Functional disparity between human PAWP and PLCζ in the generation of Ca^{2+} oscillations for oocyte activation

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Submitted on February 25, 2015; resubmitted on May 8, 2015; accepted on June 19, 2015.

Abstract: Mammalian oocyte activation is mediated by cytosolic calcium (Ca^{2+}) oscillations initiated upon delivery of a putative ‘sperm factor’ by the fertilizing sperm. Previous studies suggest the identity of this sperm factor as the testis-specific phospholipase C-ζ (PLCζ). Recently, a post-acrosomal sheath WW domain-binding protein (PAWP) has been proposed as an alternative sperm factor candidate, following a report that human PAWP protein and cRNA elicited Ca^{2+} oscillations in mouse and human oocytes. Those Ca^{2+} oscillations were inhibited by a PAWP-derived peptide corresponding to a functional PPGY binding motif. Herein, using a series of human PAWP expression constructs, we demonstrate that both human PAWP protein and cRNA are, in our experiments, unable to elicit Ca^{2+} release following microinjection into mouse oocytes. Parallel experiments performed with human PLCζ elicited the characteristic Ca^{2+} oscillations present at mammalian fertilization, which produced oocyte activation and embryo development. Furthermore, sperm-induced Ca^{2+} oscillations were not inhibited by the PAWP-derived PPGY peptide following in vitro fertilization or intracytoplasmic sperm injection. Thus, the functional disparity with PLCζ leads us to conclude that human PAWP is neither sufficient nor necessary for the Ca^{2+} oscillations that initiate mammalian oocyte activation at fertilization.

Keywords: PAWP / phospholipase C-ζ / fertilization / oocyte activation / sperm factor.

Introduction

Before fertilization, mammalian oocytes remain arrested at the second metaphase of meiosis (MII arrest), a phenomenon requiring alleviation before subsequent embryogenesis may proceed. This meiotic arrest is released through a series of collective events termed oocyte activation, including cortical granule exocytosis, second polar body emission, pronuclear formation and subsequent cell division. In all species studied to date, oocyte activation is initiated by fluctuations in oocyte cytosolic calcium levels (Ca^{2+}), which in mammals manifest as a series of Ca^{2+} transients, termed Ca^{2+} oscillations. In mammals, these oscillations are mediated by the fertilizing sperm, inducing inositol 1,4,5-trisphosphate (IP3)-mediated Ca^{2+} release shortly following gamete fusion (Kline and Kline, 1992; Stricker, 1999; Swann and Yu, 2008). There is general scientific consensus that the fertilizing sperm delivers a soluble ‘sperm factor’ to the oocyte, which then initiates a characteristically prolonged series of Ca^{2+} transients in mammals.

Over the last few decades, a number of sperm-derived molecules have been implicated in the generation of Ca^{2+} release during mammalian fertilization, including a 33 kDa protein termed oscillin. However, none of these candidate proteins have been demonstrated to elicit the repetitive Ca^{2+} release in mammalian oocytes (Nomikos et al., 2013a, 2015). Ca^{2+} oscillations during mammalian oocyte activation are generated in an IP3-mediated manner, requiring that phosphatidylinositol 4,5-bisphosphate (PIP2) is hydrolysed to liberate IP3 and diacylglycerol. This is consistent with the idea that the sperm factor is a phospholipase C (PLC) (Nomikos et al., 2012, 2013a). Accordingly, accumulating data over the past decade has supported the identity of this sperm factor to be a novel, testis-specific PLC isozyme, termed PLCζ (PLCζ) (Cox et al., 2002; Saunders et al., 2002).

Microinjection of PLCζ complementary RNA (cRNA) and recombinant protein into mouse and human oocytes initiates the characteristic fertilization patterns of repetitive Ca^{2+} release, which also support early embryonic development of PLCζ-injected oocytes up to the multicellular blastocyst stage (Saunders et al., 2002; Kouchi et al., 2004; Nomikos et al., 2013b). The presence of PLCζ protein is precisely correlated with the ability of specific sperm extract fractions that have Ca^{2+} release activity (Fujimoto et al., 2004; Kurokawa et al., 2005), while the
immunodepletion of PLCζ from such extracts suppresses their ability to release Ca^{2+} (Saunders et al., 2002). Unfortunately, the only available information about a PLCζ knock-out mouse model is found in an abstract (presented in Japan at the 11th International Symposium on Spermatology; Ito et al., 2010). In this abstract, it was reported that PLCζ may have a significant role in mouse spermatogenesis, as the PLCζ knockout displayed defective sperm cell development due to the failure of spermocytes to progress beyond elongation. Unfortunately, corroboration of this study is not possible, as no other data are available on a PLCζ knock-out animal model. Interestingly, however, there is one example of peer-reviewed evidence from a transgenic mouse model supporting a role for PLCζ in Ca^{2+} release at fertilization. Transgenic mice with RNA interference-mediated reduction of PLCζ expression yielded sperm which induced prematurely ending Ca^{2+} oscillations, and while not infertile, these mice exhibited a reduced litter size (Knott et al., 2005).

