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The role and mechanism of action of sperm PLC-zeta in mammalian fertilisation

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Short title: Structure and function of sperm-specific PLCζ

Abbreviations: phospholipase C-zeta, PLCζ; calcium, Ca^{2+}; phosphatidylinositol 4,5-bisphosphate, PIP_2; inositol 1,4,5-trisphosphate, $InsP_3$; pleckstrin homology domain, PH domain; phosphatidylinositol 3-phosphate, PI(3)P; phosphatidylinositol 5-phosphate, PI(5P); intracytoplasmic sperm injection, ICSI; assisted reproductive technology, ART

Key words: Phospholipase C zeta (PLCζ), Fertilisation, Sperm, calcium oscillations, Male infertility

ABSTRACT

At mammalian fertilisation, the fundamental stimulus that triggers oocyte (egg) activation and initiation of early embryonic development is an acute rise of the intracellular free calcium (Ca^{2+}) concentration inside the egg cytoplasm. This essential Ca^{2+} increase comprises a characteristic series of repetitive Ca^{2+} oscillations, starting soon after sperm-egg fusion. Over the last fifteen years, accumulating scientific and clinical evidence supports the notion that the physiological stimulus that precedes the cytosolic Ca^{2+} oscillations is a novel, testis-specific phospholipase C (PLC) isoform, known as PLC-zeta (PLCζ). Sperm PLCζ catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) triggering cytosolic Ca^{2+} oscillations through the inositol 1,4,5-trisphosphate ($InsP_3$) signaling pathway. PLCζ is the smallest known mammalian PLC isoform with the most elementary domain organisation. However, relative to somatic PLCs, the PLCζ isoform possesses a unique potency in stimulating Ca^{2+} oscillations in eggs that is attributed to its novel biochemical characteristics. In this review, we discuss the latest developments that have begun to unravel the vital role of PLCζ at mammalian fertilisation and decipher its unique mechanism of action within the fertilising egg. We also postulate the significant potential diagnostic and therapeutic capacity of PLCζ in alleviating certain types of male infertility.

1. Introduction

Oocyte (egg) activation is the first and most critical step that initiates embryo development after fertilisation. The term 'egg activation' describes a series of biochemical and morphological events that mammalian eggs go through to prepare the egg for early embryo development after sperm-egg fusion [1]. In all species examined up-to-date, the earliest signaling event in the activation of an egg by a sperm is a large transient increase in the level of intracellular free calcium (Ca^{2+}) concentration [1-3]. In many non-mammalian species (such as frogs and sea urchins), the Ca^{2+} increase entails a single rise (one spike). Contrastingly, in mammals and ascidians the Ca^{2+} signal is delivered as a prolonged sequence of repetitive Ca^{2+} transients, known as Ca^{2+} oscillations, that start soon after gamete fusion, persisting for several hours and beyond the completion of meiosis [1, 2, 4-6]. The frequency and duration of Ca^{2+} oscillations appears to be relatively species-specific, ranging from every two minutes, to every hour [1, 7, 8]. This striking phenomenon consequently orchestrates a series of further events of egg activation, such as cortical granule exocytosis (CGE), which blocks polyspermy, meiotic resumption, and pronuclear development [1, 9, 10]. Mammalian eggs are very sensitive to the precise pattern of Ca^{2+} oscillations [11, 12]. The frequency and amplitude of Ca^{2+} oscillations are directly responsible for cell cycle progression, with varying Ca^{2+} transients resulting in different rates of cell cycle progression [13, 14]. Considering that the rate of cell cycle progression of human oocytes following fertilization has been postulated as an indicator of normal embryogenesis [15], the profile of Ca^{2+} oscillations during mammalian fertilisation may not only be necessary and sufficient for egg activation to occur but also equally important for subsequent embryogenic events [16].

Several lines of evidence suggest that the generation of Ca^{2+} oscillations in mammalian eggs is a direct consequence of inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} release [1, 4, 17]. The involvement of the universal Ca^{2+}-mobilizing messenger, $InsP_3$, and its $InsP_3$ receptor ($InsP_3R$) was demonstrated by experiments in mouse and hamster eggs, where blocking or down-regulating the expression of $InsP_3Rs$ led to the inhibition of Ca^{2+} oscillations and egg activation [18-20]. Furthermore, microinjection of $InsP_3$ or adenophostin A, an $InsP_3$ analogue, lead to Ca^{2+} oscillations in all mammalian eggs examined, demonstrating the necessity of this signaling pathway in progression of the Ca^{2+} release and egg activation process at mammalian fertilisation [1, 21, 22].

2. Sperm factor hypothesis and a novel PLC as the trigger of Ca^{2+} oscillations at fertilisation

The vigorously-contested scientific debate over the precise mechanism of how a single sperm elicits the generation of Ca^{2+} oscillations in the unfertilized egg dates over several decades. Many theories had been proposed to explain the genesis of the Ca^{2+} transients that successfully trigger egg activation during mammalian fertilisation (for review see [1]). Over the years, the weight of evidence has steadily shifted significantly in support of the 'sperm factor' hypothesis as the most appropriate model for egg activation in mammalian, and a number of marine invertebrate, species. This theory proposed that the sperm contains a soluble factor that, upon sperm-egg fusion, readily diffuses into the egg, and is functionally capable of triggering Ca^{2+} release from intracellular stores [5]. Experimental support for this hypothesis came from studies demonstrating that injection of sperm cytosolic extracts into mammalian eggs triggered a prolonged series of Ca^{2+} oscillations, indistinguishable to those seen at fertilisation, and this physiological stimulus initated all the necessary events for successful egg activation [5, 23]. Moreover, the development of the clinical, *in vitro* fertilisation (IVF) technique, intracytoplasmic sperm injection (ICSI), a procedure that injects intact sperm directly into the egg cytoplasm, provided further support for the sperm factor hypothesis [24]. ICSI-injection of whole sperm in mammalian eggs results in egg activation and embryo development to term [25, 26].

Over the years, many candidates have been proposed as potential sperm factors. Initially, small molecules such as $InsP_3$ [27], nitric oxide (NO) [28] or nicotinic acid adenine dinucleotide phosphate (NAADP) [29] were considered as sperm factors but, although these molecules can induce some form of Ca^{2+} release from intracellular stores in some non-mammalian eggs, none of these substances could fully mimic the characteristic series of Ca^{2+} oscillations observed during IVF of mammalian eggs [1, 30]. *In vitro* PLC activity assays on mammalian cytosolic sperm extracts revealed that these extracts possess many fold higher PLC enzymatic activity compared with other somatic tissues that express several PLC isoforms [31]. More interestingly, although PLCs are enzymes that are generally characterized for their low Ca^{2+} sensitivity, the sperm extracts showed remarkable PLC activity even at 0.1μM Ca^{2+} concentration, the basal cytoplasmic Ca^{2+} levels found in the egg at the time of fertilisation [31]. Chromatographic fractionation studies suggested that the sperm factor should be a protein of ~30-100 kDa in size [1, 31, 32]. Microinjection of recombinant proteins corresponding to all the known and most characterized PLC isoforms at

the time, failed to trigger the pattern of Ca^{2+} oscillations observed during fertilisation [32, 33]. All these observations were consistent with the hypothetical existence of a novel, unidentified PLC isoform as the soluble sperm factor that triggers Ca^{2+} oscillations and activation of mammalian eggs, particularly since during this period the sequence of the human genome had not yet been reported.

