLCζ and PAWP in an attempt to provide an unbiased opinion of the mounting debate surrounding the relative importance of these two proteins (Table 1).

PHOSPHOLIPASE C ZETA (PLCζ)

While initial belief was that the SOAF was most likely to be a sperm PLC, largely due to the intimate association between PLC molecules and the generation of IP3, it was evident that none of the known PLC isoforms at the time could induce Ca^{2+} release in an oocyte in a manner similar to that observed during normal fertilization. However, experiments using a mouse testis expressed sequence tag (EST) database finally resulted in the identification of a novel PLC isoform, referred to as PLCζ [7]. PLCζ is the smallest known PLC, and since derivation has been studied on a global scale given its potential clinical worth. Initial evidence of its dominant role as the SOAF began when complimentary RNA (cRNA) synthesized against PLCζ was microinjected into mouse MII-arrested oocytes. Resultant data revealed a normal Ca^{2+} release profile and successful development to the blastocyst stage [7]. Further proof that PLCζ was the only sperm component accountable for Ca^{2+} release was the failure to generate Ca^{2+} activity when PLCζ was depleted from sperm extracts. Moreover, fractionation experiments concluded that following ICSI, PLCζ was the only sperm protein responsible for the Ca^{2+} oscillations observed [7]. Knott et al. [62] provided further supporting evidence by reporting that PLCζ-deficient

147 sperm, created via the use of RNA interference technology in transgenic mice, resulted in Ca^{2+}
148 oscillations that ended prematurely upon injection into mouse oocytes.
149
150 The structure of PLCζ is significant in executing its role in oocyte activation as each domain (X
151 and Y catalytic domains, EF hands and a C2 domain) plays an integral part in exerting function [5, 25, 27,
152 49, 53]. Removal or mutation of these domains results in failure to evoke Ca^{2+} oscillations, or abnormal
153 profiles of Ca^{2+} release [25, 49]. However, it would be presumptuous to disregard the involvement of the
154 oocyte, as PLCζ is only active when introduced into the ooplasm. The oocyte, therefore, has acquired a
155 vital role in triggering the enzymatic activity of PLCζ upon fertilization [11, 49]. Indeed, the
156 hypothesized interaction of PLCζ with receptor-bound vesicles within the oocyte containing PIP_2 for
157 hydrolysis [55] suggests critical communication between PLCζ and oocyte components (Figure 1). An
158 important future goal for researchers is therefore to identify the specific receptors within the oocyte that
159 interact with PLCζ in order to acquire functional ability. Such receptors may clearly represent useful
160 diagnostic markers or therapeutic targets, as not all cases of OAD or TFF may be attributable to sperm
161 deficiency.
162
163 *Clinical links between PLCζ and male-factor infertility*
164 In addition to the compelling body of functional and biochemical evidence that has been accrued
165 thus far, a number of clinical studies have also helped to consolidate the role of PLCζ as the dominant
166 SOAF candidate. Identifying the spatial expression of PLCζ in human sperm was first necessary in order
167 to interpret its involvement in human infertility. PLCζ expression was evident in three separate
168 localization patterns within the human sperm head: the acrosomal region, the equatorial segment, and the
169 post-acrosomal region, possibly suggesting that different populations of PLCζ might play differential
170 functional roles that is not limited to oocyte activation [11, 51, 63]. The first clinical association between
171 PLCζ and male infertility showed that PLCζ-deficient sperm from infertile men failed to evoke Ca^{2+}
172 oscillations when microinjected into mouse oocytes [57]. However, the ability to induce Ca^{2+} oscillations

in oocytes was rescued when such sperm were injected coincident with PLCζ mRNA, thus providing robust evidence that the infertile phenotype was correlated to PLCζ deficiency [57].

Genetic links between abnormal PLCζ and infertility have provided further support for the fundamental role of PLCζ in both clinical settings [58, 59], and potentially in the breeding of agricultural livestock [60]. The first genetic data involved the identification of two mutant PLCζ isoforms in an infertile non-globozoospermic male [58, 59]. Genetic analysis of the genomic DNA encoding PLCζ revealed the substitution of Proline for Histidine at position 398 (H398P) and of Histidine for Leucine at position 233 (H233L) within the Y and X catalytic domains, respectively. These mutations disrupted secondary structure of the PLCζ protein resulting in failure to initiate Ca^{2+} release, or the production of an abnormal pattern of Ca^{2+} oscillations when cRNA derived from PLCζ^{H398P} and PLCζ^{H233L} was injected into mouse eggs, respectively [58, 59]. More recently, Pan et al. [60] reported genetic variants in the PLCζ promoter of Chinese Holstein bulls, but failed to provide robust evidence of a direct effect of these variants upon specific PLCζ parameters.