Mammalian PLCζ exhibits unique combinations of physiological and biochemical properties mediated through its distinctive structure (Nomikos et al., 2013a). It has been demonstrated that PLCζ is released from mouse sperm into the fertilized oocyte 90 min after ICSI (Yoon and Fissore, 2007). This study suggested that upon fertilization, the sperm factor activity and PLCζ immunoreactivity are both released by the sperm simultaneously, which suggests that PLCζ may represent the Ca^{2+} oscillation-inducing factor within mammalian sperm (Yoon and Fissore, 2007). More importantly, clinical reports have linked reduced PLCζ expression levels and abnormal forms of PLCζ with certain cases of human male infertility where oocyte activation is deficient (Yoon et al., 2008; Heytens et al., 2009; Kashir et al., 2010, 2012a; b; Nomikos et al., 2011a).

Recent efforts have identified a novel post-acrosomal sheath WW domain-binding protein, termed PAWP (Wu et al., 2007a), which has been proposed as an alternative candidate for the sperm factor (Wu et al., 2007a, b; Aarabi et al., 2010, 2014a, b). Recombinant bovine PAWP initiated cell cycle progression and pronuclear formation upon microinjection into MII-arrested porcine, bovine, macaque as well as Xenopus oocytes (Wu et al., 2007a). In addition, injection of recombinant PAWP was reported to cause Ca^{2+} release in Xenopus oocytes (Aarabi et al., 2010). PAWP is proposed to mediate Ca^{2+} release via interaction with YAP (yes-associated protein) which can modulate oocyte PLCs such as PLCγ through an SH3 motif binding interaction (Aarabi et al., 2014a). In both human and mouse oocytes, human PAWP protein or cRNA was reported to induce Ca^{2+} oscillations that could be blocked by the co-injection of a competitive peptide inhibitor of PAWP, the sequence of which corresponds to a functional PPXY consensus binding motif found in Group-I WW-domain-containing proteins (Aarabi et al., 2014a). The same peptide was also found to block Ca^{2+} oscillations after ICSI, which would strongly suggest that PAWP plays a functional role in physiological oocyte activation (Aarabi et al., 2014a).

Due to the absence of independent corroborative data on PAWP, we recently examined the claims of a Ca^{2+} signalling role reported by Aarabi et al. (2014a), using mouse PAWP. Microinjection of recombinant mouse PAWP protein and cRNA (in 3 distinct iterations; untagged, YFP- or luciferase-tagged versions) were all consistently unsuccessful at eliciting Ca^{2+} release in mouse oocytes, even at supra-physiological levels (Nomikos et al., 2014). Such a lack of response was in stark contrast to mouse PLCζ, which was able to successfully cause Ca^{2+} oscillations in parallel experiments. Furthermore, PAWP expectedly did not hydrolyse PIP₂, but importantly was unable to modulate PLC enzymatic activity in vitro (Nomikos et al., 2014). These discordant observations raise questions about the previous results from Aarabi et al. (2014a), as well as the proposed mechanism(s) of PAWP action. However, the previous investigation was limited to an analysis of mouse PAWP, and considering the observed species-specific properties of Ca^{2+} release at oocyte activation, the characterization of human PAWP would therefore be prudent.

In this study, we generated a series of human PAWP expression constructs (Fig. 1) to enable the direct comparison of the ability of human PAWP and human PLCζ to initiate cytosolic Ca^{2+} oscillations in mouse oocytes. Our results indicate that neither recombinant human PAWP protein nor different constructs of human PAWP cRNA can induce intracellular Ca^{2+} oscillations after injection into mouse oocytes. In contrast, all oocytes injected with human PLCζ responded by displaying prominent Ca^{2+} oscillations. Further, we found that a PAWP-derived PPGY peptide, previously proposed to block sperm-induced Ca^{2+} release by binding specifically to WWI-domain-containing proteins, had no effect on Ca^{2+} oscillations in mouse oocytes after IVF or ICSI procedures. Our experiments indicate that recombinant human PAWP is not able to cause Ca^{2+} release in unfertilized mouse oocytes.

