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ORIGINAL RESEARCH

Sperm-specific post-acrosomal WW-domain binding protein (PAWP) does not cause Ca^{2+} release in mouse oocytes

Michail Nomikos[1,†*], Jessica R. Sanders[1,†], Maria Theodoridou[1,2], Junaid Kashir[1], Emily Matthews[1], George Nounesis[2], F. Anthony Lai[1], and Karl Swann[1]

[1]Institute of Molecular and Experimental Medicine, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK [2]National Center for Scientific Research 'Demokritos', 15310 Aghia Paraskevi, Greece

*Correspondence address. E-mail: mixosn@yahoo.com

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ABSTRACT: Mature mammalian oocytes undergo a prolonged series of cytoplasmic calcium (Ca^{2+}) oscillations at fertilization that are the cause of oocyte activation. The Ca^{2+} oscillations in mammalian oocytes are driven via inositol 1,4,5-trisphosphate (IP_3) generation. Microinjection of the sperm-derived phospholipase C-zeta (PLCζ), which generates IP_3, causes the same pattern of Ca^{2+} oscillations as observed at mammalian fertilization and it is thought to be the physiological agent that triggers oocyte activation. However, another sperm-specific protein, 'post-acrosomal WW-domain binding protein' (PAWP), has also been reported to elicit activation when injected into mammalian oocytes, and to produce a Ca^{2+} increase in frog oocytes. Here we have investigated whether PAWP can induce fertilization-like Ca^{2+} oscillations in mouse oocytes. Recombinant mouse PAWP protein was found to be unable to hydrolyse phosphatidylinositol 4,5-bisphosphate *in vitro* and did not cause any detectable Ca^{2+} release when microinjected into mouse oocytes. Microinjection with cRNA encoding either the untagged PAWP, or yellow fluorescent protein (YFP)-PAWP, or luciferase-PAWP fusion proteins all failed to trigger Ca^{2+} increases in mouse oocytes. The lack of response in mouse oocytes was despite PAWP being robustly expressed at similar or higher concentrations than PLCζ, which successfully initiated Ca^{2+} oscillations in every parallel control experiment. These data suggest that sperm-derived PAWP is not involved in triggering Ca^{2+} oscillations at fertilization in mammalian oocytes.

Key words: PAWP / PLCζ / fertilization / oocyte activation / sperm factor

Introduction

The activation of mature MII arrested oocytes involves a series of early events that initiate embryo development, following fusion of the sperm and oocyte plasma membranes. Key events of oocyte activation include second polar body emission, cortical granule exocytosis and pronuclear formation (Stricker, 1999; Carroll, 2001; Ducibella and Fissore, 2007). All the events of oocyte activation and subsequently early embryonic development during mammalian fertilization are triggered by a characteristic series of large cytoplasmic Ca^{2+} transients known as Ca^{2+} oscillations (Stricker, 1999; Ducibella and Fissore, 2007). This sperm-mediated Ca^{2+} release is caused via the generation of increased inositol 1,4,5-trisphosphate (IP_3) (Miyazaki *et al.*, 1993; Lee *et al.*, 2006). It is now widely accepted that mammalian sperm delivers some specific protein factor(s) into the oocyte cytoplasm after gamete fusion and that such factor(s) triggers the prolonged Ca^{2+} oscillations (Carroll, 2001; Lee *et al.*, 2006; Kashir *et al.*, 2010; Nomikos *et al.*, 2013a). Intracytoplasmic sperm injection (ICSI) has also been shown to trigger Ca^{2+} oscillations in mouse and human oocytes and this observation too is consistent with the idea that the sperm contains an intracellular activating factor (Tesarik and Sousa, 1994; Nakano *et al.*, 1997). The nature and identity of the 'sperm factor' and how it causes increased IP_3 production have been the key issues to be resolved in this field (Dale *et al.*, 2010; Nomikos *et al.*, 2013a).

Over the last decade, several lines of evidence suggest that the physiological sperm factor responsible for generating Ca^{2+} oscillations and subsequent oocyte activation is a testis-specific isoform of phospholipase C, named PLC-zeta, PLCζ (Saunders *et al.*, 2002; Cox *et al.*, 2002; Kouchi

[†] These authors contributed equally to this work.

et al., 2004; Yoon et al., 2008; Kashir et al., 2010; Nomikos et al., 2013a; 2013b). Microinjection of PLCζ cRNA or recombinant PLCζ protein into mouse oocytes triggered Ca^{2+} oscillations similar to those seen at fertilization (Saunders et al., 2002; Kouchi et al., 2004; Yoda et al., 2004). Injection of cognate PLCζ cRNA into human, bovine and pig oocytes also triggered a prolonged series of Ca^{2+} oscillations (Rogers et al., 2004; Yoneda et al., 2006; Cooney et al., 2010), leading to meiotic resumption, pronuclear formation and development up to the blastocyst stage (Saunders et al., 2002; Yoda et al., 2004; Lee et al., 2006; Nomikos et al., 2013a). By measuring the expression of luciferase-tagged PLCζ following microinjection of its mRNA into mouse oocytes, it has been estimated that the amount of PLCζ protein required to initiate Ca^{2+} oscillations is ~30 fg in mouse oocytes, which is within the estimates (20–50 fg) for the amount of PLCζ in a single mouse sperm (Nomikos et al., 2005; 2011a). PLCζ has been reported to be ~130 fg/sperm in bulls (Cooney et al., 2010). PLCζ has also been capable of explaining Ca^{2+} oscillations and oocyte activation after ICSI in the mouse. Extracts of the perinuclear matrix of mouse sperm were shown to contain an oocyte-activating factor and this factor was identified as PLCζ (Fujimoto et al., 2004). Further evidence that PLCζ is important in fertilization comes from findings that its levels in human sperm are either severely low, or completely absent in sperm samples associated with failed fertilization after ICSI (Heytens et al., 2009; Kashir et al., 2010). These data strongly suggest that PLCζ is the agent used by sperm to induce Ca^{2+} oscillations and oocyte activation at fertilization (Saunders et al., 2002; Nomikos et al., 2013a).

PLCζ is not the only protein factor that has been proposed to underlie oocyte activation at fertilization. Post-acrosomal WW-domain binding protein (PAWP) is a sperm-specific protein found in the post-acrosomal region of the perinuclear matrix, that underlies the plasma membrane in the sperm head (Wu et al., 2007). The post-acrosomal region of the perinuclear matrix is at the posterior end of the sperm head, and considered to be the first region of the sperm that is exposed to the oocyte cytoplasm after gamete fusion. PAWP can also be extracted from the perinuclear matrix of mammalian sperm, and microinjection of recombinant PAWP protein into porcine, bovine, and monkey oocytes has been reported to trigger oocyte activation as judged by pronuclear formation (Wu et al., 2007). In bull sperm, PAWP is present at ~80 fg/sperm, which in molar equivalents is similar to reported levels of PLCζ in bull sperm (Cooney et al., 2010). PAWP shows sequence homology to the N-terminal half of WW-domain-binding protein 2, while its C terminal half is rich in proline residues (Wu et al., 2007). In addition, the C-terminal region contains a functional PPXY consensus binding site for Group-I WW domain-containing proteins, alongside numerous unique repeating motifs (YGXPPXG) (Wu et al., 2007). The ability of PAWP to activate oocytes was blocked upon co-injection with competitive peptides corresponding to the PPXY motif derived from PAWP (Wu et al., 2007). Furthermore, in porcine oocytes injected with antibodies or competitive inhibitors against PAWP, sperm-induced pronuclear formation was not observed after ICSI (Wu et al., 2007). More significantly, it has been proposed that PAWP may be the physiological agent that activates oocyte via causing Ca^{2+} release, because PAWP injection was reported to activate *Xenopus* eggs through an associated rise in intracellular Ca^{2+} (Aarabi et al., 2010). It was also shown that PPXY-containing peptides blocked Ca^{2+} release and activation at fertilization in *Xenopus* eggs (Aarabi et al., 2010). However, to date, no studies have yet been published on whether PAWP can cause Ca^{2+} oscillations similar to those observed at fertilization in mature mammalian oocytes.