The evident relationship between PLCζ deficiency and human infertility understandably prompted researchers to investigate the applications of this fundamental protein in the clinic. The development of specific techniques such as immunofluorescence and Western blotting now allows us to investigate relative levels and localization patterns in human semen samples, and thus create a bioassay for oocyte activation ability. More importantly, a pure and active form of recombinant human PLCζ (hrPLCζ) will clearly help restore oocyte activation ability in cases of OAD or TFF, thus representing a valuable pharmaceutical agent.

PLCζ as a novel therapeutic option for OAD

ICSI is widely considered to be a landmark treatment option for severe male infertile conditions and remains a robust and effective technique. However, 1-5 % of ICSI cycles are still known to fail and

OAD is widely considered to be the root cause for such failures. At present, the only way to treat such cases is via the use of artificial oocyte activators (AOAs), and while some clinics deploy such techniques, there is widespread concern over the potentially deleterious effects these chemical agents may have upon the resultant embryo [64]. Production of an active and purified hrPLCζ protein would be a much more desirable treatment option than AOAs as it represents a far safer and more endogenous option [11, 65]. However, the purification process remains the biggest obstacle to this quest and while several attempts have shown clear promise, particularly in the form of mammalian lysates or purified proteins from bacterial cell lines, there are still numerous concerns over existing proteins with respect to their potential clinical application [56, 65, 66]. The most promising study recently generated hrPLCζ from a bacterial cell line using a Nus-A tagged protein which, when microinjected into mouse oocytes exhibited calcium oscillations identical to normal fertilization [12]. The same hrPLCζ also rescued the activation ability of mouse oocytes previously administered with mutant PLCζ^{H398P} and PLCζ^{H233L} cRNA [12]. While concerns remain with respect to the relative size of the Nus-A tag and its absolute requirement for activity and stability, these findings do provide very strong evidence that PLCζ is the strongest SOAF candidate to date. However, over recent years, a series of reports have emerged which question the dominance of PLCζ, and instead, have put forward an alternative candidate protein.

POST-ACROSOMAL WW-DOMAIN BINDING PROTEIN (PAWP)

Almost half a decade since its discovery, PLCζ appeared dominant in its role as the putative SOAF responsible for oocyte activation. A host of studies from many independent laboratories have validated its role as a SOAF in a reproducible and consistent manner, both from molecular and clinical perspectives. However, the role of PLCζ is currently challenged by the emergence of an alternative SOAF candidate, post-acrosomal WW-binding domain protein, or PAWP. This new candidate is a sperm-specific protein presumed to alleviate meiotic arrest and initiate pronuclear formation following fertilization, thus prompting a presumptive role in activating the oocyte [9]. The structure of PAWP is distinct in that the N-terminal exhibits sequence homology to WW domain-binding protein 2, a proline-

rich C-terminal consisting of a PP*X*Y consensus binding site for group-1 WW domain-containing proteins (PY motifs) and an unknown repeating motif (YG*X*PP*X*G) [9, 54]. The precise signalling mechanism of PAWP within the oocyte remains highly speculative but recent data suggests that PAWP binds to group-1 WW domain proteins such as yes-associated protein (YAP), expressed in the oocyte, and that it interacts with the SH3 domain of oocyte phospholipase C gamma (PLCγ) in order to modulate the phosphoinositide signalling pathway (Figure 1) [9, 14]. This proposal remains, however, purely theoretical and it is therefore crucial to elucidate the precise signalling mechanism involved before the relative role of PAWP in oocyte activation can be considered in a robust manner. However, current data do appear to show that PAWP, at least, does exhibit the accepted developmental and functional characteristics of a SOAF.