Materials and Methods

Following the report by Aarabi et al. (2014a) on human PAWP, we specifically requested the provision of the research materials described in their study (human PAWP plasmid, antibody and peptide) for proposed experiments to be independently conducted in our laboratory. However, our request for access to these materials was declined. Therefore, we obtained the human PAWP-pET28a vector, polyclonal PAWP antibody and synthetic PAWP peptide from commercial suppliers, as described below.

Cloning of human PAWP expression constructs

Human PAWP (GenBank™ accession number BC022546) was amplified by polymerase chain reaction (PCR) from a pET28a-hPAWP plasmid (Protein-tech) using Phusion polymerase (Finnzymes) and appropriate primers to incorporate a 5′-EcoRI site and a 3′-NotI site, and cloned into pETMM60 to enable bacterial protein expression. The primers used were: 5′-CAG TGAATTCGATGGCGGTGAATCAGAGCCACACCG-3′ (forward) and 5′-GAGTGCGGCCCGCTTAAGAATGGACCTGAGAAGAGGAGGC-3′ (reverse). To prepare the pCR3-hPAWP plasmid, human PAWP was PCR amplified as above using the appropriate primers to incorporate a 5′-EcoRI

![Figure 1](https://academic.oup.com/molehr/article-abstract/21/9/702/1019058/219792/256956) Schematic representations of the various PAWP constructs generated for cRNA and recombinant protein production. The various amino acid sequence lengths are indicated for each construct.
site and a 3′-NotI site, and cloned into a pCR3 vector, or a modified pCR3 vector containing a C-terminal firefly (Photinus pyralis) luciferase tag. The primers used were: 5′-CAGTGATTCTACGCCGCTAATCATGAGCC CACCG-3′ (forward) and 5′-GAGTGGCGCCTTAAGAATGAGGCT TGAAAGAAGGGG-3′ (reverse). Successful cloning of the above constructs was confirmed by dideoxynucleotide-sequencing (Applied Biosystems Big-Dye Ver 3.1 chemistry and model 3730 automated capillary DNA sequencer).

cRNA synthesis
Following linearization of human PAWP and PLCζ plasmids, cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and then polyadenylated using the poly(A) tailing kit (Pierce) at 4°C on ice. Soluble NusA-6xHis fusion protein was purified on Ni-NTA resin followed by Aarabi et al. (2014a). The peptide was capped in exactly the same way as described commercially and purchased from Lifetein LLC (South Plainfield, New Jersey, USA). The peptide was capped in exactly the same way as described by Nomikos et al. (2014).

Protein expression and purification
For NusA-6xHis-fusion protein expression (Nomikos et al., 2013b), E. coli (BL21-CodonPlus (DE3)-RILP; Stratagene) was transformed with the appropriate pETMM60 expression plasmid construct, and cultured at 37°C until A600 reached 0.6. Protein expression was induced for 18 h, 16°C with 0.2 mM isopropyl β-D-thiogalactopyranoside (ForMedium). Cells were harvested (6000g for 10 min), resuspended in PBS containing a protease inhibitor mixture (EDTA-free; Roche) and sonicated (4°C for 15 s) on ice. Soluble NusA-6xHis fusion protein was purified on Ni-NTA resin following standard procedures (Qiagen), and eluted with 250 mM imidazole. Eluted proteins were dialysed overnight (10 000 MWCO; Pierce) at 4°C until further required.

Peptide synthesis
A PAWP-derived 16-residue amino acid sequence, PPVRYGSPPPPYEAPT (PPGY), corresponding to the synthetic peptide that was previously reported to block Ca2+ oscillations after ICSI (Aarabi et al., 2014a) was synthesized commercially and purchased from Lifetein LLC (South Plainfield, New Jersey, USA). The peptide was capped in exactly the same way as described by Aarabi et al. (2014a).

Human sperm processing
Surplus semen samples were donated by individuals undergoing routine semen assessment at the Wales Fertility Institute, University Hospital of Wales, Cardiff, following informed, written consent. This study was ethically approved by the local research ethics committee (REC reference number 08/WSE/02/20). Sperm were isolated from semen through density gradient washing, prepared using PureSperm 40/60 (Nidacon) and the washed sperm were isolated following the manufacturers protocol, as previously described (Kashir et al., 2011). Following isolation, sperm were resuspended in PBS containing a cocktail of protease inhibitors (Roche), and sperm concentration was determined microscopically using an improved Neubauer haemocytometer.