3. PLCζ; the only native substance that can induce Ca^{2+} release and activation of mammalian eggs

In 2002, our laboratory in the then University of Wales College of Medicine (now Cardiff University School of Medicine) identified a novel testis-specific PLC isoform, which was termed PLCζ [34]. Microinjection of *in vitro*-transcribed cRNA encoding PLCζ into mouse eggs resulted in the observation of fertilisation-like Ca^{2+} release events, and subsequent early embryonic development up to the multicellular blastocyst stage [34]. Interestingly, the estimated expression levels of PLCζ required for the initiation of Ca^{2+} oscillations was very closely comparable to the estimated amount of this enzyme contained in a single mouse sperm [34]. Moreover, the Ca^{2+} oscillation-inducing activity of sperm extracts was completely abolished following PLCζ immunodepletion from these extracts by specific anti-PLCζ antibodies [34]. Microinjection of recombinant mouse PLCζ protein into mouse eggs also triggered Ca^{2+} oscillations similar to those observed after sperm extract injection [35]. Furthermore, RNA interference (RNAi) experiments producing severe disruption of PLCζ expression in transgenic mice testes yielded sperm with otherwise normal proeprties that most notably induced prematurely terminating Ca^{2+} oscillations with a significantly reduced litter size [36]. Subsequent studies have also identified further mammalian orthologues of PLCζ in human, hamster, monkey and horse sperm [4, 37-40], while non-mammalian testis-specific PLCζ homologues have been identified in the chicken [41] and fish [42, 43]. Additional support for the fundamental role of PLCζ at mammalian fertilisation came from a number of genetic and clinical reports that directly linked certain types of human male infertility with PLCζ deficiencies (abnormally low expression levels or mutated forms of PLCζ) in the sperm of IVF patient couples presenting with oocyte activation deficiencies (OAD) and thus total fertilisation failure i.e. infertility [44-49].

It is now well characterised and widely believed that upon sperm-egg membrane fusion, PLCζ is delivered from the sperm head into the egg cytoplasm and this then catalyses the hydrolysis of its membrane-bound substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2)

which is located on the membrane of an intracellular vesicle compartment [1, 34, 50]. The resulting liberation of $InsP_3$ stimulates opening of the $InsP_3R$, a Ca^{2+} release channel in the endoplasmic reticulum resulting in Ca^{2+} oscillations, causing egg activation and subsequent early embryonic development [1, 34, 50]; (Figure 1).

It is worth noting that over the previous 2 decades other proteins have been proposed as potential sperm factor candidates, including a 33 kDa protein (prematurely/imprudently termed 'oscillin') [51], a truncated form of the c-kit receptor (tr-kit) [52] and more recently, the postacrosomal sheath WW domain-binding protein (PAWP) [53, 54]. However, as various studies have demonstrated, none of these molecules induce the Ca^{2+} oscillations observed at fertilisation and nor fulfil all the physiological criteria for the sperm factor [1, 55-60]. Thus, all the mounting experimental and clinical evidence emanating from a number of different laboratories in the world indicate that the only identified molecule up to date, which is capable of initiating Ca^{2+} oscillations during mammalian fertilisation, is the testis-specific PLCζ [50, 61].

4. PLCζ structure and domain organization

Mammalian phosphoinositide-specific phospholipase C (PI-PLC) comprises a family of ubiquitous intracellular enzymes that play an essential role in activating intracellular signal transduction pathways to regulate various cellular functions [4, 62]. PI-PLCs directly participate in the phosphoinositide signalling pathway, catalysing the hydrolysis of PIP_2 and giving rise to two important second messenger molecules; $InsP_3$ and diacylglycerol (DAG). Fourteen distinct mammalian PLC isoforms have thus far been identified and are grouped into six distinct classes [β (1-4), γ (1,2), δ (1-4), ε, ζ, and η (1,2)], based on domain organisation and mode of activation. PLC isoforms also differ in tissue distribution, expression levels, Ca^{2+} sensitivity, catalytic regulation, and cellular localisation [4, 62, 63].

Despite its superior Ca^{2+} oscillation-inducing activity in eggs relative to somatic PLCs, sperm PLCζ is the smallest PLC isoform with the most basic domain organization. PLCζ enzymes in all the species characterized up to date, share a similar size of 70-75 kDa [4, 50, 64]. PLCζ demonstrates a typical PLC domain structure consisting of four EF hand motifs, followed by the characteristic X&Y catalytic domains and a single C2 domain at its C-terminus. These domains are common to all PLC isoforms (β, γ, δ, ε, ζ and η) [50]. The catalytic X&Y domains form the active site in all PLCs and their sequence is the most highly conserved domain between the different PLC isoforms relative to the other regulatory

domains. The sequence similarity among all PLC isoforms is 60%, but much higher among the isoforms of the same class. The catalytic X&Y domains of PLCζ shares a 64% sequence similarity with that of PLCδ1 and by structural homology, is predicted to be organised in eight repetitive beta sheet/alpha helix sequences, forming a distorted barrel [4, 50]. Mutagenesis of conserved active site residues within the XY catalytic domain of PLCζ resulted in loss of enzymatic activity, and thus its inability to induce Ca^{2+} release in mammalian eggs [1, 50]. In all PLC isoforms, a discrete region separates the X and Y catalytic domains, known as the XY-linker. In PLCζ, this is an unstructured linker region with a distinctive cluster of basic amino acid residues not found in the XY-linker regions of the other PLC isoforms, and playing an important regulatory role in PLCζ function [1, 34, 50].

A notable structural difference of sperm PLCζ to somatic PLC isoforms is it's lack of a pleckstrin homology (PH) domain [34]. PH domains are well defined structural modules of ~120 amino acid residues long, which have been identified in more than 100 different proteins [1, 4]. All PLC isoforms except PLCζ and PLCε possess a PH domain. It is believed that the PH domain facilitates the binding of PLCs to biological membranes [62, 65]. The PH domain of PLCδ1 mediates the binding of this enzyme to the cell plasma membrane by its high-affinity and -specificity binding to its membrane-bound substrate, PIP_2 [66]. With the significant absence of a PH domain, PLCζ is most closely related in domain structure to PLCδ1 enzyme exhibiting a 33% identity and 47% similarity [34]. Due to the lack of a PH domain, PLCζ appears to employ a unique mechanism to target it's biological membrane substrate, involving synergy of the positively-charged N-terminal lobe EF-hand domain with the basic amino acids at the C-terminal end of its XY-linker region, that coalesce through electrostatic interactions with its negatively-charged substrate, PIP_2 [67-69].