In this study, we have directly compared the ability of mouse PAWP and mouse PLCζ to generate Ca^{2+} oscillations in mouse oocytes. We find that recombinant PAWP protein, or a variety of tagged and untagged versions of PAWP cRNA are comprehensively unable to elicit any detectable increase in intracellular Ca^{2+} concentration after microinjection into mouse oocytes. This total lack of PAWP effect on oocyte Ca^{2+} concentration was evident despite PAWP being expressed at levels higher than PLCζ. In contrast, 100% of the oocytes injected with the various versions of PLCζ responded robustly by generating the cytoplasmic Ca^{2+} oscillations that are an unmistakeable characteristic of mammalian fertilization.

Materials and Methods

Cloning of PAWP expression constructs

Mouse PAWP (GenBank™ accession number BC119520) was amplified by polymerase chain reaction (PCR) from a pCR-BluntII-TOPO-PAWP plasmid (GE Healthcare) using Phusion polymerase (Finnzymes, Fisher Scientific, Loughborough, UK) and the appropriate primers to incorporate a 5′-SalI site and a 3′-NotI site and was cloned into pETMM60 to enable bacterial protein expression. The primers used were: 5′-CACCGTCGACATGG CAGTGAACCAGAACC-3′ (forward) and 5′-GGAAGCGGCCGCTCAC ATCTTAGAGCGGGGAGAGTGG-3′ (reverse). For pCR3-PAWP plasmid, mouse PAWP was amplified by PCR from the pCR-BluntII-TOPO-PAWP plasmid in the same manner using Phusion polymerase (Finnzymes) and the appropriate primers to incorporate a 5′-EcoRV site and a 3′-NotI site, and was cloned into a pCR3 vector, or a modified pCR3 vector containing an N′-terminal eYFP tag. The primers used were: 5′-AGCTGATATCATGG CAGTGAACCAGAACC-3′ (forward) and 5′-GGAAGCGGCCGCTCACA TCTTAGAGCGGGGAGAGTGG-3′ (reverse).

For pCR3-PAWP-luciferase, a three-step cloning strategy was used. Mouse PAWP was amplified by PCR from pCR-BluntII-TOPO-PAWP in the same manner using Phusion polymerase (Finnzymes) and the appropriate primers to incorporate a 5′-EcoRV site and a 3′-NotI site in which the stop codon had been removed and cloned into the pCR3 vector. The primers used were: 5′-AGCTGATATCATGGCAGTGAACCAGAACC-3′ (forward) and 5′-GGAAGCGGCCGCGCACATCTTAGAGCGGGGAGA GTGG-3′ (reverse). Finally, the firefly (*Photinus pyralis*) luciferase open reading frame was amplified from the pGL2 plasmid (Promega) with primers incorporating NotI sites and the product was cloned into the NotI site of the pCR3-PAWP plasmid. The primers used were: 5′-CACTGCGGCC GCGATGGAAGACGCCAAAAACATAAAGA-3′ (forward) and 5′-GCAG GCGGCCGCTTACAATTTGGACTTTCCGCCCTTC-3′ (reverse).

Successful cloning of the above expression vector constructs was confirmed by dideoxynucleotide-sequencing (Applied Biosystems Big-Dye Ver 3.1 chemistry and model 3730 automated capillary DNA sequencer by DNA Sequencing & Services™).

Protein expression and purification

For NusA-6xHis-fusion protein expression, *Escherichia coli* [BL21-CodonPlus(DE3)-RILP; Stratagene] was transformed with the appropriate pETMM60 plasmid, cultured at 37°C until A_{600} reached 0.6 and protein expression induced for 18 h, at 16°C with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (ForMedium). Cells were harvested (6000*g* for 10 min), resuspended in phosphate-buffered saline (PBS) containing a protease inhibitor mixture (EDTA-free; Roche) and sonicated 4 × 15 s on ice. Soluble NusA-6xHis-tagged fusion protein was purified on nickel nitrilotriacetic

acid resin following standard procedures (Qiagen), and eluted with 250 mM imidazole. Eluted proteins were dialysed overnight [10 000 molecular weight cut-off (MWCO); Pierce] at 4°C against 4 l of PBS, and concentrated with centrifugal concentrators (Sartorius; 10 000 MWCO).

SDS–PAGE and western blotting

Recombinant proteins were separated by SDS–PAGE as described previously (Nomikos *et al.*, 2005; 2011a, b). Separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) using a semidry transfer system (Trans-Blot SD; Bio-Rad) in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% v/v methanol) at 20 V for 1 h. Membranes were incubated overnight at 4°C in Tris-buffered saline, 0.1% Tween 20 containing 5% non-fat milk powder, and probed with a Penta-His monoclonal antibody (Qiagen) (1 : 5000 dilution). Detection of horseradish peroxidase-coupled secondary antibody was achieved using enhanced chemiluminescence detection (Amersham Biosciences).

Assay of PLC activity

Phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolytic activity of recombinant PLC proteins was assayed as described previously. The final concentration of PIP_2 in the reaction mixture was 220 μM, containing 0.05 μCi of [^{3}H]PIP_2. The assay conditions were optimized for linearity, requiring a 10-min incubation of 20 pmol of PLCζ protein sample at 25°C. In assays to determine dependence on PIP_2 concentration, 0.05 μCi of [^{3}H]PIP_2 was mixed with cold PIP_2 to give the appropriate final concentration. In assays examining Ca^{2+} sensitivity, Ca^{2+} buffers were prepared by EGTA/$CaCl_2$ admixture, as described previously (Nomikos *et al.*, 2005; 2011a, b).

cRNA synthesis

Following linearization of wild-type and chimeric PLCζ plasmids, cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and then was polyadenylated using the poly(A) tailing kit (Ambion), as per the manufacturer's instructions.

Preparation and handling of gametes

Female mice were superovulated and mature MII oocytes were collected 13.5–14.5 h after injection of human chorionic gonadotrophin and maintained in droplets of M2 media (Sigma) or H-KSOM under mineral oil at 37°C. Microinjection and experimental recordings of Ca^{2+} release or luciferase expression were carried out with mouse oocytes in Hepes-buffered media (H-KSOM) as described previously (Swann, 2013). All compounds were from Sigma unless stated otherwise. All procedures using animals were performed in accordance with the UK Home Office Animals Procedures Act and were approved by the Cardiff University Animals Ethics Committee.

