MECHANISMS OF PLCZ INDUCED CA^{2+} OSCILLATIONS IN MOUSE EGGS AT FERTILISATION

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SUMMARY

All the events of egg activation in mammalian eggs are triggered physiologically by transient increases in cytosolic free Ca^{2+} referred to as $Ca^{2+}{}_i$oscillations. These oscillations are initiated by the sperm derived PLC isoform, PLCζ. PLCζ releases Ca^{2+} by hydrolysing its substrate $PI(4,5)P_2$ to produce IP_3, however, many of the mechanisms by which PLCζ elicits Ca^{2+}release in eggs are poorly understood. The results of this thesis confirm that whilstPLCζ cRNA and recombinant protein is able to cause $Ca^{2+}{}_i$oscillations in mouse eggs the sperm derived protein PAWP does not cause any Ca^{2+} release in any circumstances. It is shown that EF hand domain and XY linker of PLCζ are important in determining its $Ca^{2+}{}_i$ releasing ability by enabling PLCζ binding to its substrate $PI(4,5)P_2$ through electrostatic interactions. The C2 domain of PLCζ was also found to play a crucial role in the Ca^{2+} releasing ability of PLCζ, possibly by binding to lipids or proteins in the target membrane. The Ca^{2+}releasing ability of eggs is acquired during oocyte maturation and a dramatic increase in PLCζ sensitivity of oocytes occurs after germinal vesicle breakdown.A variety of markers for PLCζ's substrate $PI(4,5)P_2$ including fluorescent $PI(4,5)P_2$ and gelsolin based fluorescent probes suggests that this $PI(4,5)P_2$ is localised to intracellular vesicles that could derive from Golgi apparatus. Attempts are made to measure PI turnover in these intracellular compartments of eggs during PLCζ induced $Ca^{2+}{}_i$ oscillations using several probes. The results of this thesis suggest that PLCζ releases Ca^{2+} by a novel IP_3 based signalling pathway that involves an intracellular source of $PI(4,5)P_2$.

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This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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MANUSCRIPTS

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LIST OF ABBREVIATIONS

µl	microlitre
µg	microgram
µM	micromolar
µm	micrometre
2-AG	2-arachidonoylglycerol
Ach	acetyl choline chloride
AC-lectin	*Amaranthus Caudatus* lectin
ADP	adenosine diphosphate
APC/C	anaphase promoting complex/cyclosome
ATP	adenosine triphosphate
BAPTA	1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid)
BFA	brefeldin A
BMP	bone morphogenetic protein
BMX	isobutyl- 1-methylxanthine
BRET	bioluminescence resonance energy transfer
BSA	bovine albumin serum
Ca^{2+}	calcium
Ca^{2+}_i	cytosolic free calciumconcentration
$CaCl_2$	Calcium chloride
CaM	calmodulin
CaMKII	calmodulin kinase II
cAMP	cyclic adenosine monophosphate

CB-SLIP	Click Beetle split luciferase IP_3 probe
CCD	charged coupled device
Cdc25A	cell division cycle 25 homolog A
CDK1	cyclin dependent kinase 1
CDP-DAG	cytidine diphosphate diacylglycerol
CDP-DAG synthase	phosphatidate cytidyl transferase
CERT	Ceramide transfer protein
CFP	cyan Fluorescent protein
cGMP	cyclic guanine monophosphate
CHO	Chinese hamster ovary cells
CKAR	C kinase activity reporter
CO_2	carbon dioxide
COPI	coat protein I
CSF	cytostatic factor
CTP	cytidine triphosphate
D609	tricyclodecan-9-yl-xanthogenate
DAG	diacylglycerol
DAGR	diacylglycerol reporter
DAN	fluorophore 2-dimethylamino-6-acyl-naphthalene
DGK	DAG kinase
$DiIC_{18}(3)$	1,1'-Dioctadecyl-3,,3,3',3'-Tetramethylindocarbocyanine Perchlorate
DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
EC_{50}	half maximal effective concentration

EDTA	ethylenediaminetetraacetic
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Emi 2	endogenous meiotic inhibitor 2
ER	endoplasmic Reticulum
EST	expressed sequence tag
FAD^{++}	flavin-adenine dinucleotide
fg	femtograms
FIRE	fluorescent IP_3 responsive element
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
F-SLIP	Firefly split luciferase IP_3 probe
Gd3+	gadolinium
GEF	guanine nucleotide exchange factors
GFP	green fluorescent protein
Golgi	Golgi apparatus
Grb2	growth receptor bound protein 2
GV	germinal vesicle
GVBD	germinal vesicle breakdown
H_2O	water
hCG	human chorionic gonadotrophin
HCO_3^-	bicarbonate
HKSOM Hepes	buffered potassium simplex optimised medium
hr	hour
IBC	IP_3 binding core

ICCR	IP_3 induced calcium release
ICSI	intra-cytoplasmic sperm injection
Ig	immunoglobulin
IP_2	inositol bisphosphate
IP_3	inositol trisphosphate
IP_3R	inositol trisphosphate receptor
IP_4	inositol tetrakisphosphate
IRIS	IP_3R based IP_3 sensor 1
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
K^+	potassium
KCL	potassium chloride
KCL	potassium chloride
kDa	kilo Daltons
KH_2PO_4	potassium phosphate (monobasic)
LH	lutenising hormone
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyltransferase
mg	milligram
MAGL	monoacylglycerol lipase
MAPK, ERK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C-kinase substrate
MDCK	Madin-Darby canine kidney epithelial cells
$MgCl_2$	magnesium chloride

$MgSO_4$	magnesium sulphate
MI spindle	first meiotic spindle
MII	metaphase of the second meiotic division
mM	millimolar
MPF	maturation promoting factor
Na	sodium
Na/ HCO_3^-	sodium/bicarbonate co-transporter
Na_2HPO_4	di-sodium hydrogen phosphate dihydrate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
Nedd4	neural precursor cell expressed developmentally down-regulated protein 4
NH_4Cl	ammonium chloride
NLS	nuclear localisation sequence
nM	nanomolar
nm	nanometer
NusA	N-utilization substance
OCRL1	inositol polyphosphate 5-phosphatase
OGBD	Oregon green BAPTAdextran
OSBP	oxy-sterol binding protein
PA	phosphatidic acid
PAP/LPP	3-*sn*-phosphatidate phosphohydrolase
PAS	post acrosomal sheath
PAWP	post acrosomal WW domain binding protein

PAWP	post acrosomal sheath WW domain- binding protein
PB	polar body
PBS	phosphatebuffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDBμ	phorbol 12,13-dibutyrate
PDE	phosphodiesterase
PFA	paraformaldehyde
pg	picograms
PGCs	primordial germ cells
pH	potential of hydrogen
PH domain	pleckstrin homology domain
PI(3)P	phosphatidylinositol (3)-phosphate
PI(3,4)P_2	phosphatidylinositol (3,4)-bisphosphate
PI(3,4,5)P_3	phosphatidylinositol (3,4,5)-trisphosphate
PI(4)P	phosphatidylinositol (4)-phosphate
PI(4,5)P_2	phosphoinositol (4,5) bisphosphate
PI(5)P	phosphatidylinositol (5)-phosphate
PI, PtdIns	phosphatidylinositol
PI-4 kinase	1-phosphatidylinositol 4-kinase
PIP-5 Kinases	phosphatidylinositol 4-phosphate-5 kinase
PITP	phosphatidylinositol transfer protein
PKA	protein kinase A
PKC	protein Kinase C

PLC	phospholipase C
PLCζ	phospholipase C zeta
PLD	phospholipase D
Plk1	polo-like kinase 1
PMA	phorbol-12-myristate-13-acetate
PMSG	pregnant mare's serum gonadotrophin
PMT	photomultiplier tube
PN	pronuclei
Prophase I	prophase of the first meiotic division
PPGF	Platelet-derived growth factor
psi	pounds per square inch
PTK	protein tyrosine kinase
PVA	polyvinyl alcohol
RNA	ribose nucleic acid
sec	seconds
SERCA	sarco/endoplasmic reticulum Ca^{2+} ATPase
SH domain	Src Homology domain
SMS	sphingomyelin synthase
SNAP-25	soluble N-ethylmaleimide-sensitive factorattachment protein 25
SNARE	soluble N-ethylmaleimide-sensitive factor attachment receptor
SOAF	sperm-derived oocyte activating factor
TGFβ	transforming growth factor beta
TGN	trans-Golgi network
t-SNARE	t- soluble N-ethylmaleimide-sensitive factor attachment protein

	receptor
WBP	WW domain binding protein
WEE2	wee 1-like kinase 2
WT	wild type
Xyk	*Xenopus* tyrosine kinase
YAP	yes-associated protein
YFP	yellow fluorescent protein
ZP	zona pellucida sperm binding protein

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1. INTRODUCTION

During fertilisation calcium (Ca^{2+}) signalling inside the egg is both necessary and sufficient for initiating all further developmental events (Stricker 1999). In mammals this Ca^{2+} signalling takes the form of transient repetitive increases in cytosolic Ca^{2+} concentrations (Ca^{2+}_i) called Ca^{2+}_i oscillations that occur as a result of the introduction of a sperm derived protein into the egg cytoplasm following sperm egg fusion (Cuthbertson *et al.* 1981; Swann 1994). Despite many different "sperm factor" candidates being proposed the most evidence indicates a sperm specific Phospholipase C (PLCζ) is responsible for initiating Ca^{2+}_i oscillations in mammalian eggs (Saunders *et al.* 2002).

The work presented in this thesis aims to investigate the mechanism of action for PLCζ in eliciting Ca^{2+}_i oscillations in mouse eggs. This introduction will provide an overview of previous research conducted within this field and within the areas relevant to it. Firstly, the physiological and cellular events of egg formation and maturation will be discussed including how the egg becomes receptive for fertilisation. The process of fertilisation will then be covered with particular focus on egg activation and the role of Ca^{2+}_i oscillations. Next, a summary will be provided on the sperm factor theory and possible sperm factor candidates with a full discussion on the evidence for PLCζ in this role. Finally the structure and function of PLCζ including what is already understood about its activity will be reviewed paying particular attention to the upstream and downstream signalling cascades involved in its activity and the initiation of Ca^{2+} release.

Gametogenesis

Sexual reproduction in all animal species requires the joining of two haploid gametes through a process of fertilisation to produce a diploid zygote. In order for this process to occur the specialised gametes must first be produced from an initial undifferentiated somatic germ cell. This process is known generally as gametogenesis and occurs very differently in males and females. Male gametogenesis, also referred to as spermatogenesis, results in the production of many small sperm produced throughout the whole adult life of the male. In contrast female gametogenesis, or oogenesis, results in relatively few large eggs which are produced prior to birth and stored within the ovaries. There is also considerable variation in how these processes occur between different species. Here the mechanisms of mammalian spermatogenesis and oogenesis will be discussed with particular focus on its relevance to events at fertilization.

Spermatogenesis

Spermatogenesis is the general term used to describe the process by which a diploid germ cell forms specialised haploid sperm cells. However, this process can be split into two main stages, first the formation of haploid spermatid and then the differentiation of these spermatids into mature spermatozoa by the process of spermiogenesis (see fig 1.1).

Like oocytes the precursor cells of spermatozoa are determined early on in development as undifferentiated primordial germ cells (PGCs) which must migrate to the developing gonad (see oocyte formation) (Chiquoine 1954). Once PGCs arrive at the developing testes the cells divide mitoticallyand are then referred to as spermatogonia (McLean *et al.* 2003). These cells remain connected to each other via cytoplasmic bridges through incomplete cytokinesis which allows the development of multiple sperm to be synchronised(Togawa 1971). These bridges also allow sharing of gene products between developing sperm (Ventela *et al.* 2003). Spermatogonia also interact with specialised somatic cells of the testes called Sertoli cells which produce hormones to inhibit the development of female reproductive organs (Hai *et al.* 2014). Sertoli cells also respond to hormones produced by the pituitary gland and the interstitial cells within the testes (Rato *et al.* 2016). Futhermore Sertoli cells inhibit further proliferation of the spermatogonia. This spermatogonia enters a dormant state until puberty (Chen and Liu 2015).

At puberty some of the spermatogonia enter meiosis initiating a cycle of spermatid formation and maturation (lasting approximately 60 days in humans) which occurs in waves throughout the life of the male (Heller and Clermont 1963). Sertoli cells associate with the germ cells during the process by providing nutrients, stimulating cell division and responding to hormonal stimuli (Griswold 1995). Once spermatogonia complete two rounds of meiosis, four haploid cells are produced which are referred to as round spermatids (Sá *et al.* 2012). It is at this point the differentiation process of spermiogenesis begins which results in the production of mature spermatozoa competent for fertilisation (Clermont 1993). Spermogenesis involves several key events with the first being the development of the Acrosome (Abou-Haila and Tulsiani 2000). The acrosome contains the enzymes necessary for breaking down the envelopes surrounding the egg and is formed from Golgi apparatus derived vesicles which conjugate to form a cap around the nucleus (Bedford and Nicander 1971). The dramatic growth of microtubules from the centriole gives rise to the flagellum which allows the sperm to propel itself through the female

reproductive tract (Afzelius *et al.* 1995). Finally the cytoplasmic bridges are broken as residual bodies are pinched off forming individual spermatozoa (mature sperm cells) (Dietert 1966). Sperm are stored in the seminiferous tubules until they are ejaculated during copulation.

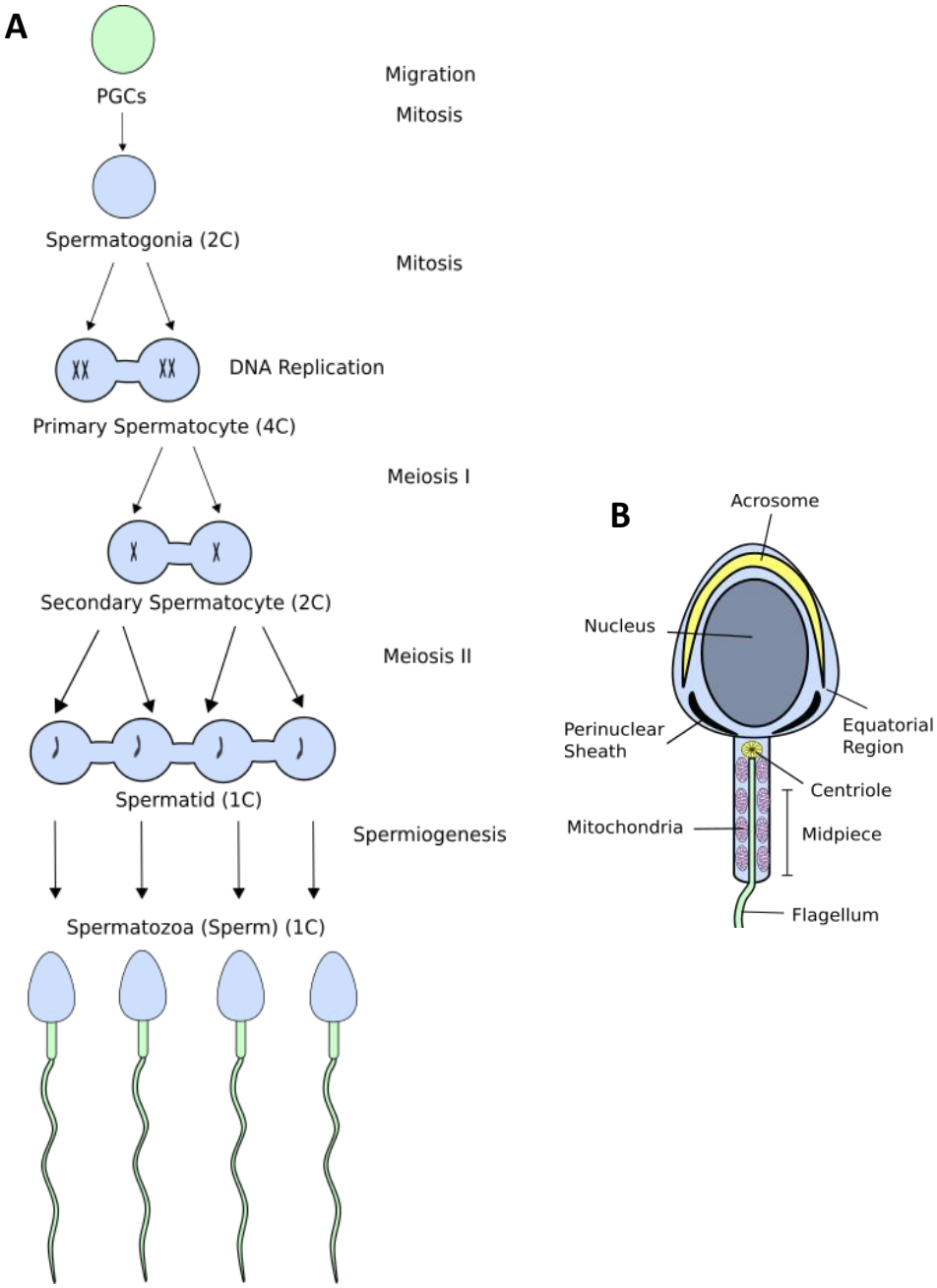


Figure 1.1 Schematic showing the process of spermatogenesis and the structure of a sperm head

A) Schematic showing the overall process of spermatogenesis. Primordial germ cells (PGCs) migrate to gonadal ridge where they proliferate. Following hormonal stimulation these cells become committed to form spermatozoa. At this point these cells are called spermatogonia. Spermatogonia undergo DNA replication followed by two rounds of meiosis whilst remaining joined by cytoplasmic bridges. These haploid spermatids then undergo a differentiation process of spermiogenesis to produce mature spermatozoa. **B)** Diagram showing the structure of a mature spermatozoa head as a cross section.

Oogenesis

Oocyte Formation

Oocytes are derived from diploid primordial germ cells (PGCs) which after production in the embryo, must migrate to the developing ovary for proliferation and differentiation (Monk and McLaren 1981). The precursors for PGCs are determined very early on in development before gastrulation (Jeon and Kennedy 1973). During this time the embryo consists of only three separate cell lineages, trophectoderm, epiblast and primitiveendoderm. It is from these layers which all embryonic and extra-embryonic cell types are differentiated (Kojima *et al.* 2014). The precursors for PGCs first form in the distal section of the epiblast sitting next to a differentiated section of trophectoderm called the extraembryonic ectoderm (Spiegelman and Bennett 1973; Lawson and Hage 1994). This differentiation occurs as a result of inductive cell signals produced by surrounding cells and includes proteins that are members of the transforming growth factor β (TGFβ) super-family, specifically bone morphogenetic proteins (BMPs) (Tam and Zhou 1996; Lawson *et al.* 1999; Ying *et al.* 2001). On the onset of gastrulation PGC precursors move through the primitive streak before residing in the extraembryonic mesoderm. At this point true PGCs become evident (Lawson and Hage 1994)

Later, PGCs migrate to the genital ridge where the developing gonads are found, at which time sexual differentiation has only just begun (Sanchez and Smitz 2012). Here the PGCs proliferateby mitosis until they colonize the ovary (Monk and McLaren 1981). This occurs due to a combination of intracellular derived transcription factors and cellular signals from surrounding somatic cells (Pesce *et al.* 2002). At this point the PGCs are considered germ cells and referred to as oogonia. During this process of proliferation the oogonia aggregate to form clusters which are connected by intercellular bridges called Oogonium clusters (Gondos 1973). By this stage some of somatic cells of the ovary have differentiated to form epithelial pre-granulosa cells which will later form the somatic cells of primordial follicle. The oogonium clusters organise with the pre-granulosa cells to form an ovarian cord which is separated from the rest of the developing ovary which remains this way until the onset of meiosis (Byskov 1986).

Oogoniain this state are still diploid and must undergo meiosis before haploid oocytes are produced. Before the oogonia enters the meiotic cell cycle, its DNA is replicated so each chromosome has two sister chromatids connected by axial proteins (Xu *et al.* 2005).Once the prophase stage of the first round of meiosis begins (Prophase I) the chromosomes

become visible and homologues pair along the equatorial plane (Hunt *et al.* 1995). The homologous chromosomes become intimately bound transversely by a selection of filamentous proteins collectively referred to as the synaptonemalcomplex (Sym *et al.* 1993). The synapses formed between chromosomes by the synaptonemalcomplex assists in the process of "crossing-over" where sections of chromosomal DNA is spliced and exchanged with the homologous chromosome adjoined to it in a cross-wise fashion (Tung and Roeder 1998). The process of crossing over, along with the formation of the synaptonemal complex, is also thought to assist in the correct segregation of chromosomes during metaphase (Newnham *et al.* 2010).

Before prophase I can be completed the cell cycle is arrested and the cell is now referred to as a primary oocyte. An oocyte can remain in this quiescent state for many years until it is ready to be ovulated (Baker and Franchi 1972). This extended period of arrest allows the oocyte to undergo massive hypertrophy and growth whilst accumulating many proteins and lipids that will later form an energy source for the early embryo (Kaplan *et al.* 1982). At this stage the oocyte still contains 4 chromatids so is ideally suited for producing high quantities of RNA (Lima-De-Faria and Borum 1962). As a result, the primary oocyte becomes very active in RNA synthesis (Kaplan *et al.* 1982). Furthermore the chromatids change structure as they elongate and de-condense in regions, this exacerbates their transcriptional power (Clarke and Masui 1983; Kleckner *et al.* 1991). This DNA is encased ina large nucleus called the germinal vesicle which is characteristic to this stage of oogenesis, therefore, a primary oocyte may also be referred to as a germinal vesicle (GV) oocyte (Clarke and Masui 1983). Towards the end of the growth phase transcription slows and much of this RNA produced is degraded or translated during oocyte maturation(Kaplan *et al.* 1982).

During the growth phase the oocyte also becomes associated with the surrounding pre-granulosa cells to form a structure known as the primordial follicle. This consists of one oocyte surrounded by layers of pre-granulosa cells which interact via intercellular signals (Pepling 2006). As the oocyte grows the follicle surrounding it develops and different layers of the granulosa cells become apparent (Thibault *et al.* 1975). The cumulus cells of the follicle remain in physical contact with the oocyte through the whole of the growth phase and later maturation phase where they play an important role in driving both these processes (Gilula *et al.* 1978).

The oocyte is surrounded by a glycoprotein envelope known as the zona pellucida. Projections from the cumulus cells form and penetrate through the zona pellucida so to

produce gap junctions (Sotelo and Porter 1959; Anderson and Albertini 1976; Bleil and Wassarman 1980b). This allows effective paracrine signalling between these cells which enable oogenesis and follicle development to be coordinated (Byskov *et al.* 1997).

In order forcells to enter the next stage of the cell cycle sufficient levels of the M-phase kinase, maturation promoting factor (MPF), are crucial (Hashimoto and Kishimoto 1988). MPF is a heterodimer containing cyclin B and cyclin dependent kinase 1 (CDK1) where cyclin B acts the regulatory subunit for the catalytic CDK1 (Labbe *et al.* 1989; Jessus and Beach 1992). As a complex, MPF is able to regulate cell cycle progression through the phosphorylationof target proteins (Doree *et al.* 1983). To maintain the oocytes in cell cycle arrest the levels of MPF are kept low to prevent further cell cycle progression. This is done by increased levels of cyclic adenosine monophosphate (cAMP) (Hashimoto and Kishimoto 1986). cAMP is able to activate protein kinase A (PKA) which in turn activates Wee2 which stimulates meiotic arrest via CDK1 inactivation (Bornslaeger *et al.* 1986; Han *et al.* 2005). The cumulus cells may play a role in maintaining meiotic arrest of the oocyteby producing cyclic guanosine monophosphate (cGMP) which enters the oocytethrough intercellular gap junctions(Hubbard and Terranova 1982). This maintains high cAMP levels by preventing its degradation (Norris *et al.* 2009). cGMP prevents cAMP degradation by inhibiting the enzymes that are responsible for hydrolysing cGMP, phosphodiesterases (PDE) (Norris *et al.* 2009). In mouse oocytes it is the isoform PDE3A that is involved in this process however inrat oocytes it is PDE5 and PDE1 isoforms that hydrolyse cAMP (Norris *et al.* 2009; Egbert *et al.* 2016). Oocytes will remain in this arrested state until hormonal signals produced at puberty initiate the oocyte maturation process. When Lutenising hormone (LH) increases the gap junctions close as a result of epidermal growth factor receptor kinase (Norris *et al.* 2010). This produces a decrease in cGMP in the oocyte which leads to an increase in PDE activity and therefore a decrease in cAMP levels (Norris *et al.* 2009).