5. Essential role of the EF hand domains in the Ca^{2+} sensitivity and membrane targeting of PLCζ

Arguably the most critical physiological feature of PLCζ allowing it to be enzymatically very active in mammalian eggs relative to other somatic PLCs, is its uniquely high Ca^{2+} sensitivity [35, 70]. PLCζ is ~100-fold more Ca^{2+} sensitive compared with its PLCδ1 counterpart, displaying an EC_{50} of ~80nM [70], well within the range of reported resting Ca^{2+} concentrations in mammalian eggs, ~120nM, explaining why PLCζ's biochemical activity is observed very soon after its release from the sperm into the egg cytoplasm [70].

We have previously shown that deletion of the EF-hand domains dramatically increases the EC_{50} of PLCζ from ~80nM to ~30μM [70], while replacement of PLCζ EF-hand domains with that from PLCδ1 results in a ~10-fold decrease in the Ca^{2+} sensitivity of PLCζ, without significantly affecting its enzymatic activity [71]. In addition, in parallel with our empirical data, mathematical modelling approaches have strongly suggested that the EF-hand motifs are the major determinants of the high Ca^{2+} sensitivity of PLCζ, which in turn leads to its unsurpassed effectiveness in triggering high frequency Ca^{2+} oscillations in mammalian eggs [71].

Recently, we investigated whether replacement of the EF-hand domains of rat PLCδ1 with mouse PLCζ, in a PLCδ1 construct lacking the PH domain can confer to this chimeric enzyme the high Ca^{2+} sensitivity of PLCζ. We generated the ΔPH/PLCδ1/EFζ construct, as shown in Figure 2A comprising the N-terminal 150 aa of PLCζ (containing its EF-hand domains) fused to a PLCδ1 deletion construct lacking the first 284 aa (comprising both its PH & EF-hand domain regions). This ΔPH/PLCδ1/EFζ construct encodes a 624 aa chimaeric protein that was expressed using the pETMM60 vector and isolated by affinity chromatography as previously described [69, 71-73] enabling characterization of the purified ~129kDa NusA-tagged PLC chimaera (Fig. 2B). The PIP_2 hydrolytic enzyme activity of PLCζ, PLCδ1 and ΔPH/PLCδ1/EFζ was determined (Fig. 3A & Table 1) and indicates that chimaeric protein displayed a ~31% reduced enzymatic activity (2015±51 nmol/min/mg) compared to PLCδ1. To investigate the effect of replacing EF-hand domains of PLCδ1 with that of PLCζ on the Ca^{2+} sensitivity of PLCδ1 (lacking the PH domain), we assessed the [^{3}H]PIP_2 hydrolysis at different Ca^{2+} concentrations ranging from 0.1nM to 0.1mM [69, 71-73]; (Figure 3B, Table 1). The resulting EC_{50} values for PLCζ (73 nM) and PLCδ1 (5.96 μM) were in good agreement with those obtained at previous studies [70, 72, 73], while the EC_{50} for ΔPH/PLCδ1/EFζ (4.71 μM) was slightly lower compared to PLCδ1, but ~64-fold higher compared to the EC_{50} of PLCζ. Our experiments suggest that the EF-domains of PLCζ on their own are not sufficient to confer the high Ca^{2+} sensitivity of PLCζ to a PLCδ1-like protein lacking the PH domain, indicating that the EF-domains play a vital role in conferring the high Ca^{2+} sensitivity of PLCζ but the overall PLCζ protein tertiary structure is an additional essential requirement.

Interestingly, the N-terminal lobe of the EF-hand domains of PLCζ contains a cluster of basic amino acid residues. We recently demonstrated that sequential neutralisation of these

basic residues within the first EF-hand domain of PLCζ significantly diminishes the PIP_2-binding properties of PLCζ [69]. We surmised that PIP_2 might be attracted to the negatively-charged PIP_2-containing component of the intracellular vesicular membrane through electrostatic interactions and thus, both the EF-hand and the XY-linker regions which are rich in basic residues, are essential for this interaction. This molecular interaction provides a tether that facilitates proper PIP_2 substrate access to and binding with the PLCζ active site [69].

6. The regulatory role of the non-conserved XY-linker region

The XY-linker region of PLCζ is extended in length and contains more positively-charged amino acid residues relative to its PLCδ1 counterpart [34, 50]. Notably, the XY-linker is the most non-conserved region of the PLCζ domain sequences determined from different species, but in all cases this linker region contains a number of positively-charged residues in close proximity to the Y catalytic domain [4, 50]. The specific physiological rationale for this XY-linker diversity is still unclear. Perhaps the diversity in this sequence may explain the species-specific differences in specific patterns of Ca^{2+} oscillations observed for various mammalian PLCζ enzymes, as well as the divergent relative potencies of these enzymes in triggering cytosolic Ca^{2+} oscillations when expressed in mouse eggs [4, 6]. Notably, we have demonstrated that the XY-linker plays a key role in the functional regulation of PLCζ enzyme activity and also in its important ability to contribute to the interaction with its membrane-resident lipid substrate, PIP_2 [67, 68, 74], Finally, the XY-linker region contains a predicted nuclear localization signal (NLS) sequence that may play a role in the regulation of at least mouse PLCζ [75].

7. The vital role of PLCζ C2 domain is highlighted by male infertility-linked point mutation

All PLC isoforms possess a C2 domain, following the XY catalytic domain. These domains comprise ~120 amino acid residues and play an important role in the Ca^{2+}-dependent subcellular membrane targeting of several lipid-metabolizing enzymes, such as PLCδ1 and cPLCA2 [76]. In PLCδ1, the structurally closest protein to PLCζ, the C2 domain interacts with the membrane phospholipid, phosphatidylserine (PS) to form a C2-Ca^{2+}-phosphatidylserine quaternary complex, which enhances its enzymatic activity [77].

The exact physiological role of PLCζ C2 domain is still unresolved. There is experimental evidence for low-affinity binding of this domain to membrane phospholipids; phosphatidylinositol 3-phosphate [PI(3)P] and phosphatidylinositol 5-phosphate [PI(5P)] [68, 78]. We have previously demonstrated that deletion or replacement of the C2 domain of PLCζ with the corresponding domain of PLCδ1 abolishes the Ca^{2+} oscillation-inducing activity in intact eggs, without affecting the in vitro PIP_2 hydrolytic properties of this enzyme [70, 71]. These observations suggest a disparate, vital (but apparently non-hydrolytic) role of the C2 domain in PLCζ function. Further support for this suggestion came from the identification of a male infertility-linked point mutation located in the C2 domain of PLCζ [49]. This is the first PLCζ infertility-linked mutation to be reported in a domain other than the XY catalytic domain. In this mutant, an isoleucine (I) residue is substituted with a phenylalanine (F) residue in 489 position (I489F) of human PLCζ sequence [49]. Analysis of this mutation revealed that at physiological concentrations this PLCζ mutant is unable to trigger Ca^{2+} release in mouse eggs [79]. However, microinjection of non-physiological levels could rescue the infertile phenotype, leading to Ca^{2+} oscillations and egg activation. Further biochemical characterization suggested that the I489F had no effect on the enzymatic properties of PLCζ, but dramatically reduced its in vitro binding to PI(3)P- and PI(5)P-containing liposomes [79]. These findings highlight the critical role of this domain in PLCζ function due to its novel membrane binding/localisatio characteristics (Figure 4). Further investigation is required to delineate the physiological significance of PLCζ-PI(3)P and -PI(5)P interactions and to identify other potential egg-derived binding partners of this domain that might regulate PLCζ function within the egg.