PAWP was first reported in 2007 [9]; immunocytochemical staining of bull testicular tissue with an antibody raised against recombinant PAWP (recPAWP) revealed the expression of PAWP in the elongated spermatid population. PAWP was first detected in stage 9 of the bull spermatogenic cycle, reaching maximal levels during stages 11 and 12. The localization of PAWP was restricted to the PAS-PT of mature sperm from different species and demonstrated by immunofluorescent or immunogold labelling methods [9]. These crucial findings were also confirmed by the work of Wu et al. [45] and Ito et al. [46]. In addition to the developmental features of PAWP, Wu et al. [9] analysed its functional characteristics by microinjecting recPAWP and alkaline PT extract into porcine, bovine and *Xenopus* oocytes. These microinjections consistently formed a pronucleus and alleviated oocytes from MII-arrest [9]. This encouraging finding provided a prerequisite step with which to expand investigations into the involvement of PAWP with calcium signalling and oocyte activation. Only recently Aarabi et al. [14] demonstrated that PAWP was able to induce calcium oscillations and oocyte activation when PAWP cRNA or recPAWP were microinjected into human and mouse oocytes. Furthermore, the release of Ca^{2+} was prevented following the microinjection of a human sperm coincident with a competitive inhibitor, PAWP-derived *PPGY* peptide, suggesting that PAWP is necessary for oocyte activation [14]. Recent

work has also shown an association between the expression of human and bull PAWP and sperm quality and fertility capabilities following ICSI and artificial insemination [47, 61]. Collectively therefore, PAWP appears to have fulfilled the relevant conditions to classify as a strong candidate for the SOAF. Interestingly, however, while stressing the importance of PAWP for oocyte activation, Aarabi et al. [63] also questioned the role of PLCζ, and this has led to much debate and confusion.

CONFLICTS BETWEEN OLD AND NEW SOAF CANDIDATES

According to Aarabi et al. [63], much controversy lies within the expression profile of PLCζ during spermatogenesis as there is insufficient evidence regarding its protein translation at present. One particular study reported that PLCζ mRNA in the boar and mouse was first expressed in round spermatids and possibly translated during the elongated spermatid stage [43]. To date, there has been no follow up study to confirm this hypothesis. Another pressing issue is related to the localization profile of PLCζ as findings from Aarabi et al. [63] showed that human PLCζ was located either over the sperm membrane or acrosomal region but not as a component of the PAS-PT. This was evident from a fractionation experiment using non-ionic detergent as PLCζ was absent from the pellet – the fraction in which the PAS-PT is retained and therefore should contain the SOAF [63]. Conversely, in the mouse, PLCζ was indeed present in the pellet, but was localized to the isolated sperm tails instead of sperm heads [44]. Further results from immunoperoxidase staining and immunogold labelling on human testicular germ cells detected PLCζ at the round spermatid stage of spermatogenesis but then showed that PLCζ progressively diminished throughout the remainder of spermiogenesis. Furthermore, in vitro fertilization (IVF) experiments using boar sperm indicated that PLCζ was no longer present following fertilization suggesting that PLCζ was released along with the acrosomal cap [63]. The same study [63] also detected PLCζ localization over the surface of the sperm head, specifically in the post-acrosomal region in mouse sperm and both the post-acrosomal and acrosomal regions in human sperm. Further investigation deduced that PLCζ is not strictly a testis-specific protein, but was also expressed in the epididymis. Collectively, the findings from Aarabi et al. [63] led to the conclusion that PLCζ cannot be the primary SOAF

candidate as it originates from the developing acrosome instead of the forming PAS-PT and is therefore merely an acrosomal component lost during fertilization. In addition to this, the purported developmental expression in the epididymis was said to correlate to its localization over the entire surface of the sperm head, and explained the disappearance of PLCζ following boar sperm IVF, in which PLCζ was lost along with the acrosomal and surface proteins. These lines of evidence reported by Aarabi et al. [63] appear to be rational but a recent study has now reported the subcellular localization of PLCζ for the first time, and these new data create further contention. According to Escoffier et al. [52], PLCζ is indeed localized to the PT of the equatorial and post-acrosomal regions of human sperm. This reaffirmed PLCζ's position as a dominant SOAF candidate as it already exhibits appropriate functional characteristics. Furthermore, PLCζ was predominantly located in the equatorial region of the PT, where the sperm fuses with the oocyte. Zanetti and Mayorga [67] showed that the equatorial region remains unchanged following the acrosome reaction, thus contradicting the findings of Aarabi et al. [63]. It is also worth noting that an earlier study involving mouse and porcine sperm confirmed that PLCζ originated in the perinuclear matrix, an oocyte-penetrating region [48].