Oocyte maturation

Through childhood all primary oocytes are maintained in prophase I of the first round of meiosis. These oocytes are encased within the primordial follicle where they undergo a period of hypertrophy and produce many of the proteins and machinery required for the zygote to develop post fertilisation. However, at this stage the oocyte still contains four times the genetic material than is necessary and is not yet receptive for fertilisation (Ducibella and Buetow 1994). During the onset of puberty, hormonal stimulus acts to trigger the maturation of a small selection of oocytes of which only one will be ovulated

(Channing *et al.* 1978). This hormonal cycle of ovulation occurs throughout the individual's life until menopause is reached which in humans is approximately 40-60 years of age. The process by which the oocyte develops into a mature egg and becomes competent for fertilisation is known as oocyte maturation (Edwards 1965). Oocyte maturation involves nuclear maturation in the form of haploidisation and chromosome reduction as well as cytoplasmic maturation which involves translational, post-translational and organellular modifications (Donahue 1968) (see fig 1.2).

During prophase I arrest the oocyte is prevented from progressing in the cell cycle and completing meiosis I by maintaining low levels of MPF (Hashimoto and Kishimoto 1986). When oocyte maturation begins due to the presence of gonadotrophins such as LH, levels of MPF increase dramatically and CDK1 becomes activated allowing it to phosphorylate target proteins (Channing *et al.* 1978; Motlik and Kubelka 1990). Interestingly, removing GV oocytes from the follicle leads to spontaneous maturation in the absence of LH which suggests that the granulosa cells must play a role in maintaining low levels of MPF and therefore preventing premature maturation (Thibault 1977).

The first cellular change that occurs following the resumption of meiosis I is the condensation of the chromosomes and breakdown of the nuclear envelope, also known as germinal vesicle breakdown (GVBD), a process that could be caused by the phosphorylation of nuclear lamins (Calarco *et al.* 1972; Lenart and Ellenberg 2003). At this time the two chromatids of each chromosome become visible and can be seen held together at the centromere and crossed over at chiasmata (Holm 1986). The first meiotic spindle (MI spindle) forms around the chromosomes that are still fairly central within the oocyte. Unlike other cellular divisions the meiotic divisions of the oocyte are asymmetric, that is to say one large oocyte produced containing half of the genetic material of the mother cell and the other half of the DNA is extruded in a small cell referred to as the polar body(Brunet and Verlhac 2011). This process ensures that the majority of cytoplasm and cellular organelles that have been produced during the growth phase are conserved within a single oocyte of higher quality (Maro and Verlhac 2002). As the point of cytokinesis in a cell is dependent on the location of the spindle, in order for this asymmetric division to occur the MI spindle must migrate to the oocyte cortex (Longo and Chen 1985). It is believed that this relocation of the spindle is dependent on transport along a meshwork of filamentous microtubule F-actinorganised by Formin 2 (Verlhac *et al.* 2000; Dumont *et al.* 2007; Azoury *et al.* 2008; Schuh and Ellenberg 2008). The migration of the MI spindle stimulates the formation of a cortical actomyosin domain which is a F-actin rich region

within a ring of myosin II (Longo and Chen 1985). Anaphase is then initiated by activation of anaphase-promoting complex (APC/C) which stimulates Seperase activity through degradation of its inhibitor Securin (Terret *et al.* 2003). Seperase is responsible for pulling homologous chromosomes apart to opposite poles of the oocyte (Terret *et al.* 2003). Completion of meiosis I following cytokinesis leads to the formation of the first polar body (Sato 1979). Meiosis II begins immediately after this and as the chromosomes are already condensed the second meiotic spindle (MII spindle) and a second actomyosin domain can both form straight away at the cortex. The oocyte now contains only one chromosome from each homologous pair however each of these contains 2 sister chromatids which are allelic but not necessarily identical as they have recombined sections of their DNA through crossing over(Holm 1986). During Meiosis II these chromatids are separated through equatorial chromosome reduction, however, the oocyte does not complete meiosis at this time but instead arrests in metaphase of meiosis II (MII arrested) whilst it acquires the ability for activation (Kubiak 1989). The MII arrested oocyte is now referred to as a metaphase II (MII) oocyte or an egg. The egg is arrested with the spindle fully formed and the chromosomes aligned however only once the egg has been fertilised by a sperm the egg will complete meiosis II and extrude the second polar body. As is the case for prophase I arrest, stabilising levels of MPF are essential for maintaining MII arrest, however in this case maintaining high levels of MPF is required (Hashimoto and Kishimoto 1988).This maintenance of MPF levels is as a result of a complex of proteins referred to as the cytostatic factor (CSF)(Kubiak *et al.* 1993). A main component of CSF has now been identified as high levels of active endogenous meiotic inhibitor 2 (Emi 2) (Shoji *et al.* 2006). Despite a high level of cyclin B degradation by the anaphase promoting complex a huge amount of cyclin B is also synthesised therefore preventing cell cycle progression to anaphase (Kubiak *et al.* 1993). Cdk1 is also maintained in an active state through phosphorylation by the phosphatase cell cycle division cycle 25 homologue A (Cdc25A) (Oh *et al.* 2013).

Alongside these nuclear alterations, structural and organelle changes are also necessary to produce a mature oocyte that is competent for fertilisation (see fig 1.3). This self-organising process is termed cytoplasmic maturation and is essential in preparing the egg for fertilisation (Watson 2007). One such alteration is the reorganisation of mitochondria that is diffuse throughout the cytoplasm in an immature GV oocyte to a polarised distribution in a mature egg (Van Blerkom and Runner 1984). Eggs and early embryos produce virtually all their ATP via oxidative phosphorylation prior to implantation therefore

require a huge amount of mitochondria, more than 100,000 per egg in fact (Biggers *et al.*1967). At the onset of meiosis I during germinal vesicle breakdown (GVBD) mitochondria begin to localise around the forming MI spindle in the centre of the oocyte (Van Blerkom and Runner 1984). As the MI spindle migrates to the oocyte cortex the mitochondria move with it however are excluded from the polar body as it is extruded (Dalton and Carroll 2013). As the egg enters meiosis II the mitochondria remain clustered around the spindle, however, no longer form a ring around the spindle (Dalton and Carroll 2013). By the time the egg is arrested in metaphase II the mitochondria remain concentrated within the spindle containing hemisphere of the egg they are somewhat more diffuse (Calarco 1995; Yu *et al.* 2010; Dalton and Carroll 2013).

Other organelles become polarised in a similar way, for example the endoplasmic reticulum (ER), which acts to store Ca^{2+} that is required for signalling at fertilisation. Throughout maturation oocytes increase their capacity to release Ca^{2+} from the ER and part of this is dependent on their redistribution to the cortex during MII phase (FitzHarris *et al.* 2003). This reorganisation is cell cycle dependent and doesn't begin until GVBD. The ER changes to form a dense network that is continuous with the nuclear envelope to surrounding the spindle and meiotic apparatus in a ring with the mitochondria packed against it (FitzHarris *et al.* 2007; Mann *et al.* 2010; Dalton and Carroll 2013). Like the mitochondria, the ER moves to the cortex with the spindle (FitzHarris *et al.* 2007; Mann *et al.* 2010). Microtubules specifically the microtubule associated motor protein dynein are responsible for locating the ER to the spindle whereas microfilaments are involved in forming and maintaining ER clusters at the cortex (FitzHarris *et al.* 2007). As there is a close correlation between ER distribution and the eggs ability to release Ca^{2+} it is possible there is a spatio-temporal connection between these (Shiraishi *et al.* 1995). Indeed levels of inositol trisphophate(IP_3) receptors on the ER appear to increase in sensitivity, increase in number by approximately 2 fold and change in distribution during oocyte maturation (Mehlmann *et al.* 1996; Fissore *et al.* 1999; Lee *et al.* 2006).

It has been reported that Golgi apparatus (Golgi) distribution also changes during oocyte maturation though it is debated whether the redistribution of Golgi is linked to that of the ER during meiosis (Payne and Schatten 2003). In a GV oocyte the Golgi appear conspicuous throughout the cytoplasm as small stacks called "mini-Golgi" which fragment during GVBD to form vesicular structures in the centre of the oocyte (Moreno *et al.* 2002). These Golgi fragments spread across the oocyte during MI and this distribution is maintained through MII arrest (Moreno *et al.* 2002). Despite this observation the true role of this Golgi

fragmentation in oocyte maturation and how it increases the egg's capacity for fertilisation is not yet fully understood.

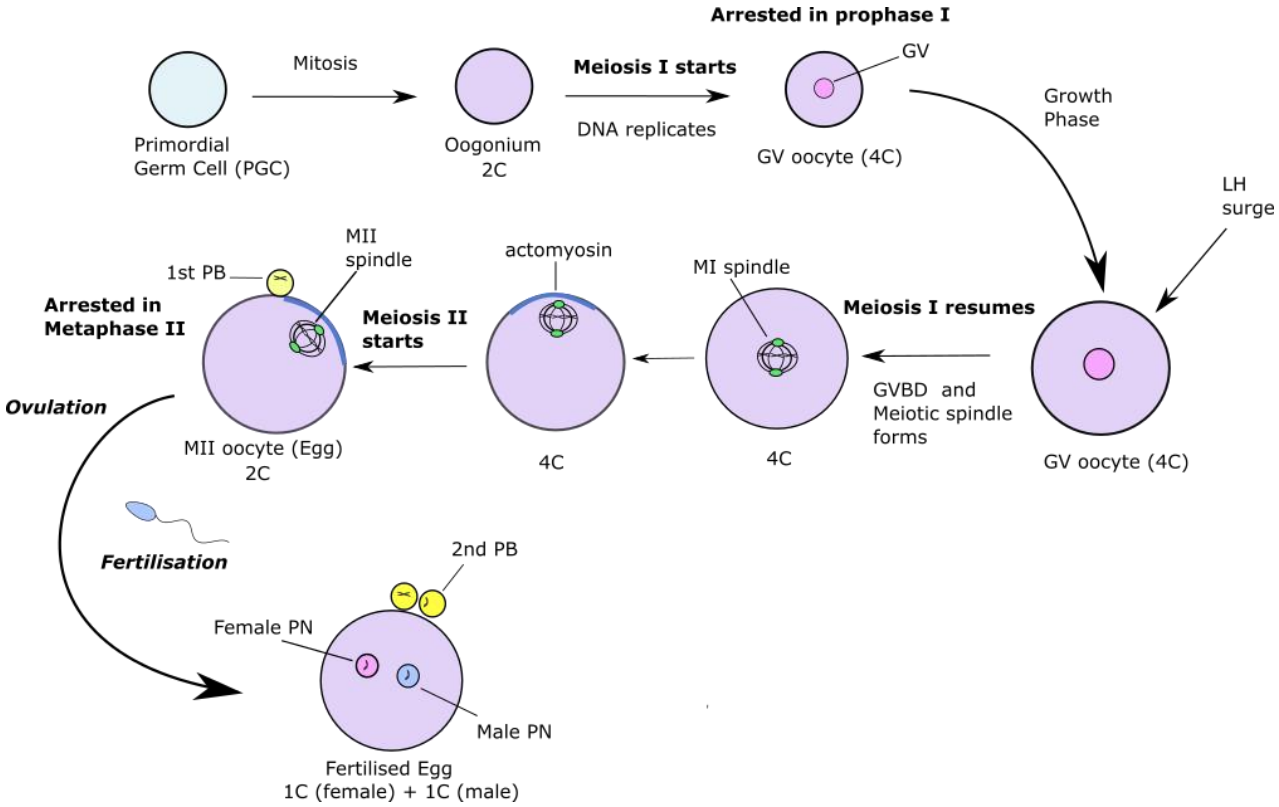


Figure 1.2 Schematic showing the overall process of oogenesis

Primordial germ cells (PGCs) migrate to gonadal ridge where they proliferate. Following hormonal stimulation these cells become committed to form oocytes and are therefore referred to as oogonia. The first round of meiosis then begins and the DNA is replicated. The oocyte is arrested in prophase of this first meiosis and possesses a visible GV. These GV oocytes enter a growth phase for many years until sexual maturity when a surge of LH resumes meiosis and the GV breaks down. The oocytes then enter the second round of meiosis however become arrested in metaphase until ovulation and fertilisation. At fertilisation meiosis II is completed resulting in the formation of male and female pronuclei (PN) and extrusion of the second polar body.

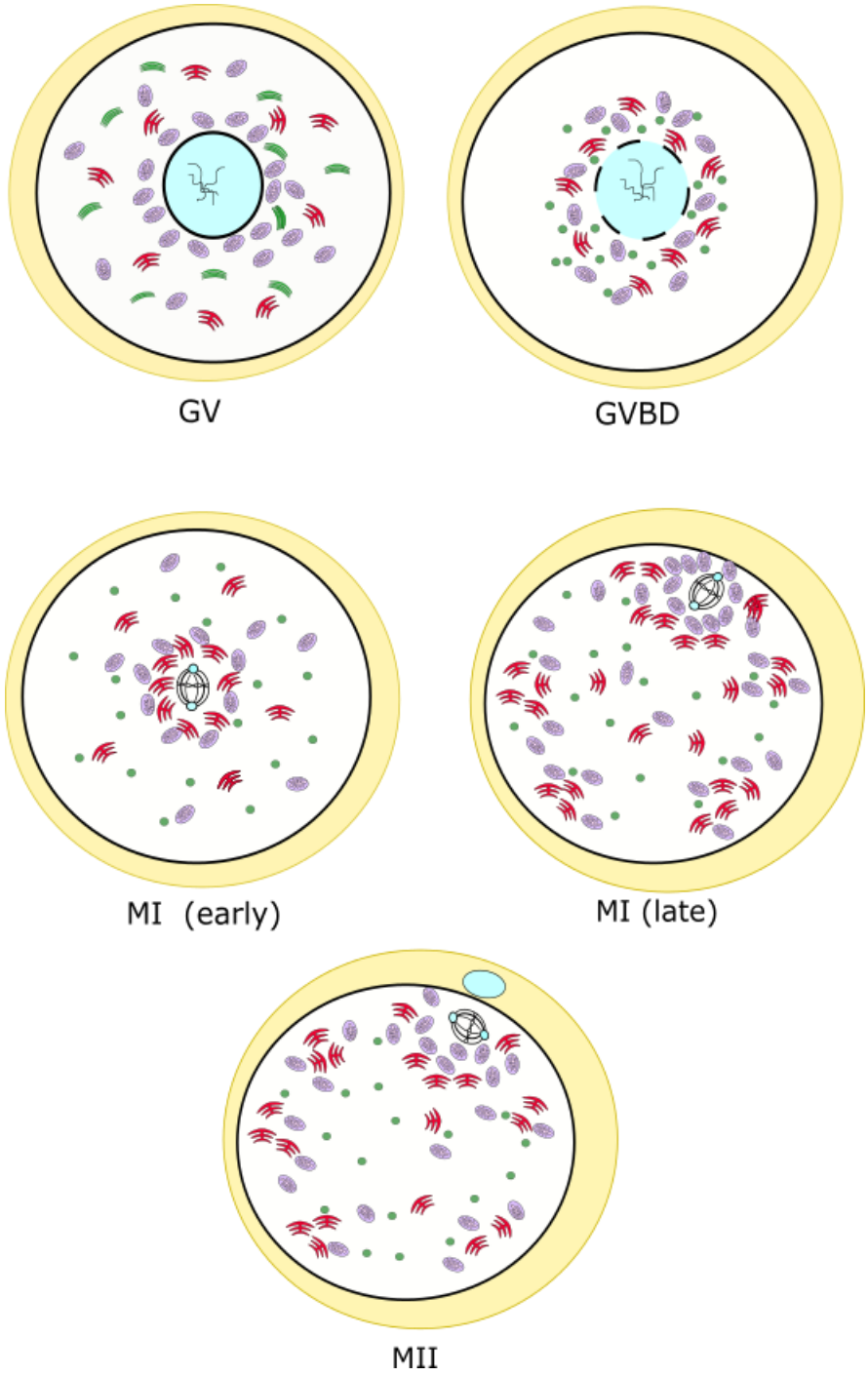


Figure 1.3 Schematic showing the change in structure and organelle distribution during cytoplasmic maturation of oocytes

In GV oocytes mitochondria (purple) and ER (red) are spread throughout the cytoplasm. The Golgi (green) is in the form of small stack structures called “mini-Golgi”. As the GV breaks down the Golgi fragments further into vesicles and become concentrated towards the centre of the egg with the ER and mitochondria. Eventually the ER and mitochondria surround the newly formed spindle. These organelles follow the spindle as it migrates to the cortex and begin the form clusters of ER and mitochondria that are concentrated to the cortex of the egg.

Fertilisation

In order for sexual reproduction to take place the gametes must first fuse so that the genetic material may be unified. Though there are many carefully orchestrated mating behaviours found in the animal kingdom to bring the egg and sperm together the actual joining of these two gametes is remarkably similar amongst species. The term fertilisation can be considered as a collective phrase that encompasses all the events that occur during this union of gametes, as a result fertilisation could be considered to start with the sperm's approach to towards the egg and end with the production of a fertilised zygote. Fertilisation therefore consists of 4 main stages the approach of the sperm towards the egg, the penetration of the vitelline layers, the fusion of the egg and sperm plasma membrane and finally ending in egg activation.

Sperm physiological changes

On its path towards the egg the sperm encounters many obstacles that reduce the chances of it fertilising the egg, however there are also many factors within the female reproductive tract that increase the chances of fertilisation. In order for the sperm and egg to effectively fuse they both must be biochemically competent for fertilisation. For the egg this process occurs prior to ovulation in the ovary during oocyte maturation (see above). Though the sperm undergoes a differentiation process within the testis, at ejaculation the sperm is actually not competent for fertilisation therefore freshly ejaculated sperm is not able to fertilise the egg. Instead, the sperm resides in the female reproductive tract for many hours before fertilisation during which time sperm undergoes a series of biochemical changes which enable it to become competent in fertilising the egg (Austin 1951a; Chang 1951). These changes are collectively referred to as capacitation and during this time the sperm switches from being in a relatively low state of metabolic activity to a much more active state (Austin 1952; Hicks *et al.* 1972; de Lamirande *et al.* 1997). Despite acquiring the ability to be motile during the maturation process in the testis, during its storage in the epididymis the sperm is maintained in an immotile state partly due to a low external and internal pH (Bishop and Mathews 1952). It isn't until the sperm is exposed to the seminal fluid at ejaculation that it becomes motile as a result of high bicarbonate and Ca^{2+} conditions that increase cAMP and possibly intracellular pH (Okamura *et al.* 1985; Visconti 2009). However, this first phase of motility is somewhat different from the movement of the sperm after capacitation. Initially the sperm flagellum moves in a slow, symmetrical, high frequency, low amplitude whiplash motion, however, after capacitation the activity of the flagellum changes so it beats in high amplitude asymmetrical strokes (Yanagimachi

1970). This alteration is referred to ashyperactivation and is required in order for the sperm to reach the fertilisation site and penetrate of the zona pellucida (Yanagimachi 1970; Stauss *et al.* 1995; Ho *et al.* 2009).

Both phases of sperm motility are a result of many biochemical alterations that take place in the sperm during the capacitation period. A number of signalling pathways are involved but theserevolve mainly around the activation of Protein Kinase A (PKA) (Visconti 2009).The early events of capacitationinvolve significant alterations to the permeability of the sperm plasma membrane (Harrison and Gadella 2005). As a result there is an influx of bicarbonate (HCO_3^-) across the plasma membrane through the Sodium/ bicarbonate (Na^{2+}/HCO_3^-) co-transporter which leads to an increase in intracellular pH (Demarco *et al.* 2003). A sperm specific HCO_3^-/Ca^{2+} sensitive adenylylcyclase then becomes active and is able to catalyse the synthesis of cAMP which activates PKA which allows it to phosphorylate many target proteins (Okamura *et al.* 1985; Lefievre *et al.* 2002). The cellular alkalization and activation of PKA have several important effects. First of all, the increase in pH leads to the hyperpolarization of the plasma membrane specifically in the tail via the activation of potassium (K^+) channels (Navarro *et al.* 2007). One such channel is I_{ksper} which is responsible for the majority of K^+ influx (Navarro *et al.* 2007). In mouse sperm this channel seems to be regulated strongly by intracellular pH, however in humans I_{ksper} appears to be regulated more strongly in response to increases in Ca^{2+} influx (Brenker *et al.* 2014). The hyperpolarization of the plasma membrane leads to voltage dependent Ca^{2+} influx via the CatSper channel in a process which is thought to be modulated by PKA activation (Kirichok *et al.* 2006). This influx of Ca^{2+} is vital for capacitation and hyperactivation and if sperm is not exposed to external Ca^{2+} it is neither motile nor able to fully capacitate (Yanagimachi and Usui 1974).

Initial PKA activation stimulates the sperm flagellum to increase its beat frequency and prolonged PKA activation induces hyperactivation (Morgan *et al.* 2008).PKA activation also has downstream effects that occur later in capacitation. For example, in the presence of extracellular (bovine serum albumin) BSA, HCO_3- and Ca^{2+} there is an increase in tyrosine phosphorylation (Visconti *et al.* 1995). During this process BSA is required as a cholesterol acceptor for depleting plasma membrane localised cholesterol on the sperm head therefore increasing membrane fluidity (Visconti *et al.* 1999). However, stimulation of an intracellular $Ca^{2+}{}_i$ increase in the sperm can overcome the need for changes in cAMP or tyrosine kinase stimulation. For example sperm treated with Ca^{2+} ionophore is able to fertilise eggs despite the lack of cAMP signalling (Tateno *et al.* 2013). Following capacitation

the sperm undergoes one final stage of development which involves the release of the sperm's acrosome contents. This occurs in response to an increase in sperm intracellular Ca^{2+} via Ca^{2+} influx through the CatSper channel as with hyperactivation (Kirichok *et al.* 2006). The acrosome is an organelle located at the apex of the sperm head which forms a subcellular cap which contains a cocktail of enzymes and binding proteins (Srivastava *et al.* 1965; Nicander and Bane 1966). During a process known as the acrosome reaction the plasma membrane fuses with acrosome outer membrane resulting the exocytosis of acrosomal contents extracellularly in the form of lots of small vesicles (Franklin *et al.* 1970). Many of these enzymes are necessary to enable the sperm to penetrate the zona pellucida (Huang *et al.* 1981). It was previously argued that the acrosome reaction was not initiated until the sperm came in contact with the zona pellucida where the enzymes that enable its penetration are released (Cherr *et al.* 1986). More significantly, it was believed that sperm that underwent the acrosome reaction before it reached the zona pellucida had lost the ability to bind and penetrate it and as a result unable to fertilise the egg (Bleil and Wassarman 1983). However, it has now been shown that acrosome reacted sperm is able to penetrate the zona pellucida and fertilise the egg (Inoue *et al.* 2011). Contrary to previous beliefs it appears 95% of the sperm have already undergone the acrosome reaction by the time it reaches the site of fertilisation (La Spina *et al.* 2016). Therefore the acrosome reaction may occur independently of the presence of the zona pellucida and could be viewed as the last event to take place in a series of biochemical changes that occur in the sperm before it interacts with the egg.

