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

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

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

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

Submitted on May 23, 2014; resubmitted on July 7, 2014; accepted on July 18, 2014

ABSTRACT: Mature mammalian oocytes undergo a prolonged series of cytoplasmic calcium (Ca\textsuperscript{2+}) oscillations at fertilization that are the cause of oocyte activation. The Ca\textsuperscript{2+} oscillations in mammalian oocytes are driven via inositol 1,4,5-trisphosphate (IP\textsubscript{3}) generation. Microinjection of the sperm-derived phospholipase C-zeta (PLC\textsubscript{ζ}), which generates IP\textsubscript{3}, causes the same pattern of Ca\textsuperscript{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\textsuperscript{2+} increase in frog oocytes. Here we have investigated whether PAWP can induce fertilization-like Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} increases in mouse oocytes. The lack of response in mouse oocytes was despite PAWP being robustly expressed at similar or higher concentrations than PLC\textsubscript{ζ}, which successfully initiated Ca\textsuperscript{2+} oscillations in every parallel control experiment. These data suggest that sperm-derived PAWP is not involved in triggering Ca\textsuperscript{2+} oscillations at fertilization in mammalian oocytes.

Key words: PAWP / PLC\textsubscript{ζ} / 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\textsuperscript{2+} transients known as Ca\textsuperscript{2+} oscillations (Stricker, 1999; Ducibella and Fissore, 2007). This sperm-mediated Ca\textsuperscript{2+} release is caused via the generation of increased inositol 1,4,5-trisphosphate (IP\textsubscript{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\textsuperscript{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\textsuperscript{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\textsubscript{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\textsuperscript{2+} oscillations and subsequent oocyte activation is a testis-specific isoform of phospholipase C, named PLC-zeta, PLC\textsubscript{ζ} (Saunders et al., 2002; Cox et al., 2002; Kouchi...
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 PLC\(\varepsilon\) 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 PLC\(\varepsilon\). In contrast, 100% of the oocytes injected with the various versions of PLC\(\varepsilon\) 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\(^{\text{TM}}\) accession number BC119520) was amplified by polymerase chain reaction (PCR) from a cCR-BluntII-TOPO-PAWP plasmid (GE Healthcare) using Phusion polymerase (Finnzymes, Fisher Scientific, Loughborough, UK) and the appropriate primers to incorporate a 5'-Sall site and a 3'-Not site and was cloned into pETMM60 to enable bacterial protein expression. The primers used were: 5'-CACCCTCGACATGG CAGTGAACCAAGC-3' (forward) and 5'-GGAAAGCGCGGAGAGACG-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'-Not 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 CAGTGAACCAAGC-3' (forward) and 5'-GGAAAGCGCGGAGAGACG-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'-Not site in which the stop codon had been removed and cloned into the pCR3 vector. The primers used were: 5'-AGCTGATATCATGG CAGTGAACCAAGC-3' (forward) and 5'-GGAAAGCGCGGAGAGACG-3' (reverse).

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

Protein expression and purification

For NusA-6xHis-fusion protein expression, Escherichia coli [BL21-CodonPlus(DE3)-RILP; Stratagenec] 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 \(\beta\)-D-1-thiogalactopyranoside (IPTG) (ForMedium). Cells were harvested (6000g for 10 min), resuspended in phosphate-buffered saline (PBS) containing a protease inhibitor mixture (EDTA-free; Roche) and sonicated 4 x 15 s on ice. Soluble NusA-6xHis-tagged fusion protein was purified on nickel nitrotriacetic
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 1 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 horse-radish peroxidase-coupled secondary antibody was achieved using enhanced chemiluminescence detection (Amersham Biosciences).

**Assay of PLC activity**

Phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolytic activity of recombinant PLC proteins was assayed as described previously. The final concentration of PIP2 in the reaction mixture was 220 μM, containing 0.05 μCi of [3H]PIP2. 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 PIP2 concentration, 0.05 μCi of [3H]PIP2 was mixed with cold PIP2 to give the appropriate final concentration. In assays examining Ca2+ sensitivity, Ca2+ buffers were prepared by EGTA/CaCl2 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. Following microinjection and isolation by affinity chromatography, the purified protein samples were characterized. Figure 2 shows NusA-tagged PLCζ protein samples were characterized. Figure 2 shows NusA-tagged PLCζ and PLCζ1 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 PLCζ1 ~ 146 kDa). These major bands were also confirmed by the penta-His antibody after immunoblot analysis (Fig. 2, right panels). For PLCζ and PLCζ1, 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 PIP2 hydrolytic enzyme activity for each recombinant protein was determined by the standard [3H]PIP2 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) Ca2+ concentrations. The enzymatic activities of PLCζ and PLCζ1 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 Ca2+ and 355 ± 35 nmol/min/mg at 1 mM Ca2+. The specific activity for PLCζ1 was 411 ± 16 nmol/min/mg at

