Male infertility-linked point mutation reveals vital binding role for the C2 domain of sperm PLCζ

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Abbreviations: phospholipase C-zeta, PLCζ; calcium, Ca^{2+}; phosphatidylinositol 4,5-bisphosphate, PIP_2; inositol 1,4,5-trisphosphate, IP_3

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ABSTRACT

Sperm-specific phospholipase C zeta (PLCζ) is widely considered to be the physiological stimulus that evokes intracellular calcium (Ca^{2+}) oscillations that are essential for the initiation of egg activation during mammalian fertilization. A recent genetic study reported a male infertility case that was directly associated with a point mutation in PLCζ C2 domain, where an isoleucine residue had been substituted with a phenylalanine (I489F). Here in, we have analysed the effect of this mutation on the *in vivo* Ca^{2+} oscillation-inducing activity and the *in vitro* biochemical properties of human PLCζ. Microinjection of cRNA or recombinant protein corresponding to PLCζ^{I489F} mutant at physiological concentrations completely failed to cause Ca^{2+} oscillations and trigger development. However, this infertile phenotype could be effectively rescued by microinjection of relatively high (non-physiological) amounts of recombinant mutant PLCζ^{I489F} protein, leading to Ca^{2+} oscillations and egg activation. Our *in vitro* biochemical analysis suggested that PLCζ^{I489F} mutant displayed similar enzymatic properties but dramatically reduced binding to PI(3)P and PI(5)P-containing liposomes compared to wild-type PLCζ. Our findings highlight the importance of PLCζ at fertilization and the vital role of the C2 domain in PLCζ function, possibly due to its novel binding characteristics.

INTRODUCTION

In mammalian oocytes (eggs), the fertilizing sperm evokes a series of pre-programmed biochemical and morphological events collectively known as 'egg activation'. It is now well established in all mammalian species studied, that the earliest step of egg activation involves marked increases in the levels of the egg cytosolic calcium concentration [Ca^{2+}], which are both necessary and sufficient for activation and early embryonic development [1-3]. Despite recent controversies, a gamete-specific phospholipase C (PLC) isoform, PLCζ is widely considered as the physiological stimulus that induces the characteristic series of large cytoplasmic Ca^{2+} transients, known as Ca^{2+} oscillations, within the fertilizing oocyte [3-8]. Sperm PLCζ is delivered from the fertilizing sperm into the egg cytoplasm, catalyses the hydrolysis of its membrane-bound phospholipid substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2), triggering the cytoplasmic Ca^{2+} oscillations through the inositol 1,4,5-trisphosphate ($InsP_3$) signaling pathway [3, 4, 9]. PLCζ appears to be the smallest currently known mammalian PLC isozyme, with the most basic domain organization amongst all PLC isoforms. PLCζ consists of four EF hand domains at the N-terminus, the characteristic X and Y catalytic domains in the centre, followed by a C-terminal C2 domain [4, 9]. Thus, PLCζ possesses a similar domain organization to PLCδ1 with the remarkable exception that it lacks an N-terminal pleckstrin homology (PH) domain [9]. The notable lack of a PH domain, makes it unclear how this sperm-specific PLC isoform directly interacts and targets biological membranes. A recent report, suggested that the N-terminal lobe of the EF-hand domain of PLCζ together with its positively charged XY-linker, has an essential role to provide a tether that facilitates proper PIP_2 substrate access and binding in the PLCζ active site [10]. However, the exact mechanism that PLCζ targets the PIP_2-containing membrane is still unknown.

Evidence for the clinical importance of PLCζ has been provided by studies that have directly linked reduced expression levels and abnormal forms of PLCζ with male infertility [5, 11-15]. A very recent genetic study, using whole-exome sequencing analysis identified a homozygous missense mutation in the PLCζ gene of two infertile brothers from Tunisia, presenting oocyte activation failure [8]. This mutation was located in the C2 domain of PLCζ, where an isoleucine at position I489 had been replaced with a phenylalanine (I489F), (Fig. 1A) [8]. Interestingly, this is the first male infertility-linked PLCζ point mutation to be reported within the C2 domain of PLCζ, a domain which although is well known to be essential for PLCζ function, its exact role still remains unclear. It was shown that I489F mutation results in

some loss of Ca^{2+} oscillation-inducing activity of PLCζ in eggs, but the degree of loss of activity was not quantified and the poor ability of the PLCζ mutant to trigger Ca^{2+} oscillations was not extensively characterized [8].

In the present study we introduced the infertility-linked PLCζ I489F mutation into human PLCζ sequence and we analysed the effect of this mutation on both the *in vivo* Ca^{2+} oscillation-inducing activity and the *in vitro* biochemical/enzymatic properties of human PLCζ. For comparison, cRNA encoding luciferase-tagged versions of wild-type and $PLC\zeta^{I489F}$ mutant or bacterially-expressed recombinant proteins were microinjected into unfertilised mouse and bovine eggs. Circular Dichroism (CD) spectroscopy was used to investigate whether the I489F mutation interferes with the proper folding of the C2 domain. The enzymatic and biochemical properties of $PLC\zeta^{WT}$ and $PLC\zeta^{I489F}$ mutant were analyzed using an *in vitro* $[^3H]PIP_2$ hydrolysis and liposome binding assays.

MATERIALS AND METHODS

Plasmid construction

Human PLCζ-luciferase in pCR3 vector [5] was subjected to site-directed mutagenesis (QuikChange II; Stratagene) to generate the $PLC\zeta^{I489F}$ mutant. $PLC\zeta^{WT}$ and $PLC\zeta^{I489F}$ mutant were amplified by PCR from the corresponding pCR3 plasmid using Phusion polymerase (Finnzymes) and the appropriate primers to incorporate a 5'-SalI site and a 3'-NotI site and were cloned into a modified pET expression vector (pETMM41). The primers used for the amplification of $PLC\zeta^{WT}$ and $PLC\zeta^{I489F}$ mutant were: 5'-CCTAGTCGACATGGAAATGAGATGGTTTTTGTC-3' (forward) and 5'-CTAAGCGGCCGCTCATCTGACGTACCAAACATAAA-3' (reverse). Similarly to the full -length PLCζ constructs, the C2 domains (480-608aa) of $PLC\zeta^{WT}$ and $PLC\zeta^{I489F}$ mutant were amplified by PCR from the aforementioned corresponding pCR3 plasmids using Phusion polymerase (Finnzymes) and the appropriate primers to incorporate a 5'-SalI site and a 3'-NotI site and were cloned into pETMM41 vector. The primers used for the amplification of $C2\zeta^{WT}$ and $C2\zeta^{I489F}$ mutant were: 5'-CACCGTCGACATGCCAATTACACTTACAATAAGG-3' (forward) and 5'-CTAAGCGGCCGCTCATCTGACGTACCAAACATAAA-3' (reverse). Successful mutagenesis and cloning of the above constructs was confirmed by dideoxynucleotide sequencing (Applied Biosystems Big-Dye Version 3.1 chemistry and model 3730 automated capillary DNA sequencer by DNA Sequencing & ServicesTM).

cRNA synthesis

Luciferase-tagged PLCζ^{WT} and PLCζ^{I489F} constructs were linearized by restriction digests and then cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and a poly(A)tailing kit (Ambion), as per manufacturer instructions.

Protein expression and purification

E.coli (BL21-CodonPlus(DE3)-RILP; Stratagene) cells were transformed with the appropriate pETMM41 construct and cultured at 37°C until the A600 reached 0.6. Then protein expression was induced for 18 hours at 16°C with 0.1 mM isopropyl *β*-D-thiogalactopyranoside (IPTG; ForMedium). Induced cells were then harvested by centrifugation at 6,000 *g* for 10 minutes at 4°C and resuspended in ice-cold amylose column buffer [10 mM tris HC1 pH 7.4, 200 mM NaCl, l mM EDTA and protein inhibitor mixture (Roche)]. Then the resuspended cells were sonicated four times for 15 seconds on ice. After 20 minutes centrifugation at 20,000 *g* at 4°C, to remove the insoluble proteins, the soluble MBP-tagged fusion proteins were purified by affinity chromatography using amylose resin column following standard procedures (New England Biolabs). Eluted proteins were then dialyzed and concentrated using centrifugal concentrators (Sartorius; 10,000 molecular weight cut-off).

SDS-PAGE and Western blot

Recombinant MBP fusion protein were separated by SDS-PAGE and immunoblot analysis was performed as described previously [10]. Proteins were probed with a monoclonal penta-His antibody (1:5,000 dilution).

Preparation and handling of mouse oocytes

Mature MII oocytes (eggs) were collected from female MF1 mice (Envigo Ltd) of 6-8 weeks old, 15 hours after injection with 10 IU human chorionic gonadotrophin (hCG). Approximately 48 hours before hCG injection mice were injected with 10 IU pregnant mare's serum gonadotrophin. Following collection, cumulus cells were removed using hyaluronidase treatment and eggs were maintained in M2 media (Sigma Aldrich) under mineral oil at 37°C until use. Injected eggs were transferred for development in KSOM Media (Embryomax by Millipore). All animal work was conducted according to Home Office Licensing procedures and approved by the Animals Ethics Committee at Cardiff University.