Sperm-Egg Interaction

By the end of capacitation the number of sperm that gets within close proximity to the fertilisation site has been reduced to a few hundred (Ahlgren 1975). If ovulation has not yet taken place the motility of the remaining sperm is arrested in the oviduct just below the fertilisation site until ovulation (Suarez 1987). If ovulation has occurred sperm motility becomes guided in the response to chemical signals called chemo-attractants released by the egg and its surrounding cells (Eisenbach 1999). In fact *in vitro* experiments have shown that sperm motility is enhanced when it is exposed to follicular fluid (Falcone *et al.* 1991). The chemotactic response of sperm is suggested to act as a selection mechanism as not all sperm are responsive to the chemo-attractants at the same time and it may only be the sperm of the optimum state that responds (Eisenbach and Giojalas 2006). In humans for example cumulus cells surrounding the egg release progesterone (Oren-Benaroya *et al.* 2008). Progesterone acts as a chemoattractant by opening the CatSper Ca^{2+} channel on the

sperm plasma membrane which causes Ca^{2+} influx and enhances sperm motility (Strunker *et al.* 2011). However, in other mammalian species such as mouse, progesterone doesn't appear to function as a chemoattractant and does not stimulate CatSper channel activity (Strunker *et al.* 2011). Within humans many different molecules have been shown to have chemo-attractant properties and it is likely that many stages are involved. Other processes such as thermotaxis and rheotaxis may also be involved (Bahat and Eisenbach 2006; Eisenbach and Giojalas 2006; Miki and Clapham 2013).

On approach to the egg the sperm faces two more obstacles before it is able to bind to the egg plasma membrane. First is the thick layer of cumulus cells surrounding the egg. The cumulus cells are held together in an extracellular matrix bound by hyaluronic acid (Talbot 1984). Human sperm digests this matrix using a sperm plasma membrane enzyme called PH20 (Sabeur *et al.* 1997). This enzyme has hyaluronidase activity and is able to break down some of the hyaluronic acid which dissociates the cumulus cells from the egg. This, along with the burrowing action of the sperm is sufficient for the sperm to reach the surface of the egg's zona pellucida (Lin *et al.* 1994).

Sperm binding to the zona pellucida is mediated by the glycoproteins that make up the zona pellucida which act as receptors for the sperm (Bleil and Wassarman 1980a). The zona pellucida of mammals is made up of either three or four different glycoproteins depending on the species. For example humans, hamsters and pigs have 4 glycoproteins – ZP1, ZP2, ZP3 and ZP4 in their zona pellucida however mice only have three as the *ZP4* gene acts as a pseudogene (Bleil and Wassarman 1980b; Hasegawa *et al.* 1991; Lefièvre *et al.* 2004; Izquierdo-Rico *et al.* 2009). There are species differences within these ZPs and human sperm is unable to recognise mouse derived ZPs. However, human sperm is able to bind to the zona pellucida of transgenic mouse eggs expressing human ZPs (Baibakov *et al.* 2012). In humans it appears all 4 ZPs bind to sperm in some way however is likely that there are different ligands on the sperm plasma membrane for these (Tsubamoto *et al.* 1999; Chakravarty *et al.* 2008; Chiu *et al.* 2008; Ganguly *et al.* 2010; Avella *et al.* 2014). Genetic knockout studies of these glycoproteins have also shown that they may not all be essential for fertilisation in the physiological situation (Avella *et al.* 2013). In mice ZP2 and ZP3 are the key glycoproteins responsible for sperm binding (Bleil and Wassarman 1988; Mortillo and Wassarman 1991; Avella *et al.* 2014). In humans some light has been shed on the possible sperm ligands that interact with the ZPs of the zona pellucida however it remains unclear the precise importance or interaction of these proteins with ZPs (Gupta 2015).

Once the sperm has penetrated the zona pellucida it enters the peri-vitelline space and comes in contact with the plasma membrane of the egg. In order for the sperm to successfully fuse with the egg plasma membrane and deliver its contents into the egg cytoplasm, the plasma membranes of the two cells must recognise and bind to each other. This occurs in a two-step binding process by a receptor mechanism. The sperm plasma membrane contains immunoglobulin IZUMO found in the equatorial region of the sperm surface which binds to its receptor JUNO on the egg plasma membrane (Inoue *et al.* 2005; Bianchi *et al.* 2014). IZUMO1 is found in the inner acrosomal membrane until the acrosome reaction, which highlights the importance of the acrosome reaction in producing sperm capable for fertilisation (Fukuda *et al.* 2016). When IZUMO binds to JUNO a complex is formed which causes a conformational change of JUNO (Aydin *et al.* 2016; Ohto *et al.* 2016). JUNO is then shed from the membrane which could act as a plasma membrane block to polyspermy,however, it is unclear exactly how these membranes fuse following this process (Bianchi and Wright 2014).

Following fusion of the egg and sperm plasma membranes the cytoplasmic contents of the sperm are released into the egg cytoplasm along with the genetic material of the sperm. This diffusion of sperm contents initiates a series of biochemical, cytoplasmic and nuclear events in the egg collectively termed “egg activation” which kick start the developmental process and signify successful fertilisation (Loeb 1914)

Role of Ca^{2+} Signalling at Egg Activation

Importance of Ca^{2+}_i signalling in egg activation

Successful egg activation requires the careful co-ordination of many interconnecting signalling pathways which enables early development to begin. Despite the cellular complexity involved during egg activation all subsequent events are triggered by one simple ion signal, calcium (Ca^{2+}) (Steinhardt and Epel 1974). In all known animal species a cytosolic increase in Ca^{2+} (known as Ca^{2+}_i) is both necessary and sufficient to initiate physiological egg activation (Stricker 1999). There is evidence to suggest that this is even the case in some higher flowering plant species (Digonnet *et al.* 1997). As a result Ca^{2+} is considered as the universal signalling molecule that initiates egg activation and has been proven necessary for this process in many species including mammals, sea urchins, frogs, medaka fish, ascidians, insects and bivalves (Steinhardt *et al.* 1974; Ridgway *et al.* 1977;

Cuthbertson *et al.* 1981; Eisen and Reynolds 1984; Speksnijder *et al.* 1989; Taylor *et al.* 1993; Stricker 1999). Inhibiting Ca^{2+}_i using Ca^{2+} chelators such as ethylene glycol tetraacetic acid (EGTA) or 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) inhibits egg activation (Kline 1988; Kline and Kline 1992; Xu *et al.* 1996). Likewise parthenogenetic egg activation can be initiated by injecting Ca^{2+} or the adding the Ca^{2+}releasing agonist Ca^{2+} ionophore to eggs (Steinhardt *et al.* 1974).

Despite its universality the profile of Ca^{2+}_i increase varies considerably between different species as does the mechanism by which it is initiated. For example, some species such as limpets rely solely on Ca^{2+} influx to increase Ca^{2+}_i uniformly through the cytosol (Deguchi 2007). Other species use a combination of Ca^{2+} influx and Ca^{2+} release from intracellular Ca^{2+} stores for example bivalves such as mussels have an initial influx of Ca^{2+} into the egg following sperm egg fusion which stimulates Ca^{2+}release from the stores which cross the egg as a wave (Deguchi *et al.* 1996). Sea urchin eggs also have an initial burst of Ca^{2+} influx however this is mediated by an action potential triggered by sperm-egg fusion and is distinct from the Ca^{2+}_i wave that initiates egg activation. The wave itself is a propagating wave-front that is stimulated by the release of Ca^{2+} from intracellular Ca^{2+} stores (Whitaker and Steinhardt 1982). Similarly frog eggs (genus *Xenopus*) also release Ca^{2+} as a single wave from internal stores at fertilisation, however, as the egg is so large this wave takes several minutes (5 to 6) to cross the egg before returning to a basal level of Ca^{2+}_i (Busa and Nuccitelli 1985). Interestingly newt eggs which are relatively close evolutionarily to *Xenopus* are fertilised using physiological polyspermy therefore require an incremental increase in Ca^{2+}_i by the fusion of many sperm. Each of these sperm causes Ca^{2+} release from the stores which propagates as a localised wave (Harada *et al.* 2011; Iwao 2012). Mammalian eggs are unique as they release Ca^{2+} from internal Ca^{2+} stores however, unlike sea urchins and frogs; this release occurs as repetitive transient increases called Ca^{2+}_i oscillations that last for many hours (Igusa *et al.* 1983; Igusa and Miyazaki 1986). Ascidians also show an increase in Ca^{2+}_ias oscillations however these are very different in pattern to mammalian eggs. In ascidians a large, singular Ca^{2+}_i wave is triggered from the site of the sperm-egg fusion site and it isn't until about 10 minutes later that high frequency Ca^{2+}_i waves begin to propagate from the vegetal hemisphere (Speksnijder *et al.* 1990). Mammalian eggs in contrast oscillate at a higher amplitude and a lot lower frequency with one Ca^{2+}_i wave about every 10 minutes for mouse eggs (Deguchi *et al.* 2000). Though Ca^{2+}_i increases as a wave-front in mammalian eggs unlike other species these Ca^{2+}_i waves can propagate relatively quickly and last approximately 1 minute each (Deguchi *et al.* 2000).

Furthermore the dynamics of Ca^{2+}_i in mammalian eggs change with time. For example in mouse and hamster eggs the first Ca^{2+}_i originates from the sperm entry site and spreads across the egg (Miyazaki *et al.* 1986; Deguchi *et al.* 2000). This increase is larger and takes longer to reach the Ca^{2+}_i peak compared to subsequent Ca^{2+}_i oscillations which occur much quicker and originate from indiscriminate areas of the egg (Miyazaki *et al.* 1986; Deguchi *et al.* 2000). There is clear pacemaker activity in mammalian eggs where during the interval between oscillations the basal Ca^{2+} appears to increase gradually before reaching a threshold where it dramatically and quickly increases before the Ca^{2+}_i then returns to a basal level as the Ca^{2+}stores refill (Swann and Yu 2008).

Though mammalian egg activation is purely dependent on release of Ca^{2+} from the endoplasmic reticulum in practice Ca^{2+} influx is also required in order to maintain Ca^{2+}_i oscillations (Miao *et al.* 2012). The Ca^{2+} oscillations of fertilising eggs will terminate if the eggs are put Ca^{2+} free media (Kline and Kline 1992). This is because during each Ca^{2+}_i increase a small amount of Ca^{2+} leaves the egg via efflux therefore Ca^{2+} influx is needed to replenish this (Igusa and Miyazaki 1983). As a result mouse eggs are not able to oscillate if they are kept in Ca^{2+} free media but can oscillate if both Ca^{2+} efflux and influx are prevented using the inhibitor Gadolinium (Gd^{3+}) at millimolar concentrations (Bird and Putney 2005; Wakai *et al.* 2013). This means the frequency of Ca^{2+}_i oscillations may be influenced by the amount of Ca^{2+} influx. In pig eggs for example store operated Ca^{2+} influx across the plasma membrane in response to Ca^{2+} release from the ER plays a role in determining the frequency of Ca^{2+}_i oscillations at fertilisation (Wang *et al.* 2015). The evidence for this comes from experiments where the Ca^{2+} channels involved in store operated Ca^{2+} influx, Orai and Stim 1, have been downregulated at fertilisation (Wang *et al.* 2015). This data shows that downregulation of Orai and Stim1 leads to a reduction in the number of Ca^{2+}_i oscillations in these eggs at fertilisation (Wang *et al.* 2015). Interestingly however too much Ca^{2+} influx may be disadvantageous as during oocyte maturation there is naturally a down regulation of Orai and Stim1 expression which leads to a reduction in the amount of Ca^{2+} influx in preparation of fertilisation (Cheon *et al.* 2013; Lee *et al.* 2013). This may be because too much Ca^{2+} influx can lead to an over stimulated Ca^{2+} response where the frequency of the Ca^{2+}_i oscillations at fertilisation are too high which could be detrimental to early embryo development (Lee *et al.* 2013). In mouse eggs store operated Ca^{2+} influx via Stim 1 and Orai doesn't seem to play a role as inhibitors of these channels have no effect on the frequency of Ca^{2+}_i oscillations at fertilisation (Takahashi *et al.* 2013). Other Ca^{2+} influx channels may play a role however, for example the T-type Ca^{2+} channel

CaV 3.2 which has been found necessary to maintain Ca^{2+}_i oscillations (Bernhardt *et al.* 2015).

Much of the male factor infertility that is accounted for by sperm defects can be overcome with the use of intracytoplasmic sperm injection (ICSI) which allows the sperm to be directly injected into the cytoplasm of the egg (Palermo *et al.* 1992; Tesarik *et al.* 1994). However, despite the use of this technology, even with those sperm that have normal morphology in around 3% of cases a patient's sperm sample is unable to fertilise any eggs following the use of ICSI resulting in a situation called "total fertilisation failure"(Flaherty *et al.* 1998). It has been suggested that this effect could be due to the sperm's inability to cause sufficient Ca^{2+}release in order to trigger egg activation (Yoon *et al.* 2012). In fact fertilisation failure can be rescued with the addition of Ca^{2+}ionophore which acts to dramatically increase $Ca^{2}{}_i$ by causing Ca^{2+} influx (Tesarik and Testart 1994). It is important to note that the Ca^{2+}_i increase elicited by Ca^{2+} ionophore is not comparable to the Ca^{2+}_i oscillations seen at fertilisation as Ca^{2+} ionophore causes a large Ca^{2+}_i increase that is sustained for a long time (Tesarik and Testart 1994). Even though eggs can be activated in this way it is not yet been established whether the development of these embryos is impaired compared to those eggs that are activated with healthy sperm alone. This is especially relevant because the frequency of Ca^{2+}_i oscillations at fertilisation has been shown to be important for successful later development (Ducibella *et al.* 2002; Ozil *et al.* 2006). However, the exact downstream effects of Ca^{2+}_i signalling on later development are yet to be determined.

<u>Ca^{2+} release at fertilisation is induced by IP_3</u>

There are many pathways that are able to induce Ca^{2+}release from internal stores in mammalian cells one of these is the IP_3 signalling cassette. The IP_3 pathway is part of the phosphoinositide metabolic pathway and centres on the production of IP_3 at membranes following the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) by phospholipase C (PLC) (Berridge *et al.* 1983; Berridge 1984; Berridge and Irvine 1984). IP_3 acts as a secondary messenger that diffuses through the cytoplasm and binds to the IP_3 receptor (IP_3R) on the endoplasmic reticulum (ER) (Supattapone *et al.* 1988; Snyder and Supattapone 1989). The IP_3R is a Ca^{2+} releasing channel consisting of 4 subunits with 3 different isoforms (Snyder and Supattapone 1989; Danoff *et al.* 1991; Maeda *et al.* 1991; Nakagawa *et al.* 1991). IP_3 binds to the receptor's IP_3 binding domain which causes a conformational change allowing cytosolic Ca^{2+} to bind also which opens the channel

allowing Ca^{2+} to be released from the lumen of the ER (Mignery and Sudhof 1990; Chan *et al.* 2007). This process is known as IP_3 induced Ca^{2+}release (IICR) and was first characterised in rat pancreatic aciner cells (Streb *et al.* 1983) however has since been recorded in many other cell types (Bonis *et al.* 1986; Delfert *et al.* 1986; Muto *et al.* 1986). IICR is also the mechanism by which the majority of $Ca^{2+}$is released at fertilisation particularly in those eggs that experience Ca^{2+}_i increase as a wave (Miyazaki and Ito 2006). For example if IP_3 is injected into sea urchin eggs this causes a Ca^{2+}_i wave like that seen at fertilisation (Slack *et al.* 1986). Similar effects are seen when *Xenopus* (Larabell and Nuccitelli 1992), ascidian (Albrieux *et al.* 1997) and Medaka fish eggs (Iwamatsu *et al.* 1988) are injected with IP_3. Furthermore an increase in IP_3 quantity has been recorded at fertilisation in *Xenopus* eggs (Snow *et al.* 1996). IICR seems to have the samerole in mammalian eggs as Ca^{2+}release is also recorded when IP_3 is injected into mouse (Kline and Kline 1994), bovine (Fissore *et al.* 1992), rabbit (Fissore and Robl 1993) and hamster eggs (Miyazaki 1988). A single IP_3 injection causes a single large release of Ca^{2+} from the stores, that occurs within seconds (Miyazaki 1988). Ca^{2+}_i oscillations can be induced however only when IP_3 is repeatedly injected into eggs or a caged IP_3 is injected which can be photo-activated (Jones and Nixon 2000). The type I IP_3R (IP_3R1) is the prominent isoform in mammalian and *Xenopus* eggs (Xu *et al.* 1994; Runft *et al.* 1999). Experiments inhibiting IICR by targeting this receptor activity have provided more evidence for its role in mammalian egg activation (Xu *et al.* 1994; Fissore *et al.* 1999). In hamster if the type I IP_3Rs are downregulated using a monoclonal antibody against the receptor no Ca^{2+}_i oscillations are seen at fertilisation (Miyazaki *et al.* 1992). What's more, during fertilisation IP_3R undergo proteolysis in response to IP_3 binding, an effect that is mimicked by the addition of the IP_3 analogue Adenophostin A (Jellerette *et al.* 2000). IP_3R can be depleted in immature oocytes using Adenophostin A prior to *in vitro* maturation to produce MII eggs lacking IP_3Rs however these eggs do not oscillate when they are fertilised by IVF (Brind *et al.* 2000). This suggests that IP_3 is the only signalling molecule necessary to initiate Ca^{2+} release in mammalians eggs at fertilisation. Interestingly the Ca^{2+}released as a product of IICR acts to further activate the IP_3R in a positive feedback loop until a certain Ca^{2+}_i level (Iino and Endo 1992). In this model Ca^{2+}_i acts as a conventional agonist of the IP_3R at low levels of IP_3 (Mak *et al.* 1998). However, when the cytosolic IP_3 levels increase this reduces the affinity of Ca^{2+}_i for the inhibitory site of the IP_3R (Mak *et al.* 1998). If the IP_3 agonist Adenophostin A is injected into mouse eggs the Ca^{2+}_i oscillations stimulated are not low frequency like those seen at fertilisation (Jellerette *et al.* 2000). It is therefore possible that fluctuations in cytosolic IP_3 levels may

regulate the pace and pattern of the oscillations and enable eggs to oscillate at low frequency for many hours (Swann and Yu 2008).

The IP_3R in eggs may be regulated by multiple pathways. Firstly its expression may be controlled at the transcriptional and post-transcriptional level. IP_3R activity can also be altered post-translationally as a result of protein phosphorylation which can either stimulate its activity or decrease its activity (Roderick and Bootman 2003). Due to the Ca^{2+} sensitivity of the channel it is also suspected that cytosolic Ca^{2+}_i aswell as the levels of available IP_3 may also play a role in regulating the channel activity and therefore a role in determining oscillation pattern (Iino and Endo 1992). However, dynamic measurements of cytosolic IP_3 are yet to be recorded in eggs.

Soluble sperm factor theory

Though it is clear that the fusion of the sperm to the egg is required in order to elicit mammalian Ca^{2+}_i oscillations by the production of IP_3, exactly how this signal is triggered was a matter of debate for some years. In most other cell types IP_3 is produced at the plasma membrane in response to external stimuli via stimulation of a receptor on the extracellular face of the plasma membrane (Streb *et al.* 1983). However, this does not seem to be the case in eggs during fertilisation in the vast majority of cases. In some species the initial Ca^{2+}_i signalling can be a result of the physical interaction between the egg and sperm for example in sea urchins sperm fusion to the egg causes a jump in capacitance which produces an inward current (McCulloh and Chambers 1991). This leads to a depolarization of the membrane which triggers an action potential and a transient increase in Ca^{2+}_i via influx (Shen and Steinhardt 1984). It is important to note however this Ca^{2+} influx is distinct from the larger Ca^{2+}_i wave that occurs around 15 seconds later that is responsible for egg activation (Shen and Buck 1993) . Mammalian eggs do also experience a jump in capacitance during sperm fusion however as the membrane is already very depolarized with a potential of -30 to -40 V there is no action potential and therefore no Ca^{2+} influx occurs (Lee *et al.* 2001) . Injecting mouse eggs with dextran linked fluorescent dyes prior to sperm egg fusion has shown that the cytoplasm of the sperm diffuses into the egg and there is a delay of 1 to 2 minutes before Ca^{2+}_i oscillations begin (Lawrence *et al.* 1997; Lee *et al.* 2001). This provides evidence that the signal transduction for IICR is not a result of sperm-egg membrane interaction, instead is initiated by some factor present in the sperm (Swann 1990b). This is supported by the success of the clinical procedure ICSI where a sperm is injected directly into an egg to fertilise it therefore bypassing all membrane

interaction (Tesarik *et al.* 1994). Damaging the sperm head prior to its injection into the egg seems to be important in determining whether it can cause Ca^{2+}_i oscillations and fertilise the egg (Dozortsev *et al.* 1995). It has been suggested that this damage to the sperm plasma membrane is necessary to allow sufficient release of Ca^{2+}release inducing "sperm factor" into the egg (Swann *et al.* 1994). Furthermore sperm-egg fusion defective mice are infertile even though sperm-egg binding is unimpaired (Kaji *et al.* 2000). This evidence suggests that the cytosolic components of the sperm must be released into the egg in order to cause Ca^{2+} release giving rise to the "sperm factor hypothesis" (Swann 1990b). This is the idea that in mammals the sperm head contains a soluble sperm factor that is released into egg cytoplasm following sperm-egg fusion and initiates the Ca^{2+} release by the production of IP_3 (Swann 1990b). There is now an overwhelming amount of evidence supporting this theory. For example if mammalian soluble sperm extracts from a range of species are injected into hamster, mouse, bovine and human eggs you see Ca^{2+}_i oscillations similar to that seen at fertilisation, with these eggs activating fully even developing up to blastocyst (Swann 1990b; Homa and Swann 1994; Swann 1994; Wu *et al.* 1997). Furthermore these sperm extracts were shown to cause a mass increase in IP_3 in sea urchin egg homogenates like that seen at fertilisation (Jones *et al.* 1998). Interestingly sperm extracts are able to cause Ca^{2+}release and oscillations in species other than that they are derived from and even work across animal phylum. For example sperm extracts from boars or pigs can even cause Ca^{2+}release in marsupials, marine worms and sea urchin eggs (Parrington *et al.* 1999; Witton *et al.* 1999; Stricker *et al.* 2000). This would suggest that the Ca^{2+}releasing sperm factor may be universal at least amongst mammals and possibly conserved in other animal groups also.

Though the sperm factor was initially thought to be soluble in all mammalian species it seems in the sperm of some species, for example mouse, this is not the case and may only be extractable using reducing agents (Kimura *et al.* 1998). This insoluble fraction that was able to cause Ca^{2+}_i oscillations was termed sperm derived oocyte activating factor (SOAF)(Kimura *et al.* 1998). Due to the differences in solubility between the hamster derived sperm factor and the mouse derived SOAF it was suggested that these were distinct entities. However, it is now recognised that the SOAF and sperm factor as actually the same thing but just with different solubility properties (Fujimoto *et al.* 2004). It has also been established that the sperm factor is most likely to reside the perinuclear theca of the sperm head which is also the first part of the sperm to fuse with the egg plasma membrane (Kimura *et al.* 1998).