**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 pETM60 vector and purified as NusA-tagged fusion protein (Fig. 1). In addition to PAWP, both the NusA-tagged mouse PLCζ and rat PLCζ1 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 for 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 PLCζ1 recombinant proteins analysed by SDS–PAGE and immunoblot detection using an anti-His (penta-His) mouse monoclonal antibody. The transient 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 PLCζ1 ~ 146 kDa). These major bands were also confirmed by the penta-His antibody after immunoblot analysis (Fig. 2, right panels). For PLCζ and PLCζ1, 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 PIP2 hydrolytic enzyme activity for each recombinant protein was determined by the standard [3H]PIP2 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) Ca2+ concentrations. The enzymatic activities of PLCζ and PLCζ1 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 Ca2+ and 355 ± 35 nmol/min/mg at 1 mM Ca2+. The specific activity for PLCζ1 was 411 ± 16 nmol/min/mg at
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 PLC\(\zeta\) or PLC\(\delta\), we pre-incubated PLC\(\zeta\) and PLC\(\delta\) 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 PLC\(\zeta\) and PLC\(\delta\), at either low or high Ca\(^{2+}\) concentrations.

### Comparison of Ca\(^{2+}\) oscillation-inducing activities of PAWP and PLC\(\zeta\) in unfertilized mouse oocytes

We then directly compared the Ca\(^{2+}\) oscillation-inducing activities of recombinant NusA-tagged PLC\(\zeta\) and PAWP proteins in unfertilized mouse oocytes. Figure 1 and Figure 2 illustrate the 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 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), PLC\(\zeta\) (B) and PLC\(\delta\) (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).
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 PLC$\zeta$ 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 PLC$\zeta$, 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 \(\mu\)g/\(\mu\)l in pipette) \(n = 25\) oocytes, and in (B) NusA-PLC\(_{\gamma}\) recombinant protein was injected (0.5 \(\mu\)g/\(\mu\)l 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 \(\mu\)g/\(\mu\)l, \(n = 28\)) (left panel) and untagged PLC\(_{\gamma}\) cRNA (0.04 \(\mu\)g/\(\mu\)l, \(n = 24\)) (right panel). In (B), Ca\(^{2+}\) levels are shown in an MII oocyte following microinjection of YFP-PAWP cRNA (1.5 \(\mu\)g/\(\mu\)l, \(n = 23\)) (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).
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 PLC_ζ in relation to their relative expression levels in mouse oocytes (as we have demonstrated previously with PLC_ζ), 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 PLC_ζ cRNA-injected mouse oocytes at a luminescence reading of 3.9 counts per second, corresponding to protein expression of ~59 fg PLC_ζ/oocyte (Fig. 7, bottom trace). Recombinant PLC_ζ-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, PLC_ζ, 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 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 PLC_ζ-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 PLC_ζ-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 PLC_ζ-luciferase cRNA. The image represents 30 min of light 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 PLC\(\zeta\)- 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 (Wue et al., 2007). The expression range calculated for our PAWP-luc experiments was also from \(~100 \text{ fg}\) to \(~2 \text{ pg/oocyte}\). For the YFP-PAWP experiments, we do not have an absolute calibration. However, since fluorescent proteins need to be expressed at \(~1 \mu\text{M}\) to be detectable (Niswender et al., 1995) the final expression levels of YFP-PAWP in our experiments would likely be \(~10 \text{ pg/oocyte}\). 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.

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 \(\mu\text{g/}\mu\text{l}\)) where the luminescence at the end of all such traces was calculated at 56 cps giving a PAWP-luciferase protein amount of \(~1.6 \text{ pg per oocyte}\) (\(n = 58\)). Bottom panel shows recordings from an oocyte microinjected with PLC\(\zeta\)-luciferase (firefly) RNA (1 \(\mu\text{g/}\mu\text{l}\)), where the average luminescence at the end of all such traces was calculated as 18 cps, giving a PLC\(\zeta\)-luciferase protein amount of \(~0.36 \text{ pg per oocyte}\) (\(n = 57\)). The image shows the luminescence signals from the oocytes injected with the different cRNAs for PAWP-luciferase or PLC\(\zeta\)-luciferase with 30 min of integration starting at 4 h.
ultimately work through a Src-like kinase, and hence PLC\(\gamma\) (Wu et al., 2007). Previous studies have shown that artificial stimulation of the PLC\(\gamma\) 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 PLC\(_\gamma\) was fully effective, and using the same methods and set of tagged constructs that are fully functional with PLC\(_\gamma\), 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 PLC\(_\gamma\)-mediated signaling 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 PLC\(_\gamma\), 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 \(\approx 5\) 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\(^{2+}\) signaling 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 PLC\(_\gamma\), our current data suggests that PAWP cannot be used as an agent to induce artificial oocyte activation in mammals.

**Acknowledgements**

We thank Matilda Katan (University College London) for providing the rat PLC\(\delta I\) clone.

**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.

**Funding**

This work was supported by a Wellcome Trust grant (number 090063/Z/09/Z). J.R.S holds a research scholarship supported by Cardiff University School of Medicine.

**Conflict of interest**

All authors declare that no conflict of interest exists.

**References**


Miyazaki S, Shirakawa H, Nakada K, Honda Y. Essential role of the inositol 1,4,5-trisphosphate receptor/Ca\(^{2+}\) release channel in Ca\(^{2+}\) waves and
PAWP fails to trigger oocyte Ca\(^{2+}\) oscillations

947