Preparation and handling of bovine oocytes

Ovaries were collected after slaughter from the local abattoir and transported to the laboratory at 24 ^{o}C in PBS (Sigma P4417) within 1 hour. Cumulus oocyte complexes were collected by slashing the surface of the ovary with a scalpel in Medium 199 – Hepes buffered and the solution was passed through a 100-micron mesh filter in order to retain the cumulus oocyte complexes (COC). COC were placed in maturation media for 22h [16]. After completion of maturation COC were vortexed for 3-4 min in hyaluronidase in order to isolate the mature eggs, which were immediately washed and transferred in M2 media until use.

Protein microinjection and measurements of intracellular Ca^{2+}

Eggs were in incubated in M2 media containing Cal-520 AM (5 μM) for 30 minutes at 37 ^{o}C before the experiment. Eggs were held in M2 for microinjection [17]. Recombinant PLCζ^{WT} and PLCζ^{I489F} mutant fusion proteins were diluted in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4) prior to introduction into oocytes using a high pressure injection method. Alexa Fluor 594 (1 mM) was used as a loading control to ensure the equal protein volume injection. Eggs were imaged in HKSOM media using a Nikon TE2000 inverted epifluorescence microscope connected to a cooled intesified CCD camera (Photek,UK) and fluorescence was recorded by photon counting software (Photek,UK). In cases where luciferase expression was measured the fluorescence (to quantify Ca^{2+} changes) was recorded alternately using a 10 second switching cycle [18]. The fluorescence signal was normalised to relative fluorescence by plotting absolute fluorescence divided by basal level fluorescence (F/F0). All egg experiments were conducted within a three (3) week period.

cRNA microinjection and measurements of intracellular Ca^{2+} and luciferase expression

Eggs were held in M2 for microinjection. cRNA was diluted in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4) and in the case of cRNA also mixed with 1 mM Oregon Green BAPTA dextran OGBD (Life Technologies) prior to introduction into oocytes using a high pressure injection method [17]. Bolus injection calculated the amount of injection solution microinjected that was approximately 3-5% of oocyte volume. Eggs were imaged in HKSOM media containing 100 μM luciferin using a Nikon TE2000 inverted epifluorescence microscope connected to a cooled intensified CCD camera (Photek,UK) and both luminescence and fluorescence were recorded by photon counting software (Photek, UK). Luminescence (quantifying luciferase expression) and fluorescence (quantifying Ca^{2+} changes) were recorded alternately using a 10 second switching cycle [18] with these 2 signals being plotted

individually for each oocyte over the same time scale. The fluorescence signal was normalised to relative fluorescence by plotting absolute fluorescence divided by basal level fluorescence (F/F0) and luminescence was plotted as a running average over 5 minutes. All egg experiments were conducted within a 3-month period.

CD spectroscopy

CD spectra of MBP, MBP-C2ζ^{WT} and MBP-C2ζ^{I489F} were recorded on an Aviv model 215 instrument (Aviv Biomedical Inc., Lakewood, NJ) using a 0.1-cm quartz cell at 4 °C. Proteins were dissolved in 100 mM NaF, 20 mM KH_2PO_4/NaOH, pH 7.0, at a concentration of ~ 0.15 mg/ml. Concentrations were determined based on the absorbance at 280 nm assuming extinction coefficients derived from the amino acid composition [19]. Secondary structure content was analysed using the CDsstr algorithm [20] as implemented on DichroWeb [21] using the SMP180 reference spectra [22].

Thermal stability was monitored at 221nm in 0.5 °C intervals from 4 °C to 70 °C (MBP) or up to a maximum temperature when protein aggregation was observed as indicated by a sharp increase of the dynode voltage with settings resulting in an average heating rate of ~ 30 °C/h. Apparent melting temperatures T_m and van't Hoff's enthalpies ΔH_{vH} were estimated from non-linear curve fitting assuming a 2-state folded-to-unfolded transition as described [23] with the ellipticity of the unfolded state set as that observed for MBP.

Molecular modelling

Structural models of MBP, MBP-C2ζ^{WT} and MBP-C2ζ^{I489F} were generated using SWISS-MODEL with the corresponding parts of the PDB coordinates 3mq9 (residues 5 to 366) and 1djg (rat PLCδ1, residues 496 to 624) as templates [24].

PIP_2 hydrolysis assays

The PIP_2 hydrolytic activity of recombinant MBP-tagged PLCζ proteins was determined as described previously [5, 10, 25]. The final PIP_2 concentration in the reaction mixture was 220 µM, containing 0.05 µCi of [^{3}H]PIP_2. For the assays examining the Ca^{2+} dependence of PLC enzymatic activity, the Ca^{2+} buffers were prepared by EGTA/$CaCl_2$ admixture, while in assays to determine the dependence on substrate PIP_2 concentration, 0.05 µCi of [^{3}H]PIP_2 was mixed with cold PIP_2 to give the appropriate final concentration [10, 26].

K_m and EC_{50} values of Ca^{2+} dependence for PIP_2 hydrolysis for the MBP-tagged PLCζ recombinant proteins were determined by non-linear regression analysis (GraphPad Prism 5).

Liposome preparation and binding assays.

Unilamellar liposomes were prepared as previously described [10, 26]. For the protein-liposome binding experiments, liposomes (100 µg) were incubated with 1 µg of each MBP-tagged recombinant protein for 30 min at room temperature and centrifuged for 5 hours at 4 °C. Supernatants and pellets were analysed either by SDS-PAGE and Coomassie Brilliant Blue staining or by the [^{3}H]PIP_2 hydrolysis assay, as previously described [10].

RESULTS

Microinjection of cRNA encoding PLCζ^{I489F} mutant completely failed to trigger Ca^{2+} oscillations when expressed at physiological concentrations in mouse eggs

To investigate the impact of I489F mutation on the *in vivo* Ca^{2+} oscillation-inducing activity of human PLCζ, we used site-directed mutagenesis to generate the PLCζ^{I489F} mutant. To enable direct comparative analysis with PLCζ^{WT} and to verify that this construct was faithfully expressed as protein in cRNA-microinjected unfertilized mouse eggs, we produced this mutant as a luciferase-fusion construct, as previously described [10, 26, 27]. Microinjection of cRNA encoding luciferase-tagged PLCζ^{WT} (PLCζ^{WT}-LUC) caused prominent Ca^{2+} oscillations in all injected eggs (~4.0 spikes in the first hour of oscillating), similar to those observed during fertilization, following successful protein expression to a level indicated by a luminescence reading of 0.06 c.p.s., (Fig. 1B left panel, Table 1). In contrast, microinjection of cRNA corresponding to PLCζ^{I489F}-LUC mutant failed to cause any Ca^{2+} oscillations at equivalent protein expression levels (0.06 c.p.s.), (Fig. 1B right panel, Table 1). Interestingly, expression of PLCζ^{I489F}-LUC mutant at significantly higher levels (0.74 c.p.s.) led to low frequency Ca^{2+} oscillations (~2.3 spikes in the first hour of oscillating), (Fig. 1C, Table 1), suggesting that overexpression of PLCζ^{I489F}-LUC, which leads to overload of this mutant within the egg cytoplasm, can induce Ca^{2+} release from intracellular stores.

Microinjection of MBP-PLCζ^{I489F} protein is less effective at triggering Ca^{2+} oscillations in mouse eggs

PLCζ^{WT} and PLCζ^{I489F} mutant were then subcloned into the pETMM41 expression vector to allow prokaryotic expression of these constructs, as 6xHis-MBP-fusion recombinant proteins. We have previously demonstrated that NusA is a powerful fusion partner for PLCζ, significantly enhancing the bacterial expression, protein solubility, as well as the purified recombinant protein stability over time [5, 10]. In this study, we demonstrate for first time that in addition to NusA, MBP tag is an extremely effective protein tag for PLCζ. Our comparative experiments with NusA tag showed that MBP is an effective protein fusion partner for PLCζ preserving its stability over time (data not shown).

Optimal protein production for these MBP-fusion PLCζ constructs required induction of protein expression with 0.1 mM IPTG for 18 h at 16 ^{0}C. Following bacterial expression in *E. coli* and isolation by amylose resin affinity chromatography, the purified recombinant proteins were analysed by SDS-PAGE and immunoblot analysis using an anti-His (penta-His) monoclonal antibody (Fig. 2A). The dominant protein band with mobility corresponding to the predicted molecular mass for MBP-PLCζ^{WT} and MBP-PLCζ^{I489F} mutant was observed for both recombinant fusion proteins (~118 kDa). These major bands were also confirmed by immunoblot analysis by the penta-His antibody (Fig. 2A right panel). Some additional, fainter, low molecular weight bands could be observed, which were also detected by the penta-His antibody and were probably the result of protease degradation occurring during the various stages of protein expression and purification. Microinjection of MBP-PLCζ^{WT} into mouse eggs at a concentration of 0.0375 mg/ml revealed that it possesses a potent ability to trigger cytoplasmic Ca^{2+} oscillations (Fig. 2B; upper panel), matching that observed after microinjection of native sperm extracts [28]. Microinjection of the MBP protein alone was unable to induce Ca^{2+} release. Interestingly, microinjection of MBP-PLCζ^{I489F} mutant protein at equivalent levels that MBP-PLCζ^{WT} triggered physiological Ca^{2+} oscillations (0.0375 mg/ml), was either unable to induce any Ca^{2+} oscillations (13/23 eggs) or could only trigger very low frequency Ca^{2+} oscillations (10/23 eggs), (Fig. 2B; lower panel).