There has been several suggested sperm factor candidates. For example a truncated form of the tyrosine kinase receptor c-kit expressed in the testis called tr-kit was found to cause egg activation when injected into mouse eggs (Sette *et al.* 1997). It was proposed that tr-kit releases Ca^{2+} by activating egg derived PLCγ to produced IP_3 (Sette *et al.* 1998). Tr-kit was thought to promote the formation of a multi-molecular complex of PLCγ, the protein-tyrosine proto-oncogene Fyn and the RNA binding protein Sam 68 which subsequently enables PLCγ to release Ca^{2+} (Sette *et al.* 2002; Paronetto *et al.* 2003). However, tr-kit has not yet been shown to cause Ca^{2+}_i oscillations in any mammalian eggs. Furthermore nucleotide sequencing has shown that tr-kit is only functionally expressed in mouse sperm and in many other mammalian species specifically rat, pig and human tr-kit is considered a pseudogene (Sakamoto *et al.* 2004). The sperm structural protein post-acrosomal sheath WW-domain binding protein (PAWP) has also recently been suggested as a potential sperm factor candidate however the effects of PAWP at causing egg activation are yet to be confirmed (Wu *et al.* 2007). The evidence for a role of PAWP in egg activation will be discussed in detail in the introduction to Chapter 3.

PLCζ as a sperm factor in inducing Egg Activation

Characterisation of a novel PLC that induces egg activation

Due to the high levels of biochemical PLC activity in sperm extracts it was suggested that the sperm factor itself could be a PLC (Jones *et al.* 1998; Rice *et al.* 2000). However, the known PLC isoforms expressed in the sperm were not present in the fraction of sperm extract that has the ability to cause Ca^{2+}_i oscillations (Wu *et al.* 2001). Furthermore injection of recombinant protein of these isoforms into eggs or the addition to sea urchin egg homogenates is unable to cause Ca^{2+} release or the Ca^{2+}_i oscillations like that seen at fertilisation (Jones *et al.* 2000). This therefore led to the search for a novel PLC isoform that had the characteristics of a sperm factor. Indeed a search through the mouse expressed sequence tag (EST) database for a PLC like sequence that is expressed in the mouse spermatid lead to the discovery of a novel PLC (Saunders *et al.* 2002). This PLC was coined PLCζ and is specific to the testis as expression tests using northern blots were unable to detect expression in any other mouse tissues (Saunders *et al.* 2002). Expression of PLCζ in the sperm has since been characterised in many other species including human, monkey, horse, hamster pig, chicken, medaka fish, rat and cows (Cox *et al.* 2002; Coward *et al.* 2005; Yoneda *et al.* 2006; Ito *et al.* 2008; Ross *et al.* 2008; Young *et al.* 2009; Bedford-Guaus *et al.* 2011; Sato *et al.* 2013).

PLCζ has many key structural, biochemical and behavioural features which make it distinct from other PLCs (see fig 1.4) however the most critical of these is its ability to cause Ca^{2+}_i oscillations when introduced into eggs just in the way sperm extracts do (Saunders *et al.* 2002). This is a vital characteristic in determining if PLCζ is the true physiological sperm factor, as the egg activating sperm factor must be-able to elicit Ca^{2+}_i oscillations and subsequent egg activation in eggs at the levels of protein found in the sperm. If PLCζ cRNA or recombinant protein is injected into mouse eggs a series of Ca^{2+}_i oscillations are triggered that look very similar to those seen at fertilisation (Cox *et al.* 2002; Saunders *et al.* 2002; Kouchi *et al.* 2004) (see fig 1.5). These parthenogenetically activated eggs activate fully as indicated by resumption of meiosis, the formation of pronuclei and even develop normally up to blastocyst stage (Cox *et al.* 2002; Saunders *et al.* 2002). Similar developmental outcomes have also been recorded when PLCζ is injected into human and pig eggs (Rogers *et al.* 2004; Yoneda *et al.* 2006) . Furthermore, over expression of PLCζ in the mouse ovary results in spontaneous activation of oocytes within the ovary (Yoshida *et al.* 2007). Despite possessing these egg activating properties, for PLCζ to be considered a valid sperm factor candidate it is crucial that it elicits Ca^{2+}_i oscillations at physiological levels of protein present in the sperm and is able to enter the egg at sperm-egg fusion. Immunocytochemistry has shown that PLCζ resides in the equatorial region and perinuclear theca of the sperm head (Yoon *et al.* 2008; Heytens *et al.* 2009; Escoffier *et al.* 2015a). This is significant as the perinuclear theca is the first part of the sperm to fuse with the eggand therefore the sperm factor must reside here (Kimura *et al.* 1998). Importantly approximately 50 fg of mouse PLCζ protein is a sufficient amount to cause physiological like Ca^{2+}_i oscillations and this is within the physiological range found in a single mouse sperm (Saunders *et al.* 2002). In porcine sperm this amount of PLCζ is higher at 350 fg (Kurokawa *et al.* 2005). Other PLCs are not able to cause Ca^{2+} release or oscillations at this level. PLCδ1 concentrations must exceed 1pg in order to cause any Ca^{2+}_i oscillations in mouse eggs (Kouchi *et al.* 2004) whereas PLCβ1 or PLCγ are not able to induce any Ca^{2+} release in mouse eggs even at 5pg (Jones *et al.* 2000).

To determine if PLCζ is exclusively responsible for the Ca^{2+}_i oscillations elicited by the sperm it was important to establish what the egg activating activity of sperm is in the absence of PLCζ. To do this immuno-depletion experiments have been completed where PLCζ was depleted in sperm extracts using an anti-PLCζ antibody (Saunders *et al.* 2002). Immunoblot analysis comparing anti-PLCζ extracts with controls shows that when sperm extracts undergo this antibody treatment PLCζ is successfully removed from the supernatant

(Saunders *et al.* 2002). These PLCζ depleted extracts are not able to cause fertilisation-like Ca^{2+}_i oscillations like those elicited by the control antibody treated sperm extract following microinjection into mouse eggs (Saunders *et al.* 2002). Furthermore using a sea urchin egg homogenate assay it's been shown that these PLCζ depleted sperm extracts do not possess any Ca^{2+}releasing activity (Saunders *et al.* 2002). These results are further supported by sperm fractionation studies which have shown that only the sperm fraction containing PLCζ is able to elicit Ca^{2+}_i oscillations in eggs (Fujimoto *et al.* 2004; Kurokawa *et al.* 2004). It appears that PLCζ causes this Ca^{2+}release through the production of IP_3 in the same way as sperm does at fertilisation because injection of PLCζ into mouse eggs causes a rapid down-regulation of IP_3R just like that seen at IVF fertilisation (Lee *et al.* 2010). When eggs are fertilised by IVF using sperm from a RNA interference transgenic mice with a reduced amount of PLCζ the Ca^{2+}_i oscillations recorded terminate prematurely compared to IVF controls (Knott *et al.* 2005). When these transgenic mice were mated fertility was compromised indicated by a reduced litter size (Knott *et al.* 2005).

The functional effects of PLCζ in model systems such as these are further strengthened by clinical data on male factor infertility. Immunoblots and immunofluorescence have shown reduced levels of PLCζ in the sperm and specifically in equatorial region can be correlated to a sperm's failure to fertilise and activate the egg even following ICSI (Yoon *et al.* 2008; Heytens *et al.* 2009; Escoffier *et al.* 2015b). Furthermore, deficiencies in PLCζ can be rescued with the addition of Ca^{2+} ionophore following ICSI resulting in full egg activation (Taylor *et al.* 2010; Chithiwala *et al.* 2015). Using a mouse model of male infertility has shown that heat treated sperm which isn't able to fertilise an egg by ICSI is able to fertile eggs if co-injected with recombinant PLCζ protein (Sanusi *et al.* 2015). Immunofluorescence has shown that sperm from patients suffering from recurrent failure for egg activationhas reduced overall levels of PLCζ however there is considerable variation in PLCζ levels amongst control sperm (Kashir *et al.* 2013). This suggests that in a fertile population there may be variance in sperm's egg activating ability (Kashir *et al.* 2013). Taken together this data offers very strong evidence for PLCζ as the physiological sperm factor needed to trigger egg activation at fertilisation in mammals.

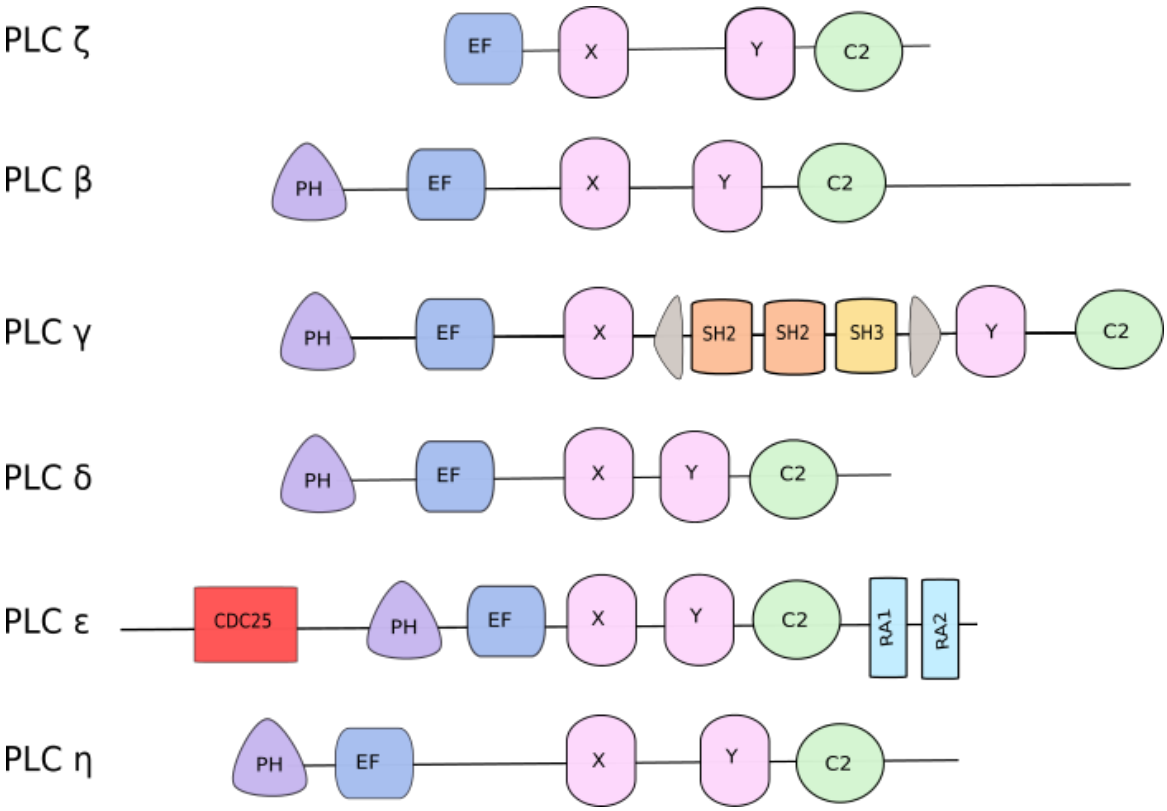


Figure 1.4 Schematics showing the structural domains of different PLC isoforms

PH = Pleckstrin homology domain

EF = EF hands domain

X = X catalytic domain

Y = Y catalytic domain

C2 = PKC-homology type II

SH2= Src homology 2 domain

SH3 = Src homology 3 domain

RA1 = Ras associated 1 domain

RA2= Ras associated 2 domain

CDC25= Ras guanine exchange factor domain

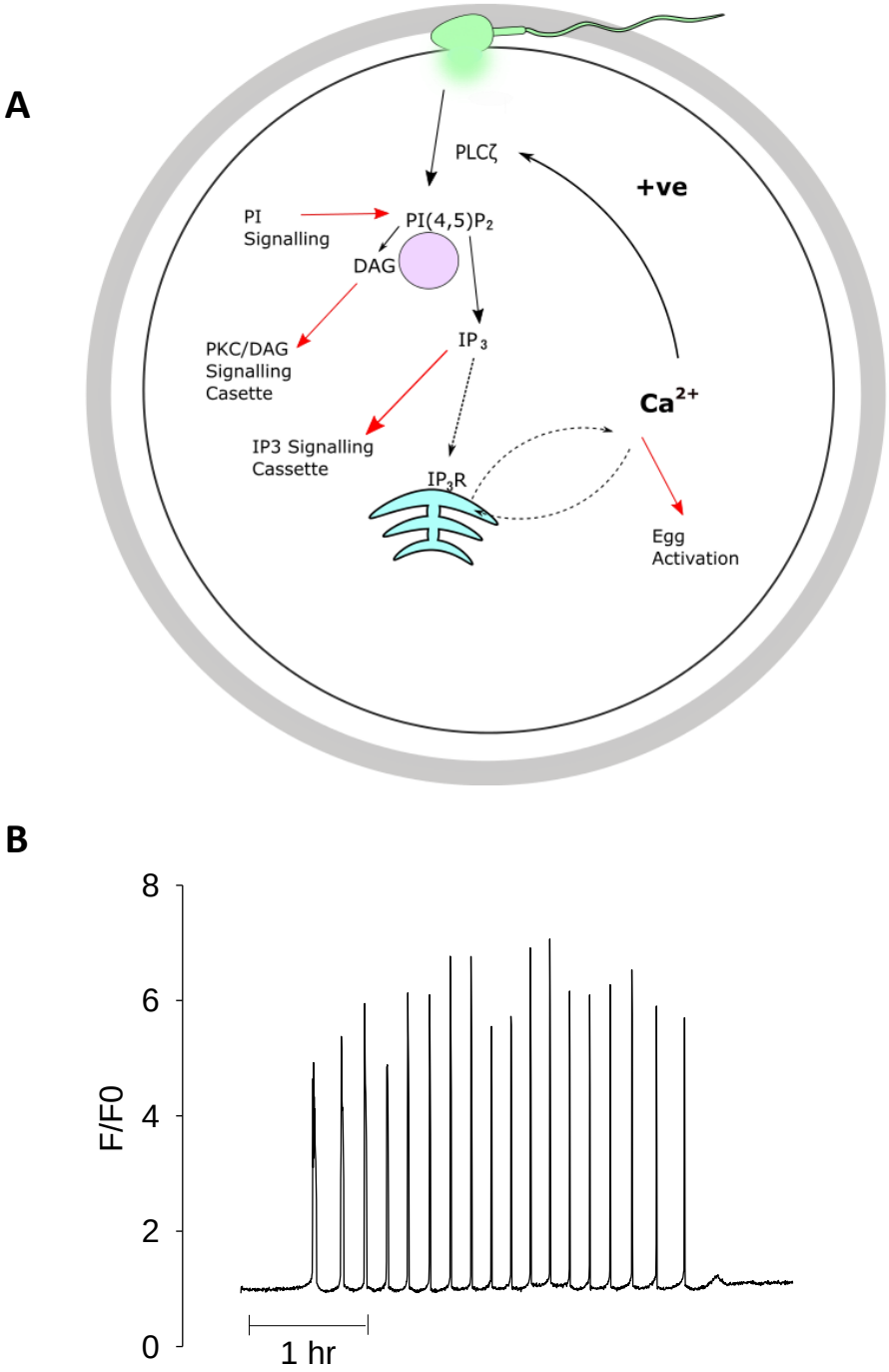


Figure 1.5 Diagram summarising how PLCζ releases Ca^{2+}_i in mammalian eggs and an example of Ca^{2+}_i oscillations initiated by PLCζ

A) Diagram summarising how PLCζ releases Ca^{2+}_i in mammalian eggs and how this pathway is connected to other signalling pathway (red arrows). PLCζdiffuses into the egg at fertilisation and hydrolyses $PI(4,5)P_2$ (on unknown vesicles) to produce IP_3 and DAG. IP_3 diffuses through the cytoplasm and binds to the IP_3R on the ER to release Ca^{2+}. This leads to egg activation and also positively feeds back onto PLCζ activity to release more Ca^{2+}. **B)** Trace showing Ca^{2+}_i oscillations in a mouse egg injected with PLCζ cRNA (0.02 µg/µl) and the Ca^{2+}_i indicator Rhod-2 dextran (pipette concentration= 1mM, Life technologies).

Structure of PLCζ

There are 13 known isoforms of PLC found in mammals which can be classified depending on their structure and the mechanism by which they are activated (see fig 1.4) (Suh *et al.* 2008). All PLCs have the same basic structure which enables them to catalyse $PI(4,5)P_2$ hydrolysis and induce IP_3 formation and Ca^{2+}release (Majerus *et al.* 1986). The active site for this enzyme comprises of two catalytic domains named X and Y which are separated by a large lopped linker region (Hicks *et al.* 2008). These X and Y domains are highly conserved and essential for the catalytic activity to all PLCs however these isoforms can vary greatly depending on the additional regulatory subunits which can drastically alter their mechanism of action (Hicks *et al.* 2008). For example the SH domain of PLCγ allows activation by tyrosine kinases (Kadamur and Ross 2013).

Though the enzymatic activity of PLCζ is very potent it possesses the most elementary domain structure of all PLC isoforms (Saunders *et al.* 2002). PLCζ is unique, as unlike all other PLC isoforms, it does not possess a pleckstrin homology (PH) domain which is able to bind phosphatidylinositols and therefore attach the enzyme to the plasma membrane where its substrate is located (Lemmon *et al.* 1995; Saunders *et al.* 2002). This makes PLCζ the smallest PLC isoform at only about 70 – 74 kDa in mammals depending on species (Cox *et al.* 2002; Saunders *et al.* 2002). Despite its elementary structure each domain has very specific structure and function which is vital for the activity of PLCζ in eggs.

X and Y Catalytic Domains

The active site for all PLCs is made up of the X and Y domains which consist of two barrel like structures comprising of alternating β sheets and α helices (Suh *et al.* 2008). This region is very well conserved between all PLC isoforms,in fact PLCζ has approximately 64% homology with its closest related PLC isoform PLCδ1 of which it shares many structural characteristics (Saunders *et al.* 2002). The residues responsible for the catalytic activity of the active site have been identified for PLCδ1 and all these residues (His^{311}, Glu^{341}, Asp^{343}, His^{356} and Glu^{390}) appear to be conserved or conservatively replaced in PLCζ (Ellis *et al.* 1998; Nomikos *et al.* 2011a). For example the mutation in Asp^{343} residue in the catalytic domain of PLCδ1 renders the enzyme catalytically inactive and the same effect is seen if the analogous residue of PLCζ (D^{210R}) is mutated which ablates its ability to elicit Ca^{2+}_i oscillations (Ellis *et al.* 1998; Nomikos *et al.* 2011a). The loss of Ca^{2+}_i inducing ability seen following this mutation confirms that the ability of PLCζ to elicit effective Ca^{2+} release by the hydrolysis of $PI(4,5)P_2$ is dependent on the active site (Swann *et al.* 2006). Several

mutations in the catalytic domain of PLCζ have also been associated with male infertility as they seem to reduce the sperms' egg activating ability. A maternally inherited point mutation in the Y domain of PLCζ (H398P) has been discovered which is associated with male infertility (Heytens *et al.* 2009). This does not seem to affect the localisation of PLCζ in the sperm however it dramatically reduces its Ca^{2+}releasing activity. For example injection of recombinant PLCζ protein containing this mutation is unable to cause Ca^{2+}_i oscillations (Heytens *et al.* 2009; Kashir *et al.* 2011).This mutation appears to be conserved across mammalian species as a homolog for this mutation (H435P) has been identified in mouse PLCζ (Nomikos *et al.* 2011a). This mutated PLCζ is not able to hydrolyse $PI(4,5)P_2$ *in vitro* or elicit Ca^{2+} release when injected into mouse eggs (Nomikos *et al.* 2011a). Another mutation in the catalytic domain of PLCζ (H233L) has also been identified in male fertility patients which is thought to affect the capability of PLCζ to interact with other proteins and therefore does not trigger normal Ca^{2+}release in the egg (Kashir *et al.* 2012). Collectively these data show that the catalytic domain of PLCζ is crucial for its ability to hydrolyse $PI(4,5)P_2$ and trigger Ca^{2+}release.

X-Y linker

The X and Y catalytic domains are joined by a long amino acid looped chain called the X-Y linker. This region is much less conserved between different PLC isoforms and can even vary between species (Swann *et al.* 2006). In most PLCs (β, γ, δ and η) the X-Y linker is negatively charged and acts to mediate auto-inhibition of enzymatic activity by preventing $PI(4,5)P_2$ access to the active site through steric hindrance and electrostatic repulsion however this does not appear to be the case with PLCζ (Hicks *et al.* 2008; Gresset *et al.* 2010). In PLCζ the X-Y linker is longer in length and contains many more basic residues than PLCδ1 (Essen *et al.* 1996; Cox *et al.* 2002; Saunders *et al.* 2002). As a result the X-Y linker of PLCζ is positively charged which seems to give it a specific role in regulating the activity of PLCζ and in targeting it to its substrates (Nomikos *et al.* 2011c). The X-Y linker provides PLCζ with a novel enzymatic mechanism as deleting the X-Y linker decreases the capacity for PLCζ to hydrolyse $PI(4,5)P_2$ *in vitro* which subsequently decreases the enzymes Ca^{2+}_i inducing ability *in vivo* (Nomikos *et al.* 2011c). There is evidence to suggest that the basic residues in PLCζ's X-Y linker are responsible for this enzymatic control as neutralising these positively charged residues in this region of mouse PLCζ depletes the Ca^{2+}_i inducing ability of PLCζ in eggs and decreases its ability to hydrolyse $PI(4,5)P_2$ *in vitro* (Nomikos *et al.* 2011b). It has been suggested that the positive residues in the X-Y linker of PLCζ help target it to membrane bound $PI(4,5)P_2$ therefore anchoring it in place and increasing its

enzymatic function (Nomikos *et al.* 2011b; Nomikos *et al.* 2011c). Indeed the X-Y linker has been found to have a high affinity for $PI(4,5)P_2$ *in vitro* dependent on the presence of basic residues in the region (Nomikos *et al.* 2011b) . It is also possible that species differences in the X-Y linker of PLCζ could be account for the differences in potency and rate of activity seen between species (Saunders *et al.* 2007). Furthermore as the X-Y linker is found close to the active site it may play other roles in regulating the catalytic activity of PLCζ that are not yet understood (Nomikos *et al.* 2012).

EF-hand Domains

All PLCs contain a region of EF motifs that are primarily responsible for the enzymes Ca^{2+} sensitivity and activating the protein (Nomikos *et al.* 2005). For example the EF hands of PLCδ1 links the PH domain with the catalytic domain and mediates the enzymatic activity through binding Ca^{2+} (Essen *et al.* 1996). PLCζ is unique amongst PLCs as it is active at relatively low levels of Ca^{2+}_i (10-100 nM) with optimal activity around 1μM which means PLCζ operates at half its maximum enzymatic activity at egg basal levels of Ca^{2+}_i of around 100 nM (Kouchi *et al.* 2004; Nomikos *et al.* 2005). This activity is further stimulated by the increase in Ca^{2+}_i which could form a positive feedback loop with IP_3 and sustainthe Ca^{2+}_i oscillations at fertilisation (Miyazaki and Ito 2006). This is in great contrast to PLCδ1 which is not activated until around 1 μM Ca^{2+}_i and has peak activity around 50 μM which makes PLCζ extremely more sensitive to Ca^{2+} than PLCδ1 (Nomikos *et al.* 2005). PLCζ has two pairs of EF hand pair-wise lobesconstructed of 4 helix loop helix motifs found at the N-terminus of the protein (Saunders *et al.* 2002). These motifs appear to be fundamental for PLCζ to elicit Ca^{2+}_ioscillations as deleting one or both of these EF hand domains ablates its Ca^{2+}_i inducing activity in eggs however the hydrolytic activity of PLCζ remains unaffected (Kouchi *et al.* 2005; Nomikos *et al.* 2005). It appears that this disruption of activity is at least in part due to a reduction in Ca^{2+} sensitivity. When the EF hands are removed the EC_{50} of PLCζ changes from 80 nM to 30 μM (Nomikos *et al.* 2005). This dramatic decrease in Ca^{2+} sensitivity means that PLCζ is no longer able to hydrolyse $PI(4,5)P_2$ at the basal levels of Ca^{2+}_i present in the egg (Nomikos *et al.* 2015). The EF hands may also have a role in targeting PLCζ to the intracellular membranes as, unlike PLCδ1, PLCζ lacks the PH domain which allows effective targeting of PLCs to $PI(4,5)P_2$ at the plasma membrane (Rebecchi and Pentyala 2000). The role that the EF hand domain plays in membrane targeting to $PI(4,5)P_2$ is still unclear.