Sperm immunoblotting
Sperm were aliquoted in single-use aliquots, such that each aliquot contained a total of 5 × 10^6 sperm, and resuspended in 5 × SDS gel sample loading buffer. Each single-use aliquot was frozen on dry ice, and stored at −80°C until required. For SDS-PAGE and immunoblotting, single aliquots were thawed and briefly heated at 100°C, before loading onto 4–20% pre-cast gradient gels (BioRad) for SDS-PAGE as previously described (Nomikos et al., 2005).

Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore) using a semidytransfer system (Trans-Blot SD; BioRad) in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% v/v methanol) at 18 V for 1 h. Membranes were incubated overnight at 4°C in blocking buffer (Tris-buffered saline, 0.1% Tween-20, 5% non-fat milk proteins). Membranes were incubated with an anti-PAWP polyclonal antibody raised in rabbit (Proteintech) (1:50 000), followed by probing with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:50 000), both diluted in blocking buffer and incubated at room temperature for 1 h. Antibody detection was achieved using enhanced chemiluminescence (ECL Select; GE LifeSciences).

Sperm immunofluorescence
Sperm obtained following density gradient washing were fixed by incubation in 10% neutral-buffered formalin solution (Sigma) for 10 min at room temperature, following which the fixative was diluted with an equal volume of PBS, and sperm were centrifuged at 500g for 10 min. The supernatant was discarded, and the fixed-sperm pellet was resuspended in PBS containing protease inhibitors, and stored at 4°C until further required.

Immunofluorescence was performed as previously described (Grasa et al., 2008; Kashir et al., 2011). Briefly, fixed sperm samples were added to PAP moulds (Vector laboratories) drawn on to slides coated with 0.01% (w/v) poly-l-lysine solution (Sigma), allowing the samples to settle onto the slide. Cells were permeabilised with PBS-1% Triton X-100 at room temperature for 1 h. Non-specific antigen binding sites in permeabilised cells were blocked with PBS-5% bovine serum albumin (BSA; Sigma) for 1 h at room temperature. Cells were incubated with an anti-PAWP polyclonal antibody raised in rabbits diluted in PBS-5% BSA (1:300; Proteintech) overnight at 4°C, following which cells were incubated with AlexaFluor-488-conjugated anti-rabbit secondary antibody, diluted in PBS-5% BSA (1:100; Life Technologies) at room temperature for 1 h. Cells were washed three times with PBS between each aforementioned step. Cells were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; DAPI; Vector Laboratories) and slides stored at 4°C until imaging. Slides were imaged using a 63× oil-immersion lens, and a Zeiss Axiosvert 200 fluorescence microscope utilizing a brightfield filter to image sperm cells, and a fluorescein isothiocyanate (FITC) filter for PAWP immunofluorescence. Images were captured using an AxioCam MRC camera, and processed using ImageJ. Representative images were also obtained by confocal microscopy, using a Leica TCS SP5 microscope and a 100× oil-immersion lens. PAWP (green) fluorescence was captured at a wavelength of 488 nm, alongside differential interference (DIC) images. Images were captured and analysed using the LAS AF software package (Leica).

Preparation and handling of gametes
MII oocytes were collected from 6- to 8-week-old female MF1 mice (Harlan UK Ltd) that were superovulated by injection of pregnant mare’s serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) 48 h apart. Approximately 15 h after hCG injection, mature MII oocytes were collected and held in M2 media (Sigma-Aldrich) under oil at 37°C until microinjection. Sperm were collected from the epididymis of ~10-week-old male C57/CBA F1 hybrid mice. Sperm was suspended in T6 media containing bovine serum albumin (BSA) (16 mg/ml) for 3 h to allow for capacitation prior to use. All procedures using animals were approved by the Cardiff University Animals Ethics Committee and were carried out under a UK Home Office Licence.

Microinjection and measurement of intracellular Ca2+ and luciferase expression
Prior to, and during microinjection, mouse oocytes were held in M2 media. PAWP was introduced into oocytes by high pressure microinjection of either recombinant protein or cRNA in KCl-Hepes injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4); this was then mixed with an equal amount of a...
fluorescent Ca$^{2+}$ indicator either Oregon Green Bapta Dextran, (OGBD; 1 mM) (Life Technologies) or Rhod dextran (1 mM) (Life Technologies). Where the PPGY peptide was microinjected, the peptide was diluted in KCl-Hepes and mixed with OGBD in the same way. The amount of solution injected was between 3 and 5% of the oocyte volume estimated from the diameter of cytoplasmic displacement caused by a bolus injection. Following microinjection, the zona pellucida of the oocytes was removed using Tyrode's acid solution (Sigma-Aldrich), and transferred to HKSOM media for imaging (Saunders et al., 2002; Nomikos et al., 2013b). For oocytes that were fertilized by IVF, 5 mM glucose was added to the HKSOM prior to imaging and sperm suspended in T6 containing BSA was added once imaging had commenced.