To investigate whether it was possible to rescue the low frequency Ca^{2+} oscillations, MBP-PLCζ^{I489F} recombinant protein was microinjected at higher levels. We found that a 2-fold increase in the amount of MBP-PLCζ^{I489F} mutant (0.075 mg/ml) microinjected into mouse eggs was able to rescue the defective Ca^{2+} oscillation-inducing phenotype and trigger egg activation, as indicated by cleavage of the 2-cell stage (Fig. 3). These findings agree with our previous observations, suggesting that overload of PLCζ^{I489F} mutant within the mouse egg cytoplasm, can induce Ca^{2+} oscillations.

MBP-PLCζ^{WT} protein is more potent in triggering Ca^{2+} oscillations in bovine eggs than MBP-PLCζ^{I489F}

To examine whether our previous observations regarding the ability of PLCζ^{I489F} mutant to trigger Ca^{2+} oscillations in mouse eggs are consistent in eggs of a different species, we compared the abilities of MBP-PLCζ^{WT} and MBP-PLCζ^{I489F} recombinant proteins to induce Ca^{2+} oscillations in bovine eggs. The optimal concentration for MBP-PLCζ^{WT} to induce a physiological pattern of Ca^{2+}oscillations in bovine eggs was 0.15 mg/ml. In contrast, microinjection of MBP-PLCζ^{I489F} mutant protein at this concentration was able to trigger very low frequency Ca^{2+} oscillations in all microinjected eggs (Fig. 4).

I489F does not alter the folding and the thermal stability of PLCζ C2 domain

To investigate whether I489F mutation within the C2 domain of PLCζ interferes with the proper folding of this domain, we analysed the MBP-tagged wild type and mutant C2 domains (C2ζ^{WT} and C2ζ^{I489F}) by CD spectroscopy. Attempts to produce an untagged or 6xHis-tag version of the C2 domain of PLCζ^{WT} using the bacterial expression system proved unsuccessful, as the protein appeared to be completely insoluble, accumulating into inclusion bodies. Thus, C2ζ^{WT} and C2ζ^{I489F} (Fig. 5A) were cloned into pETMM41 expression vector to allow bacterial expression of these domains as 6xHis-MBP-fusion recombinant proteins. The presence of MBP tag significantly enhanced the expression of soluble C2 domains and the affinity-purified MBP-tagged C2 domains after SDS-PAGE and immunoblot analysis using the penta-His antibody, displayed the predicted molecular mass (~59 kDa); (Fig. 5B). It is worth noting, that prior the CD experiments removal of the MBP moiety from the C2 domains was attempted but this resulted in rapid degradation of the proteins. Thus, the intact MPB-C2 domains were used for our CD studies, while the MBP moiety alone served as a control for our experiments. Spectra recorded at 4 ^{0}C were indistinguishable from each other, but different from that of MBP alone (Fig. 6A). Deconvolution of the spectra resulted in 39 % α helix / 18 % β strand and 35 % α helix / 24 % β strand for MBP by itself and the two fusion proteins, respectively. These values are in very good agreement with those of homology based models of MBP (41 % α helix, 17 % β strand) and a PLCζ C2 domain combined with MBP (35 % α helix, 27 % β strand). The thermal stability was measured by monitoring the CD signal at 221nm (Fig. 6B). All samples showed a steep decrease in ellipticity. In contrast to MBP, the fusion proteins precipitated upon unfolding as indicated by an increase of light scattering.

Assuming a two-state folded-to-unfolded transition and extrapolating to the ellipticity observed for unfolded MBP, fitting of the data resulted in melting temperature T_m = 60.5 ± 0.5 ^{0}C and van't Hoff's enthalpies of ~560 and ~250 kJ/mol for MBP and the two fusion proteins, respectively. For MBP, these values agree with those previously reported [29].

I489F does not alter the *in vitro* enzymatic properties of human PLCζ

We then examined the impact of I489F mutation on the *in vitro* enzymatic properties of PLCζ. To determine the specific PIP_2 hydrolytic enzyme activities for PLCζ^{WT} and PLCζ^{I489F} a micellar [^{3}H]PIP_2 hydrolysis assay was used as previously described [10, 27, 30]. The histogram of Fig. 7A and Table 2 reveal that the enzymatic activities of PLCζ^{WT} and PLCζ^{I489F} were almost identical (960±43 vs 953±49 nmol/min/mg), suggesting that I489F mutation has no effect on the ability of PLCζ to hydrolyse *in vitro* PIP_2. In order, to investigate the effect of I489F mutation on Ca^{2+} sensitivity of PLCζ enzyme activity we assessed the ability of PLCζ^{WT} and PLCζ^{I489F} to hydrolyse [^{3}H]PIP_2 at different Ca^{2+} concentrations ranging from 0.1 nM to 0.1 mM. The resulting EC_{50} values for PLCζ^{WT} (66 nM) and PLCζ^{I489F} (60 nM) were very similar (Fig. 7B, Table 2). In addition, calculation of the Michaelis-Menten constant *Km*, for PLCζ^{WT} and PLCζ^{I489F} also yielded in comparable values (81 vs 93 μM), (Table 2), indicating that I489F mutation has no effect on the Ca^{2+} sensitivity or on the *in vitro* enzymatic affinity of PLCζ for its substrate, PIP_2.

I489F dramatically reduces the binding of PLCζ to PI(3)P and PI(5)P

The only specific binding partners for PLCζ C2 domain that have been *in vitro* identified up to date are PI(3)P and PI(5)P [31, 32]. To examine the binding properties of PLCζ^{I489F} to PIP_2, PI(3)P and PI(5)P we employed two different approaches, a liposome-binding (pull-down) and a liposome-binding/enzyme assay, as previously described [10, 26]. For these experiments, we prepared unilamellar liposomes, which were composed of PC:CHOL:PE (4:2:1) with incorporation of either 1% PIP_2, 5% PI(3)P or 5% PI(5)P. For diminishing the non-specific protein binding to highly charged lipids, the liposome binding experiments were performed in the presence of a near-physiological concentration of $MgCl_2$ (0.5mM) [26]. The MBP moiety alone served as the negative control for our experiments. As shown in Fig. 8, MBP did not exhibit any specific liposome binding in the absence or presence of PIP_2, PI(3)P and PI(5)P, whereas our positive control MBP-PLCζ^{WT} displayed robust binding to liposomes containing either 1% PIP_2, 5% PI(3)P or 5% PI(5)P. On the other hand,

MBP-C2ζ^{WT} showed significant binding only to liposomes containing either 5% PI(3)P or 5% PI(5)P. In contrast, although MBP-PLCζ^{I489F} was able to bind strongly to liposomes containing 1% PIP_2, its binding to liposomes containing either 5% PI(3)P or 5% PI(5)P significantly diminished (Fig. 8). Similarly, MBP-C2ζ^{I489F} was unable to bind liposomes containing 5% PI(3)P or 5% PI(5)P. These findings clearly suggest that I489F mutation clearly affects the binding of PLCζ C2 domain to PI(3)P and PI(5)P. For more quantitative analysis, a liposome-binding/enzyme assay, was employed to analyze the binding of MBP-PLCζ^{WT} and MBP-PLCζ^{I489F} to PIP_2, PI(3)P and PI(5)P. Thus, 1 μg of recombinant protein corresponding to MBP-PLCζ^{WT} and MBP-PLCζ^{I489F} were incubated with liposomes containing either 1% PIP_2, 5% PI(3)P or 5% PI(5)P. After centrifugation, the supernatant were separated for the precipitated liposomes and the PIP_2 hydrolytic activity determined using the standard [^{3}H]PIP_2 hydrolysis assay. Based on the % of the PIP_2 hydrolytic activity pre- and post-liposome binding, we estimated the relative binding of each PLCζ protein to the different phosphoinositide specific-containing liposomes [10]. As shown at histograms in Fig. 9, the binding of PLCζ^{WT} and PLCζ^{I489F} to PIP2-containing liposomes was almost identical, while the binding of PLCζ^{I489F} to PI(3)P- and PI(5)P-containing liposomes had been reduced by ~50% compared to PLCζ^{WT}, suggesting that I489F mutation indeed dramatically reduces the binding of PLCζ to PI(3)P and PI(5)P.

DISCUSSION

Mounting experimental and clinical evidence strongly supports the notion that sperm-specific PLCζ is the sole physiological stimulus of egg activation during mammalian fertilization [4, 8, 11-14, 33-36]. Sperm-delivered PLCζ triggers the repetitive Ca^{2+} oscillations within the fertilized egg, by catalysing the hydrolysis of PIP_2 stimulating the $InsP_3$ signalling pathway [3, 9]. Although PLCζ is the smallest PLC isoform with the most basic domain structure organization, its discrete biochemical properties contribute to its supreme effectiveness in triggering the Ca^{2+} signalling phenomenon within the fertilized mammalian eggs [9].

The past few years, clinical reports have directly linked defects in human PLCζ with documented cases of male infertility. Firstly, Yoon et al., [11] reported a number of infertile patients presenting oocyte activation failure, providing evidence that was due to absence of reduced levels of PLCζ within their sperm [11]. Then, the first direct link between male

infertility and a mutation on PLCζ gene came from a study that reported a mutation on the catalytic domain of PLCζ (H398P) of a patient who failed fertilization after ICSI [12]. Interestingly, another study reported a second PLCζ mutation on the same heterozygous infertile patient, also in the catalytic domain (H233L) [14]. Recently, Escoffier et al., [8] reported a homozygous missense mutation in the PLCζ gene of two infertile brothers from Tunisia, presenting egg activation failure [8]. This mutation was located in the C2 domain of PLCζ where an Ile had been replaced with a Phe residue (I489F), (Fig. 1A) making it the first PLCζ mutation located in a different domain than the catalytic.