8. Localisation of PLCζ in the sperm

PLCζ has been identified in sperm of numerous species and generally appears localised at distinct subcellular regions of the sperm head, with potentially differential functional roles for each specific population [64]. PLCζ has been identified in acrosomal and post-acrosomal regions of mouse and porcine sperm, with a tail population also identified in porcine sperm [80-82]. In equine sperm, PLCζ was reported in the acrosome, equatorial segment, head mid-peace, as well as the principal piece of the flagellum [40]. In humans, three distinct populations of PLCζ have been identified in the acrosomal, equatorial and post-acrosomal regions of the sperm head, with a potentially additional tail localisation [44, 47, 83-86]. However, it remains unclear whether such populations are physiologically valid.

Specific PLCζ localisation remains a concern, with numerous studies identifying variable patterns within the same mammalian species, often using the same antibody probe.

A previous study suggested that PLCζ was localised within the acrosome of mouse and human sperm, with further PLCζ populations present on the sperm surface [87]. However, using the same antibodies, multiple studies indicated PLCζ localisation to the post-acrosomal region of the mouse sperm [80, 88], as well as to the acrosomal, equatorial, and post-acrosomal regions of human sperm [44, 84-86, 89]. Indeed, there only seems to be consensus regarding PLCζ localisation in mouse sperm. This variance in PLCζ localisation is not only limited to observations between different species, with a study reporting significant variance in localisation patterns observed in sperm between human subjects [86].

Perhaps such variance is attributable to differences in immunolocalisation protocols used by different studies, or due to limited specificity of the polyclonal antibodies that have been used in all such studies to date, particularly in relation to human sperm. Recently developed epitope-specific PLCζ polyclonal antibodies to human, mouse and porcine PLCζ, have exhibited reliable consistency in results throughout multiple studies for both recombinant and native PLCζ [90-92]. Further to such progress, it has recently been demonstrated that specific antigen unmasking/retrieval protocols are required to enhance the visualisation efficacy of PLCζ in mammalian sperm, perhaps due to strong intra- or inter-molecular conformation(s)/interaction(s) between discrete domains of monomeric and/or oligomeric PLCζ [92]. Following the application of these specific antibodies and enhanced protocols, PLCζ has been identified in the acrosomal and post-acrosomal, acrosomal and equatorial, as well as, post-acrosomal and equatorial compartments of mouse, human and porcine sperm, respectively. Furthermore, use of these antibodies has consistently observed potential tail localisation in all species [92]. These recent observations from application of new PLCζ-specific probes and protocols suggest that previously published results regarding PLCζ subcellular localisation in sperm of mammalian species may require further detailed evaluation. For example, Grasa *et al*., [89] and Young *et al*., [88] demonstrated in human and mouse sperm, respectively, that prior to capacitation acrosomal populations of PLCζ were prominent, shifting to a predominantly post-acrosomal localisation following capacitation. While such results remain supportive of PLCζ's proposed identity as the mammalian sperm factor, it is important that further high-resolution localisation analysis enables a concensus view to emerge.

9. Localisation of PLCζ in the egg and the search for a putative 'egg factor'

Another unique biochemical feature of sperm PLCζ is its localisation in mammalian eggs. Somatic PLC isoforms are known to localise to the plasma membrane where they hydrolyse their membrane-bound substrate PIP_2 [50]. However, there is no detectable PLCζ localisation in the plasma membrane of eggs [93]. Moreover, the depletion of plasma membrane PIP_2 using a targeted inositol lipid phosphatase has no effect upon PLCζ- or sperm-induced Ca^{2+} oscillations, even though such a PIP_2-depletion strategy effectively abolishes PLCδ1-induced Ca^{2+} oscillations [93]. Further, immunocytochemical approaches suggest that the introduced sperm PLCζ becomes localised to small vesicles throughout the egg cytoplasm [93]. Interestingly, PIP_2 is also detected in such cytoplasmic vesicles. This putative intracellular PIP_2 source is very significant because the expression of an inositol lipid phosphatase fused with catalytically-inactive PLCζ in mouse eggs (to specifically target and deplete this vesicular PIP_2), potently inhibited both sperm- and PLCζ-induced Ca^{2+} oscillations [93]. This indicates that the egg cytosolic Ca^{2+} oscillations induced by PLCζ or sperm rely upon hydrolysis of PIP_2 from intracellular vesicles primarily, and not the plasma membrane, which in itself represents an entirely new aspect of PI-mediated Ca^{2+} signalling.

Immediately after PLCζ is injected into eggs it is capable of triggering Ca^{2+} oscillations, hence PLCζ appears to be autonomously active. However, relative to the egg, PLCζ is present at >1000 times higher concentration in sperm where it appears to be either enzymatically inactive or compartmentalised. Transfection studies have shown that PLCζ can be stably expressed in CHO cells at concentrations 100-1000 times that found in an egg during fertilization, and yet this does not cause any change in Ca^{2+} homeostasis [91]. However, when these PLCζ-expressing CHO cells, or extracts from these cells, are injected into mouse eggs, Ca^{2+} oscillations are induced [91]. These data suggest that PLCζ may only be active in eggs, and this further implies that eggs possess an essential factor(s) that is specifically required for PLCζ to hydrolyse PIP_2. The target molecule or 'receptor' for PLCζ is likely to be on the egg vesicles that contain PIP_2 and if this target interacts very specifically with PLCζ then this hypothesis may also explain why other mammalian PLC isoforms e.g. PLCδ1, are orders of magnitude less effective in triggering Ca^{2+}oscillations when injected into mouse eggs. The identification of an egg receptor for PLCζ will have

major implications for understanding wider cases of infertility and for animal reproductive technologies.