C2 domain

The final functional domain present in all PLCs is the PKC-homology type II (C2) domain which normally behaves in a Ca^{2+} dependent way to form Ca^{2+}-phospholipid complexes which help mediate membrane association (Medkova and Cho 1999). The C2 domain of PLCδ1 consists of a sandwich of 8 well conserved B sheets linked by peptide loops and contains approximately 120 residues responsible for phospholipid binding, for example through its interaction with phosphatidylserine (Lomasney *et al.* 1999). Despite a lack of understanding regarding the role of C2 domain in PLCζ its role in eliciting Ca^{2+} release in eggs is undisputed as deletion of the C2 domain of PLCζ completely ablates itsCa^{2+}_i releasing activity with only a small effect on enzyme activity with no effect on Ca^{2+} sensitivity (Nomikos *et al.* 2005). It has been suggested that the C2 domain of PLCζ may help sequester it to the membrane through phospholipid binding like PLCδ1 which is able to bind to phosphatidylserine contained in the plasma membrane (Rizo and Südhof 1998). The C2 domain of PLCζ does bind to PI(3)P and PI(5)P *in vitro* however this binding is relatively low affinity and more work needs to be done to determine why C2 domain is so vital for the activity of PLCζ (Kouchi *et al.* 2005).

PLCζ Mode and Mechanism of Action

Signalling cascade for PLCζ

All isoforms of phosphoinositide specific phospholipase C cause Ca^{2+}release by the production of IP_3 via the hydrolysis of $PI(4,5)P_2$ and have an absolute requirement for Ca^{2+}_i in order to work (McDonald and Mamrack 1989). These enzymes work as an integral part of the phosphoinositide and IP_3 signalling cassettes triggering the same signalling events (Putney 1987). However, the mechanism by which a particular PLC isoform becomes activated differs and is often determined by variations in molecular structure and regulatory units. For example PLCγ is activated by endogenous stimulation of tyrosine kinase receptors by growth factors (Margolis *et al.* 1990). This stimulation induces relocation of PLCγ to the plasma membrane and tyrosine phosphorylation of PLCγ via the SH2 and SH3 domains which is correlated with $PI(4,5)P_2$ turnover (Piiper *et al.* 1997; Kim *et al.* 1999). PLCβ also requires endogenous receptor stimulation to become activated stimulation of heterotrimeric G-protein coupled receptors is necessary to activate G proteins which targets the plasma membrane so it may become activated (Taylor *et al.* 1991; Jhon *et al.* 1993). Likewise PLCε also requires G-protein coupled receptor binding to become activated however possesses an additional downstream role as an exchange factor

through stimulation by small GTPases which could produce a feedback loop (Song *et al.* 2001; Wing *et al.* 2001; Wing *et al.* 2003). PLCη may also be mediated by G-Protein coupled receptors as it activated in this way however even in the absence of endogenous signalling will still bind to the plasma membrane via its PH domain (Hwang *et al.* 2005; Nakahara *et al.* 2005; Stewart *et al.* 2007). This would suggest that not all PLCs need exogenous receptor binding in order to become active. This is certainly true for PLCδ which can be activated in response to Ca^{2+}release by other PLCs such as PLCβ but does not itself require exogenous stimulation (Guo *et al.* 2010). It has been suggested that PLCδ may also be mediated by phosphatidic acid (PA) or guanine nucleotide exchange factors (GEFs) which could form an auto-amplification loop (Pawelczyk and Matecki 1999; Baek *et al.* 2001). PLCζ is unusual in the respect that until now no endogenous stimulation or G-protein signalling have been affiliated with its activation and despite the close structural relationship with PLCδ1, PLCζ is around 10 times more sensitive to Ca^{2+} (Nomikos *et al.* 2005). Therefore, on its introduction into the egg, PLCζ is able to hydrolyse $PI(4,5)P_2$ immediately without any further stimulation (Saunders *et al.* 2002). However, accessory proteins or binding proteins associated with PLCζ activity other than $PI(4,5)P_2$ have yet to be identified.

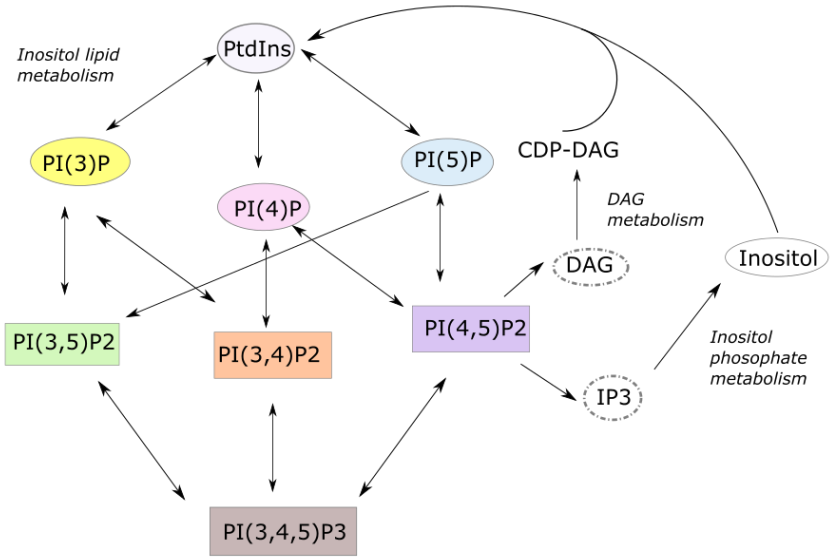
All PLCs use $PI(4,5)P_2$ as their substrate to cause Ca^{2+}release, albeit with varying affinities. This is significant as $PI(4,5)P_2$ has a particularly important role within the phosphoinositide pathway as it not only produces secondary signalling messengers it also acts as the metabolic precursor for other signalling phosphoinositides such as $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (see fig 1.6) . Furthermore it is able to act as a lipid messenger as part of the $PI(4,5)P_2$ signalling cassette by regulating a variety of cellular processes such as membrane trafficking, cytokinesis, exocytosis and regulation of ion channels (Field *et al.* 2005; Klein *et al.* 2008; Tan *et al.* 2015).

The parent molecule for all phosphoinositide is known as Phosphatidylinositol (PI, PtdIns) (Thompson *et al.* 1963). This possesses free hydroxyl groups on its inositol ring that are available for phosphorylation through inositol lipid metabolism where the ring becomes phosphorylated at different positions to produce phosphatidylinositol intermediates (Thompson *et al.* 1963) (see fig 1.6). Inositol lipid metabolism is tightly regulated by a whole range of kinases and phosphatases which synthesise and degrade phosphoinositides continuously (Hsu and Mao 2015). An example of one such enzyme is a Rho-GAP-domain containing phosphatase called OCRL1 which is able to hydrolyse $PI(4,5)P_2$, $PI(3,4,5)P_3$ and inositol phosphates (Attree *et al.* 1992).

$PI(4,5)P_2$ is produced from PI via 2 metabolic stages which are carried out by lipid kinases and phosphatases (Toker 1998). First the hydroxyl group at 4' position on the inositol ring of PI is phosphorylated by PI-4 kinases to produce PI(4)P, this is then phosphorylated further at the 5' position by PIP-5 kinases to produce $PI(4,5)P_2$ (Toker 1998). This occurs either directly in the plasma membrane or $PI(4,5)P_2$ is synthesised in the ER where it is not available for PLC hydrolysis (Helms *et al.* 1991). Therefore $PI(4,5)P_2$ can transported to cellular membranes, such as the plasma membrane, by specialist phosphatidylinositol transfer proteins (PITPs) (Cunningham *et al.* 1995). PITPs also transport parent Ptdlns to the plasma membrane where it can metabolised to produce $PI(4,5)P_2$. This $PI(4,5)P_2$ is available then for hydrolysis following activation of PLC to release two second messengers (see fig 1.5). The phosphodiester bond is cleaved to produce IP_3 which contains the inositol head groups, and diacylglycerol (DAG) which consists of the glycerol backbone and acyl chains (Berridge 1984; Kirk *et al.* 1984). Whilst DAG continues to reside on the plasma membrane where it feeds into the DAG/ protein kinase C signalling pathway (see below), IP_3 is soluble and diffuses through the cytoplasm. It is this IP_3 that initiates Ca^{2+}release via binding to IP_3R as part of the IP_3 signalling cassette (Berridge and Irvine 1984) (see above and fig 1.5). The Ca^{2+} released then further stimulates the hydrolysing activity of PLCζ producing a positive feedback loop which allows the Ca^{2+} release to continue for prolonged periods of time (Nomikos *et al.* 2005). However, inositol phosphates such as IP_3 may enter back into the phosphoinositide pathway as they are metabolised to produce free inositol by entering the inositol phosphate metabolic pathway (Joseph and Williams 1985; Rubiera *et al.* 1988). Degradation of IP_3 in this way not only makes free inositol available for the synthesis of new phosphoinositides it also acts as an OFF mechanism for IP_3 induced Ca^{2+} release. There are two pathways by which IP_3 is metabolised either it is broken down by phosphatases to produce IP_2 or phosphorylated in a $Ca^{2+}{}_i$ dependent manner by kinases to produce IP_4 (Hughes and Putney 1988). As $Ca^{2+}{}_i$ oscillations at mammalian fertilisation are able to persist for many hours there must be a high turnover of phosphoinositide and continuous periodic production of IP_3 like that in sea urchin eggs (Berridge *et al.* 1983; Ciapa *et al.* 1992). It is quite probable that the rapid metabolism of phosphoinositide as well as synthesis and degradation of specific intermediates at discrete membrane domains may be crucial for regulating the oscillatory activity of eggs at fertilisation and strict regulation of the enzymes in this pathway are vital (Yu *et al.* 2012). In addition most phosphoinositides have characteristic binding domains, therefore it has been suggested the

regional localisation of specific phospholipids could provide membrane recognition sites for other proteins or lipids (Lemmon 2008).

A



B

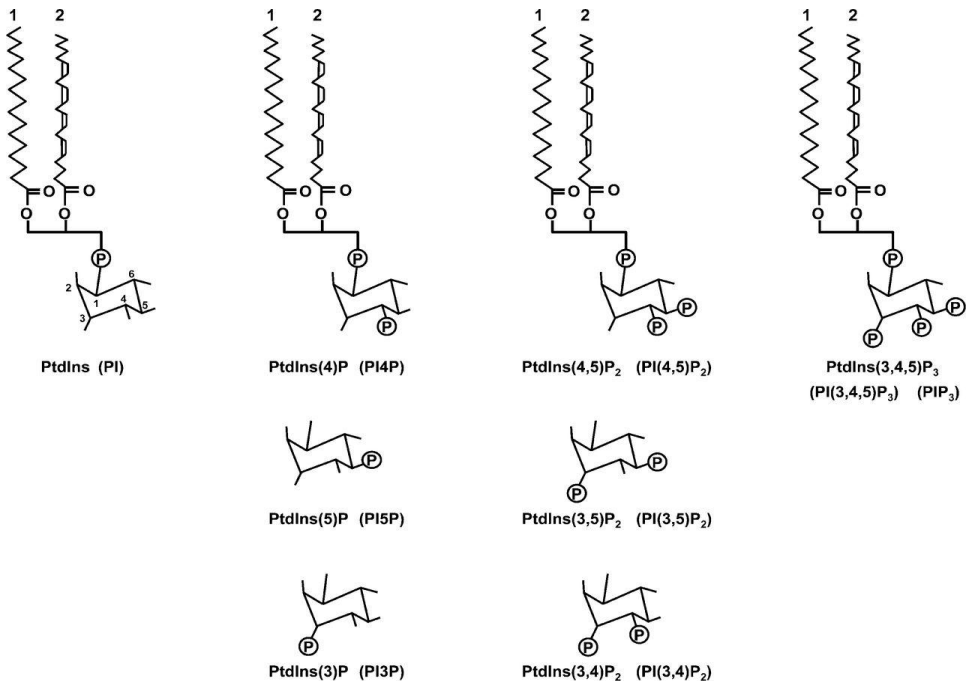


Figure 1.6 Diagram summarising the phosphoinositide metabolism cycle

A) Schematic showing how each phosphoinositide is connected by phosphorylation and de-phosphorylation that is indicated by arrows. **B)** Schematic showing the phosphoinositol head structure of different phosphoinositides and how these are attached to the glycerol backbone and fatt acid tails. Taken from *P.T. Hawkins et al. Biochm. Soc. Trans. 2006;34:647-662 ©2006 by Portland Press Ltd*

Spatial and Temporal Dynamics of PLCζ

In mammalian eggs PLCζ causes Ca^{2+}_i oscillations at basal levels of Ca^{2+}_i following no further exogenous stimulation (Saunders *et al.* 2002). These oscillations last several hours before they are abruptly cease after approximately 4 hours (Cox *et al.* 2002; Saunders *et al.* 2002). Even though PLCζ activity is so potent and enduring in eggs the same is not true for somatic cells. If CHO cells are transfected with PLCζ they do not experience Ca^{2+}_i oscillations, however if these cells are then introduced into mammalian eggs Ca^{2+}_i oscillations can be recorded in the eggs (Phillips *et al.* 2011). Furthermore transgenic mice that over-express PLCζ ectopically experience no alterations in their somatic cells however in female mice the oocytes within the ovaries are found to activate prematurely (Yoshida *et al.* 2007). This would suggest that there must be something unique about the egg physiology and cell biology that allows PLCζ to be active at eliciting Ca^{2+} release in these cells. These differences are not surprising given that a mammalian egg is arrested in the metaphase of meiosis 2 and has undergone much organelle reorganisation compared to a somatic cell in interphase of mitosis (Kubiak 1989). What is surprising however, is the that the single substrate for PLCζ, $PI(4,5)P_2$ is abundant in most somatic cells as well as eggs even though PLCζ is unable to utilise this pool of $PI(4,5)P_2$ to produce IP_3 in somatic cells (Phillips *et al.* 2011). This would suggest that PLCζ is temporally and spatially regulated by factors present in the egg in a cell dependent manner (Swann and Yu 2008).

There is evidence that the spatial activity of PLCζ in eggs differs considerably to that of other PLCs. If Venus tagged PLCζ cRNA is injected into eggs its localisation appears diffuse within the cytoplasm in contrast to other PLC isoforms which hydrolyse $PI(4,5)P_2$ at the plasma membrane (Yoda *et al.* 2004). It is important to notethat in order to record a signal from a fluorescent protein there must be over 100 nM of it present in the cell, however PLCζ is physiologically active at 1-10 nM (Niswender *et al.* 1995; Saunders *et al.* 2002). This means that this fluorescently tagged PLCζ must be overexpressed by 10 to a 100 times physiological level in these experiments. This cytoplasmic localisation has been further supported using immunostaining for cMyc tagged PLCζ which is shown to localises to small vesicles throughout the cytoplasm (Yu *et al.* 2012). Immunostaining for endogenous $PI(4,5)P_2$ in the egg shows that $PI(4,5)P_2$ can also be found in a similar vesicular pattern in the egg with considerable overlap with PLCζ (Yu *et al.* 2012). Furthermore it appearsPLCζ hydrolysesthis vesicular $PI(4,5)P_2$ as oppose to $PI(4,5)P_2$ at the plasma membrane.

Depleting the levels of PI(4,5)P_2 at the plasma membrane does not affect Ca^{2+}_i oscillations at fertilisation or initiated by PLCζ injection. However, Ca^{2+}_i oscillations induced by high levels of PLCδ1 are prevented following this plasma membrane PI(4,5)P_2 depletion (Yu *et al.* 2012). In addition fertilisation causes no detectable decrease in PI(4,5)P_2 levels at the plasma membrane which would be expected if PLCζ was hydrolysing this pool of PI(4,5)P_2 (Halet *et al.* 2002). Furthermore when PLCζis injected into eggs, DAG is not produced at the plasma membrane like it is following injection of PLCδ1 (Yu *et al.* 2008).This would suggest that unlike other PLC isoforms PLCζ does not hydrolyse PI(4,5)P_2 at the plasma membrane but instead hydrolyses an intracellular pool of PI(4,5)P_2 (Yu *et al.* 2012). It is unclear however what enablesPLCζ to discriminate between PI(4,5)P_2 found at the plasma membrane and vesicular PI(4,5)P_2 or if this is a dependent on addition factors present in the egg.

Approximately 4- 5 hours after fertilisation mouse Venus tagged PLCζ can be seen to translocate to the newly reformed pronucleus which coincides with the termination of the Ca^{2+}_i oscillations (Yoda *et al.* 2004). This translocation is reliant on a short basic motif found within the X-Y linker of mouse PLCζ called the nuclear localisation signal (NLS)(Larman *et al.* 2004). If the basic residues within this motif are neutralised then PLCζ does not become localised to the nucleus and therefore continues to hydrolyse PI(4,5)P_2 for longer than it should do (Larman *et al.* 2004). Furthermore Ca^{2+}_i oscillations have been recorded during the 1st mitosis of a fertilised mouse zygote which could be a result of PLCζ re-entering the cytoplasm following nuclear envelope breakdown (Larman *et al.* 2004).

It is likely that PLCζ activity is regulated in a combinatorial manner with the aid of addition "egg factors" and alterations in conformation as a result of interactions of the functional domains in the structure of PLCζ. However, it is still unknown precisely which lipids or proteins within the egg regulate the activity of PLCζ and may determine its unique localisation.

Downstream signalling events at Egg Activation

Egg activation is a series of complex cellular changes orchestrated by a variety of signalling pathways. Ca^{2+}_i signalling is the initial event at egg activation which in turn triggers a whole series of downstream signalling events that may themselves be crucial for the later events of egg activation (Jones 2007). As a result many of the enzymes involved in these later events of egg activation are stimulated by an increase in Ca^{2+}_i in a dose dependent manner (Jones 2007). Therefore, due to the low frequency nature of the Ca^{2+}_i oscillations at

fertilisation many of the enzymes in these pathways are activated periodically along with each Ca^{2+}_i transient (Swann and Yu 2008). This section will discuss some of the pathways and enzymes that are involved in these later signalling events of egg activation and their importance in allowing the fertilised zygote to develop.

Cortical Granule Exocytosis

At fertilisation it is necessary that the sperm reaches the egg in order to successfully fuse with the egg and fertilise it. However, it is highly disadvantageous for more than one sperm fuses with a single egg as this can create a tetraploid zygote which has surplus genetic material and couldsubsequently be lethal (Bender *et al.* 1989). It is therefore crucial that protective mechanisms are put in place to ensure only one sperm fertilises a single egg and all other sperm are prevented from doing so in a series of processes collectively termed as blocks to polyspermy (Horvath *et al.* 1993). For some species, particularly those that have their eggs externally fertilised, for example marine based invertebrates, a fast block to polyspermy is required as many sperm will be surrounding the egg prior to fertilisation (Jaffe 1976; Goudeau and Goudeau 1989). Fast blocks to polyspermy normally take the form of a depolarization of the egg plasma membrane as a direct result of the first sperm plasma membrane fusing with the egg plasma membrane and therefore take effect immediately (Jaffe 1976; Goudeau and Goudeau 1989). However, no such fast block has been recorded in mammalian eggs (Jaffe *et al.* 1983). This may be because mammalian eggs are fertilised internally thereforeonly 100-200 reach the site of fertilisation where the egg resides (Ahlgren 1975). The reduction in the number of sperm greatly reduces the risk of polyspermy but a biochemical block to polyspermy is also required (Conrad *et al.* 1971). This block occurs relatively slowly and is mediated by the release of cortical granules into the peri-vitelline space (Fukuda and Chang 1978; Cherr *et al.* 1988; Raz *et al.* 1998). Cortical granules are small secretory vesicles of between 0.1 µm and 1µm that are bound to the plasma membrane prior to fertilisation and are formed from the Golgi apparatus during oocyte maturation (see above) (Gulyas 1980; Cherr *et al.* 1988; Ducibella *et al.* 1988). When a Ca^{2+}_i increase is induced during fertilisationcortical granules are exocytosed into the peri-vitelline space so their contents can be released (Kline and Stewart-Savage 1994). Measurements of the plasma membrane capacitance of hamster eggs immediately following sperm fusion have shown that the majority of cortical granules are exocytosed during the first Ca^{2+}_i increase and cease by the fourth Ca^{2+}_i oscillation (Kline and Stewart-Savage 1994). This process causes many biochemical changes so no more sperm can fuse (Braden *et al.* 1954). The most understood of these is modifications to the ZPs known as

the zona reaction (Moller and Wassarman 1989; Dolci *et al.* 1991; Miller *et al.* 1993). Proteolytic cleavage of ZPs such as the cleavage ZP2 to ZPf2 by ZP2 proteinase prevents further binding of sperm and the penetration of already bound sperm through the matrix (Moller and Wassarman 1989).

It is well established that cortical granule exocytosis is a Ca^{2+}_i dependent process and is closely coupled to the Ca^{2+}_i oscillations that occur at fertilisation (Kline and Kline 1992; Kline and Stewart-Savage 1994). Microinjection of IP_3 into unfertilised eggs leads to the zona reaction and can be blocked by antibodies against IP_3Rs (Kurasawa *et al.* 1989; Xu *et al.* 1994). However, it still remains to be determined exactly how Ca^{2+}release causes the release of cortical granules.

The involvement of the protein kinase C (PKC) signalling cassette has been suggested as this has been found to translocate to the plasma membrane during fertilisation in response to Ca^{2+}_i (Eliyahu and Shalgi 2002). Cortical granule exocytosis induced by Ca^{2+} ionophore can be blocked by the addition of PKC inhibitors (Ducibella and LeFevre 1997). However, it appears PKC signalling may not be sufficient in causing cortical granule exocytosis in the physiological situation as phorbol ester produces a pattern of cortical granule exocytosis that is different from that seen at fertilisation (Ducibella and LeFevre 1997). Moreover, different PKC inhibitors do not inhibit fertilisation induced cortical granule exocytosis and stimulating Ca^{2+}release using ionomycin which does not induce PKC signalling still causes cortical granule exocytosis (Ducibella and LeFevre 1997; Eliyahu and Shalgi 2002).

Other Ca^{2+}_i activated proteins have also been suggested to play a role in cortical granule exocytosis. For example Calmodulin (CaM) and Calmodulin dependent kinase II (CaMKII) are both located in the cortical region of the egg and have key roles in egg activation events such as cell cycle resumption (Knott *et al.* 2006). However, the role of CaMKII in this process is far from clear. Introduction of constitutively active CaMKII into mouse eggs does cause some cortical granule exocytosis this is not to the extent seen at fertilisation (Gardner *et al.* 2007; Backs *et al.* 2010). It is possible that CaM/CaMKII is not required for cortical granule exocytosis but still plays a role in assisting the process via it's interaction with other proteins. The mechanism by which cortical granules dock to the plasma membrane and become released appears to be dependent on a class of proteins known as soluble NSF-attachment protein receptors (SNAREs) (Ikebuchi *et al.* 1998). Cleavage of the t-SNARE SNAP-25 using botulinum neurotoxin A in mouse eggs prior to their fertilisation appears to inhibit cortical granule exocytosis (Ikebuchi *et al.* 1998).