Here, we introduced the infertility-linked I489F PLCζ mutation in human PLCζ sequence and we assessed the effects of this mutation upon the *in vivo* Ca^{2+} oscillation-inducing activity and the *in vitro* biochemical and enzymatic properties of human PLCζ. Our study provides evidence and extends the previous work of Escoffier et al., [8] by revealing that (i) PLCζ I489F mutation dramatically reduces the Ca^{2+} oscillation-inducing activity of PLCζ in mouse and bovine eggs (Figs 1B, 2B, 4). Although microinjection of physiological levels of PLCζ mutant are either unable or triggered very low frequency Ca^{2+} oscillations in mouse and bovine eggs, a 2-fold increase in the amount of PLCζ microinjected in mouse eggs was capable of rescuing the defective Ca^{2+} oscillation-inducing phenotype, triggering egg activation of these $PLC\zeta^{I489F}$ microinjected eggs (Fig. 3). This suggests that by overloading the egg with significantly higher amounts of this PLCζ mutant can lead to successful oocyte activation, explaining the infertility of the two heterozygous brothers carrying this mutation. (ii) CD spectroscopy showed that the I489F C2 mutation has no effect on the proper folding and the thermal stability of this domain (Fig. 6). This was consistent with our observations regarding the enzymatic properties of $PLC\zeta^{I489F}$ mutant, which were almost identical with the enzymatic properties of $PLC\zeta^{WT}$ (Fig. 7, Table 2), suggesting that I489F mutation has no effect on the ability of PLCζ to hydrolyse PIP_2. (iii) More importantly, our liposome binding experiments revealed that I489F mutation dramatically reduces (~50%) the binding of PLCζ to PI(3)P and PI(5)P (Figs. 8, 9), two phosphoinositides, which have been previously reported to interact with PLCζ *in vitro* [31, 32]. To the best of our knowledge, PLCζ C2 domain is the first C2 domain amongst the C2 domains of all PLC isoforms that it has been shown to directly interact with PI(3)P and PI(5)P, *in vitro*. Although it is difficult to predict which amino acid residues play a role on these interactions, we have shown that I489 is a key residue for efficient binding of PLCζ to both PI(3)P and PI(5)P. High resolution three-dimensional structure analysis of PLCζ

C2 domain by X-ray crystallography could help to reveal the critical binding sites for these interactions. It is now also necessary to understand the physiological role of the binding of PLCζ to PI(3)P and PI(5)P and also to extensively search for any other unidentified membrane egg proteins, which might interact with the C2 domain of PLCζ, assisting with the proper localization and targeting of this enzyme within the egg cytoplasm.

It has been proposed, at least for mouse PLCζ, that its nuclear translocation ability regulates the cell-cycle dependent Ca^{2+} oscillations [3]. It is worth noting that there is no scientific evidence for the C2 domain playing a role in the nuclear sequestration of PLCζ, as it has been clearly demonstrated that the nuclear localization signal (NLS) of mouse PLCζ is located within the XY linker region, close to the start of the Y domain [3].

Understanding the complex mechanism of action of PLCζ requires further investigation. However, based on our previous and recent findings we propose that after sperm-egg fusion and the delivery of PLCζ from the sperm into the egg cytoplasm, the high Ca^{2+} sensitivity of this prototypic PLC is conferred by its EF hand domains, allowing it to be active at resting egg Ca^{2+} levels [27, 31, 37]. PLCζ may then associate with a specific intracellular membrane by interaction of the C2 domain with either PI(3)P, PI(5)P or another unidentified egg membrane protein. Finally, the positively charged XY linker together with the first EF hand domain provide a tether to facilitate proper PIP_2 substrate access, in order the catalytic XY domain to proceed with the catalysis of the hydrolysis of PIP_2 to produce $InsP_3$ (Fig. 10) [10].

In conclusion, the identification of the first male infertility-linked PLCζ point mutation located in the C2 domain of PLCζ, provides the first clinical support for the vital role of this domain on PLCζ function. As our study proposes, this is purely due to its novel binding properties of this domain to PI(3)P and PI(5)P or other unidentified egg factor. In addition, the identification of another male infertility-linked PLCζ mutation necessitates the use of recombinant PLCζ protein in a clinical setting with the aim to rescue such cases of oocyte activation failure.

AUTHOR CONTRIBUTIONS

M.N., K.S. and F.A.L. devised the project strategy. M.N., K.B., K.S. and F.A.L. designed the experiments, which were performed by M.N., P.S., J.S., K.B., B.L.C., L.B., M.L.,

and Z.S. M.N. prepared the first manuscript draft, which was revised and approved by all authors.

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REFERENCES

1 Stricker, S. A. (1999) Comparative biology of calcium signaling during fertilization and egg activation in animals. Dev Biol. **211**, 157-176

2 Runft, L. L., Jaffe, L. A. and Mehlmann, L. M. (2002) Egg activation at fertilization: where it all begins. Dev Biol. **245**, 237-254

3 Nomikos, M., Kashir, J., Swann, K. and Lai, F. A. (2013) Sperm PLCzeta: from structure to Ca2+ oscillations, egg activation and therapeutic potential. FEBS Lett. **587**, 3609-3616

4 Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K. and Lai, F. A. (2002) PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development. **129**, 3533-3544

5 Nomikos, M., Yu, Y., Elgmati, K., Theodoridou, M., Campbell, K., Vassilakopoulou, V., Zikos, C., Livaniou, E., Amso, N., Nounesis, G., Swann, K. and Lai, F. A. (2013) Phospholipase Czeta rescues failed oocyte activation in a prototype of male factor infertility. Fertil Steril. **99**, 76-85

6 Nomikos, M., Sanders, J. R., Kashir, J., Sanusi, R., Buntwal, L., Love, D., Ashley, P., Sanders, D., Knaggs, P., Bunkheila, A., Swann, K. and Lai, F. A. (2015) Functional disparity between human PAWP and PLCzeta in the generation of Ca2+ oscillations for oocyte activation. Mol Hum Reprod. **21**, 702-710

7 Satouh, Y., Nozawa, K. and Ikawa, M. (2015) Sperm postacrosomal WW domain-binding protein is not required for mouse egg activation. Biol Reprod. **93**, 94

8 Escoffier, J., Lee, H. C., Yassine, S., Zouari, R., Martinez, G., Karaouzene, T., Coutton, C., Kherraf, Z. E., Halouani, L., Triki, C., Nef, S., Thierry-Mieg, N., Savinov, S. N., Fissore, R., Ray, P. F. and Arnoult, C. (2016) Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. Hum Mol Genet. **25**, 878-891

9 Nomikos, M. (2015) Novel signalling mechanism and clinical applications of sperm-specific PLCzeta. Biochem Soc Trans. **43**, 371-376

10 Nomikos, M., Sanders, J. R., Parthimos, D., Buntwal, L., Calver, B. L., Stamatiadis, P., Smith, A., Clue, M., Sideratou, Z., Swann, K. and Lai, F. A. (2015) Essential Role of the EF-hand Domain in Targeting Sperm Phospholipase Czeta to Membrane Phosphatidylinositol 4,5-Bisphosphate (PIP2). J Biol Chem. **290**, 29519-29530

11 Yoon, S. Y., Jellerette, T., Salicioni, A. M., Lee, H. C., Yoo, M. S., Coward, K., Parrington, J., Grow, D., Cibelli, J. B., Visconti, P. E., Mager, J. and Fissore, R. A. (2008) Human sperm devoid of PLC, zeta 1 fail to induce Ca(2+) release and are unable to initiate the first step of embryo development. J Clin Invest. **118**, 3671-3681