Microinjection and measurement of intracellular Ca^{2+} and luciferase expression

Mouse oocytes were washed in M2 and microinjected with cRNA diluted in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4). The volume injected was estimated from the diameter of cytoplasmic displacement caused by the bolus injection. All injections were 3–5% of the oocyte volume. Oocytes were microinjected with the appropriate cRNA, mixed with an equal volume of 1 mM Oregon Green BAPTA dextran, OGBD (Molecular Probes) or 1 mM Rhod Dextran (Molecular Probes) in the injection buffer. For simple fluorescence recordings, oocytes were maintained in H-KSOM following microinjection and imaged on a Nikon Eclipse Ti-U microscope equipped with a cooled CCD camera (Coolsnap HQ?, Photometrics, USA) (Gonzalez-Garcia *et al.*, 2013). When both luminescence and fluorescence were to be recorded, oocytes were maintained in H-KSOM containing 100 μM luciferin, and imaged on a Nikon TE2000 or a Zeiss Axiovert 100 microscope equipped with a cooled intensified CCD cameras (Photek Ltd, UK). The luminescence (for luciferase expression) and fluorescence (for Ca^{2+} measurements) from oocytes were collected by switching back and forth between the two modes on a 10 s cycle (Campbell and Swann, 2006; Swann *et al.*, 2009). These two signals are then displayed as two separate signals over the same time-period. The fluorescent light emitted by the Ca^{2+} indicator is shown in relative units. The amount of luciferase was estimated from the luminescence from oocytes that were lysed in a luminometer, and this was compared with luminescence from recombinant luciferase protein used to generate a standard curve (Nomikos *et al.*, 2005; Swann *et al.*, 2009). A conversion factor between the luminescence from the luminometer and the luminescence from the recording system was then calculated. All live imaging experiments on oocytes were made during a 3-month period.

Results

Expression and enzymatic characterization of recombinant PAWP protein

Initial attempts to express and purify PAWP either as an untagged or as a 6xHis-tag protein using prokaryotic expression proved unsuccessful as the protein appeared to be completely insoluble, accumulating into inclusion bodies. We have recently demonstrated that NusA is an extremely effective fusion protein partner for PLCζ, significantly increasing the bacterial expression and yield of soluble PLCζ protein, as well as enhancing the stability of the purified fusion protein over time (Nomikos *et al.*, 2013b; Theodoridou *et al.*, 2013). Thus, mouse PAWP was cloned into the pETMM60 vector and purified as NusA-tagged fusion protein (Fig. 1). In addition to PAWP, both the NusA-tagged mouse PLCζ and rat PLCδ1 that we have previously characterized (Theodoridou *et al.*, 2013; Nomikos *et al.*, 2014) were expressed and purified using the same bacterial expression system, and served as comparative standards for our studies. As described in 'Materials and Methods', optimal protein production for all NusA fusion constructs required induction of protein expression with 0.1 mM IPTG for 18 h at 16°C. Following induced expression in *E. coli* and isolation by affinity chromatography, the purified protein samples were characterized. Figure 2 shows NusA-tagged PAWP, PLCζ and PLCδ1 recombinant proteins analysed by SDS–PAGE and immunoblot detection using an anti-His (penta-His) mouse monoclonal antibody. The dominant protein band with mobility corresponding to the predicted molecular mass for each construct was observed for all recombinant proteins (PAWP ~98 kDa, PLCζ ~134 kDa and PLCδ1 ~146 kDa). These major bands were also confirmed by the penta-His antibody after immunoblot analysis (Fig. 2; right panels). For PLCζ and PLCδ1, additional low molecular weight bands could be observed, which were also detected by the penta-His antibody, and are probably the result of protease degradation occurring during the stages of protein isolation.

The specific PIP_2 hydrolytic enzyme activity for each recombinant protein was determined by the standard [^{3}H]PIP_2 hydrolysis assay. The histogram in Fig. 3 summarizes the enzyme-specific activity values obtained for each recombinant protein at 1 μM (left panel) and 1 mM (right panel) Ca^{2+} concentrations. The enzymatic activities of PLCζ and PLCδ1 are in agreement with our previous observations, revealing that PLCζ exhibits a specific activity of 512 ± 58 nmol/min/mg (mean ± SEM) at 1 μM Ca^{2+} and 355 ± 35 nmol/min/mg at 1 mM Ca^{2+}. The specific activity for PLCδ1 was 411 ± 16 nmol/min/mg at

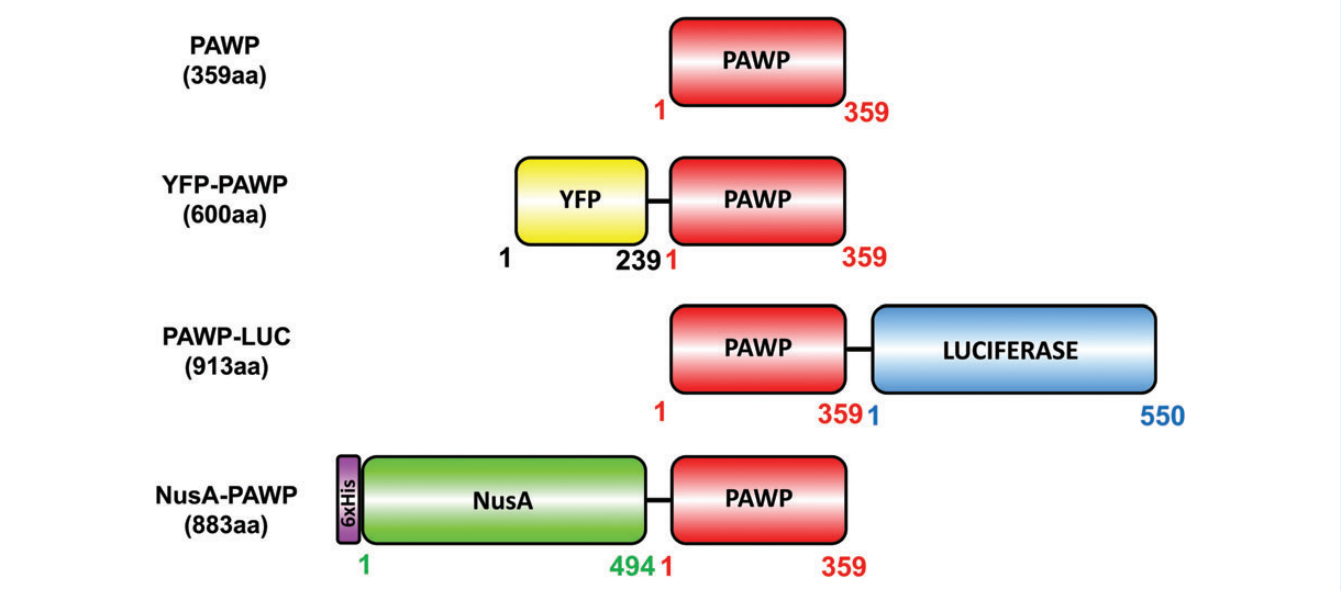

Figure 1 Schematic representations of the various PAWP expression plasmid constructs generated for cRNA and recombinant protein production, together with the respective amino acid sequence lengths of NusA, YFP, firefly luciferase and mouse PAWP.