Oocytes fertilized by ICSI had sperm microinjected into the cytoplasm using custom-made ICSI pipettes (Yoshida and Perry, 2007) which penetrated the oocyte plasma membrane by a piezo-pulse delivered by a Prime Tech piezo manipulation system (Intracel, Royston, UK). The AM Ca$^{2+}$ indicator PE3-AM (fura2LeakRes-AM, Sigma) was used in some of the ICSI fertilized oocytes to measure changes in cytosolic Ca$^{2+}$ concentrations (Igarashi et al., 2005). Oocytes were incubated in 10 μM PE3-AM in M2 media for ~30 min before being transferred into M2 media and then equilibrated for ~30 min prior to sperm injection.

Where only fluorescence was measured, a Nikon Eclipse TI-U microscope was used with a cooled CCD CoolSnap HQ2 camera (Photometrics, USA) (Gonzalez-Garcia et al., 2013) to record the fluorescence signal. In oocytes where luminescence as well as fluorescence was recorded, luciferin (100 μM, Sigma) was added to the HKSOM prior to imaging with a Nikon TE2000 microscope and a cooled intensified CCD camera (Photek). Luciferase protein expression and cytosolic Ca$^{2+}$ changes, quantified by luminescence and fluorescence, respectively, were measured concurrently by switching back and forth between the two modes every 10 s (Campbell and Swann, 2006; Swann et al., 2009). Luminescence and fluorescence signals were plotted separately over the same time period for each oocyte. Fluorescence emitted by the Ca$^{2+}$ indicator was normalized to relative fluorescence units by dividing each intensity by the basal starting fluorescence (F/F0). However for PE3, the fluorescence signals were displayed as the excitation ratio of 350 nm/380 nm (Igarashi et al., 2005). Luminescence was plotted as a running average over a 5 min period.

**Results**

**Expression of native and recombinant human PAWP protein**

Expression and distribution of native PAWP in human sperm was examined by immunoblot and immunofluorescence analysis on ejaculated, density gradient-washed sperm from male donors. A commercially available, anti-PAWP polyclonal antibody positively detected a single, immunoreactive, ~32 kDa protein corresponding to human PAWP (Fig. 2A, right panel). Immunofluorescence analysis revealed native hPAWP localization predominantly in the post-acrosomal region of the sperm head with some additional tail staining (Fig. 2B).

Similar to mouse PAWP (Nomikos et al., 2014), attempts to express and purify human PAWP as a 6xHis-tagged fusion protein proved unsuccessful, as the protein appeared to be >90% insoluble, accumulating into inclusion bodies (data not shown). Thus, recombinant human PAWP was cloned into the pETTM60 vector, expressed as NusA-6xHis-tagged fusion protein and purified by Ni-NTA affinity chromatography. We recently showed that NusA is an effective bacterial fusion protein partner for human PLCζ, significantly improving the expression of soluble PLCζ protein in E. coli, and enhancing the temporal stability of the expressed and purified protein (Nomikos et al., 2013b; Theodoridou et al., 2013). Optimal protein production for NusA-6xHis-tagged hPAWP required induction of protein expression with 0.1 mM IPTG for 18 h at 16 °C.

Expression and purity of affinity-purified NusA-6xHis-tagged hPAWP protein was confirmed by SDS-PAGE (Fig. 3Aa) and immunoblot detection, using an anti-His (penta-His) mouse monoclonal antibody (Fig. 3Ab) and the anti-PAWP polyclonal antibody (Fig. 3Ac). The predicted molecular mass for NusA-6xHis-tagged hPAWP is ~92 kDa. The corresponding protein with the appropriate molecular mass was observed in both the gel and immunoblots (Fig. 3A). Using a [3H]PIP2 hydrolysis assay (Nomikos et al., 2013b; Theodoridou et al., 2013), we found that recombinant hPAWP protein did not exhibit any in vitro PIP2 hydrolytic enzyme activity. Additionally, hPAWP at either low or high Ca$^{2+}$ concentrations (data not shown), had no effect on the in vitro PIP2 hydrolytic enzyme activities of human PLCζ and rat PLCζ, suggesting that hPAWP does not act as a generic activator of PLC enzymatic activity.