12 Heytens, E., Parrington, J., Coward, K., Young, C., Lambrecht, S., Yoon, S. Y., Fissore, R. A., Hamer, R., Deane, C. M., Ruas, M., Grasa, P., Soleimani, R., Cuvelier, C. A., Gerris, J., Dhont, M., Deforce, D., Leybaert, L. and De Sutter, P. (2009) Reduced amounts and abnormal forms of phospholipase C zeta (PLCzeta) in spermatozoa from infertile men. Hum Reprod. **24**, 2417-2428
13 Kashir, J., Heindryckx, B., Jones, C., De Sutter, P., Parrington, J. and Coward, K. (2010) Oocyte activation, phospholipase C zeta and human infertility. Hum Reprod Update. **16**, 690-703
14 Kashir, J., Konstantinidis, M., Jones, C., Lemmon, B., Lee, H. C., Hamer, R., Heindryckx, B., Deane, C. M., De Sutter, P., Fissore, R. A., Parrington, J., Wells, D. and Coward, K. (2012) A maternally inherited autosomal point mutation in human phospholipase C zeta (PLCzeta) leads to male infertility. Hum Reprod. **27**, 222-231
15 Nomikos, M., Elgmati, K., Theodoridou, M., Calver, B. L., Cumbes, B., Nounesis, G., Swann, K. and Lai, F. A. (2011) Male infertility-linked point mutation disrupts the Ca2+ oscillation-inducing and PIP(2) hydrolysis activity of sperm PLCzeta. Biochem J. **434**, 211-217
16 Chung, J. T., Keefer, C. L. and Downey, B. R. (2000) Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI). Theriogenology. **53**, 1273-1284
17 Swann, K., Campbell, K., Yu, Y., Saunders, C. and Lai, F. A. (2009) Use of luciferase chimaera to monitor PLCzeta expression in mouse eggs. Methods Mol Biol. **518**, 17-29
18 Campbell, K. and Swann, K. (2006) Ca2+ oscillations stimulate an ATP increase during fertilization of mouse eggs. Dev Biol. **298**, 225-233
19 Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. Protein Sci. **4**, 2411-2423
20 Johnson, W. C. (1999) Analyzing protein circular dichroism spectra for accurate secondary structures. Proteins. **35**, 307-312
21 Whitmore, L. and Wallace, B. A. (2008) Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. Biopolymers. **89**, 392-400
22 Abdul-Gader, A., Miles, A. J. and Wallace, B. A. (2011) A reference dataset for the analyses of membrane protein secondary structures and transmembrane residues using circular dichroism spectroscopy. Bioinformatics. **27**, 1630-1636
23 Greenfield, N. J. (2004) Analysis of circular dichroism data. Methods Enzymol. **383**, 282-317
24 Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Gallo Cassarino, T., Bertoni, M., Bordoli, L. and Schwede, T. (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. **42**, W252-258
25 Nomikos, M., Elgmati, K., Theodoridou, M., Georgilis, A., Gonzalez-Garcia, J. R., Nounesis, G., Swann, K. and Lai, F. A. (2011) Novel regulation of PLCzeta activity via its XY-linker. Biochem J. **438**, 427-432
26 Theodoridou, M., Nomikos, M., Parthimos, D., Gonzalez-Garcia, J. R., Elgmati, K., Calver, B. L., Sideratou, Z., Nounesis, G., Swann, K. and Lai, F. A. (2013) Chimeras of sperm PLCzeta reveal disparate protein domain functions in the generation of intracellular Ca2+ oscillations in mammalian eggs at fertilization. Mol Hum Reprod. **19**, 852-864
27 Nomikos, M., Blayney, L. M., Larman, M. G., Campbell, K., Rossbach, A., Saunders, C. M., Swann, K. and Lai, F. A. (2005) Role of phospholipase C-zeta domains in Ca2+-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca2+ oscillations. J Biol Chem. **280**, 31011-31018
28 Swann, K. and Yu, Y. (2008) The dynamics of calcium oscillations that activate mammalian eggs. Int J Dev Biol. **52**, 585-594
29 Beena, K., Udgaonkar, J. B. and Varadarajan, R. (2004) Effect of signal peptide on the stability and folding kinetics of maltose binding protein. Biochemistry. **43**, 3608-3619
30 Nomikos, M., Theodoridou, M., Elgmati, K., Parthimos, D., Calver, B. L., Buntwal, L., Nounesis, G., Swann, K. and Lai, F. A. (2014) Human PLCzeta exhibits superior fertilization potency over mouse PLCzeta in triggering the Ca(2+) oscillations required for mammalian oocyte activation. Mol Hum Reprod. **20**, 489-498

31 Kouchi, Z., Shikano, T., Nakamura, Y., Shirakawa, H., Fukami, K. and Miyazaki, S. (2005) The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase Czeta. J Biol Chem. **280**, 21015-21021
32 Nomikos, M., Elgmati, K., Theodoridou, M., Calver, B. L., Nounesis, G., Swann, K. and Lai, F. A. (2011) Phospholipase Czeta binding to PtdIns(4,5)P2 requires the XY-linker region. J Cell Sci. **124**, 2582-2590
33 Cox, L. J., Larman, M. G., Saunders, C. M., Hashimoto, K., Swann, K. and Lai, F. A. (2002) Sperm phospholipase Czeta from humans and cynomolgus monkeys triggers Ca2+ oscillations, activation and development of mouse oocytes. Reproduction. **124**, 611-623
34 Knott, J. G., Kurokawa, M., Fissore, R. A., Schultz, R. M. and Williams, C. J. (2005) Transgenic RNA interference reveals role for mouse sperm phospholipase Czeta in triggering Ca2+ oscillations during fertilization. Biol Reprod. **72**, 992-996
35 Kashir, J., Nomikos, M., Lai, F. A. and Swann, K. (2014) Sperm-induced Ca2+ release during egg activation in mammals. Biochem Biophys Res Commun. **450**, 1204-1211
36 Amdani, S. N., Yeste, M., Jones, C. and Coward, K. (2016) Phospholipase C zeta (PLCzeta) and male infertility: Clinical update and topical developments. Adv Biol Regul. **61**, 58-67
37 Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T. and Miyazaki, S. (2004) Recombinant phospholipase Czeta has high Ca2+ sensitivity and induces Ca2+ oscillations in mouse eggs. J Biol Chem. **279**, 10408-10412

TABLE LEGENDS

Table 1

Properties of luciferase-tagged PLCζ^{WT} and PLCζ^{I489F} mutant expressed in unfertilised mouse eggs

Ca^{2+}-oscillation-inducing activity and luciferase luminescence levels (cps) in the 1st hour of spiking are summarized for mouse eggs microinjected with luciferase-tagged PLCζ^{WT} and PLCζ^{I489F} mutant. Results are expressed as means ±S.E.M.

Table 2

***In vitro* enzymatic properties of MBP-tagged PLCζ^{WT} and PLCζ^{I489F} mutant**

Summary of specific enzyme activity, *Km* and EC_{50} values of Ca^{2+}-dependence for PIP_2 hydrolysis, determined by non-linear regression analysis (GraphPad Prism 5), (see Fig. 7).

FIGURE LEGENDS

Figure 1

Effect of I489F mutation on Ca^{2+}-oscillation-inducing activity of human PLCζ in mouse eggs

(A) Schematic representation of human PLCζ domain organisation identifying the location of I489F mutation within the C-terminal C2 domain. (B, C) Fluorescence and luminescence recordings reporting the Ca^{2+} changes [fluorescence (black traces) and luciferase expression (red traces; luminescence) in cps respectively] in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged $PLC\zeta^{WT}$ and $PLC\zeta^{I489F}$ mutant (see also Table 1).

Figure 2

MBP-$PLC\zeta^{I489F}$ recombinant protein fails to induce physiological pattern of Ca^{2+} oscillations in mouse eggs, when microinjected in equivalent concentration levels to MBP-$PLC\zeta^{WT}$

(A) SDS-PAGE of affinity-purified recombinant MBP-tagged $PLC\zeta^{WT}$ and $PLC\zeta^{I489F}$ proteins (2µg) analysed by 8% SDS-PAGE and Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using the penta-His antibody (1:5,000 dilution); (right panel). (B) Patterns of Ca^{2+} oscillations in unfertilized mouse eggs following microinjection of MBP-$PLC\zeta^{WT}$ and MBP-$PLC\zeta^{I489F}$ recombinant proteins. F/F0 represents fluorescent intensity of Cal520 relative to baseline.

Figure 3

Microinjection of higher (non-physiological) levels of MBP-$PLC\zeta^{I489F}$ recombinant protein in mouse eggs induces physiological pattern of Ca^{2+} oscillations and triggers egg activation.

F/F0 represents fluorescent intensity relative to baseline. The right panel shows 2-cell stage mouse embryos 22-24h after injection of MBP-$PLC\zeta^{I489F}$ recombinant protein.

Figure 4

MBP-PLCζ^{WT} recombinant protein has greater potency in triggering Ca^{2+} oscillations in bovine eggs compared to MBP-PLCζ^{I489F}

Bovine eggs were injected with MBP-PLCζ^{WT} recombinant protein (top trace) or MBP-PLCζ^{I489F} protein (bottom trace) and Ca^{2+} oscillations recorded. F/F0 represents fluorescent intensity of Ca^{2+} dye relative to baseline.

Figure 5

Expression of wild type and I489F mutant PLCζ C2 domains as MBP-tagged recombinant proteins

(A) Schematic representation of the MBP fusion protein PLCζ C2 domains with numbers denoting their amino acid coordinates. (B) SDS-PAGE of affinity-purified recombinant MBP-tagged PLCζ C2 domains (1µg) analysed by 10% SDS-PAGE and Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using the penta-His antibody (1:5,000 dilution); (right panel).

Figure 6

CD analysis of MBP and MBP-tagged PLCζ C2 fusion proteins

(A) CD spectra were recorded at 4 ºC for MBP (black), MBP-C2ζ^{WT} (blue) and MBP-C2ζ^{I489F} (red). (B) Thermal stability was monitored at 221nm upon heating. In contrast to MBP, the fusion proteins precipitated at ~ 63 ºC. Dashed lines represent best fits assuming a two-state unfolding mechanism and an ellipticity of the unfolded state common with MBP.

Figure 7

I489F mutation does not affect the *in vitro* enzyme specific activity and the Ca^{2+} sensitivity of PLCζ

(A) PIP_2 hydrolysis enzyme activity of MBP-PLCζ^{WT} and MBP-PLCζ^{I489F} obtained with the standard [^{3}H]PIP_2 hydrolysis assay. Values are means ± S.E.M. (n=4), using two different preparations of recombinant protein and each experiment was performed in duplicate. (B) Effect of various [Ca^{2+}] on the normalized activity of MBP-PLCζ^{WT} and MBP-PLCζ^{I489F} recombinant proteins. For these assays, values are ± S.E.M. (n=4), using two different batches of recombinant proteins and with each experiment performed in duplicate (see Table 2).