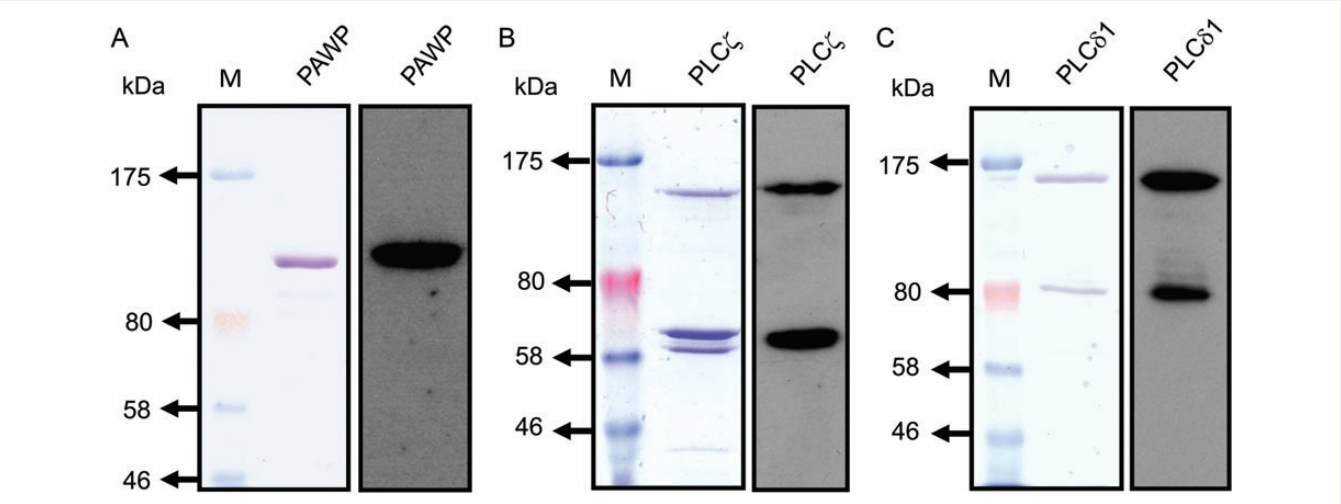

Figure 2 Expression and purification of recombinant NusA-6xHis-tagged PAWP (**A**), PLCζ (**B**) and PLCδ1 (**C**) recombinant proteins. Affinity-purified, fusion proteins (1 μg) were analysed by 7.5% SDS–PAGE followed by either Coomassie Brilliant Blue staining (left panels) or immunoblot analysis (right panels) using the Penta-His monoclonal antibody (1 : 5000 dilution).

1 μM Ca^{2+} and 2720 ± 60 nmol/min/mg at 1 mM Ca^{2+}. In contrast, PAWP did not exhibit any *in vitro* enzymatic activity at either 1 μM or 1 mM Ca^{2+} concentrations (Fig. 3).

To investigate whether PAWP has any modulatory effect on the PIP_2 hydrolytic enzyme activity of PLCζ or PLCδ1, we pre-incubated PLCζ and PLCδ1 proteins with equal (0.25 μM) or 4× fold excess (1 μM) of PAWP recombinant protein and then we tested their specific PIP_2 hydrolytic activities at 1 μM and 1 mM Ca^{2+}. As shown in Fig. 4A and B, recombinant PAWP did not show any detectable effect on the *in vitro* PIP_2 hydrolytic enzyme activities of PLCζ and PLCδ1, at either low or high Ca^{2+} concentrations.

Comparison of Ca^{2+} oscillation-inducing activities of PAWP and PLCζ in unfertilized mouse oocytes

We then directly compared the Ca^{2+} oscillation-inducing activities of recombinant NusA-tagged PLCζ and PAWP proteins in unfertilized mouse

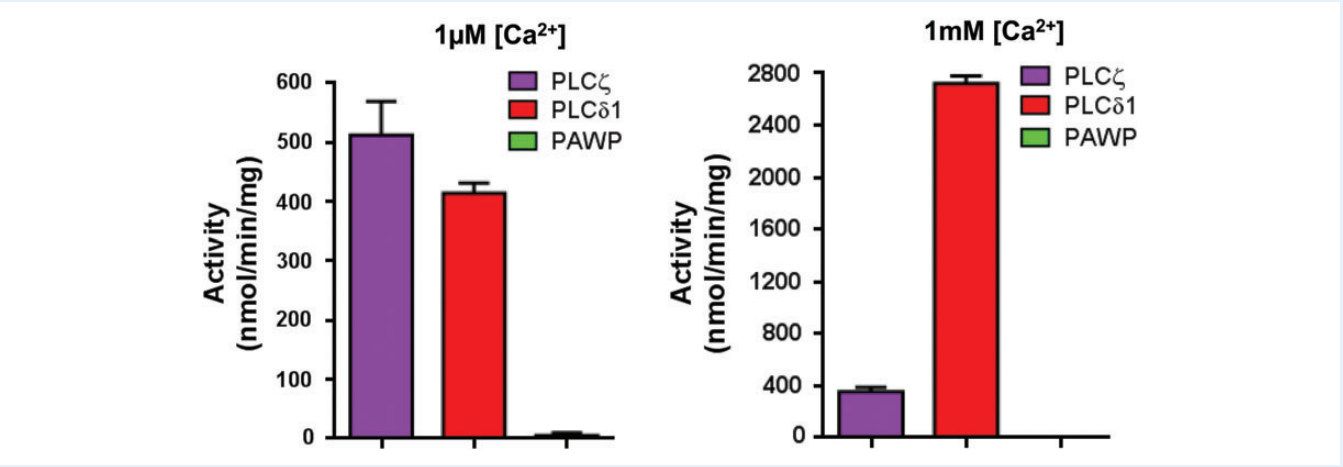

Figure 3 *In vitro* enzymatic properties of PAWP, PLCζ and PLCδ1 recombinant proteins**.** [^{3}H]PIP_2 hydrolysis activities of the purified NusA-6His-tagged PAWP, PLCζ and PLCδ1 recombinant proteins (0.25 μM). Results are mean ± SEM ($n = 4$), determined using two different preparations of recombinant protein and with each experiment performed in duplicate. In control experiments with NusA, no specific PIP_2 hydrolysis activity was observed (data not shown).