**Human PAWP does not cause Ca$^{2+}$ oscillations in unfertilized mouse oocytes**

We directly compared the Ca$^{2+}$ oscillation-induction ability of affinity-purified, recombinant NusA-6xHis-tagged human PAWP and PLCζ.
proteins in unfertilized mouse oocytes. Microinjection of two different pipette concentrations (0.05 and 0.5 \( \mu g/\mu l \)) of recombinant hPAWP protein did not cause any Ca\(^{2+}\) release in mouse oocytes (Fig. 3B, left and middle panels). In contrast, microinjection of recombinant hPLC\(_z\) protein revealed its potent ability to trigger Ca\(^{2+}\) oscillations (Fig. 3B, right panel), matching those observed after microinjection of native sperm extracts (Swann and Yu, 2008; Nomikos et al., 2012). The microinjection of NusA protein on its own does not cause any Ca\(^{2+}\) release (Nomikos et al., 2013b).

The relative Ca\(^{2+}\) oscillation-inducing activities of both human PAWP and PLC\(_z\) were assessed in mouse oocytes by designing and preparing a hPAWP expression plasmid that contained firefly luciferase at the C-terminus, analogous to the control hPLC\(_z\) expression plasmid (Fig. 1). Prominent Ca\(^{2+}\) oscillations were monitored in human PLC\(_z\) cRNA-injected mouse oocytes when the hPLC\(_z\)-luciferase expression level gave a luminescence reading of 1.8 counts per second (Fig. 4B). In contrast, microinjection of human PAWP-luciferase failed to cause any Ca\(^{2+}\) release (Nomikos et al., 2013b).

To investigate whether the lack of Ca\(^{2+}\)-oscillation-inducing activity of recombinant NusA or luc-tagged hPAWP proteins was due to the presence of fusion tags, we microinjected cRNA corresponding to an untagged version of hPAWP into unfertilized mouse oocytes. Mouse oocytes were microinjected with three different pipette concentrations (0.06, 0.3, and 1.0 \( \mu g/\mu l \)) of hPAWP cRNA. Untagged PAWP was unable to induce Ca\(^{2+}\) release in mouse oocytes at any of the concentrations (Fig. 5A). To confirm faithful protein expression of hPAWP cRNA in mouse oocytes, the recombinant hPAWP protein expressed in oocytes was detected in immunoblot using the anti-PAWP polyclonal antibody. A single band with the predicted molecular mass of \(~32\) kDa was readily detected in the immunoblot where 10 hPAWP cRNA-injected oocytes were loaded into a gel lane (Fig. 5B, right lane). In contrast, the lane where 10 uninjected oocytes were loaded on the same gel, exhibited no immunoreactivity (Fig. 5B, left lane). Analysis of the immunoreactive \(~32\) kDa PAWP protein expressed in oocytes by densitometry, compared with calibrated levels of purified recombinant NusA-hPAWP protein produced in bacteria, indicated that 400–500 fg/oocyte of PAWP protein was expressed after 4 h using 1 mg/ml cRNA.

Sperm-induced Ca\(^{2+}\) oscillations in mouse oocytes injected with PAWP-derived PPGY peptide

Aarabi et al. (2014a) recently reported that a synthetic peptide derived from the PAWP amino acid sequence blocked intracellular Ca\(^{2+}\) release induced by mouse spermatozoa. The authors suggested that this PPGY peptide binds specifically to WWI-domain-containing proteins to confer a competitive inhibitory effect on PAWP’s PPGY motif. To investigate this hypothesis, we synthesized an identical PPGY peptide to Aarabi et al. (2014a) and tested whether this peptide could...
reduce or completely block sperm-induced Ca^{2+} oscillations in mouse oocytes after IVF or ICSI. As shown in Fig. 6A and Table I, microinjection of PPGY peptide at two different pipette concentrations (0.5 and 2.5 mg/ml) did not display any effect on Ca^{2+} oscillations in oocytes fertilized by IVF using fresh mouse sperm. This absence of effect was congruent with ICSI experiments performed using this peptide where we...
also found that the PPGY peptide could not block or inhibit Ca\(^{2+}\) oscillations induced by mouse sperm after ICSI (Fig. 6B and Table II). Figure 6B (middle and right panels) shows the cytosolic Ca\(^{2+}\) oscillations in MII oocytes fertilized by ICSI with fresh sperm following injection of 2.5 mg/ml PPGY peptide before, or at the same time, as injection with fresh mouse sperm (see Table II).