Figure 8

I489F mutation reduces the binding of PLCζ to PI(3)P and PI(5)P containing liposomes

Liposome 'pull-down assays' of MBP-tagged PLCζ^{WT} and PLCζ^{I489F} proteins. Unilamellar liposomes containing either PIP_2 (1%), PI(3)P (5%), or PI(5)P (5%) were incubated with PLCζ recombinant proteins. Following liposome centrifugation, both the supernatant (s) and liposome pellet (p) were subjected to SDS-PAGE and Coomassie Brilliant Blue staining.

Figure 9

Quantitative analysis suggest that I489F mutation reduces ~50% the binding of PLCζ to PI(3)P and PI(5)P containing liposomes

Normalized binding of MBP-PLCζ^{WT} and MBP-PLCζ^{I489F} recombinant proteins to unilamellar liposomes containing (A) 1% PIP_2, (B) 5% PI(3)P and (C) 5% PI(5)P. Following centrifugation, the supernatants were assayed for their ability to hydrolyse PIP_2 *in vitro,* using the standard [^{3}H]PIP_2 hydrolysis assay, (n=4±SEM, using two different preparations of recombinant protein). Based on the % of the PIP_2 hydrolytic activity pre- and post- liposome binding the relative binding of each PLCζ protein to the liposomes was determined. Significant statistical differences (asterisks) were calculated by an unpaired Student's t-test; ****P* < 0.0005, (GraphPad, Prism 5).

Figure 10

Schematic illustration of a proposed intracellular targeting mechanism of PLCζ

Our study suggests that association of PLCζ with a specific vesicular membrane may be mediated by interaction of the C2 domain with PI(3)P, PI(5)P or an as yet unidentified membrane protein. Then, association of PLCζ with the negatively-charged PIP_2 involves electrostatic interactions with the positively-charged 1st EF-hand domain and the XY-linker region. The catalytic XY domain subsequently proceeds with the enzymatic cleavage of PIP_2. The high Ca^{2+} sensitivity of the enzyme is conferred by the EF hand domain enabling PLCζ to be active at resting nanomolar Ca^{2+} levels (Figure modified from [10]).

TABLES

Table 1

Construct	Pipette cRNA concentration (μg/μl)	Number of eggs injected	Number of eggs oscillating	Mean number of spikes 1st hr of spiking	Average expression in 1st hr of spiking (cps)
PLCζ^{WT}-LUC	0.015	20	19/20	4 ± 0.290	0.06 ± 0.007
PLCζ^{I489F}-LUC	0.015	19	1/19	0.15± 0.016	0.06 ± 0.004
PLCζ^{I489F}-LUC	0.1	16	15/16	2.3 ± 0.250	0.74 ± 0.038

Table 2

PLCζ protein	PIP_2 hydrolysis enzyme activity (nmol/min/mg)	Ca^{2+} dependence EC_{50} (nM)	*Km* (μM)
PLCζ^{WT}	960±43	66	81
PLCζ^{I489F}	953±49	60	93

FIGURES

Figure 1

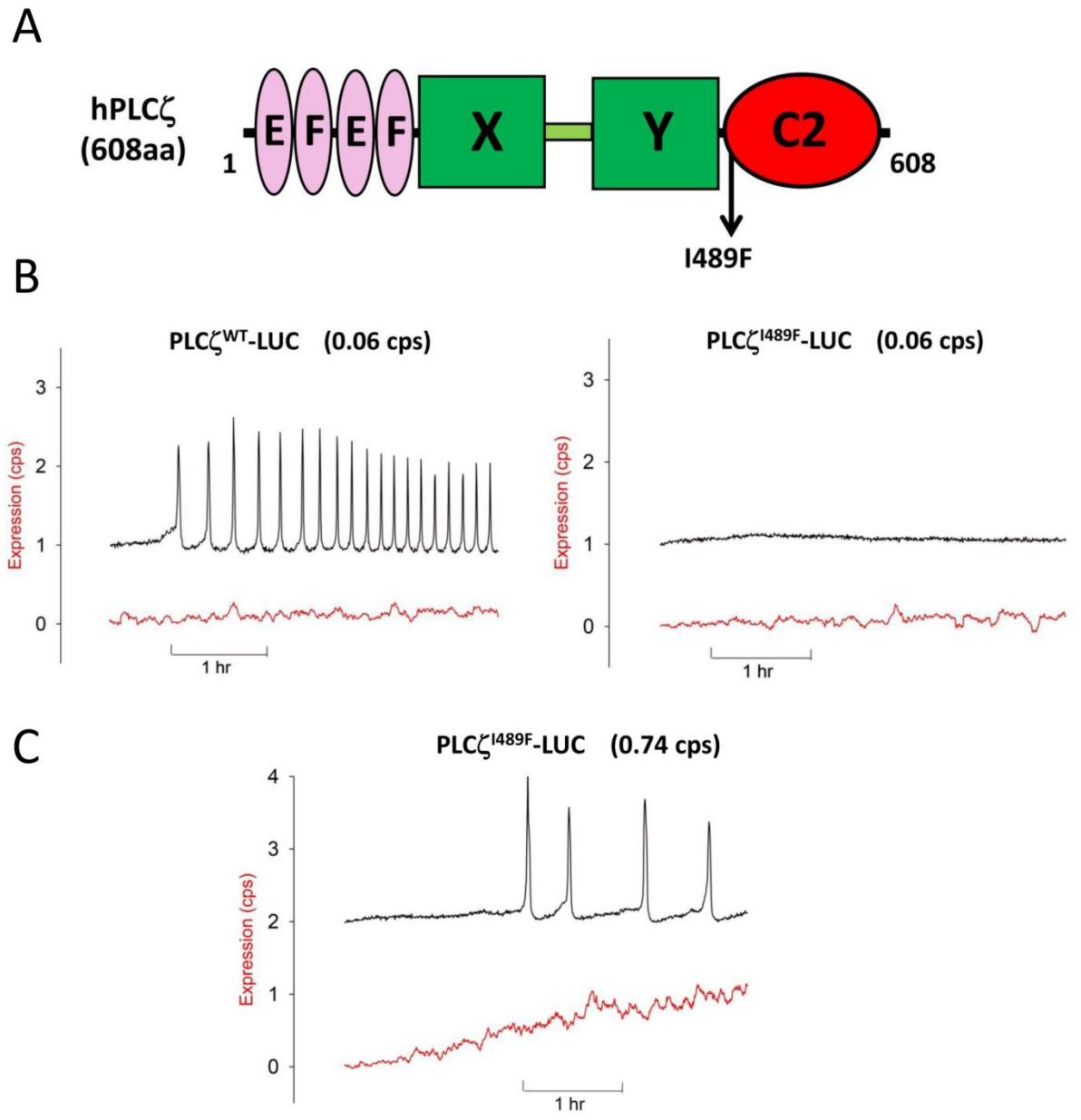
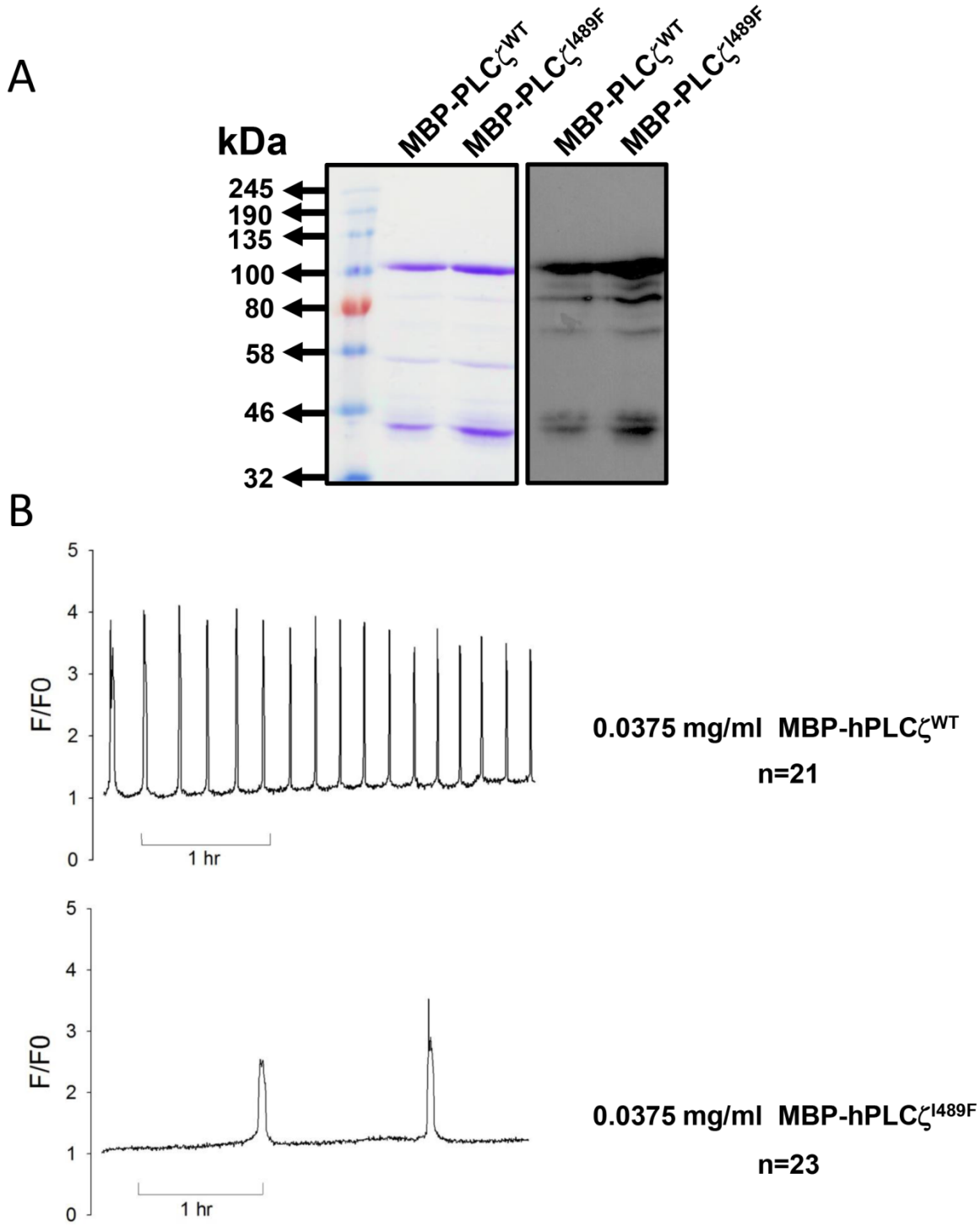