CAMKII pathway and the resumption of Meiosis

Prior to fertilisation, a mammalian egg is maintained in an arrested state within metaphase of the second round of meiosis (Borum 1967). Once the egg has been fertilised meiosis may be completed leading to the extrusion of a second polar body following cytokinesis and eventually the formation of a male and female pronuclei (Austin 1951b). This process relies on a complex of signalling pathways that are stimulated downstream of the Ca^{2+}_i oscillations (Jones 2007). As with meiotic arrest prior to fertilisation the resumption of the cell cycle is dependent on the levels of intracellular MPF (see above). During metaphase II arrest prior to fertilisation the levels of MPF are sustained due to presence of high levels of CSF components such as Emi2 (see above) which inhibits the APC/C (Madgwick and Jones 2007). However, at fertilisation the increase in Ca^{2+}_i triggers a chain of events that alter the levels of MPF (see fig 1.7). The increase in cytosolic Ca^{2+}_i is detected by the Ca^{2+} sensing messenger protein CaM which binds to the free Ca^{2+} ions in the cytoplasm. This leads to the activation of the enzyme CaMKII through a process of autophosphorylation (Zhu *et al.* 1996; Madgwick *et al.* 2005). CaMKII activation at fertilisation appears to be a shared feature among vertebrates however only CamKIIγ isoform is present in mouse eggs (Backs *et al.* 2010). Due to the low frequency pattern of Ca^{2+}_i oscillations, CaMKIIγ becomes activated with each Ca^{2+}_i transient producing peaks in CaMKII activity (Markoulaki *et al.* 2004). It has been shown that CaMKII activity is directly correlated to the levels of cytosolic free Ca^{2+}_i and that CaMKII does not become activated at fertilisation in the absence of cytosolic Ca^{2+}_i increase (Markoulaki *et al.* 2004). In vertebrates the activity of CaMKII has been shown to be vital for meiotic resumption and injection of recombinant CaMKII protein into mouse eggs causes parthenogenetic egg activation (Knott *et al.* 2005; Backs *et al.* 2010). This indicates that CaMKII is the key intermediate messenger linking Ca^{2+}_i increase and meiotic resumption.

The mechanisms by which increased CaMKII causes cell cycle resumption are well understood in eggs. CaMKII inhibits Emi2, a component of CSF, by phosphorylating it and therefore allowing further phosphorylation by Polo-like kinase 1 (Plk1) which subsequently leads to the degradation of Emi2 (see fig 1.7) (Liu and Maller 2005; Hansen *et al.* 2006; Madgwick and Jones 2007). The degradation of Emi2 frees the E3 ubiqutinligase APC/C so it may become activated (Sako *et al.* 2014). APC/C polyubiquinates cyclin B1 and Securin which makes them available for proteolysis by the proteasome (see fig 1.7) (Pesin and Orr-Weaver 2008). As cyclin B1 is a component of the MPF, targeted destruction of cyclin B1 leads to a reduction in MPF level (Marangos and Carroll 2004).Inactivating MPF in this way

allows the cell to leave metaphase and complete the cell cycle (Fulka *et al.* 1992). The degradation of Securin leads to the activation of an enzyme called Separase which promotes separation of the chromatids (see fig 1.7) (Fulka *et al.* 1994; Hornig *et al.* 2002). CDK1 is also directly deactivated following phosphorylation by the kinase Wee1b (Oh *et al.* 2011). The meiotic cell cycle is completed with cytokinesis leading to second polar body extrusion as CaMKII activity oscillates with the increase in Ca^{2+}_i so does cyclin B1 destruction (Marangos and Carroll 2004). Each Ca^{2+}_i oscillations leads to further destruction of cyclin B1 in a prolonged manner which is necessary for full egg activation (Kubiak 1989; Marangos and Carroll 2004). However, destruction of cyclin B alone is not enough to transition the cell into interphase of the first mitosis of the zygote. For this to occur the pronuclei has to reform in a process driven by components of the mitogen activated protein kinase (MAPK) pathway, specifically MAPK3 (ERK1) and MAPK1 (ERK2) (Verlhac *et al.* 1994; Moos *et al.* 1995; Moos *et al.* 1996). Like MPF, MAPK levels are maintained at a high level during metaphase II arrest before fertilisation (Dupre *et al.* 2011). After fertilisation MAPK activity is maintained until just before pronuclear formation and does not cease completely until after pronuclear formation (Gonzalez-Garcia *et al.* 2014). Towards the end of the series of Ca^{2+}_i oscillations ERK1/ERK2 becomes dephosphorylated resulting in a conformational change leading to its inactivation which reduces MAPK signalling considerably (see fig 1.7) (Gonzalez-Garcia *et al.* 2014). Unlike cell cycle resumption which in some instances requires only a small number of Ca^{2+}_i transients before cyclin B levels are low enough to allow cell cycle progression, MAPK inactivation requires prolonged Ca^{2+}_i oscillations that last for many hours (Vitullo and Ozil 1992). Therefore in most cases a sufficient number of Ca^{2+}_i oscillations are required before the egg can become fully activated in the physiological situation. There is also a suggestion that the frequency and amplitude of Ca^{2+}_i oscillations during fertilisation could affect further developmental pre-implantation events however this is yet to be investigated fully (Ozil *et al.* 2006).

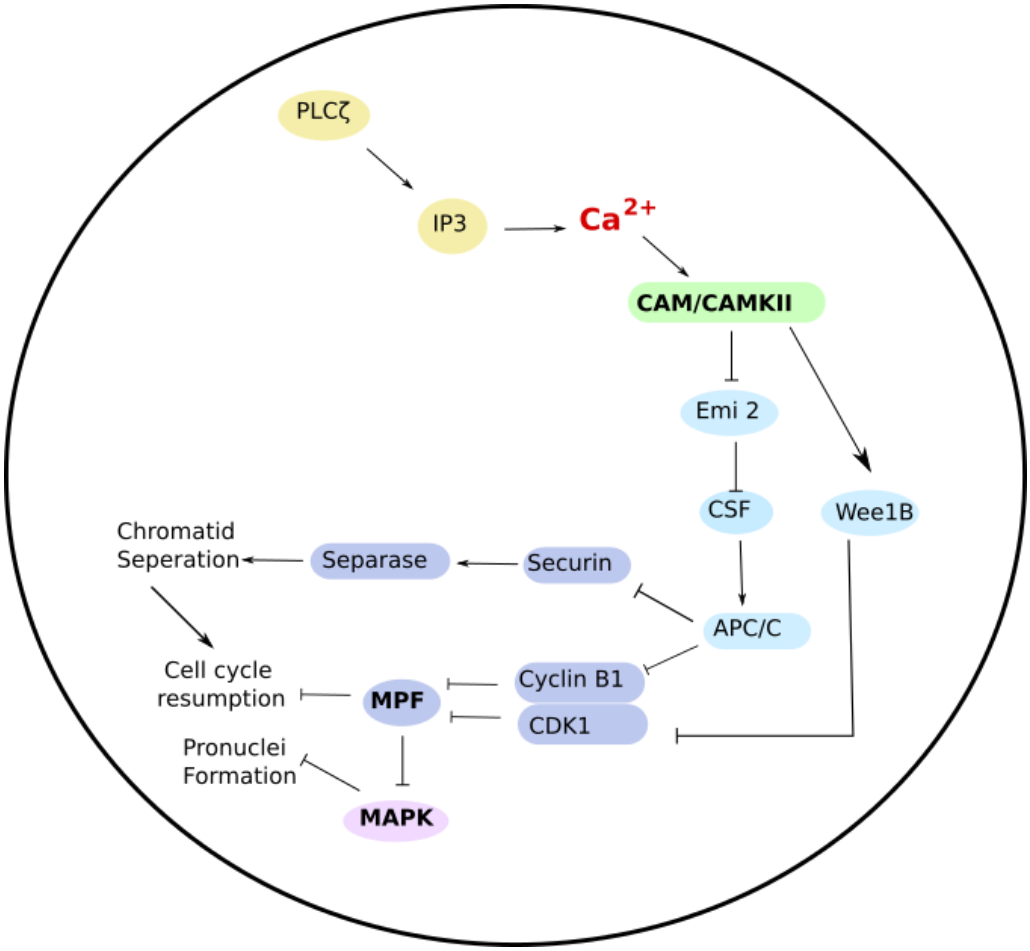


Figure 1.7 Schematic showing the molecular triggers of egg activation in mammals

PLCζ causes an increase in $Ca^{2+}{}_i$ by producing IP_3. This increase in cystosolic Ca^{2+} binds to and activates CAM/CAMKII. CAMKII phosohorylates Emi2 (a component of CSF), leading to its destruction which lifts the inhibition of APC/C. Once APC/C is activated this polyubinates cyclin B1 and Securin enabling their destruction. Cyclin B1 is a component of MPF so its destruction leads to a decrease in MPF which reduced MAPK signalling and promotes cell cycle resumption. The destruction of securin leads to the activation of separase which promotes chromatid segregation. CAMKII activation also stimulates Wee1B which directly phosphorylates and inactivates CDK1, another component of MPF.

DAG/PKC Pathway

The hydrolysis of $PI(4,5)P_2$ by PLCζ not only produces IP_3 which triggers Ca^{2+} release but also produces the lipid diacylglycerol (DAG) (Yu *et al.* 2008). DAG is a small molecule consisting of a glycerol backbone and 2 fatty acyl chains at the 1' and 2' sn positions and despites its simple structure the successful production of DAG is essential for cell survival and homeostasis (Berridge 1984; Williamson *et al.* 1985). DAG has an exceptional array of functions in lipid metabolism, maintaining membrane integrity and cellular signalling as a result many different mechanisms have evolved to regulate the levels of intracellular DAG (Carrasco and Merida 2007). Its production as a result of PLC induced hydrolysis of $PI(4,P)_2$ is just one mechanism by which DAG is generated (Hughes and Putney 1988) (see fig 1.8). Unlike IP_3, DAG remains in membranes where it acts as a lipid second messenger central to the DAG/PKC pathway (Bell *et al.* 1986). In this pathway DAG stimulates the protein kinase C- family of enzymes (PKCs) through their translocation to the plasma membrane which are then able to modulate the activity of other proteins by altering their phosphorylation (Kraft *et al.* 1982; Kraft and Anderson 1983). The PKC family contains a number of isoformsdivided into 3 main subgroups that differ in both their structural characteristics and mechanisms of activation (Ohno and Nishizuka 2002). Conventional and novel PKCs both require DAG for their activation however conventional PKCs are also Ca^{2+} dependent (Oancea and Meyer 1998). On activation PKCs undergo a conformational change which dramatically increases their affinity for the membrane causing them to translocate to the plasma membrane and bind phospholipids via their C2 domain (Snoek *et al.* 1986; Orr and Newton 1992; Newton and Keranen 1994; Mosior and Newton 1995). The C1 domain then binds to the DAG present here thus anchoring PKC for activation (Burns and Bell 1991). The Ca^{2+} sensitivity of conventional PKCs makes this process much quicker when compared to novel PKCs (Mosior and Epand 1994).

PKC has been found to be activated at fertilisation as assays indicate that the rate of phosphorylation of the substrates of PKC can be seen to increase following fertilisation induced Ca^{2+}release (Gallicano *et al.* 1993; de Barry *et al.* 1997; Tatone *et al.* 2003). The activity of conventional PKCs has been recorded at fertilisation however the activity of the enzyme appears to be $Ca^{2+}{}_i$ frequency dependent and PKC translocation to the membrane is reliant on the amount of Ca^{2+} release (Oancea and Meyer 1998; Violin *et al.* 2003). This is important as $Ca^{2+}{}_i$ oscillations at fertilisation follow a characteristic pattern which begins

with a large initial Ca^{2+}_i transient with high frequency and low amplitude oscillations superimposed onto this transient (Swann and Yu 2008). If conventional PKC tagged to a GFP (cPKC-GFP) is injected into eggs prior to fertilisation then during the initial Ca^{2+}_i increase cPKC-GFP is found to translocate to the plasma membrane indicted by an increase in GFP signal in this region (Halet 2004). However, the following Ca^{2+}_i oscillations are much lower frequency which means that though conventional PKC is recruited to the plasma membrane following Ca^{2+} release, this recruitment is significantly reduced when compared to that during higher frequency Ca^{2+}_i transients. Because there is no additive effect of PKC enzyme activity is not sustained (Halet 2004). The PKC activity during these Ca^{2+}_i oscillations have been recorded dynamically using a fluorescence resonance energy transfer (FRET) probe for PKC (Gonzalez-Garcia *et al.* 2013). In these experiments the PKC FRET probe C-kinase activity reporter (CKAR) was introduced into eggs by microinjection prior to fertilisation. Once the eggs had been fertilised the ratio of the 2 fluorophores in the FRET probe were recorded alongside the changes in Ca^{2+}_i (Gonzalez-Garcia *et al.* 2013). It was found that like Ca^{2+}_i increase,the activation of PKC was oscillatory however the rise occurred shortly after the Ca^{2+}_i transient and returned to basal level before the next Ca^{2+}release (Gonzalez-Garcia *et al.* 2013). Interestingly the activation of PKC at the plasma membrane was much lower than that recorded in cytoplasm (Gonzalez-Garcia *et al.* 2013). This would suggest that novel PKCs may also be activated elsewhere in the egg and indeed un-conventional PKCs have been found to activate at fertilisation (Tatone *et al.* 2003). For example if PLCζ is hydrolysing $PI(4,5)P_2$ on vesicles throughout the cytoplasm then the DAG produced could recruit novel PKC so it becomes activated (Yu *et al.* 2008; Yu *et al.* 2012). It has been postulated that due to the relatively small amount of activation of conventional PKC that this is not necessarily required for egg activation however there is evidence that it may possess a role in Ca^{2+} influx, for example in the elicitation of high frequency secondary Ca^{2+}_i oscillations that occur when PLCζ is over-expressed (Yu *et al.* 2008). Indeed this phenomenon is mimicked by the stimulation of PKC (Yu et al 2008). Despite the change in its dynamics it remains unclear precisely what the role of PKC is in the egg activation process.

DAG not only acts as an activator for the PKC pathway but also feeds back into the phosphoinositide cycle via the DAG metabolic pathway (Hodgkin *et al.* 1998). This means that signalling DAG produced as a result of the hydrolysis of $PI(4,5)P_2$ must be broken down in order to terminate the messenger signal and be recycled for the resynthesis of phosphoinositides (Paulus and Kennedy 1960) (see fig 1.8). DAG may be metabolised two

ways, first following its phosphorylation by DAG kinase and secondly via its hydrolysis by DAG lipases both of which reside and operate within cell membranes (Call and Rubert 1973; Morley *et al.* 1975). The latter is likely to be the most common form of DAG breakdown in cells and involves the removal of fatty acyl chains at the SN'1 position of DAG by DAG lipase resulting in production of a particular monoglycerol called 2-arachidonoylglycerol (2-AG) (Hasegawa-Sasaki 1985; Gammon *et al.* 1989). 2-AG is further metabolised bythe serine hydrolase, monoglycerollipase (MAGL) to produce the by-products glycerol and arachidonic acid (Ghafouri *et al.* 2004). DAG kinases in contrast phosphorylates DAG to form phosphatidic acid (PA) which can then be used to resynthesize phosphoinositides (Hokin and Hokin 1963). Some isoforms of DAG kinase (DAG kinase α class) possess EF hand domains making them sensitive to Ca^{2+} and therefore act as an OFF mechanism in response to Ca^{2+} release (Abe *et al.* 2003). In this pathway PA is translocated from the cell membrane where it has been produced to the endoplasmic reticulum by class II PITPs (Garner *et al.* 2012). Here PA is available for condensation with cystidinetriphosphate (CTP) by the enzymephosphatidate cytidyl transferase (CDP-DAG synthase) leading to the production of CDP-DAG (Carter and Kennedy 1966; Icho *et al.* 1985). CDP-DAG is then available to undergo head group exchange with free inositol following their condensation by the enzyme phosphatidylinositol synthase (Tanaka *et al.* 1996). This results in the production of parent phosphatidylinositol (PI) available for phosphorylation and the synthesis of other phosphoinositides including $PI(4,5)P_2$ (Tanaka *et al.* 1996).

To complicate the situation further PA and DAG may also be produced by other signalling pathways that do not require the stimulation of PLC (see fig 1.8). In fact there are over 50 structurally distinct forms of DAG which possess variations in their fatty acyl chain constituents (Hodgkin *et al.* 1998). Though PA and DAG are inconvertible the two pathways can be considered as distinct (see fig 1.8). It has therefore been proposed that all these forms of lipid exist in the cell simultaneously however maintained in different pools (Hodgkin *et al.* 1998). For example, PA may also be produced from phosphatidylcholine (PC) following the hydrolysis of its P-O bond by the enzyme phospholipase D (PLD) with choline produced as a by-product (Billah and Anthes 1990). The PA produced by this reaction has the phosphate group of the parent PC and acyl chains which are mono-saturated or saturated (Hodgkin *et al.* 1998) (see fig 1.8). This PA is then further metabolised by dephsophorylation to produce DAG by the enzyme phosphatidatephosphatase (PAP, LPP) (Martin 1988). However, as this form of DAG is

derived from PC the fatty acyl chains are mono-saturated or saturated therefore they do not serve the same function as PLC induced DAG which has polyunsaturated fatty acidchains (Hodgkin *et al.* 1998). This saturated DAG does not appear to possess much of a signalling role and is a poor activator of PKC so therefore could be referred to as "metabolic" DAG (Pettitt *et al.* 1997). However, before it is metabolised to DAG, the PA produced as a result of PLD stimulation does appear to possess a signalling role. For example this isoform of PA is able to activate the enzyme PIP5K which produces PI(4,5)P_2 from PI(4)P (Loijens *et al.* 1996). This would suggest that functional PA and signalling DAG do not always operate in the same pathways and lipid binding sites within cellular membranes must have the ability to distinguish between different molecular species of DAG.

DAG and PA production may be spatially regulated thus producing distinct localised pools of DAG that may determine its function (Hodgkin *et al.* 1998). For instance, DAG may also be produced in the Golgi apparatus as a by-product of the synthesis of the phospholipidsphingomyelin (Villani *et al.* 2008) (see fig 1.8). In this reaction phosphatidylcholine donates its phosphocholine moiety to ceramide by the enzymatic activity of sphingomyelin synthase (SMS) which produces sphingomyelin and DAG (Merrill and Jones 1990). Protein kinase C (PKC) has been found to translocate to the Golgi apparatus and becomes activated following binding to this DAG which suggests that the enzymatic activity of sphingomyelin synthase may regulate Golgi localised production of DAG and PKC activation (Villani *et al.* 2008). However, due to the strict control of this enzyme and the small specific pools of phosphatidylcholine that are likely to be metabolised during the process it is unclear how much DAG is produced via this pathway (Villani *et al.* 2008). It is also worth noting that different isofroms of sphingomyelin synthaseoperate in different cellular membranes including the plasma membrane so this form of DAG is not only produced in the Golgi (Yeang *et al.* 2011). It is clear that DAG is present in the Golgi apparatus of somatic cells as part of physiological metabolic pathways (Liu *et al.* 2009).

In addition to its role as a second messenger DAG may also assist in maintaining the structure of membranes it is associated with, particularly the Golgi apparatus. For example the cone shape of DAG means it promotes negative curvature on membranes, a process that is vital for the early formation of COPI vesicles at the Golgi (Shemesh *et al.* 2003; Cooke and Deserno 2006; Carrasco and Merida 2007). Interestingly the DAG involved in this curvature is produced by the conversion of PA by PAPs where this PA is generated from the

acylation of lysophatidic acid (LPA) by lyso PA-acyltransferase (LPAAT) (Hishikawa *et al.* 2008) (see fig 1.8). LPA has an inverted cone-shape which imposes positive curvature on the membranes it associates with (Kooijman *et al.* 2003). As DAG, PA and LPA are inter-convertible it is the correct balance between these lipids that determines the alterations in membrane curvature necessary for vesicle formation. If the enzyme PAP is inhibited using the drug propranolol thus preventing DAG production from PA, then membrane bud formation at Golgi is also inhibited (Asp *et al.* 2009).

Despite the functional differences between different pools of polyunsaturated signalling DAG and PLD induced mono/saturated metabolic DAG these two signalling pathways can stimulate each other (Hodgkin *et al.* 1998). It has been shown that when DAG is produced following cell stimulation and activation of PLC it is produced in a bisphasic manner (Nakashima *et al.* 1991). That is, there is an initial rapid rise in DAG which has found to be mainly polyunsaturated followed by a slower increase in mono/saturated DAG that is sustained for a longer time (Hodgkin *et al.* 1998). This is probably because the signalling polyunsaturated DAG produced as a result of PLC activity goes on to activate PKC (Hodgkin *et al.* 1998). PKC is able to further activate PLD through the PKC substrate myristoylated alanine-rich C-kinase substrate (MARCKS) in a number of different ways (Morash *et al.* 2000). The activation of PLD produces PC derived mono/saturated metabolic DAG which creates the second phase of DAG production (Hodgkin *et al.* 1998). It is unclear whether this biphasic response occurs during Ca^{2+}_{i} oscillations in mammalian eggs at fertilisation as DAG dynamics are yet to be measured successfully during fertilisation.

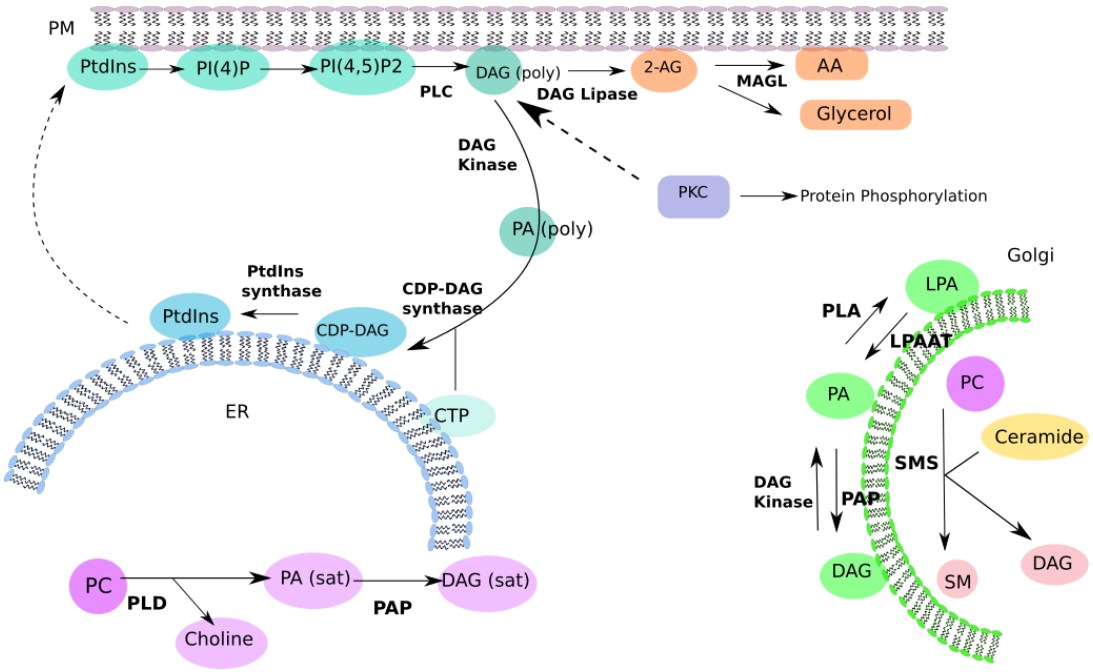


Figure 1.8 Schematic showing the different pathways of PA and DAG production

Two main forms of DAG/PA are present in cells, those that have poly-unsaturated fatty acid acyl chains (poly) called signalling DAG/PA and those that have saturated fatty acid acyl chains (sat) called metabolic DAG/PA. These two pathways of DAG/ PA production are not inter-convertible

Using Fluorescent and bioluminescent based indicators in eggs

Immunostaining is a useful tool for observing the specific localisation of proteins or markers within a cell using an antibody that specifically binds to the target of interest which becomes visible when a fluorescent conjugated secondary antibody binds it. However, this technique requires cells to be fixed and therefore does not provide a way to measure cell signalling pathways dynamically in live cells. To do this live cell indicators are required. One way to create such an indicator is to tag a targeting gene with a fluorescent protein. Fluorescent proteins emit light following the absorption of a specific excitation wavelength. The light that is emitted has a longer wavelength and lower energy than that absorbed. For example an indicator to measure IP_3 at the plasma membrane has been developed by tagging the PH domain with GFP. This indicator has been used to measure PI turnover at the plasma membrane in eggs (Halet *et al.* 2002). Howeve, these probes require high resolution imaging to look at the translocation of these probes. One way to avoid this issue is to use probes based on "Fluorescence Resonance Energy Transfer" or FRET. FRET relies on energy transfer between two fluorophores. When these two fluorophores come in close contact fluorescent light emitted from the donor fluorophore, for example CFP, excites the acceptor fluorophore, for example CFP. This produces a change in CFP-YFP signal ratio. The FRET based probe CKAR have been used to measure PKC phosphorylation in mouse egg (Gonzalez-Garcia *et al.* 2013). However, in order to measure a fluorescent signal that fluorescent protein must be present at approximately 100 nM which makes it difficult to measure low levels of fluorescent protein (Niswender *et al.* 1995).