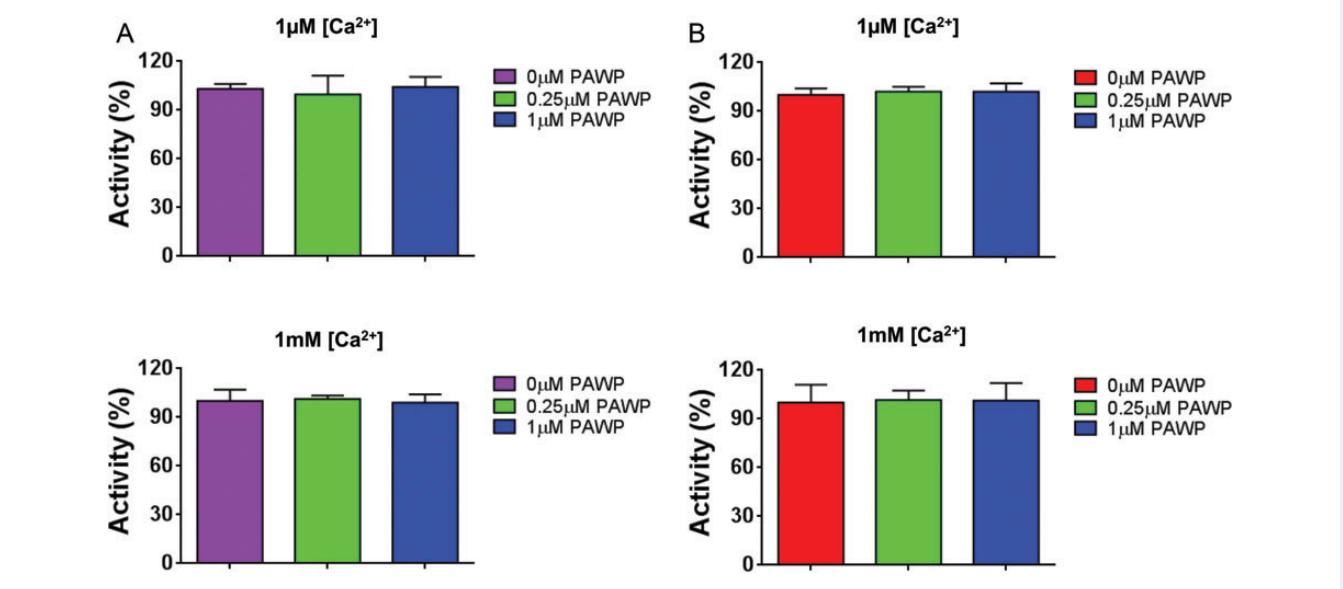

Figure 4 Effect of varying PAWP concentration on the normalized activities of PLCζ (**A**) and PLCδ1 (**B**). Results are mean ± SEM ($n = 4$), determined using two different preparations of recombinant protein and with each experiment performed in duplicate.

oocytes. As we have previously reported, NusA protein microinjection alone does not cause any Ca^{2+} changes (Nomikos *et al.*, 2013b). Microinjection of recombinant mouse PAWP protein (with a pipette concentration of 0.5 mg/ml) failed to trigger Ca^{2+} oscillations in any of the unfertilized mouse oocytes (Fig. 5A). In contrast, microinjection of recombinant mouse PLCζ protein into mouse oocytes caused a distinctive series of cytosolic Ca^{2+} oscillations (Fig. 5B) similar to those previously reported (Kouchi *et al.*, 2004).

To investigate whether the lack of Ca^{2+}-oscillation-inducing activity of recombinant NusA-tagged PAWP protein was due to the NusA moiety, we next microinjected cRNA encoding an untagged mouse PAWP into unfertilized mouse oocytes. Oocytes were microinjected with a pipette cRNA concentration of 1.25 mg/ml. As shown in Fig. 6A (left panel), untagged PAWP was unable to induce any Ca^{2+} release in mouse oocytes, in contrast with untagged PLCζ where microinjection of 0.04 mg/ml cRNA triggered high frequency Ca^{2+} oscillations similar to those observed upon microinjection of concentrated sperm extracts into mouse oocytes (Fig. 6A, right panel).

To confirm that PAWP cRNA was faithfully expressed in mouse oocytes, we microinjected mouse oocytes with cRNA encoding a YFP-PAWP fusion construct. As shown in Fig. 6B, microinjection of 1.5 mg/ml cRNA corresponding to YFP-PAWP showed high levels of expression YFP-PAWP protein in mouse oocytes (right panel), but this fusion protein was completely lacking in Ca^{2+}-oscillation-inducing

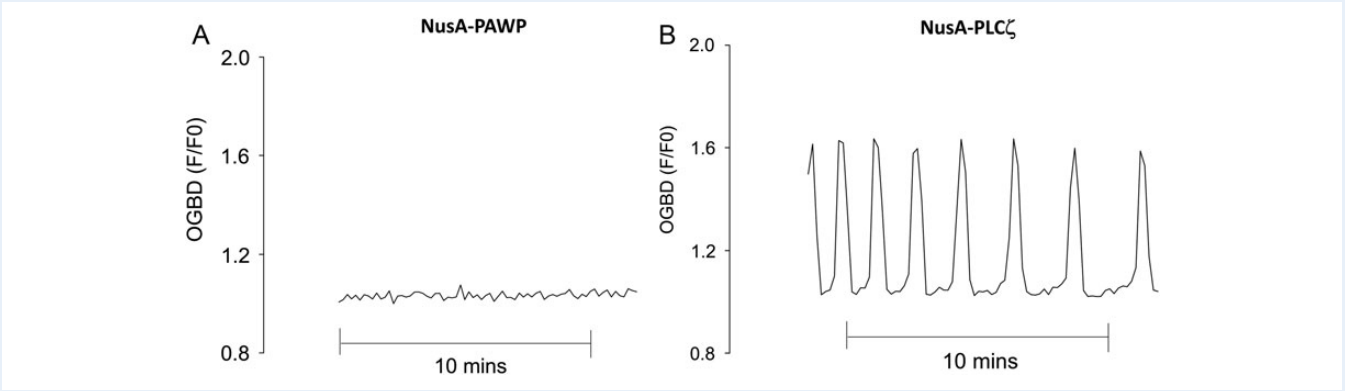

Figure 5 Samples traces are shown of Ca^{2+} levels in MII oocytes measured with OGBD (Molecular Probes) fluorescence following microinjection of proteins. Fluorescence intensities (F) are normalized by dividing by the starting or 'resting' values (F0). In (**A**), NusA-PAWP recombinant protein was injected (0.5 μg/μl in pipette) $n = 25$ oocytes, and in (**B**) NusA-PLCζ recombinant protein was injected (0.5 μg/μl in pipette) $n = 12$ oocytes (OBGC, Oregon Green BAPTA dextran.)