10. PLCζ, ICSI failure and male infertility

ICSI, a powerful IVF technique whereby a single sperm is injected into oocytes, is an effective method of assisted reproductive technology (ART) for men presenting with suboptimal parameters, such as abnormal sperm concentration, motility, or morphology. Such methodology is generally applied in cases when couples experience low fertilisation success or complete fertilisation failure following conventional IVF. However, despite relatively high rates of ICSI success, total fertilisation failure occurs in ~1–5% of ICSI cycles, usually recurring in subsequent ART cycles [94, 95]. Potential causative factors include failed sperm head decondensation, premature sperm chromatin condensation, oocyte spindle defects and sperm aster defects [46], while issues such as incorrect sperm injection/expulsion, or low gamete quality [96, 97] may also contribute. However, a failure of the oocyte activation mechanism is frequently considered to be the main contributory factor [46, 95, 98-101].

Importantly, morphologically normal sperm from several human patients have been found to fail to activate mouse eggs [45, 101], with sperm either unable to elicit Ca^{2+} release following injection into mouse eggs, or else producing significantly diminished and abnormal Ca^{2+} oscillation profiles, being severely reduced in frequency and amplitude [44, 45]. Furthermore, immunofluorescence and immunoblot analysis of such sperm exhibited reduced or absent levels of PLCζ within the sperm head, while the Ca^{2+} oscillation-inducing activity of such sperm was 'rescued' in mouse oocytes by the co-injection of PLCζ cRNA [44, 45]. Subsequent to these studies, numerous investigations performing PLCζ assessment in human sperm undergoing fertility treatment indicate that sperm with defective oocyte activation capacity are associated with reduced/absent levels of PLCζ [44-46, 86, 102, 103], while PLCζ deficiencies are also associated with sperm conditions, such as globozoospermia [45, 46, 102, 104, 105] and recurrent partial hydatidiform moles (abnormal pregnancies) [106].

Importantly, abrogation of PLCζ gene function in patients diagnosed with oocyte activation deficiency has also now been reported. Two PLCζ mutations were identified from an infertile man, whose sperm lacked the capacity to elicit Ca^{2+} oscillations that led to disrupted PLCζ activity and an infertility phenotype [45, 47]. Biochemical characterisation indicated that the mutations, both occurring in the active site domains of PLCζ, led to disruption of local protein folding, while injection of correspondingly mutated cRNA and

recombinant protein into mouse eggs resulted in highly abnormal Ca^{2+} transients that were unable to initiate oocyte activation [48, 90, 107]. Both mutations were reported to be heterozygous, with one inherited from the patient's father, with the other inherited from the patient's mother, indicating for the first time that such maternally-inherited loss-of-activity mutations contribute towards male infertility [47, 107].

Subsequently, a homozygous mutation was identified by Escoffier *et al*., (2016) from a similarly infertile patient, occurring within the C2 domain of PLCζ [49]. As discussed above, this PLCζ C2 domain mutant displays similar enzymatic activity compared to wild-type PLCζ, however exhibiting dramatically reduced binding to PI(3)P and PI(5)P-containing liposomes [79]. Single nucleotide polymorphisms (SNPs) have also been reported either within the coding sequence of PLCζ or its associated bi-directional promoter in human patients [44, 108]. Further polymorphisms have also been identified in the PLCζ gene of bulls and stallions, at promoter regions and exonic loci, which were shown to positively correlate to semen parameters and the fertility status of such sperm [109, 110]. However, as these polymorphisms were not physiologically or biochemically characterised, any potential contributory effects exerted by SNPs upon sperm PLCζ function are currently unclear.

Recent data suggest a significant level of variance in terms of total PLCζ levels and localisation patterns in human sperm from normal, fertile men [44, 45, 84, 85]. Such variance in sperm PLCζ presents an interesting problem, particularly when considering that absent/reduced levels of PLCζ have previously been implicated in cases of infertility, and that levels of PLCζ directly impact upon the frequency and amplitude of the resulting Ca^{2+} oscillations. Injection of increasing PLCζ cRNA levels in human oocytes results in increasing frequencies and amplitudes of Ca^{2+} oscillations [111], which may also exert effects upon gene expression profiles in a Ca^{2+}-dependent manner during early embryogenesis [13, 14]. Indeed, preimplantation development of oocytes activated by differential levels of human PLCζ suggests that too little, or too much, PLCζ leads to poor development to the blastocyst stage despite apparently normal oocyte activation. Thus, abnormalities in sperm PLCζ levels may underlie not only infertility through fertilisation failure, but also cases of male sub-fertility, whereby adequate PLCζ may be delivered to oocytes to cause activation only, but which may be insufficient for embryonic competence.

11. Clinical applications of PLCζ

The success of ART is reflected by estimates that such methods now account for ~7% of total birth rates in some developing countries [46]. However, several conditions such as severe male infertility (19-57% of infertility cases) remain untreatable, even after ICSI treatment [112]. It seems clear that sperm from males that fail to activate human eggs following ICSI exhibit abrogated, reduced, or aberrant forms of PLCζ. Thus, alongside a potential diagnostic parameter, PLCζ may represent an endogenous method to clinically treat cases of egg activation failure.

Currently, oocyte activation failure is clinically treated by assisted oocyte activation (AOA) methods, predominantly application of Ca^{2+} ionophores, resulting in improved rates of fertilization and successful pregnancy. However, concerns currently exist that such synthetic non-native chemicals may be potentially detrimental to embryo viability [113], considering that such methods elicit a single large Ca^{2+} increase unlike the repetitive pattern of Ca^{2+} oscillations observed at fertilisation. Indeed, computational modelling and experimental studies suggest that the temporal pattern of Ca^{2+} changes may exert specific effects upon rates of cell cycle progression, and thus, subsequent embryogenesis [114]. Thus, it is important that an endogenous, potentially safer method of assisted egg activation is established, for which PLCζ is an extremely attractive candidate. Rogers *et al*. [115] obtained parthenogenetically-generated human blastocysts by injection of PLCζ cRNA into eggs, while Yoon *et al*. [44] demonstrated that abnormalities in sperm PLCζ could be counteracted by co-injection with mouse PLCζ cRNA. Moreover, a very recent study reported the determination of the optimal concentration of human PLCζ cRNA to activate human oocytes [111].

However, while representing a significant research tool, clinical use of cRNA injections may prove problematic due to uncontrolled expression and potential reverse transcription activity inherent to mammalian oocytes [46]. Thus, the production of purified, enzymatically-active, recombinant human PLCζ protein has been a goal for many laboratories around the world, to enable dose-controlled delivery of functionally-viable PLCζ protein. To this degree, recent efforts have been able to produce such versions of human PLCζ, which is able to effectively rescue activation in mouse and human oocytes, representing a significant milestone in potential clinical applicability to reproductive medicine [90]. However, while this recombinant protein may be significantly advantageous to patients suffering from recurrent ICSI failure, it remains unclear whether a PLCζ ‘therapeutic’ may be able to aid further types of patients where normal activation at

fertilisation occurs, but subsequent initiation of early embryogenesis is poor, a causative factor for recurrent implantation failure.