Figure 2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kDa
MBP-PLCζWT
MBP-PLCζI489F
MBP-PLCζWT
MBP-PLCζI489F
245
190
135
100
80
58
46
32
B
F/F0
1 hr
0.0375 mg/ml MBP-hPLCζWT
n=21
F/F0
1 hr
0.0375 mg/ml MBP-hPLCζI489F
n=23

Figure 3

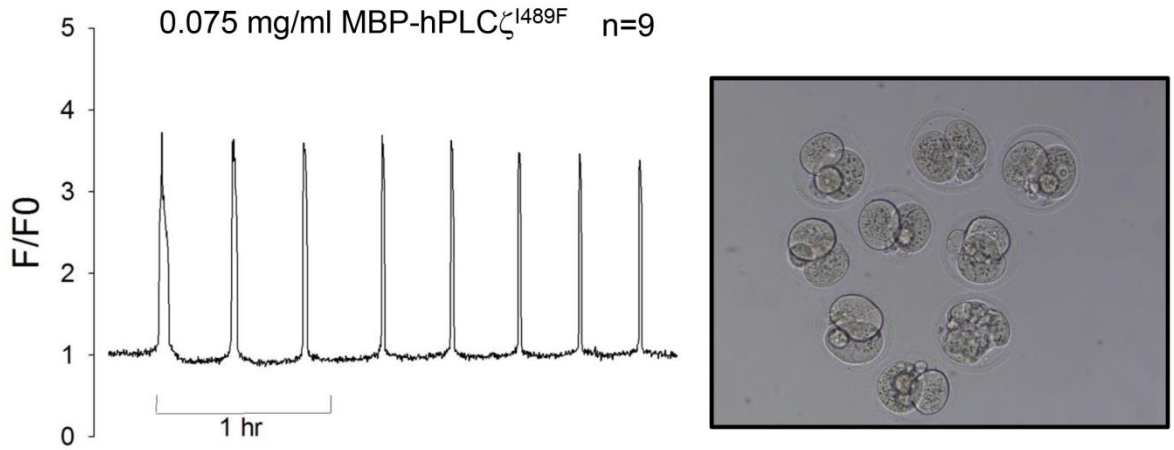

Figure 4