Table I  Effect of PPGY peptide on IVF-induced Ca\(^{2+}\) oscillations in mouse oocytes.

<table>
<thead>
<tr>
<th>Pipette concentration of PPGY peptide (μg/μl)</th>
<th>n</th>
<th>Mean number of oscillations/2 h</th>
<th>SD of the mean number of oscillations/2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>8</td>
<td>27.9</td>
<td>6.1</td>
</tr>
<tr>
<td>2.5</td>
<td>9</td>
<td>21.9</td>
<td>7.7</td>
</tr>
<tr>
<td>No peptide</td>
<td>8</td>
<td>22.4</td>
<td>5.9</td>
</tr>
</tbody>
</table>

The PAWP-derived peptide (PPGY) was injected into mouse oocytes at 0.5 and 2.5 μg/μl and its ability to alter the Ca\(^{2+}\) oscillations was observed following in vitro fertilization. The presence and absence of peptide were compared by recording the number of Ca\(^{2+}\) oscillations within the first 2 h.

Table II  Effect of PPGY peptide on ICSI-induced Ca\(^{2+}\) oscillations in mouse oocytes.

<table>
<thead>
<tr>
<th>Pipette concentration of PPGY peptide (μg/μl)</th>
<th>Injected at the same time as sperm?</th>
<th>n</th>
<th>Number of oocytes oscillated</th>
<th>% of oocytes oscillated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>Yes</td>
<td>7</td>
<td>6</td>
<td>86</td>
</tr>
<tr>
<td>2.5</td>
<td>No</td>
<td>31</td>
<td>19</td>
<td>61</td>
</tr>
<tr>
<td>No peptide</td>
<td>—</td>
<td>18</td>
<td>14</td>
<td>78</td>
</tr>
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</table>

The PAWP-derived peptide (PPGY; 2.5 μg/μl) was injected into mouse oocytes either mixed with the sperm (peptide + ICSI; n = 7) or injected separately before the injection of sperm (peptide injection followed by ICSI; n = 31) and the effect on the Ca\(^{2+}\) oscillations was compared with ICSI in mouse oocytes with no peptide injection (ICSI; n = 18).

Discussion

Oocyte activation is a fundamental step that initiates embryonic development after fertilization. In all mammalian species studied to date, the process of oocyte activation involves marked oscillations in the oocyte cytosolic concentration of ionized Ca\(^{2+}\), a phenomenon which is both
Human PAWP does not cause Ca\(^{2+}\) release in oocytes

necessary and sufficient for the successful completion of all the oocyte activation events before early embryonic development (Stricker, 1999; Nomikos et al., 2013a). The factor in the sperm that causes the Ca\(^{2+}\) oscillations is not species-specific, since fertile human sperm when injected into mouse oocytes causes prolonged Ca\(^{2+}\) oscillations (Heytens et al., 2009). For more than a decade since its discovery in 2002, a significant body of biochemical, physiological, and clinical evidence has supported PLCζ as the sperm factor, a sperm-derided molecule capable of generating the characteristic Ca\(^{2+}\) oscillations observed during fertilization (Cox et al., 2002; Saunders et al., 2002; Fujimoto et al., 2004; Kouchi et al., 2004; Knott et al., 2005; Kurokawa et al., 2005; Nomikos et al., 2005, 2007, 2011a, b; Grasa et al., 2008; Yoon et al., 2008; Heytens et al., 2009; Kashir et al., 2011, 2012a, b; Theodoridou et al., 2013).

Introduction of the sperm-specific PLCζ protein into mouse and human oocytes mimics all the fundamental characteristics which are observed when the fertilizing sperm triggers Ca\(^{2+}\) oscillations. In addition, PLCζ has been demonstrated to catalyse the hydrolysis of its membrane-bound substrate IP\(_3\), generating intracellular Ca\(^{2+}\) oscillations via the IP\(_3\) signalling pathway (Nomikos et al., 2013a). As aarabi et al. (2014a) recently reported that another sperm head protein, PAWP, which exclusively resides in the post-acrosomal sheath region of the perinuclear theca (PT), elicits Ca\(^{2+}\) oscillations and oocyte activation in human and mouse oocytes, similar to that observed during ICSI.