12. Concluding remarks

The 2002 discovery of PLCζ, the putative physiological 'sperm factor' that plays a vital role at mammalian fertilisation represented a major breakthrough in the field of reproductive biology. It is now widely accepted that PLCζ is the sole physiological stimulus that is delivered from the fertilising sperm into the egg cytoplasm, soon after sperm-egg membrane fusion, triggering the Ca^{2+} oscillations required for successful egg activation and early embryogenesis. Despite all the recent advances that have improved our knowledge about the unique biochemical properties of this gamete-specific enzyme, the precise biological mechanism of PLCζ action and regulation within the fertilising sperm and egg has not yet been fully characterised. All the recent genetic and clinical reports that have directly linked male infertility cases with reduced expression levels and mutated forms of this sperm-specific protein necessitate elucidation of the molecular mechanism that PLCζ employs to 'kick start' a new life. For example, the participation of PLCζ in a standard biochemical pathway [PI signalling pathway] that is known to be present in all types of cells in the body, but uniquely, PLCζ appears to only be fully active within eggs. It is still currently unclear the mechanism by which PLCζ is kept inactive within the sperm head but soon after its release within the egg cytosol, it is immediately able to hydrolyse its membrane-bound substrate PIP_2, inducing Ca^{2+} release and subsequent egg activation. Moreover, while the biochemical properties of PLCζ domains have been characterised, a full understanding of how all these distinct domains work together in synchrony within the egg remains incomplete. The recent genetic report that identified the infertility-linked point mutation in the C2 domain of PLCζ, clearly suggests that this domain plays a fundamental role in PLCζ function, although it is not directly involved in the hydrolysis of PIP_2. This necessitates further investigation of the exact role of this domain in PLCζ function. There is a possibility that PLCζ interacts through this domain with an unidentified egg protein or receptor that mediates PLCζ action in the egg. Unravelling and understanding the full mechanism of PLCζ action and mode of regulation, can provide the basis for creating new advances not only in clinical medicine but also in animal breeding technologies.

AUTHOR CONTRIBUTIONS

M.N. prepared the first draft of the manuscript, which was revised and approved by J.K. and F.A.L.

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TABLE LEGENDS

Table 1

***In vitro* enzymatic properties of NusA-tagged ΔPH/PLCδ/EFζ chimaera.** Summary of specific enzyme activity and EC_{50} values of Ca^{2+}-dependence for PIP_2 hydrolysis, determined by non-linear regression analysis (GraphPad Prism 5), (see Fig. 3) for PLCζ, PLCδ1 and ΔPH/PLCδ1/EFζ fusion proteins.

FIGURE LEGENDS

Figure 1

Schematic representation of egg activation triggered by sperm-specific PLCζ. Following sperm-egg membrane fusion, PLCζ is released from the sperm into the egg cytosol and targets a distinct intracellular vesicular membrane containing its membrane-bound substrate, phosphoinositide 4,5-bisphosphate (PIP_2). PLCζ-mediated PIP_2 hydrolysis produces two second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ subsequently binds to the $InsP_3$ receptor ($InsP_3$) on the endoplasmic reticulum, an interaction that triggers Ca^{2+} release from intracellular stores, which subsequently produces the characteristic pattern of Ca^{2+} oscillations that initiate egg activation (Figure modified from [1, 59]).

Figure 2

Expression of recombinant NusA-tagged ΔPH/PLCδ1/EFζ chimaeric protein. (A) Schematic representation of the domain structure of PLCζ (brown), PLCδ1 (purple) and their corresponding ΔPH/PLCδ1/EFζ chimaera. The various amino acid lengths and respective coordinates are indicated for each construct. (B) Expression of recombinant NusA-tagged ΔPH/PLCδ1/EFζ chimaeric protein. 1 μg of recombinant NusA-tagged ΔPH/PLCδ1/EFζ protein analysed by 7% SDS-PAGE and Coomassie Briliant Blue staining (left panel) and immunoblot analysis using the anti-NusA antibody (1:25,000 dilution; right panel).

Figure 3

In vitro enzyme specific activity and Ca^{2+} sensitivity of ΔPH/PLCδ1/EFζ chimaera. (A) PIP_2 hydrolysis enzyme activity of PLCζ, PLCδ1 and ΔPH/PLCδ1/EFζ proteins obtained with the standard [^{3}H]PIP_2 hydrolysis assay [70-74]. 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 PLCζ, PLCδ1 and ΔPH/PLCδ1/EFζ 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 1).

Figure 4

Schematic illustration of the proposed intracellular mechanism of action of PLCζ in mammalian eggs. After its delivery from the fertilising sperm, PLCζ associates with a specific vesicular membrane by a potential interaction of the C2 domain with PI(3)P, PI(5)P or an as yet unidentified (membrane or cytosolic) egg protein. Then, PLCζ associates with its negatively-charged substrate PIP_2 via electrostatic interactions with the positively-charged 1st EF-hand domain and the C-terminal part of the XY-linker region. The catalytic XY domain subsequently proceeds with the enzymatic cleavage of PIP_2. The high Ca^{2+} sensitivity of PLCζ, which enables it to be active at resting nanomolar Ca^{2+} levels, is conferred by its EF hand domains. It has been reported that the XY-linker of mouse PLCζ contains a nuclear localisation signal (NLS), which targets the enzyme to pronuclei in a cell-cycle dependent manner (Figure modified from [1, 69, 79].

TABLES

Table 1

Recombinant PLC protein	**PIP_2 hydrolysis enzyme activity (nmol/min/mg)**	**Ca^{2+} dependence EC_{50} (nM)**
PLCζ	532±24	73
PLCδ1	2934±36	5960
ΔPH/PLCδ1/EFζ	2015±51	4710