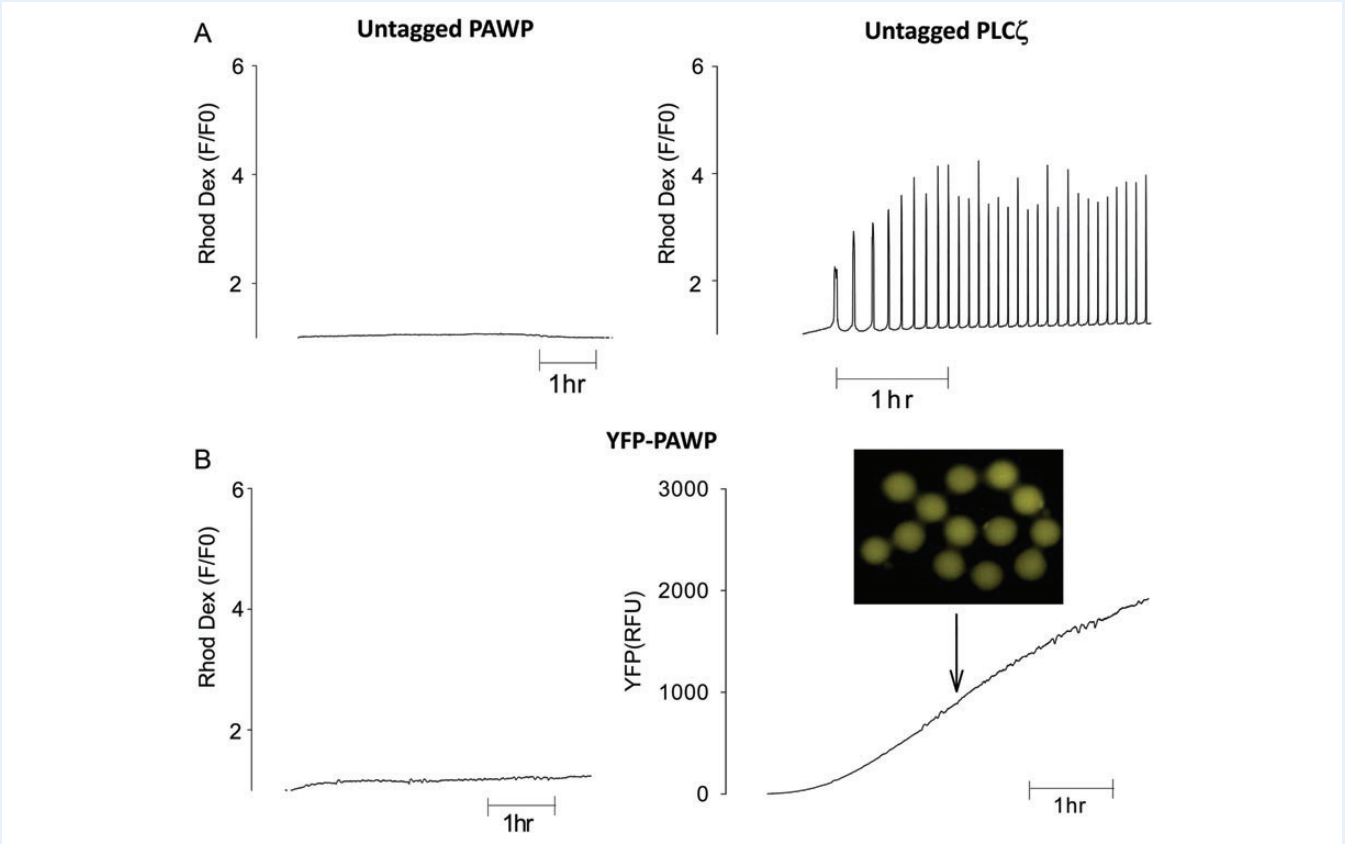

Figure 6 Sample traces of Ca^{2+} changes in MII oocytes using Rhod Dextran dye are shown following microinjection of different cRNAs. Again fluorescence intensities (F) are normalized by dividing by the starting or 'resting' values (F0). In (**A**) are examples of cRNA injections for untagged PAWP cRNA (1.25 μg/μl, $n = 28$) (left panel) and untagged PLCζ cRNA (0.04 μg/μl, $n = 24$) (right panel). In (**B**), Ca^{2+} levels are shown in an MII oocyte following microinjection of YFP-PAWP cRNA (1.5 μg/μl, $n = 23$) (left panel), and on the right-hand panel the YFP fluorescence is shown for the same oocyte, as well as an image of the group of 13 YFP-PAWP cRNA-injected oocytes all exhibiting successful recombinant expression of YFP-PAWP protein (right panel, inset).

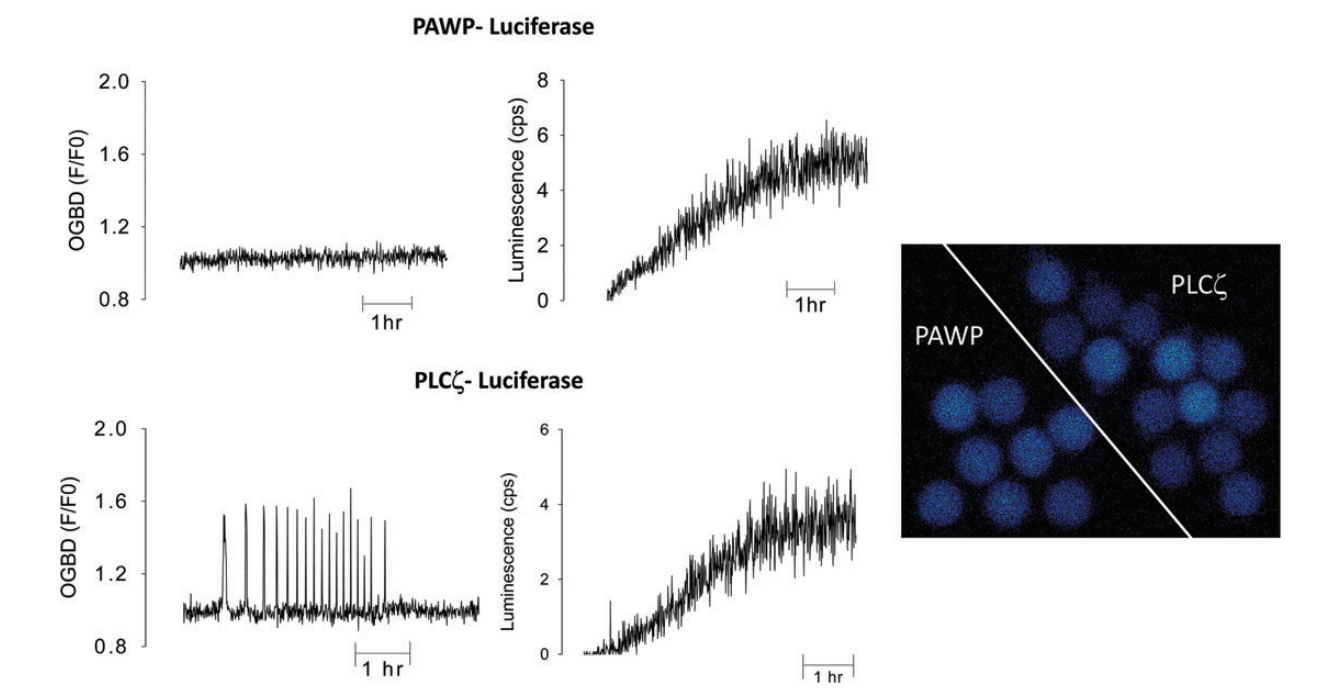

Figure 7 Sample traces of Ca^{2+} levels in oocytes measured using OGBD fluorescence alongside expression of luciferase. Top panel shows recordings from an oocyte injected with PAWP-luciferase cRNA (0.08 μg/μl). The left-hand trace is the OGBD fluorescence and the right-hand trace is the expression from the same oocyte represented by luminescence of luciferase. Average luminescence at the end of all such recordings was calculated at 5.6 cps giving a PAWP-luciferase protein amount of ~89 fg per oocyte ($n = 18$). Bottom panel shows recordings from an oocyte injected with PLCζ-luciferase (firefly) RNA (0.06 μg/μl). Again the Ca^{2+} trace is on the left and the luciferase luminescence on the right. The average luminescence at the end of all such traces was calculated at 3.9 cps giving a PLCζ-Luciferase protein amount of ~59 fg per oocyte ($n = 13$). The image shows the luminescence signals from the oocytes microinjected and recorded at the same time with PAWP-luciferase or PLCζ-luciferase cRNA. The image represents 30 min of light integration starting at 4 h.