The authors showed that recombinant human PAWP cRNA or protein is able to trigger Ca\(^{2+}\) oscillations in mouse and human oocytes, which could be blocked by co-injection of a peptide derived from the WWI domain-binding motif of PAWP, which acts as a competitive inhibitor of PAWP (Aarabi et al., 2014a). This recent report followed the initial proposal in 2007 from the same group, suggesting that PAWP promotes meiotic resumption as well as pronuclear development during fertilization (Wu et al., 2007a). Based on their studies, microinjection of PAWP protein into porcine, bovine, macaque, and Xenopus oocytes resulted in pronuclear formation, an indicative event of successful oocyte activation (Wu et al., 2007a).

However, we recently demonstrated that microinjection of recombinant mouse PAWP protein and cRNA was unable to elicit Ca\(^{2+}\) release in mouse oocytes, even at supra-physiological levels (Nomikos et al., 2014). In contrast, mouse PLCζ successfully triggered Ca\(^{2+}\) oscillations in every parallel experiment. These data beg the question as to whether human PAWP, which has been recently proposed to have potential applications in the diagnosis and treatment of male infertility, can be considered as the physiological agent that induces Ca\(^{2+}\) oscillations and oocyte activation at mammalian fertilization.

Our current findings are consistent with our previous study investigating mouse PAWP (Nomikos et al., 2014), suggesting that, as with mouse PAWP, human PAWP is not involved in the generation of Ca\(^{2+}\) oscillations and oocyte activation. Firstly, we demonstrated that the microinjection of recombinant human PAWP protein was unable to induce any form of Ca\(^{2+}\) increase in mouse oocytes (Fig 3B). Further, expression of PAWP in mouse oocytes by injecting the corresponding cRNA did not trigger any Ca\(^{2+}\) oscillations (Figs 4 and 5A). This absence of effectiveness in oocytes was repeatedly the case, regardless of whether a luciferase C-terminal tagged version of PAWP or an untagged PAWP construct was expressed. To examine a wide span of protein expression levels, we injected a range of cRNA concentrations (ranging from 0.006 to 1 mg/ml) of the untagged human PAWP construct and we demonstrated that hPAWP cRNA was faithfully expressed in mouse oocytes by immunoblot analysis using an anti-PAWP polyclonal antibody (Fig 5B). Moreover, the 16-amino acid peptide, derived from the WWI domain-binding motif of PAWP sequence that Aarabi et al. (2014a) reported to block sperm-induced Ca\(^{2+}\) oscillations, in our experiments failed to show any inhibitory effect on sperm-induced Ca\(^{2+}\) oscillations in mouse oocytes after IVF or ICSI (Fig. 6).

This is the first attempt to repeat the intriguing findings presented by Aarabi et al. (2014a) regarding human PAWP protein. However, we could not replicate any of the observations of Aarabi et al. (2014a) and consider that it would now be prudent for this topic to be further investigated by other independent research groups. Currently, compelling evidence exists to support PLCζ as the ‘sperm factor’; this evidence encompasses the distinctive molecular, structural and functional properties of this enzyme, the unique biological mechanism of action during fertilization of mammalian oocytes, in addition to its direct medical relevance to male fertility. Furthermore, such data are a result of the research of multiple laboratories worldwide that have provided independent confirmation in support of a sperm factor role for PLCζ. Certainly, we cannot exclude that PAWP may have potentially relevant value as a diagnostic and/or prognostic indicator of sperm quality, but our current data on human PAWP, together with our previous observations for mouse PAWP, strongly suggest that this protein is not involved in generating the Ca\(^{2+}\) oscillations that are an intrinsic requirement to the activation of oocytes at mammalian fertilization.

Authors’ roles

M.N., K.S. and F.A.L. devised the project strategy. M.N., J.K., K.S. and F.A.L. designed the experiments. P.A., D.S., P.K. and A.B. were involved in patient recruitment and sperm processing. The experiments were performed by M.N., J.R.S., J.K., R.S., L.B. and D.L. M.N., J.K., K.S. and F.A.L. drafted the manuscript which was revised and approved by all authors.

Funding

M.N. and J.K. hold an EU-FP7 Marie Curie Intra-European Fellowship Award (628634) and a Health Fellowship Award from the National Institute for Social Care and Health Research (NISCHR), respectively. J.R.S. and R.S. hold research scholarships supported by Cardiff University School of Medicine and the Libyan Ministry of Education, respectively.

Conflict of interest

Cardiff University holds intellectual property rights on PLCζ (named inventors are F.A.L. and K.S.). All other authors declare no conflict of interest.

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