From Aarabi et al. [63], it is evident that the credence of PLCζ as a SOAF is now under scrutiny, but there are several important factors that need to be addressed in order to confirm or refute the claims of these authors. Firstly, the unusual PLCζ localization patterns reported by Aarabi et al. [63] contrast starkly with research data arising from other groups spanning six years which have consistently shown PLCζ to be a cytosolic protein localized to three defined regions within the sperm head [11, 50, 51, 68, 69]. Secondly, there are concerns over low sample size, and the specificity of the polyclonal antibody used in these recent papers. Finally, the issue of Ca^{2+} release ability has caused significant conflict. Aarabi et al. [14] reported that the microinjection of recPAWP into mouse and human oocytes elicited calcium oscillations similar to that seen following ICSI. This represents a major line of evidence to support a potential role for PAWP in oocyte activation. However, Nomikos et al. [70] replicated their earlier experimental procedures [12] and injected recombinant versions of PLCζ and PAWP into mouse

oocytes to monitor the respective Ca^{2+} release profile elicited. Recombinant PLCζ initiated Ca^{2+} release in a manner similar to that reported previously in 100% of oocytes injected, in line with that normally observed at fertilization. However, in their hands, recombinant PAWP protein, or PAWP cRNA, did not cause Ca^{2+} release, even following modifications of rPAWP concentration or the use of various tagged and untagged forms of PAWP cRNA. This understandably has created significant confusion in the field and given the growing debate surrounding the relative importance of PLCζ and PAWP in the initiation of oocyte activation [13-15, 70, 71], it is now imperative for the two opposing groups involved to consolidate and verify their evidence, and for other research groups to carry out independent replication and verification.

CONCLUSION

The body of evidence in support of PLCζ as the dominant SOAF candidate is already impressive and continues to accrue over time, particularly in terms of its functional activity, sub-cellular location, and the clear clinical links between PLCζ deficiency and OAD. The recent emergence of PAWP as an alternative candidate has received both genuine interest but also significant scrutiny. Unfortunately, significant controversy remains in the marked conflict between the respective datasets reported by Aarabi et al. (47) and Nomikos et al. (70) with respect to the functional ability of PAWP to cause Ca^{2+} release in oocytes. Independent verification and validation is now an absolute necessity. Of course, without such authentication, it would be very unwise to dismiss PAWP outright. What has been established is that PAWP is a sperm-specific protein localized to a region of the sperm that is involved in oocyte activation, and that there appears to be a positive correlation between PAWP content in sperm and fertilization rates for patients undergoing assisted reproductive technology (ART). At present, however, the potential involvement of PAWP in the process of oocyte activation is questionable, but cannot be discounted altogether. The precise signalling pathway involved has yet to be proven. It is vital that future work aims to elucidate potential correlates between the expression and function of PAWP and specific cases of OAD and TFF. Such studies will be imperative in developing new diagnostic assays and therapeutic agents for

those couples experiencing OAD and TFF, a growing sub-set of patients who have no other remedial alternative at present but to use AOAs.