activity (left panel). The YFP-PAWP protein did not show any obvious subcellular localization pattern but appeared to remain evenly dispersed throughout the oocyte cytoplasm (Fig. 6B, right panel). Whilst none of the YFP-PAWP cRNA-injected oocytes formed pronuclei, we noticed that 5/23 oocytes did form second polar bodies after 5 h. This was similar to the untagged PAWP cRNA injections, where 4/28 oocytes formed second polar bodies.

Finally, using another approach to directly compare the Ca^{2+} oscillation-inducing abilities of PAWP and PLCζ in relation to their relative expression levels in mouse oocytes (as we have demonstrated previously with PLCζ), we generated a PAWP fusion construct in which PAWP was C-terminally tagged with firefly luciferase. This strategy to measure PAWP-luciferase luminescence in living cells enables real-time monitoring of relative protein expression, while concurrently measuring Ca^{2+} levels (Swann *et al.*, 2009). Prominent Ca^{2+} oscillations were observed in PLCζ cRNA-injected mouse oocytes at a luminescence reading of 3.9 counts per second, corresponding to protein expression of ~59 fg PLCζ/oocyte (Fig. 7, bottom trace). Recombinant PLCζ-luc triggered somewhat higher frequency Ca^{2+} oscillations when expressed at 360 fg/oocyte (Fig. 8, bottom trace). However, microinjection of PAWP-luc completely failed to cause a detectable Ca^{2+} increase in any injected oocytes, either when the recombinant protein was expressed at 89 fg/oocyte (Fig. 7, top trace), or at 1.6 pg/oocyte (Fig. 8, top trace). These data suggest that recombinant mouse PAWP protein is unable to initiate any Ca^{2+} increase in mouse oocytes. We also observed that none of the PAWP-luc injected oocytes formed pronuclei. However, we found that 6/45 PAWP-luc injected oocytes formed a second polar body. This effect was unrelated to the amount of PAWP-luc protein expressed, but the rate was marginally higher than the rate of second polar body formation with control luciferase-expressing oocytes (1/19). These data suggest there may be a minor effect of PAWP in promoting second polar body formation. However, since none of the PAWP-injected oocytes showed any sign of a Ca^{2+} increase, this minor effect is unrelated to changes in intracellular Ca^{2+} signalling.

Discussion

In all animal species studied to date, oocyte activation involves increases in the concentration of oocyte cytosolic Ca^{2+}, which are both necessary and sufficient for stimulating embryo development (Stricker, 1999). In mammals, such increases consist of a series of Ca^{2+} oscillations that last several hours (Miyazaki *et al.*, 1993; Nomikos *et al.*, 2013a). To date, only one sperm-derived molecule, PLCζ, has been shown to cause Ca^{2+} oscillations similar to those seen at fertilization (Saunders *et al.*, 2002; Lee *et al.*, 2006). Nevertheless, it has been suggested by one laboratory group that the sperm protein PAWP is the agent used by the sperm to activate development in mammals (Wu *et al.*, 2007; Aarabi *et al.*, 2010). This suggestion is partly based on the finding that

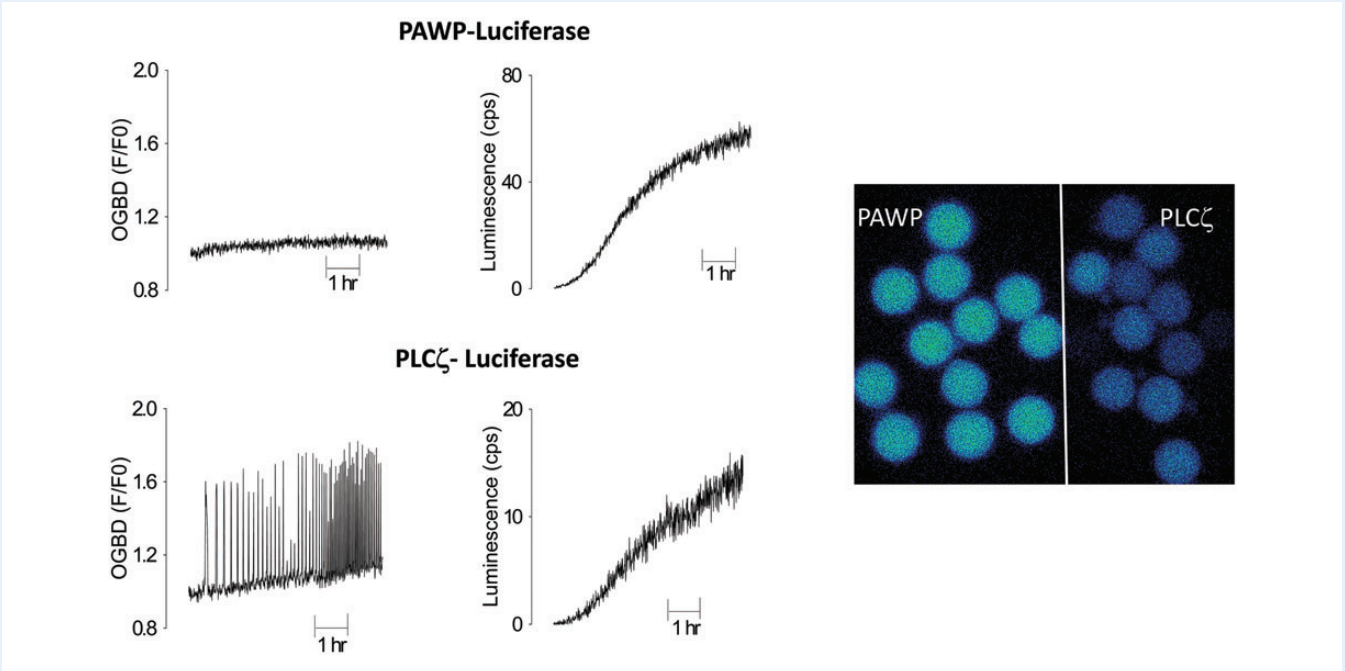

Figure 8 Sample traces of Ca^{2+} levels in oocytes measured using OGBD fluorescence alongside expression of luciferase. OGBD fluorescence and luciferase luminescence are plotted and displayed as in Fig. 7. Top panel shows recordings from an oocyte injected with PAWP-luciferase (firefly) cRNA (1.25 μg/μl) where the luminescence at the end of all such traces was calculated at 56 cps giving a PAWP-luciferase protein amount of ~1.6 pg per oocyte ($n = 58$). Bottom panel shows recordings from an oocyte microinjected with PLCζ-luciferase (firefly) RNA (1 μg/μl), where the average luminescence at the end of all such traces was calculated as 18 cps, giving a PLCζ-luciferase protein amount of ~0.36 pg per oocyte ($n = 57$). The image shows the luminescence signals from the oocytes injected with the different cRNAs for PAWP-luciferase or PLCζ-luciferase with 30 min of integration starting at 4 h.