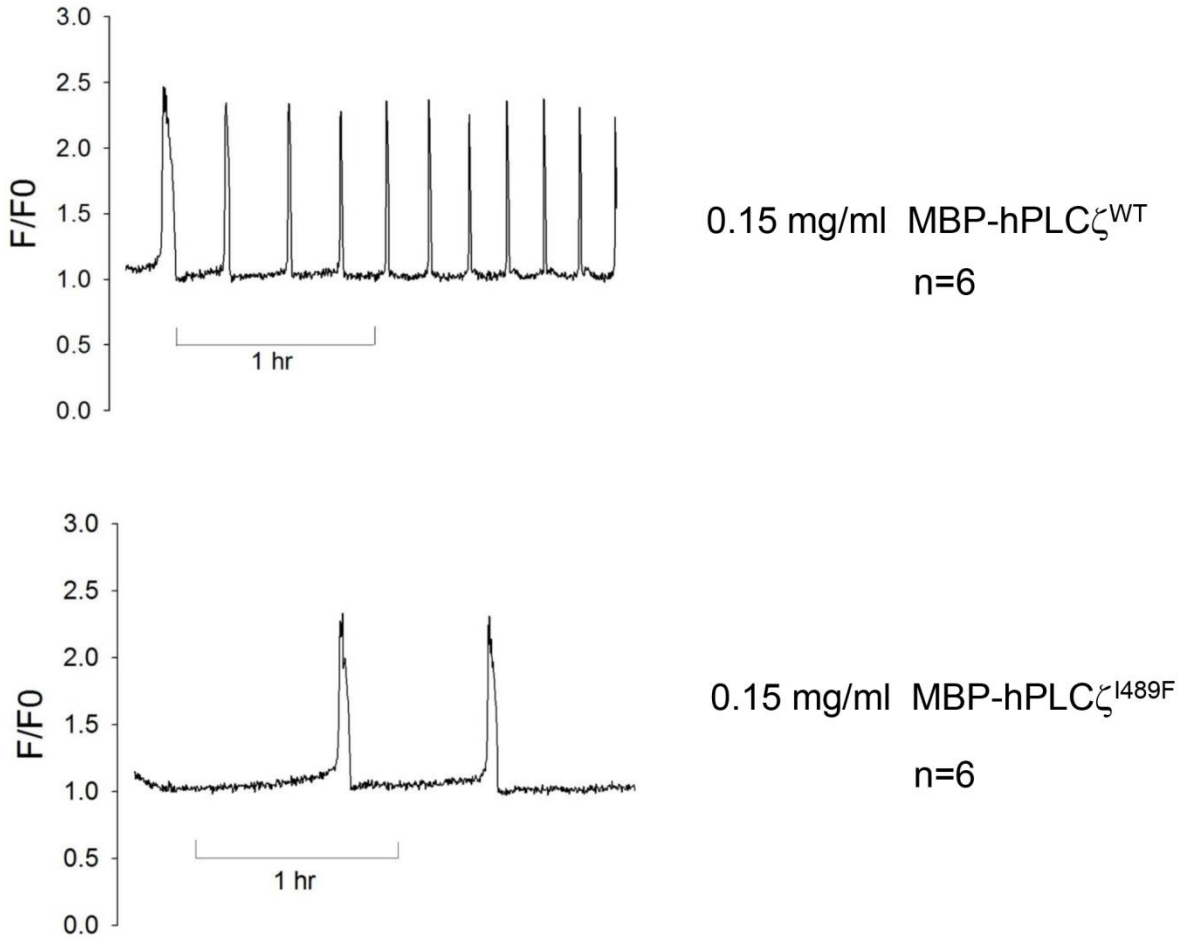

Figure 5

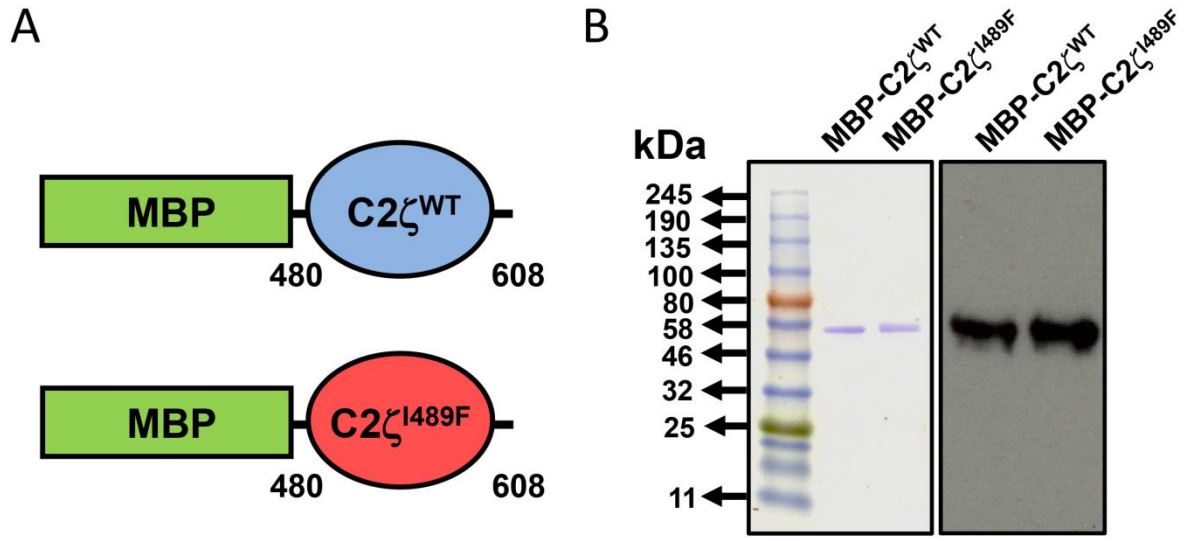

Figure 6

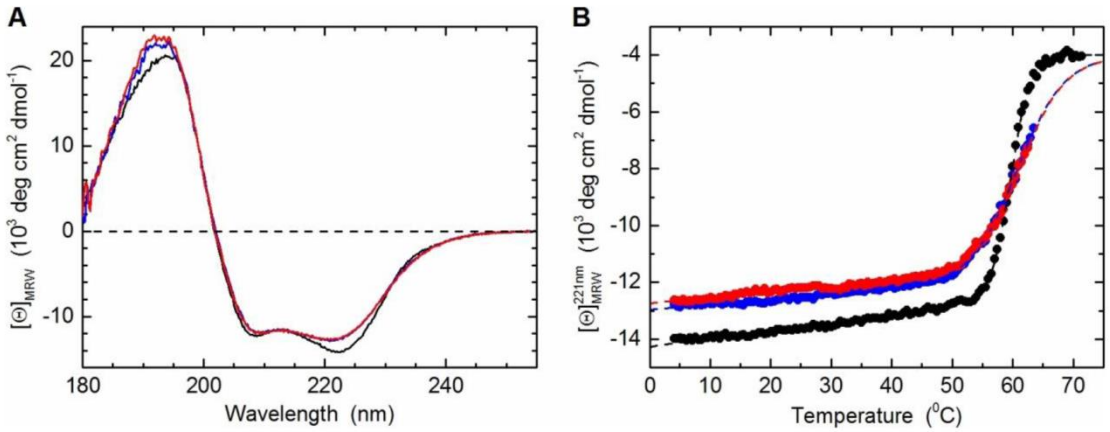

Figure 7

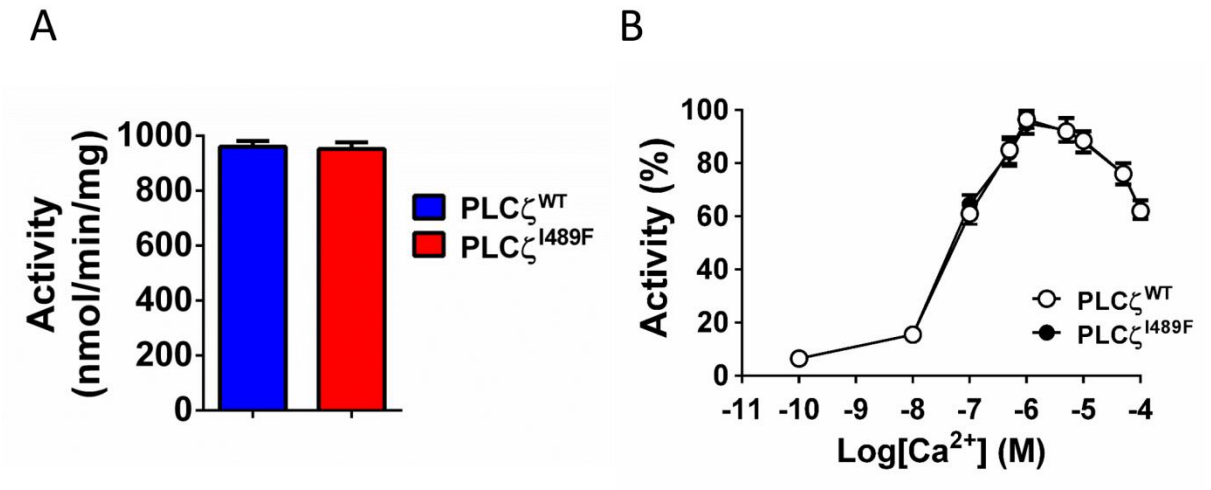

Figure 8

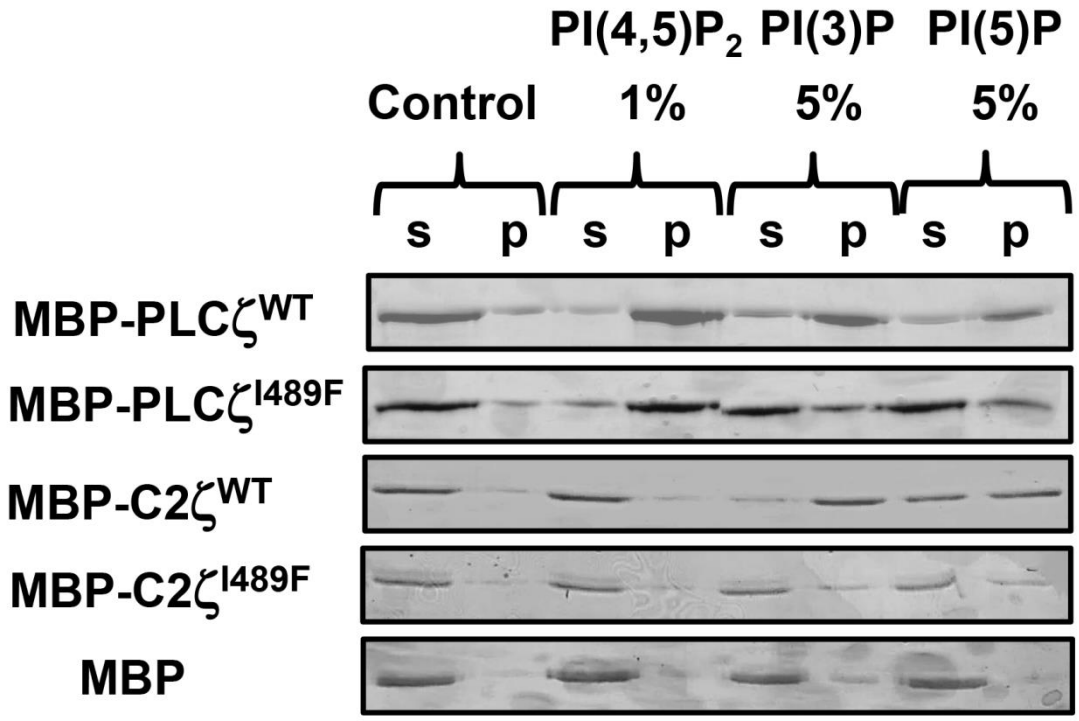

Figure 9

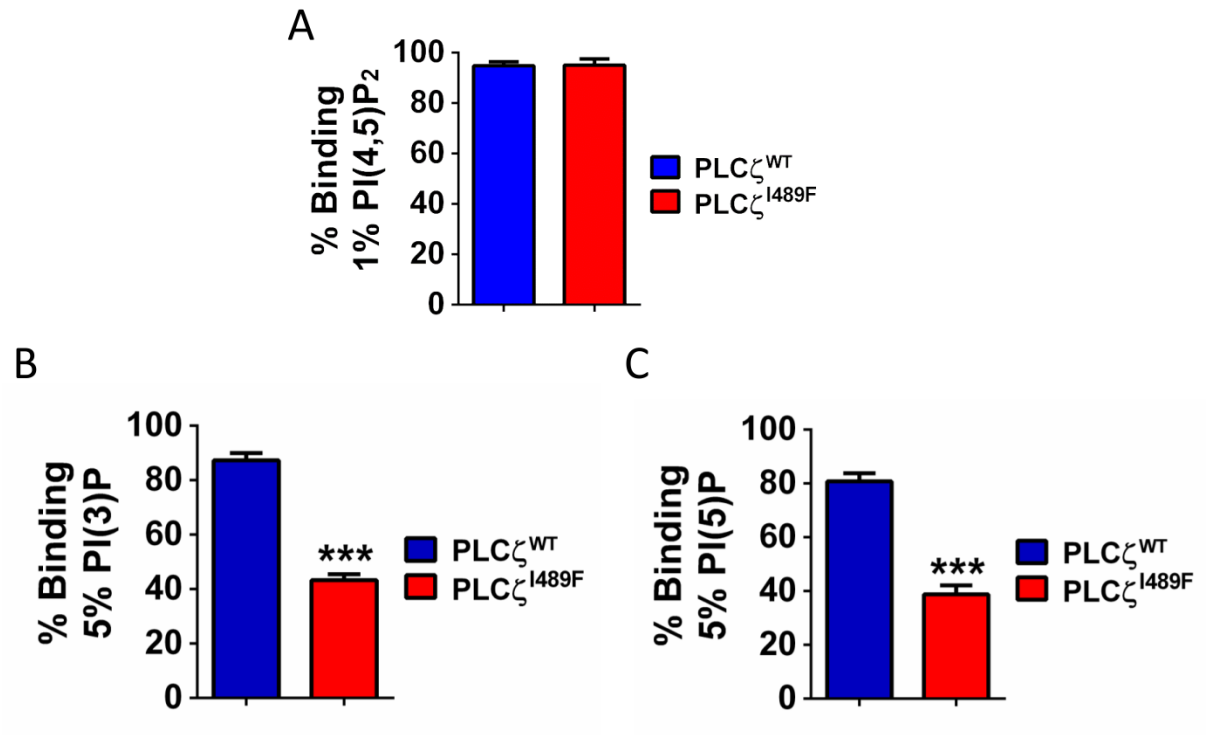

Figure 10