FIGURES

Figure 1

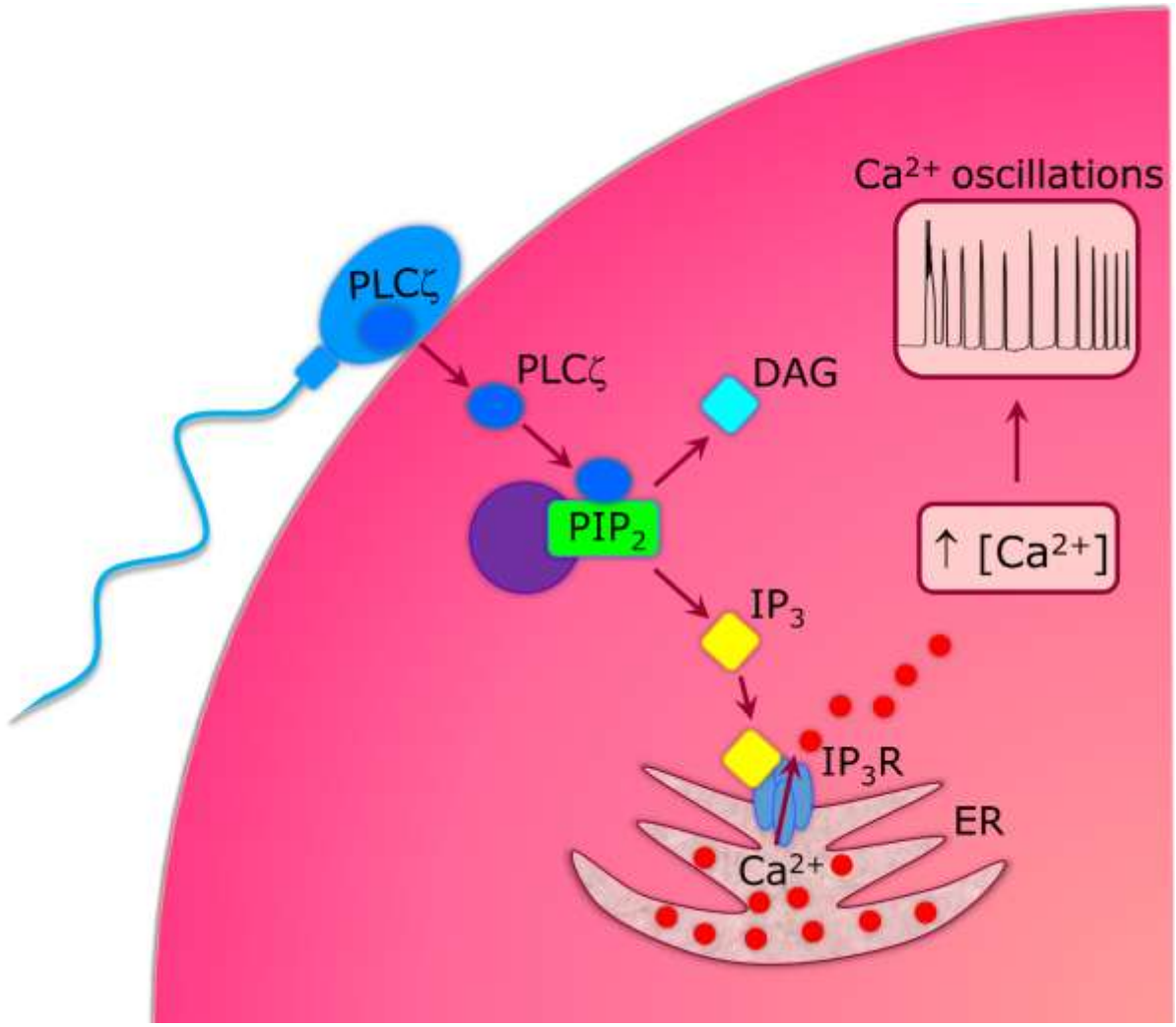
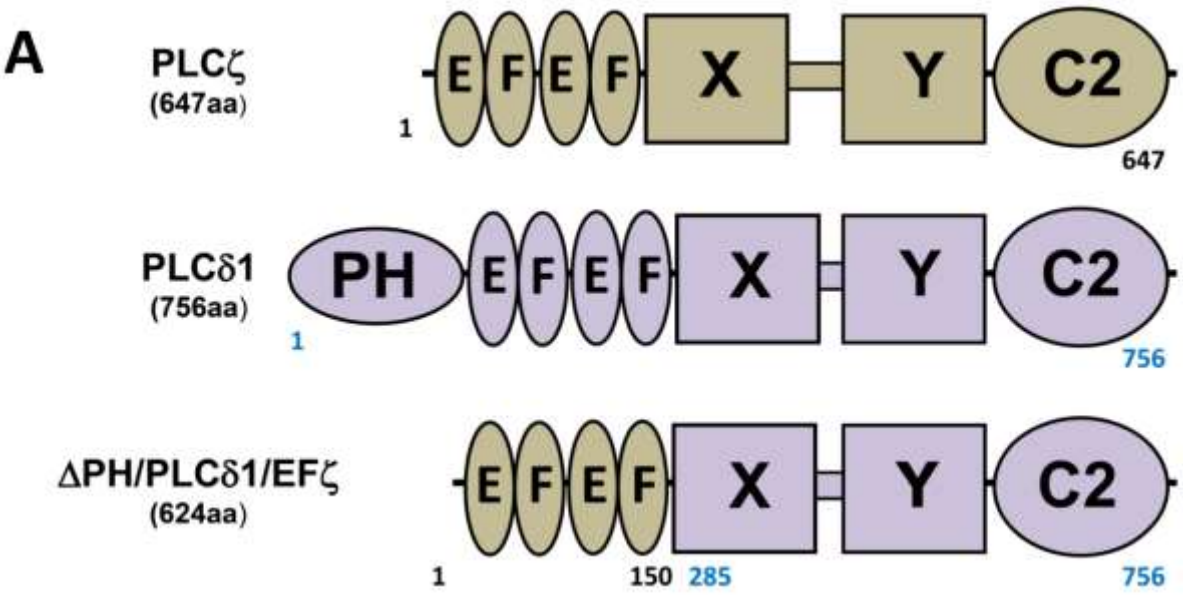


Figure 2


A
PLCζ
(647aa)
1
E F E F X Y C2
647
PLCδ1
(756aa)
PH E F E F X Y C2
1
756
ΔPH/PLCδ1/EFζ
(624aa)
E F E F X Y C2
1
150
285
756