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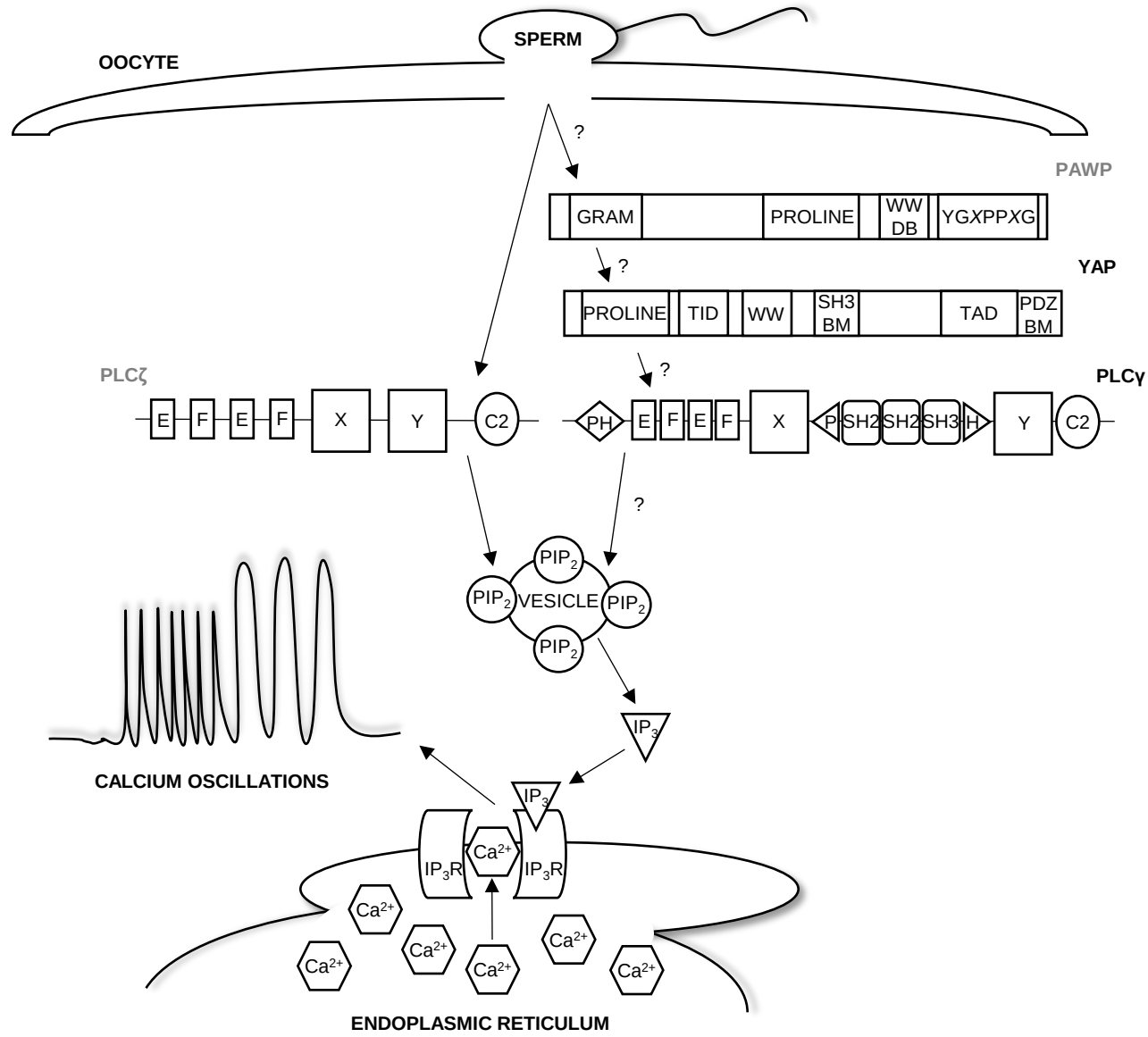
FIGURE LEGENDS

Fig 1. An overview of the established and proposed mechanisms of action for PLCζ and PAWP upon fertilization. PLCζ and PAWP are thought to induce the release of Ca^{2+} following gamete fusion by different mechanisms. Evidence shows that PLCζ hydrolyzes PIP_2-containing vesicles in order to generate IP_3, which then binds to IP_3Rs to cause the pulsatile release of Ca^{2+} from the endoplasmic reticulum. PAWP is hypothesized to bind to oocyte-borne YAP proteins, and then via the resultant SH3-BM to the SH3 domain of PLCγ in order to activate the phosphoinositide signalling pathway in a similar mechanism to PLCζ. SH3-BM: Src Homology 3 binding motif. Structural features of PLCζ and PLCγ were reproduced from Saunders et al. [7]. PAWP and YAP were derived from Universal Protein Resource. http://www.uniprot.org/ (PAWP: Q6ICG8 and YAP: P46937). Accessed 18 March 2015.

TABLE 1. Key features of PLCζ and PAWP with respect to their candidature as a SOAF.