Chemiluminescence on the other hand, requires no excitation light; instead light is produced as a by-product of a chemical reaction. This process can occur naturally in some species and is therefore known as bioluminescence for example in fireflies which produce bioluminescence by the enzyme luciferase. Luciferase uses ATP as an energy source to oxidise its substrate luciferin resulting in the production of electronically excited oxy-luciferin which leads to the emission of energy in the form of a photon so oxy-luciferin may return to its ground state. Chemiluminescent (luminescent) probes can be created through the addition of a firefly luciferase tag to the protein of interest and the addition of luciferin to the media containing the cells. Using luminescence as a measure of protein expression in this way is a lot more advantageous compared to fluorescent tagged probes as luminescence is much more sensitive and avoids the issue of background light and autofluorescence. As a result using luminescent probes it is possible to measure very low amounts of protein expressed in the egg and detect small differences in expression. For

example PLCζ expression levels have been measured in mouse eggs by tagging PLCζ cRNA with a luciferase sequence and measuring the luminescence signal (Swann *et al.* 2014). Both fluorescent and luminescence based indictors will be used in experiments demonstrated in this thesis.

Aims and Objectives

This thesis will investigate the mechanisms by which sperm derived PLCζ induces Ca^{2+}release in mouse eggs at fertilisation. This includes investigating both the structural factors of PLCζ itself that allow it to release Ca^{2+} by the IP_3 signalling cassette, as well as cellular properties of the egg that enable PLCζ to release Ca^{2+} in only this cell type. The potential role of other sperm derived proteins that could be involved in Ca^{2+} release or PLCζ activity is also examined. Finally the live cell dynamics of crucial signalling messengers such as IP_3 and DAG will attempt to be measured using a series of new genetic probes.

2. MATERIALS AND METHODS

Mice

All oocytes were collected from 6-8 week old female mice of the strain MF1 (Harlan UK Ltd). Sperm was collected from the epididymis of male C57/CBA F1 hybrid (Harlan UK Ltd) mice at approximately 10 weeks of age. All mice were kept in light/dark cycle condition with the free availability of food and water. All animal procedures were UK Home Office Licensed and approved by Cardiff University's Ethics Committee.

Collection and Handling of Gametes

GV oocytes were collected directly from the ovaries of the female mice by tissue harvest following schedule one culling using CO_2 overexposure and cervical dislocation. The ovaries were immediately transferred to warm M2 media (Sigma Aldrich) containing 100 µM IBMX (3-isobutyl-1-methylxanthine) (Sigma Aldrich) following dissection. Under a dissection microscope (SMZ1000, Nikon) ovaries were then macerated in M2 media containing 100 µM IBMX to release oocytes. The oocytes with a visible germinal vesicle were then selected and maintained in 100µM IBMX containing M2 media and incubated at 37°C until use. Where GV oocytes were *in vitro* matured (IVM) these oocytes were washed out of IBMX, transferred to a drop of clean M2 media under mineral oil (Sigma Aldrich), incubated at 37°C and left to spontaneously mature.

MII oocytes (eggs) were collected from the excised oviducts of female mice that had previously been super ovulated. For this protocol mice were primed with 10 IU (international units) of pregnant mare's serum gonadotrophin (PMSG; Intervet) before being injected with 10 IUof Human chronic gonadotrophin (hCG; Intervet) approximately 50 hours later. The eggs were then collected from the mice by tissue harvest 15 hours post hCG injection and the dissected oviducts placed directly into a warmed M2 media. The oviducts were then transferred to a petri dish of warmed M2 media containing 300 mg/ml hyaluronidase (Sigma Aldrich) under a dissection microscope. The cumulus masses containing the oocytes were torn from the oviduct using a thick needle and left in the hyaluronidasecontaining media until the oocytes dissociated from the cumulus cells. The free oocytes were then washed through 4 drops of M2 media to remove any hyaluronidase. The eggs were incubated at 37°C in a drop of M2 media under embryo tested mineral oil (Sigma Aldrich) until use.

All oocytes were transported using glass pipettes that were controlled by mouth suction. Pipette suction devices were constructed from silicone tubing containing a cotton wool plug attached to the back end of a plastic pipette tip. A custom made glass Pasteur pipette pulled using a Bunsen burner to produce a diameter of about 100 µm was then fitted onto the tubing via the plastic pipette tip. The glass pipette was changed frequently. At the other end of the tubing a sterile mouth piece was constructed using a 1 ml plastic syringe with the plunger taken out.

In Vitro Fertilisation

Eggs were fertilised by IVF using fresh sperm expelled from the excised epididymis into T6 media (1.42 mM KCL, 1.78 mM $CaCl_2$, 99 mM NaCl, 25 mM $NaHCO_3$, 0.47 mM $MgCl_2$, 0.36 mM Na_2HPO_4, 5.56 mM glucose, 24.9 mM Na lactose, 0.47 mM Na pyruvate, 10 mg/l phenol red) that contained 16 µg/µl Bovine serum albumin (BSA) (Sigma Aldrich) in a plastic tube (Falcon). Sperm were incubated at 37 °C in this media and kept at a CO_2 level of 5% for approximately 3 hours to allow for capacitation. At the time of fertilisation approximately 50µl of sperm suspended in the T6 was added to the dish containing the eggs that were being imaged. It was important that the zona pellucida of the eggs was removed prior to imaging in this instance to prevent the egg moving around during imaging.

Use of Fluorescent/ Luminescent Probes

Both fluorescent and chemiluminescent tagged constructs are useful tools as they allow quantification of the amount of a protein being expressed in a cell or determine the localization of a particular molecule within the cell. Fluorescent proteins such as GFP, YFP and CFP can be fused to specific proteins to provide a fluorescent construct. All molecular probes, proteins and cRNA were produced by Prof Tony Lai's Lab (Cardiff University) or commercially available products. This was with exception of DAN20 probe which was kindly provided by Ankona Datta (Tata Institute of Fundamental Research, Mumbai, India) and Green "Upwards" DAG probe (Montana Molecular, US). See Table 2.1 for the full list of fluorescent tags used.

Molecular Biology

Cloning of DNA expression constructs

Original DNA was amplified by polymerase chain reaction (PCR) using Phusion DNA polymerase (Finnzymes, Fischer Scientific, UK) from a plasmid containing the gene of interest in the presences of the appropriate primers. If recombinant protein was to be

produced the PCR product was then cloned into a subsequent pETMM60 plasmid which allowed bacterial expression using the corresponding primers. For the synthesis of cRNA the DNA was amplified in the same way before being sub-cloned using the appropriate primers into a PCR3 vector or a PCR3 vector that contained a firefly (*Photinus pyralis*) luciferase tag at the c terminus. Dideoxynucleotide-sequencing (Sangar sequencing) was used to confirm the expression of these constructs using the Big-Dye Terminator V 3.1 cycle sequencing kit (Applied Biosystems) and an automated capillary DNA analyser (model 3730, DNA Sequencing and Services)

cRNA synthesis

Once the PCR3 plasmids had been linearised complementary RNA was synthesised using the mMessage mMachine T7 transcription kit (Ambion). To ensure faithful translation and stability inside the cell the cRNA was polyadenylated by means of aPoly(A) tailing kit (Ambion). Once produced cRNA was either stored at - 80°C or -20 °C.

Production and purification of recombinant protein

All recombinant protein produced possessed a N-utilization substance (NusA) to increase solubility and 6x-Histidine (6x-His) tag to allow purification. NusA is a bacterial tag which acts to stabilize proteins as they are translated by promoting hairpin folding and termination. The relevant pETMM60 plasmid was transformed into Escherichia coli (BL21-CodonPlus (DE3)-RILP, Stratagene, CA, USA). These bacteria were then cultured partly in the presence of isopropyl β-D-thiogalactopyranoside (0.2 mM; Formedium, UK). Once cells had been harvested they were re-suspended in an EDTA free protease inhibitor mix (Roche) diluted in PBS and then sonicated. The recombinant protein was then purified using nickel-nitrilotriacetic affinity chromatography matrix (Ni-NTA Agrose; Qiagen) which selects for His tagged proteins. The protein was eluted and dialysed overnight before being concentrated. Recombinant protein was either used immediately, kept at 4°C for short term storage or snap froze and stored at -80°C for long term storage.

Preparation for injection

Recombinant protein and cRNA were diluted prior to microinjection in either RNase free molecular grade water (Sigma Aldrich) or HEPES buffered injection buffer (KCL HEPES) containing 120 mM KCL, 20 mM HEPES, at pH 7.4. Where Ca^{2+}_{i} was being measured this was mixed in equal parts with a Ca^{2+}_{i} indicator. Solution was mixed using a benchtop centrifuge (Spectrofuge 24D, Jencons) prior to microinjection.

Use of Peptides

The PAWP derived PPXY peptide used (PPVRYGSPPPGYEAPT) was a 16 residue amino acid sequence synthesised by a commercial company and bought from Liftein LLC (South Plainsfield, New Jersey, USA).Where peptides were microinjected they were diluted prior to microinjection in the same way as cRNA and protein, in either KCL HEPES or molecular grade RNase free water. The peptide solution was microinjected alongside a fluorescent Ca^{2+} indicator as an internal control to ensure that the peptide had entered the egg successfully.

Use of Chemicals and Drugs

Most chemicals were dissolved in Dimethylsulfoxide (DMSO) where possible at a stock of 1000 x the working concentration and stored at either -20°C or -80°C until use. D-Luciferin sodium salt (luciferin) was dissolved in molecular grade water at a 1000 times the working dilution and added to the media when required. BODIPY FL- $PI(4,5)P_2$ was diluted directly into KCL HEPES. $DiIC_{18}(3)$ crystals were dissolved in soybean oil until saturated before being centrifuged and the saturated oil removed from the remaining un-dissolved crystals. DAG kinase inhibitor was added to eggs in oil free conditions. See table 2.2 for full list of chemicals used and working concentrations of these. All chemicals were obtained from Sigma Aldrich unless otherwise stated.

Ca^{2+}_i Indicators

Fluctuations in Ca^{2+}_i were measured using fluorescent Ca^{2+}_i indictors introduced into the eggs. Dextran linked Ca^{2+} dyes for example Oregon Green 488 BAPTA-1 Dextran (OGBD; Life Technologies) and Rhod-2 Dextran (RhodDex; Life Technologies) were microinjected directly into the cytoplasm prior to imaging at a final concentration of 1mM after dilution with injection buffer such as KCL HEPES dextran linked dyes such as these are retained in the cytoplasm for long periods of time and do not appear to compartmentalize. Rhod Dextran in a lot of cases is preferable for measuring Ca^{2+} fluctuations alone because it has a high Kd (750 nm) which reduces Ca^{2+}_i buffering effects and has a large dynamic range. Rhod dextran is excited by green light (excitation 552 nm) which has less phototoxic effects to the cell and the emitted red light (581 nm) is spectrally far away from the auto-fluorescence emitted by the cell. However, when both chemiluminescence and fluorescence (for Ca^{2+}_i) were being recorded on the same system simultaneously, Rhod Dextran could not be used as the luminescence imaging system could only measure a single wavelength once recording had begun (i.e. it does not contain an automated filter wheel).

In these instances OGBD was used as it has a similar emissionspectrum (peak emission of 523 nm) to the chemiluminescence produced by the variant of Firefly and click beetle luciferase that was used (peak emission ~550 nm). See Table 2.1 for details of peak emission and excitation wavelengths for the Ca^{2+}_{i} indictors used.

Microinjection

Solution was microinjected into eggs using high pressure pulse injection via the insertion of a micropipette. Prior to the microinjection itselfthe glass micropipettes (injection pipettes) were pulled from fine glass rods containing a filament (GC150 F, 1.5 mm outer diameter and 0.86 mm inner diameter, Harvard Apparatus Ltd) using a micropipette puller (model P-30, Sutter Instrument Company) to produce micropipette with a diameter of less than 1 µm. The injection pipette was back loaded with the desired solution (dye, cRNA, recombinant protein etc.) using a micro-loader pipette tip (Eppendorf) attached to a 1 ml plastic syringe. Eggs that were to be microinjected were then transferred to Petri dish (Nunc, Thermo Fisher Scientific) containing a shallow drop of M2 media under mineral oil which was placed on the microscope stage (inverted microscope, Eclipse TE2000-S, Nikon UK Ltd). The injection pipette was then fitted to a holder (ME2H25FW, World Precision Instruments) containing a silver wire and a side port connected to a Picopump pressure injector (model PV820, World Precision Instruments) via silicone tubing. This holder was then attached to the pre-amplifier of an electrometer (IE-25A, Warner Instrument Corporation) which could be manipulated manually using hydraulic manipulators (3D fine micromanipulator, Narashige). The eggs were held stable during injection using a holding pipette that had previously been pulled from G-100 TF glass (Harvard Apparatus Ltd) using PN-30 pipette puller (Narashige). The holding pipette was attached to syringe system (Cell Tram Air 5176, Eppendorf) which allowed the eggs to be sucked onto the holding pipette. The injection pipette was inserted through the zona pellucida surrounding the egg until it reached the surface of the egg plasma membrane. A small electrical oscillation using the electrical amplifier was then delivered to the plasma membrane which allowed the injection pipette to penetrate the plasma membrane and deliver solution into the cytoplasm by a high pressure pulse (typically 20 psi for approximately 0.5 secs). The amount of solution injected was estimated by measuring the diameter of cytoplasmic displacement created by a bolus injection and was found to be approximately 3-5 % of the total egg volume.

Immunocytochemistry

Immunocytochemistry was used to look at both the endogenous localisation and the localisation of exogenously derived proteins at the subcellular level using a confocal microscopy system. The eggs were first washed in Phosphate buffered saline solution (PBS) containing 1% polyvinyl alcohol (PVA) (Sigma Aldrich) three times before being incubated in 4% paraformaldehyde (Sigma Aldrich) in PBS 1% poly vinyl alcohol (PVA) for 15 minutes. The cells were then quenched in 50mM ammonium chloride (NH_4Cl) with 0.2 % bovine serum albumin (BSA) in PBS for 15 minutes and permeabilised in a PBS buffer containing 0.2% BSA and 0.1% Triton x100 in a homemade humidity chamber for a further 15 minutes. The cells were then washed in 10% goat's serum (Sigma Aldrich) diluted in 1% PVA in PBS and incubated for 1 hour at room temperature or overnight at 4 °C. If mouse derived monoclonal was used as the primary antibody the cells were then incubated for 1 hour in "mouse-on mouse" Ig blocking agent (Invitrogen) diluted to the manufacturer's instructions in PBS 1% PVA. The cells were then incubated with the primary antibody (diluted to an appropriate concentration) in 0.2% BSA in 1% PVA PBS for a further hour. The cells were then incubated for a final hour in a fluorescently conjugated secondary antibody (Invitrogen), diluted by 1:500 in 10% goat's serum. Cells were imaged in a 5 µl drop of PBS under mineral oil in a glass bottom dish (FluoroDish, World precision instruments, inc). Between each fixing and staining step and before imaging the cells were thoroughly washed in PBS containing 1% PVA.

Preparation of Eggs for Imaging

For imagingin most cases the eggs were placed in glass bottom chambers containing a 0.9 - 1 ml drop of HEPES Buffered KSOM media (pH 7.4, made up in cell culture grade water: 10mM HEPES, 95 mM NaCl, 0.35 mM KH_2PO_4, 0.2 mM $MgSO_4$, 2.5 mM KCl, 0.01 mM EDTA, 0.2 mM Na Pyruvate, 1 mM L-glutamine 0.2 mM glucose and 10 mg/l phenol red) under mineral oil. If there was to be no further addition of drugs or sperm to the chamber the eggs were placed directly in the dish with the zona pellucida intact. However, if the eggs were to be fertilised by IVF or an additional solution was going to be added chamber, the zona pellucida were first removed by treatment in acid Tyrode's solution (Sigma Aldrich) before being washed in M2 and placed in the imaging chamber. By removing the zona pellucida the eggs were better able to stick to the glass cover slip and minimise movement.

Epifluorescence Imaging

Imaging of fluorescent proteins was carried out using an inverted epifluorescencemicroscope (see fig 2.1) (Nikon Eclipse Ti-U microscope) with a heated stage device (Warner Instruments) so the eggs could be maintained at 35 - 37°C. During epifluorescence imaging light is provided by either an LED lamp (OptoliteLEDLite, Cairns Research Ltd) or a white halogen lamp. The light passes through an excitation filter of the appropriate wavelengths. Only light within the excitation spectrum of the fluorescent protein passes through the filter and reflects off the dichoric mirror and through the objective onto the sample. The fluorescent dyes or proteins inside the egg absorb this light and emit light of a different wavelength (at a wavelenth within the emission spectrum). The emitted light passes back through the objective straight through the dichroic mirror and the emission filter to the cooled CCD camera (CoolSNAPHQz, Photometrics) which was connected to the *In Vivo* computer Software (See fig 2.2 for schematic). The filters could be automatically changed using an optical filter changer (Lambda 10-3, Sutter Instrument Company) to allow multiple fluorescent channels to be detected during one experiment by switching filters at set intervals and using a double or triple bandpass filter. Different excitation and emission filters, diochroic mirrors and light sources were used for different fluorescent dyes or proteins (See Table 2.1). When measuring fluorescence the sample was imaged through a 20x (0.75 N.A, Fluor) objective and images were acquired every 10 secs with an exposure of 100- 400 millisecs. Light exposure to the eggs was kept to a minimum by using a camera pixel binning of 8x8 which increased camera sensitivity therefore reduced photo damage.Images were saved as .tif files for further analysis offline.

In experiments where FRET based probes were used this epifluorescence imaging system was used to measure multiple wavelengths of fluorescence emission. The system was configured so that the automated filter wheel switched between allowing the yellow emitted signal or the green emitted signal through to be detected for 200 ms by the camera every 10 secs. The signal emitted by each fluorophore was recorded as a separate measurement for each time point. A ratio of these two signals was then calculated by dividing the fluorescence of the YFP signal by the CFP signal and plotting tehse values against time.

Confocal Imaging

Epifluorescence microscopy is a simple way to measure whole cell fluctuations in fluorescence over a long period of time however as the whole sample becomes illuminated

and the emitted fluorescence on all focal planes is detected therefore it is often difficult to resolve the localisation of the fluorescence clearly on the intracellular level. Confocal microscopy overcomes some of these disadvantages of standard epifluorescence imaging by eliminating out of focus light and using point illumination to focus on only one focal plane at a time. A spatial pinhole is used which means only fluorescence emitted in or very close to the focal plane passes to the detector (photomultiplier tube) and out of focus light is excluded (See fig 2.2). This means that using confocal imaging depth of field can be conferred and images of a greater resolution may be produced.

Confocal imaging was used in this project to specifically look at the intracellular localisation of fluorescence probes. Eggs were imaged at 63x oil objective using a SP5inverted microscope (Leica, Wetzler, Germany) and data was acquired through the LAS-AF software (Leica) via a computer connected to the scanner head of the microscope. The coherent light source was provided by either an Argon (488 nm) or Helium-Neon laser (643 nm or 543 nm) (at around 30% power) and the appropriate emission and excitation filters were used (see Table 2.1) using a pinhole aperture of 91 µm. Images were usually taken as equatorial or cortical cross sections of one egg at a time. Eggs were imaged at room temperature for short periods of time in a 5µl drop of M2 media under mineral oil. Images were exported as tif. files and analysed using LAS lite software (Leica).