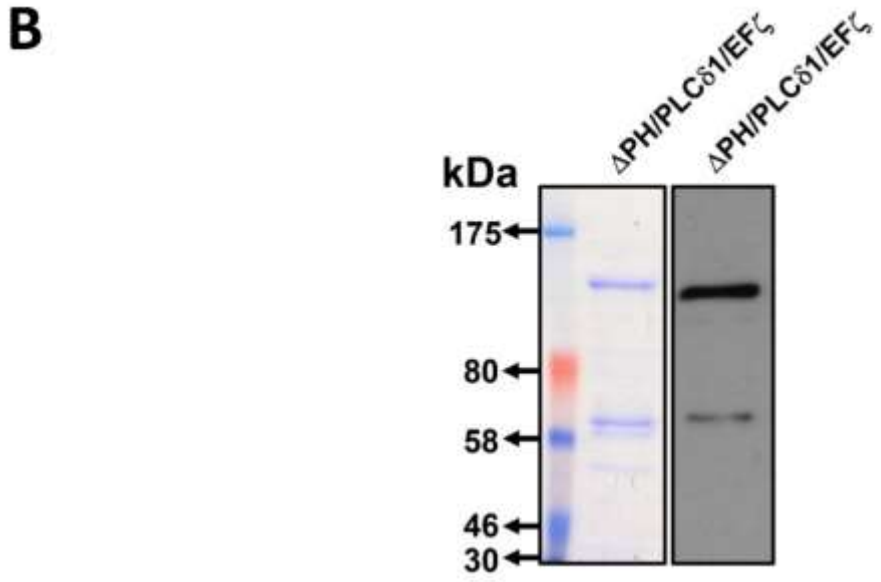

B
ΔPH/PLCδ1/EFζ
ΔPH/PLCδ1/EFζ
kDa
175
80
58
46
30

Figure 3

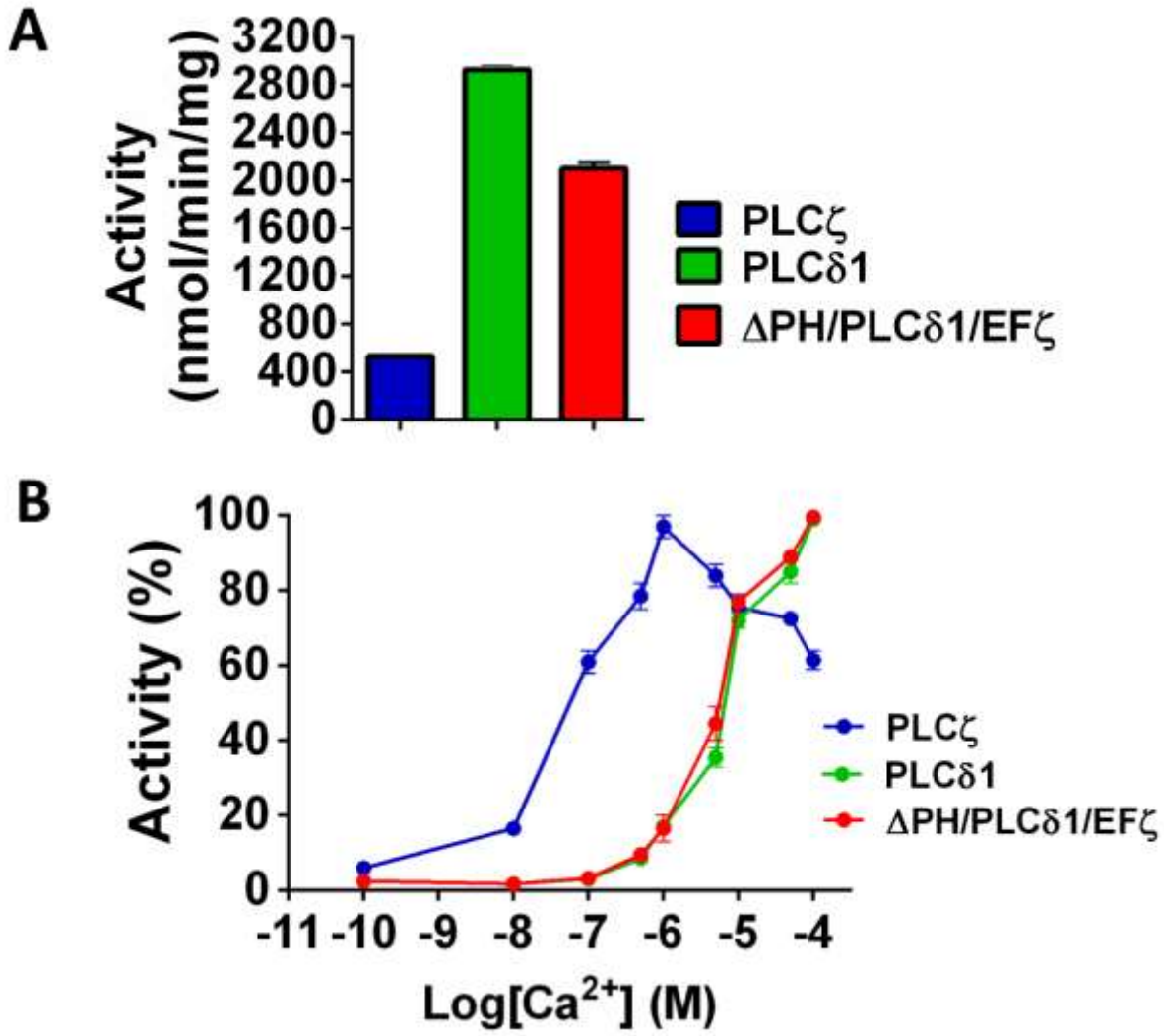
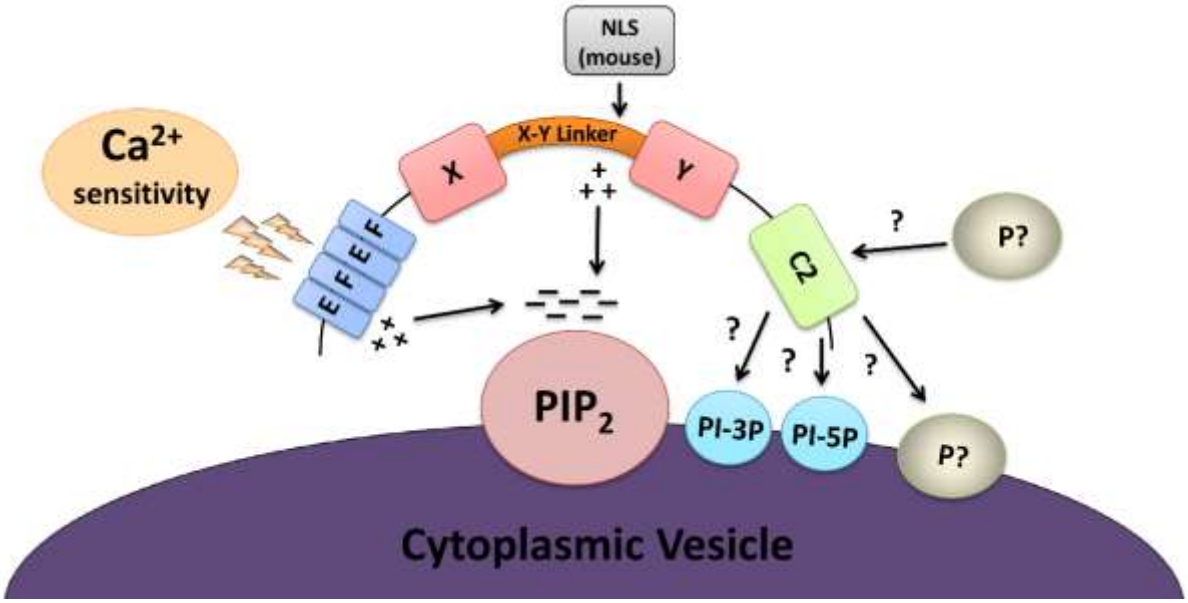

A
Activity (nmol/min/mg)
3200
2800
2400
2000
1600
1200
800
400
0
PLCζ
PLCδ1
ΔPH/PLCδ1/EFζ
B
Activity (%)
100
80
60
40
20
0
-11 -10 -9 -8 -7 -6 -5 -4
Log[Ca2+] (M)
PLCζ
PLCδ1
ΔPH/PLCδ1/EFζ

Figure 4


NLS
(mouse)
X-Y Linker
X
Y
Ca2+
sensitivity
E F E F
C2
?
P?
PIP2
PI-3P
PI-5P
P?
Cytoplasmic Vesicle