Features	PLCζ[a]	PAWP[a]	Study (Year) [Ref][b]
Expression Profile	PLCζ mRNA is first expressed in round spermatids, likely to be translated in elongating spermatids, and detectable in all subsequent stages, both in the testes and epididymis.	Expression of PAWP is restricted to the elongating spermatids and thought to be secreted later by the epididymis.	**Saunders et al. (2002) [7]; Yoneda et al. (2006) [43]; Young et al. (2009) [44]**; Wu et al. (2007a, b) [9, 45]; Ito et al. (2010) [46]; Aarabi et al. 2014b [47]
Localization Patterns	PLCζ localization patterns have been detected in the acrosomal, equatorial, post-acrosomal and/or the PAS-PT region of the sperm head.	PAWP is localized to the PAS-PT region of the sperm head only.	**Fujimoto et al. (2004) [48]; Swann et al. (2006) [49]; Yoon and Fissore (2007) [50]; Grasa et al. (2008) [51]; Escoffier et al. (2015) [52]**; Wu et al. (2007a, b) [9, 45]; Aarabi et al. (2014b) [47]
Structure	PLCζ is the smallest PLC isoform consisting of a C2 domain, four tandem EF-hand domains and X and Y catalytic domains. Each domain plays a crucial role in PLCζ function.	The structure of PAWP consists of an N-terminal which has sequence homology to WW-domain binding protein 2 and a proline-rich C-terminal with a PP*X*Y consensus binding site and an unknown repeating motif.	**Saunders et al. (2002, 2007) [7, 8]; Swann et al. (2006) [49]; Nomikos et al. (2011a, b) [25, 53]**; Wu et al. (2007a) [9]; Aarabi et al. (2010) [54]
Calcium Signalling Mechanism	PLCζ hydrolyzes PIP_2 upon introduction into the oocyte generating IP_3, IP_3 binds to IP_3R causing the release of Ca^{2+} from the ER (supported by a large body of experimental evidence).	PAWP is thought to bind to oocyte-borne YAP proteins, and interacts with the SH3 domain of PLCγ resulting in activation of the phosphoinositide signalling pathway (hypothetical only, no experimental evidence).	**Swann et al. (2006) [49]; Nomikos et al. (2011a) [25]; Swann and Lai (2013) [55]**; Wu et al. (2007a) [9]; Aarabi et al. (2014a) [14]
Oocyte Activation	MII-arrested mouse oocytes microinjected with PLCζ cRNA induced a normal Ca^{2+} release profile and encountered normal development to the blastocyst stage. Sperm extracts devoid of PLCζ failed to induce Ca^{2+} activity. Microinjection of recombinant human PLCζ protein (hrPLCζ) in either lysate or purified form induced normal Ca^{2+} oscillations in mouse oocytes.	PAWP cRNA or recombinant PAWP microinjected into porcine, bovine, *Xenopus*, mouse and human oocytes induced Ca^{2+} oscillations similar to that during ICSI and oocyte activation. Microinjection of sperm coincident with a competitive inhibitor, PAWP derived *PPGY* peptide, prevented Ca^{2+} release. (However, at least one other group has failed to replicate these results).	**Saunders et al. (2002) [7]; Kashir et al. (2011) [56]; Nomikos et al. (2013) [12]**; Wu et al. (2007a) [9]; Aarabi et al. (2014a) [14]
Links to Infertility	Sperm with reduced levels, total absence or abnormal localization patterns of PLCζ fail to evoke Ca^{2+} oscillations. Activity can be restored following the microinjection of sperm coincident with PLCζ mRNA. Two novel mutations in PLCζ, H398P and H233L, were discovered in an infertile non-globozoospermic patient and produced an abnormal Ca^{2+} release profile. Two genetic variants from bull PLCζ affected influenced semen quality traits.	Total levels of PAWP in bull and human spermatozoa are correlated with sperm quality and fertilization rates.	**Yoon et al. (2008) [57]; Heytens et al. (2009) [58]; Kashir et al. (2012a) [59]; Pan et al. (2013) [60]**; Kennedy et al. (2014) [61]; Aarabi et al. (2014b) [47]

[a] PLCζ, phospholipase C zeta; PAWP, post-acrosomal sheath WW binding protein; PAS-PT, post-acrosomal sheath-perinuclear theca; PIP_2, phosphatidylinositol 4,5-biphosphate; IP_3R, inositol-1,4,5-triphosphate receptors; Ca^{2+}, calcium; ER, endoplasmic reticulum; YAP, yes-associated protein; ICSI, intracytoplasmic sperm injection. [b] Bold text indicates references for PLCζ.


SPERM
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