PAWP can cause pronuclear formation when injected into bovine, monkey, pig and Xenopus oocytes (Wu *et al.*, 2007), and that its injection into Xenopus oocytes can cause an increase in intracellular Ca^{2+} concentrations (Aarabi *et al.*, 2010). These data specifically beg the obvious question as to whether PAWP can trigger the appropriate pattern of Ca^{2+} oscillations in a mammalian oocyte, which has yet to be clearly resolved.

Therefore, in this study, we have utilized mouse oocytes to address this question regarding PAWP's efficacy, as such cells are the most studied model system for signal transduction at fertilization in mammals, and there is extensive knowledge of the mechanism of Ca^{2+} oscillations and the downstream effectors (Miyazaki *et al.*, 1993; Lee *et al.*, 2006; Ducibella and Fissore, 2007). In an extensive series of experiments, we have found that mouse recombinant PAWP protein was unable to cause Ca^{2+} oscillations in mouse oocytes. Furthermore, expressing PAWP in mouse oocytes by injecting the corresponding cRNA did not lead to any form of Ca^{2+} increase. This was the case regardless of whether we expressed a C- or N-terminal tagged construct of PAWP, or whether we injected untagged PAWP. All of these methods have been successfully used to express PLCζ in mouse oocytes in a way that allows it to trigger sustained cytoplasmic Ca^{2+} oscillations. The amount of PAWP we introduced into mouse oocytes was at least as much, and sometimes considerably more than that used in previous studies. PAWP was previously reported to be effective in activating pig, bovine and monkey oocytes at final concentration ranges of 100 fg to 2.5 pg/oocyte (Wu *et al.*, 2007). The expression range calculated for our PAWP-luc experiments was also from ~100 fg to ~2 pg/oocyte. For the YFP-PAWP experiments, we do not have an absolute calibration. However, since fluorescent proteins need to be expressed at ~1 μM to be detectable (Niswender *et al.*, 1995) the final expression levels of YFP-PAWP in our experiments would likely be ~10 pg/oocyte. Mouse oocytes are several times smaller than pig or cow oocytes so the effective concentrations that we have used would likely be several times higher than those used in pig, bovine and monkey oocytes. We monitored expression from just after the injection of cRNA, when expression was undetectable, all the way up to maximal values (0.1–10 pg). However, we were unable to observe any Ca^{2+} oscillations throughout the entire period of expression, effectively monitoring the effects of PAWP over a wide range of concentrations from zero to many times higher than that reported in previous studies. Consequently, the current data suggest that PAWP is ineffective at causing Ca^{2+} release in mammalian oocytes over a wide range of concentrations. The only PAWP effect we noticed in some of our experiments was a slight increase in the number of oocytes that formed second polar bodies after PAWP injection. This was a small and inconsistent effect, and not associated with Ca^{2+} increases, and so was difficult to investigate any further.

PAWP is an alkaline protein that shares sequence homology to the N-terminal half of WW domain-binding protein 2, while the C-terminal half is rich in proline residues. PAWP does not have any predicted enzymatic activity, and our data suggest that it does not possess any PLC hydrolytic activity, nor the ability to act as a generic activator of PLC activity. So, it may be reasonable to assume that PAWP mediates its effects in oocytes via interaction with other proteins. It has been suggested that PAWP effects are via an interaction with Yes associated proteins that

ultimately work through a Src-like kinase, and hence PLCγ (Wu et al., 2007). Previous studies have shown that artificial stimulation of the PLCγ pathway, via exogenous expression of growth factor receptors, causes Ca^{2+} oscillations in mouse oocytes (Mehlmann et al., 1998). Hence, for PAWP to mediate its effects via this pathway, it would be expected to generate IP_3 and Ca^{2+} oscillations. However, since we found no evidence for any PAWP-induced Ca^{2+} oscillations in mouse oocytes under the same conditions where PLCζ was fully effective, and using the same methods and set of tagged constructs that are fully functional with PLCζ, it seems unlikely that PAWP mediates any of its proposed oocyte-activating ability via this pathway. It should also be noted that injecting excess SH2 domains to block PLCγ-mediated signalling in mouse oocytes, does not block Ca^{2+} oscillations in fertilizing mouse oocytes (Mehlmann et al., 1998). Hence, if PAWP mediates any potential effects via PLCγ, then its role in physiological activation during fertilization would be questionable.

Although we have seen no sign of PAWP causing a Ca^{2+} increase in mouse oocytes, previous work has shown that PAWP injection into *Xenopus* oocytes caused an increase in Ca^{2+} as measured by increases in Ca^{2+} green fluorescence (Aarabi et al., 2010). However, in the study by Aarabi et al., it was not clear whether PAWP triggered the same type of Ca^{2+} increase that has previously been reported for fertilization in this species. In *Xenopus* oocytes, the sperm stimulates a distinctive and regenerative Ca^{2+} wave that crosses the oocyte in ~5 min (Fontanilla and Nuccitelli, 1998). Such a distinctive Ca^{2+} wave is also stimulated by injection of IP_3 (Busa et al., 1985), or by mammalian cytosolic sperm extracts (Wu et al., 2001). However, the PAWP-induced Ca^{2+} increase did not show wave-like characteristics. Nevertheless, it was claimed that PAWP in *Xenopus* and mammalian oocytes is relevant to fertilization, because the ability of PAWP, or sperm, to activate oocytes is blocked by prior injection of PPXY motif-containing peptides (Aarabi et al., 2010). However, the specificity of these peptides is unclear since there were no controls used to investigate whether these peptides exert an inhibitory action upon pronuclear formation itself. It remains possible that the inhibitory effects of these peptides, in mammalian oocytes at least, are mediated downstream of Ca^{2+} signalling events which involve a range of protein kinases (Ducibella and Fissore, 2007). Since there appears to be a negligible effect of PAWP on mouse oocytes, it is difficult to test any other role for this protein in this species. Regardless of how PAWP may mediate the previously reported effects in oocytes, our present data clearly suggest that PAWP does not initiate the Ca^{2+} oscillations required to activate the mouse oocyte at fertilization. Furthermore, unlike PLCζ, our current data suggests that PAWP cannot be used as an agent to induce artificial oocyte activation in mammals.

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Author's roles

M.N., G.N., F.A.L. and K.S. devised the project strategy. M.N., F.A.L. and K.S. designed the experiments, which were performed by M.N., J.R.S, J.K., E.M., M.T. and M.N., F.A.L. and K.S. prepared the manuscript.

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Conflict of interest

All authors declare that no conflict of interest exists.

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