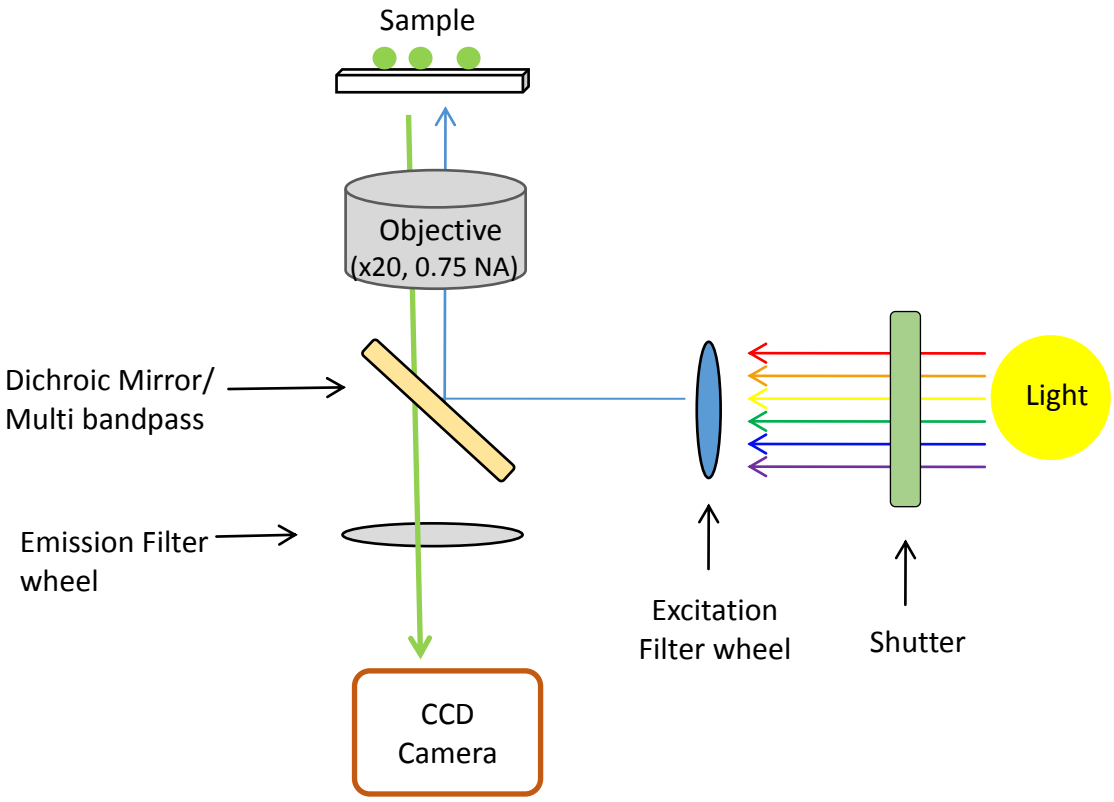


Figure 2.1 Schematic of epifluorescence imaging system using an inverted microscope

The light source is produced by either a halogen or LED lamp which is automatically controlled by an electronic gating or a motorised shutter. This light passes through an excitation filter to select the correct wavelength of light required for excitation. This light is then reflected off a dichroic mirror through a x20 objective with a numerical aperture of 0.75, onto the sample. The light emitted from the sample as a result of this excitation passes back through the objective, the dichroic mirror and an emission filter. This emission filter excludes all wavelengths of light other than that emitted from the sample (at a set level). This light is then projected onto a cooled or intensified CCD camera which reads the light signal

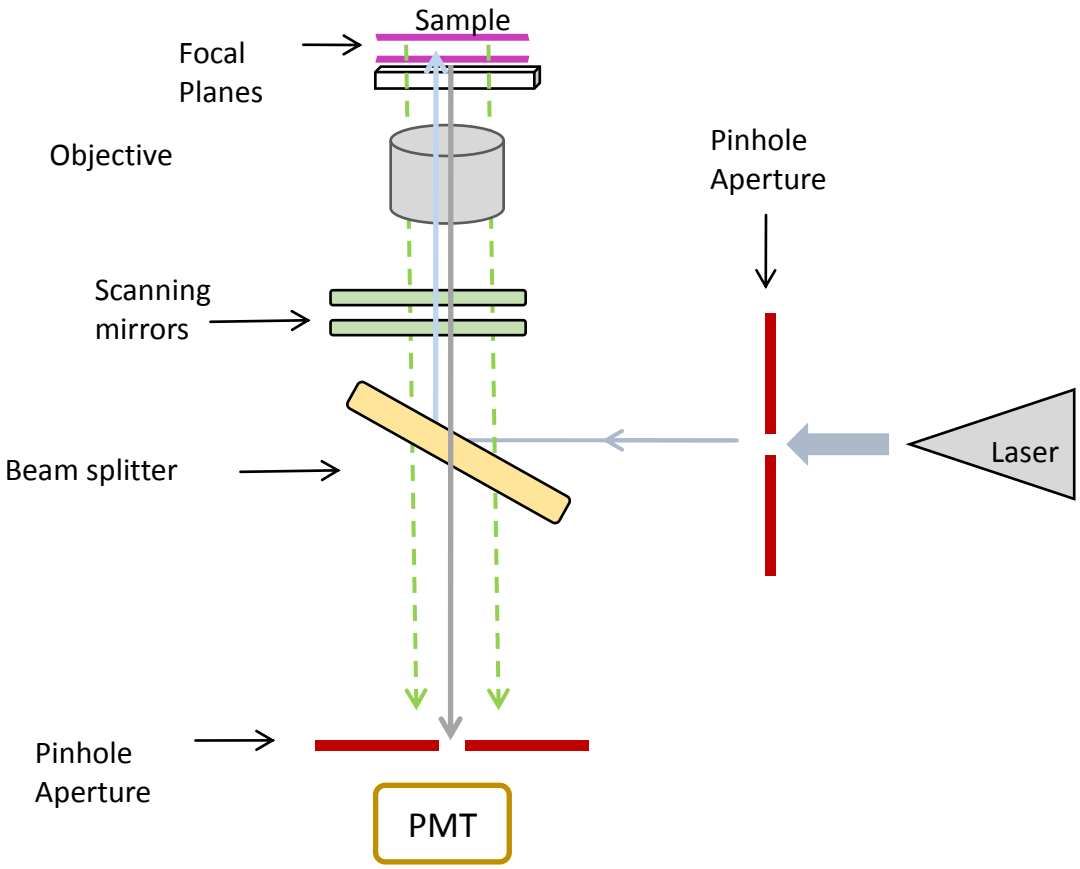


Figure 2.2 Schematic of confocal imaging system using an inverted microscope

The excitation light source is produced by a laser that emits light of only one wavelength (blue). This light then passes through a pinhole aperture which directs it onto a dichroic mirror or beam splitter. This reflected light then passes through a series of motorised scanning mirrors which scan the laser light over the sample. The light passes through the x63 objective where it can illuminate the sample. As emitted light travels back through the scanning mirrors it is de-scanned and passes directly through the dichroic mirror. Only the light from the desired focal plane passes through the pinhole aperture and is detected by the PMT (photo-multiplier tube).

Measuring Chemiluminescence

Chemiluminescence (luminescence) was measured using a different imaging system to that used for measuring fluorescence alone.In addition to operating as an epifluorescence microscope this system was also able to measure luminescence via photon counting. The eggs were imaged on an inverted microscope (TE200, Nikon or Axiovert S100, Zeiss) contained within a light proof dark box to prevent stray light entering the camera during imaging. The eggs were placed in a dish with a glass coverslip base contained within 0.9-1 ml HKSOM incubated with 100µM luciferin (Sigma Aldrich) covered with mineral oil and maintained at 35-37 °C using a heated chamber device (Intracel Ltd) attached to the microscope stage. During imaging light was directed to a cooled, intensified CCD Camera (Photek, UK) which was connected to the microscope (see fig 2.1). Light was provided for bright field and fluorescence imaging by a halogen lamp which was gated by electro mechanical shutters (Uniblitz) and controlled through the Photek Software. Fluorescence of the Ca^{2+}_{i} indicator OGBD and GFP proteins were measured on this system using a 505 nm long-pass FITC filter block and the camera sensitivity was reduced to 10%. When measuring luminescence the light source was switched off, the camera sensitivity was switched to 100% and luminescence was recorded as integrated light emission in the form of number of photons per second. Both fluorescence and luminescence were recorded simultaneously during an experiment by taking advantage of the electronic gating of the light source. At 10 sec intervals the system switched between measuring fluorescence and luminescence by opening and closing the light shutter and automatically switching camera sensitivity. This made it possible to plot luminescence and fluorescence on the same time scale for each egg in a single experiment. During all experiments eggs were imaged through a x20 objective (0.75 NA, Fluor).

Calibrating Protein Expression

Measuring the luminescence of a luciferase tagged construct enables the amount of protein expression inside the cell to be quantified. This can be done by determining the conversion factor between the value of luminescence recorded during imaging and the amount of luminescence produced by a particular amount of luciferase protein. To do this luminescence values of the eggs at the end of imaging were recorded before the eggs were removed from the imaging system and placed in a test tube containing phosphate buffered saline solution with 0.5% Triton X-100 and 1mM MgATP for lysis (Sigma Aldrich). Luminescence of the lysed eggs was then measured in a custom made luminometer consisting of a cooled photomultiplier tube (S20) connected to an amplifier (Electrontubes

Ltd) and a dark proof tube holder. Once in the tube holder 100µM luciferin was added to the test tube containing the eggs and luminescence was measured using an amplifier which was linked to some computer software (Electrontubes Ltd). Luminescence was recorded as absolute photon counts plotted against time and the mean luminescence of each egg was calculated by dividing the total photon counts recorded by number of eggs in the group. This luminescence value was compared to a calibration curve of luminescence created using known quantities of recombinant luciferase protein in lieu of eggs in the test tubes. A regression analysis was carried out to determine the relationship between the end luminescence value of the eggs recorded by the imaging system and the luminescence value measured by the luminometer. This allowed the conversion of imaging luminescence values into luminometer luminescence values to be calculated without using the luminometer. These calculated luminometer luminescence values could be entered into the equation for the recombinant luciferase calibration curve to calculate the approximate amount of luciferase protein in any eggs injected with a luciferase tagged construct.

Analysing Images

Images that were collected using simple epifluorescence recording were analysed using the acquisition software Image J. The .tif files were analysed as an image stack using the multi-measure plugin to measure fluorescence intensities. Areas were drawn around the images of eggs as “regions of interest” so individual fluorescence intensities of these eggs were calculated.Anegg-free region of interest was also taken to give a non-specific background fluorescence which was then subtracted from the absolute fluorescence for each egg to give a background corrected fluorescence value. Fluorescence was normalised by dividing the background corrected fluorescence (F) by the basal fluorescence value (F0) this F/F0 was then plotted against time.

Data analysis and Statistics

Fluorescence and luminescence measurements recorded by the luminescence system were saved as numerical values (number of photons detected) therefore the data collected from this system was analysed in a different way to that of the epifluorescence system. Data was both acquired and exported using the Photek software. Once the recording had stopped regions of interest were drawn around each egg so the fluorescence/luminescence for individual eggs could be determined as well as a random egg-free region (background). Data in the form of “count rate” for each egg was then generated and exported in the form of a text file of photon counts per second, every 10 seconds, from each region of interest.

This text file was then imported into Sigma Plot 12 Software (Systat Software inc) for further analysis. The fluorescence/ luminescence of the background area was subtracted from the egg fluorescence/luminescence value to give a background corrected count rate or fluorescence for each egg. The F/F0 was then calculated for the fluorescence data and luminescence was plotted as background corrected luminescence against time.

Average luminescence values or number of Ca^{2+}_{i} ocillations were represented by calculating the mean. For these values either standard deviations (SD) or standard error of the means (SEM) were also calculated. Stastistics were conducted to test for significant differences between groups. In this case a Pearson's test for normality was conducted first. If the data passed this test a Student's T-test for significant difference was carried out with a 95% confidence interval. If the data failed the test for normality a Mann-Whitney Ranked test for comparision between 2 groups was conducted instead again with a 95% confidence interval.

Fluorophore or indicator	Purpose/targets/ measures	Colour	Excitation wavelength (nm)	Emission wavelength (nm)
Oregon green BAPTA Dextran (OGBD)	cytosolic Ca^{2+}_i indicator	Green	494	523
Rhod- 2 Dextran	cytosolic Ca^{2+}_i indicator	Red	552	581
BODIPY FL Ceramide *(Thermo Fisher)*	Golgi target	Green	500	510
GOLGI -ID® *(Enzo Life Sciences)*	Golgi target	Green	~460	~540
AC-lectin (fluorescein)*(vector labs)*	Golgi target	Green	494	512
$DiIC_{18}(3)$ *(Thermo Fisher)*	ER target	Red-Orange	549	565
eYFP	tag	Yellow	514	527
m-Cherry	tag	Red	587	610
Td-Tomato	tag	Red	554	581
eGFP	tag	Green	484	510
eCFP	tag	Blue	435	485
Alexa Fluor 488 *(Thermo Fisher)*	conjugated antibody	Green	493	519
Alexa Fluor 594 *(Thermo Fisher)*	conjugated antibody	Red	590	617
Green "Upwards" DAG *(Montana Molecular)*	DAG	Green	~490	~520
PBP10 (Rhodamine B) *(Tocris Bioscience)*	$PI(4,5)P_2$	Red-orange	540	625
DAN20 (DAN)	$PI(4,5)P_2$	Blue and Green	405	435 - 465 (blue) 510- 540 (green)
BODIPY FL-$PI(4,5)P_2$ *(Echelon inc)*	$PI(4,5)P_2$	Green	494	512

Table 2.1 Table showing all fluorescent tags and indicators used and the corresponding maximal excitation and emission wavelengths

Chemical/drug	Molar concentration
3-isobutyl-1-methylxanthine (IBMX)	100 µM
Propranolol	300 µM
Brefeldin A (BFA)	5 µM
D-Luciferin	100 µM
Diacylglycerol Kinase Inhibitor I	10 µM
D609	100 µM
Phorbol 12,13-dibutyrate (PDBµ)	2 µM
phorbol-12-myristate-13-acetate (PMA)	1 µM
Ionomycin	100 µM
A23187	5 µM
Acetyl choline chloride (Ach)	100 µM

Table 2.2: Table showing all chemicals/drugs added to eggs and oocytes during experiments and the working molar concentration of these

3. DOES PAWP HAVE A ROLE IN INDUCING CA^{2+}_I OSCILLATIONS AND EGG ACTIVATION IN MOUSE EGGS?

Introduction

During mammalian fertilisation the initiation of early developmental events are dependent on the MII-arrested oocyte (egg) becoming activated following sperm-egg fusion. The events of egg activation are triggered by a series of transient increases in cytosolic calcium concentration (Ca^{2+}_i) known as Ca^{2+}_i oscillations (Stricker 1999). It is well established these oscillations are a result of inositol trisphosphate (IP_3) induced Ca^{2+}release from the endoplasmic reticulum (Miyazaki 1993) which is triggered by the introduction of a sperm derived factor (Swann 1990b). This sperm specific factor, also known as sperm-derived oocytes activating factor (SOAF), is located in the perinuclear theca of mature spermatozoa and is deposited into the egg during egg sperm fusion (Kimura *et al.* 1998). The perinuclear theca is the first part of the sperm to dissolve into the egg cytoplasm and only sperm extracts which contain an intact perinuclear theca are able to elicit full egg activation (Sutovsky *et al.* 2003).

Post-acrosomal sheath WW Domain- binding Protein (PAWP) is a sperm specific alkaline extractable protein located in the post-acrosomal sheath (PAS) of the perinuclear theca (Wu *et al.* 2007; Aarabi *et al.* 2014a). There are two main functional domains found in PAWP, an N-terminus which has homology with the WW domain-binding protein 2 and a proline rich unique C-terminus. The N-terminus of PAWP therefore has the ability to bind to WW domain containing proteins. WW domains are 38-40 amino acid length sequences named after the 2 conserved tryptophan residues (W) contained within them (Macias *et al.* 1996) and is formed of 3 anti-parallel β sheets which are held tighter by hydrogen bonds but does not contain any disulfide bridges (Ibragimova and Wade 1999). WW domains have the ability to mediate protein-protein interactions through the formation of protein rich modules by recognising and interacting with proline rich regions (Salah *et al.* 2012) or in some cases via phosphoserine or phosphothreonine binding (Lu *et al.* 1999). WWI domain containing proteins including yes-associated protein (YAP), neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) and dystrophin mediate protein interactions by recognising polypeptides containing the consensus PPXY motif (PY motif) where P represents proline residues and Y represents tyrosine (Chen and Sudol 1995; Sudol

and Hunter 2000). YAP is able to bind to two specific ligands called WW domain binding protein 1 (WBP1) and WW domain binding protein 2 (WBP2) both of which contain this PY motif and are very highly conserved between species (Chen *et al.* 1997). Furthermore,point substitution of the prolines or the tyrosine in the PY motif of either WBP1 or WBP2 can abolish YAP binding (Chen *et al.* 1997). WW domains can be considered as functionally similar to Src homology 3 (SH3) domains however are structurally different (Sudol 1996). In fact, YAP also contains a SH3 binding motif which enables it to bind to the SH3 domain of c-Yes, a proto-oncogene of the Src family of tyrosine kinase as well as other SH3 domain-containing proteins (Sudol 1994).

PAWP is exclusively expressed and assembled during spermatid elongation (Wu *et al.* 2007; Aarabi *et al.* 2014a) with the final equivalent amount of PAWP protein found in bull sperm estimated to be around 80 fg (Wu *et al.* 2007). Microinjection of bull PAWP recombinant protein into porcine, bovine, *Xenopus* and rhesus monkey eggs has been reported to induce full egg activation up to pronuclear formation (Wu *et al.* 2007). Resumption of meiosis was also recorded in *Xenopus* eggs following injection of PAWP indicated by the extrusion of a second polar body (Wu *et al.* 2007). Interestingly the co-injection of a competitive peptide containing the PPXY motif derived from PAWP alongside recombinant PAWP protein into *Xenopus* eggs prevented the resumption of meiosis previously observed (Wu *et al.* 2007). The PPXY containing peptide also inhibited the resumption of meiosis of eggs that had been injected with perinuclear theca alkaline extract from the sperm (Wu *et al.* 2007). Furthermore porcine eggs injectedwith anti-recombinant PAWP serum or PPXY containing peptide that were fertilised by ICSI displayed high rates of developmental arrest and lacked pronuclear formation (Wu *et al.* 2007) This effect was not seen in ICSI fertilised eggs injected with pre-immune serum or a peptide containing a point mutated version of the PPXY motif (Wu *et al.* 2007). These data suggest that pronuclear formation and successful fertilisation following perinuclear theca extract injection and ICSI is mediated by PY motif – WWI domain-mediated signalling within the egg (Wu *et al.* 2007).

PAWP is suggested to elicit egg activation by operating upstream of Ca^{2+}_i signalling. Indeed the microinjection of bull PAWP recombinant protein into *Xenopus* eggs elicits Ca^{2+}_i increase (Aarabi *et al.* 2010). However, this Ca^{2+}_i increase is not observed as Ca^{2+}_i wave crossing the egg as has previously been reported in fertilised *Xenopus* eggs (Bugrim *et al.* 2003). Nevertheless this Ca^{2+}_i change also appears to be dependent on PY motif /WWI domain interaction. The Ca^{2+}_i increase seen following the injection of either PAWP recombinant protein or *Xenopus* sperm could be prevented by the co-injection of the

peptide containing PAWP derived PPXY motif but not by a peptide with a point mutated version of this motif (Aarabi *et al.* 2010). Similar effects of PAWP have been reported in mammalian eggs. Microinjection of human PAWP recombinant protein into mouse eggs and human PAWP cRNA into human eggs has been found to elicit $Ca^{2+}{}_i$ oscillations similar to those seen in eggs fertilised using ICSI (Aarabi *et al.* 2014b). Oscillations induced by either PAWP or ICSI in both species could be blocked following the co-injection of a PAWP derived PPXY motif containing peptide (PPXY peptide) however not by a point mutated version of this motif (Aarabi *et al.* 2014b).

Together these data suggest that PAWP is able to activate eggs by stimulating the $Ca^{2+}{}_i$ signalling pathway specifically by triggering $Ca^{2+}{}_i$ oscillations in mammalian eggs, a process which is dependent on PY motif –WWI domain interaction. Far-western analysis has shown that the PPXY motif of bull PAWP recombinant protein has binding affinity to WWI domain of Yes-associated protein (YAP) however does not bind to the point mutated WW domain of YAP^{P202A} (Wu *et al.* 2007). Furthermore the binding of recombinant PAWP protein to YAP could be inhibited by co-incubation with a PPXY containing peptide but was not inhibited by a peptide containing the mutated PY motif PPGF (Wu *et al.* 2007). It has therefore been suggested that the PPXY motif found in the proline rich c-terminus of PAWP functionally binds to the WWI domain of YAP once PAWP is inside the egg cytosol (Wu *et al.* 2007; Aarabi *et al.* 2010; Aarabi *et al.* 2014b). As YAP possesses a SH3 binding motif it has been postulated, that on binding to PAWP, YAP may activate PLCγ through in a non-canonical pathway via its SH3 domain (Aarabi *et al.* 2014b). Once activated, PLCγ is able to hydrolyse $PI(4,5)P_2$ to generate IP_3 and elicit Ca^{2+} release (Aarabi *et al.* 2014b)

These results have made way for potential clinical applications of PAWP to be proposed. It appears a reduced PAWP concentration within human and bull sperm can be correlated with failure for that sperm to successfully fertilise an egg, suggesting PAWP levels could be an effective indictor of sperm quality (Aarabi *et al.* 2014a; Kennedy *et al.* 2014).It has also been suggested that PAWP could be introduced into eggs during assisted fertility to increase success rates for example in ICSI failure cases (Kennedy *et al.* 2014). Together these data suggests that PAWP could be an alternative sperm factor candidate to PLCζ and may have a role in activating mammalian eggs at fertilisation.

Aims

PAWP has been proposed as a potential and promising sperm factor candidate for triggering the initiation of $Ca^{2+}{}_i$ oscillations during mammalian fertilisation (Wu *et al.* 2007;

Aarabi *et al.* 2014b). As the Ca^{2+} releasing effects of PAWP in mammalian eggs have only be demonstrated by one research group and using a human derived form of PAWP (Aarabi *et al.* 2014b) this project aimed to reproduce the effects of PAWP previously seen and determine if mouse and human derived PAWP could cause Ca^{2+}_i oscillations in mouse eggs in a physiological manner. Furthermore this project aimed to investigate how the Ca^{2+}_i oscillations elicited by PAWP compared to those Ca^{2+}_i oscillations initiated by the introduction of the sperm derived PLCζ (Saunders *et al.* 2002) and whether these two proteins work together as cofactors to elicit Ca^{2+}_i oscillations. These experiments also intended to quantify the amount of PAWP protein that was required by the egg for Ca^{2+}_i oscillations to be triggered. Finally the role of PY motif –WWI domain interaction in eliciting Ca^{2+}_i oscillations in mouse eggs at fertilisation was further investigated with the aim to determine if the inhibition of this interaction prevented Ca^{2+}_i oscillations caused by PAWP or any other stimuli.

Results

PAWP recombinant protein does not cause Ca^{2+}_i oscillations in mouse eggs

As human PAWP recombinant protein has previously been shown to cause Ca^{2+}_i oscillations when injected into both human and mouse eggs (Aarabi *et al.* 2014b) it was important to investigate whether mouse derived PAWP recombinant protein had the ability to elicit Ca^{2+}_i oscillations in mouse eggs. For this, human and mouse derived protein was synthesised by a colleague Michail Nomikos (MN). In the paper published by (Aarabi *et al.* 2014b) human derived PAWP recombinant protein was synthesised with only a His-tag attached however on trying this the recombinant protein produced was found not to be soluble therefore in the present study a NusA tag was also added to increase solubility. To determine if mouse PAWP could elicit Ca^{2+}_i oscillations this His and NusA tagged PAWP recombinant protein (NusA-mPAWP) (fig 3.1) at a pipette concentration of 0.5 µg/µl was microinjected into MII arrested unfertilised mouse eggs along with a fluorescent Ca^{2+}_i indicator (OGBD) so changes in Ca^{2+}_i could be measured. Whilst recording the fluorescence in these eggs no changes in cytosolic Ca^{2+}_i concentrations were seen in any eggs (fig 3.2) indicting that NusA-mPAWP was not able to elicit Ca^{2+}_i oscillations. As a direct comparison a group of eggs were also microinjected with NusA tagged mouse derived PLCζ recombinant protein (NusA-PLCζ) mixed with Ca^{2+}_i dye in the same way at the same pipette concentration as NusA-mPAWP. In contrast to the results seen with PAWP, at this concentration NusA-PLCζ was able to induce Ca^{2+}_i oscillations similar to those seen as fertilisation (fig 3.2).

To ensure there were no fundamental differences in the activity of mouse and human derived PAWP human derived NusA-PAWP recombinant protein (NusA-hPAWP) (fig. 3.1) was also injected into MII arrested unfertilised mouse eggs. Eggs were microinjected with NusA-hPAWP at a pipette concentration of 0.5 µg/µl mixed with the Ca^{2+}_{i} indicator Rhod Dextran so alteration in Ca^{2+}_{i} could be measured. Separate eggs were also microinjected with a lower pipette concentration of NusA-hPAWP (0.05 µg/µl) mixed with Rhod dextran to be sure the eggs weren't overdosed with PAWP therefore preventing physiological response. In both groups of eggs no changes in cytosolic Ca^{2+}_{i} concentrations were recorded in any of the eggs during imaging (fig. 3.3). These data would suggest that PAWP recombinant protein derived from mouse or human is unable to cause Ca^{2+}_{i} oscillations in mouse eggs in the manner seen at fertilisation.

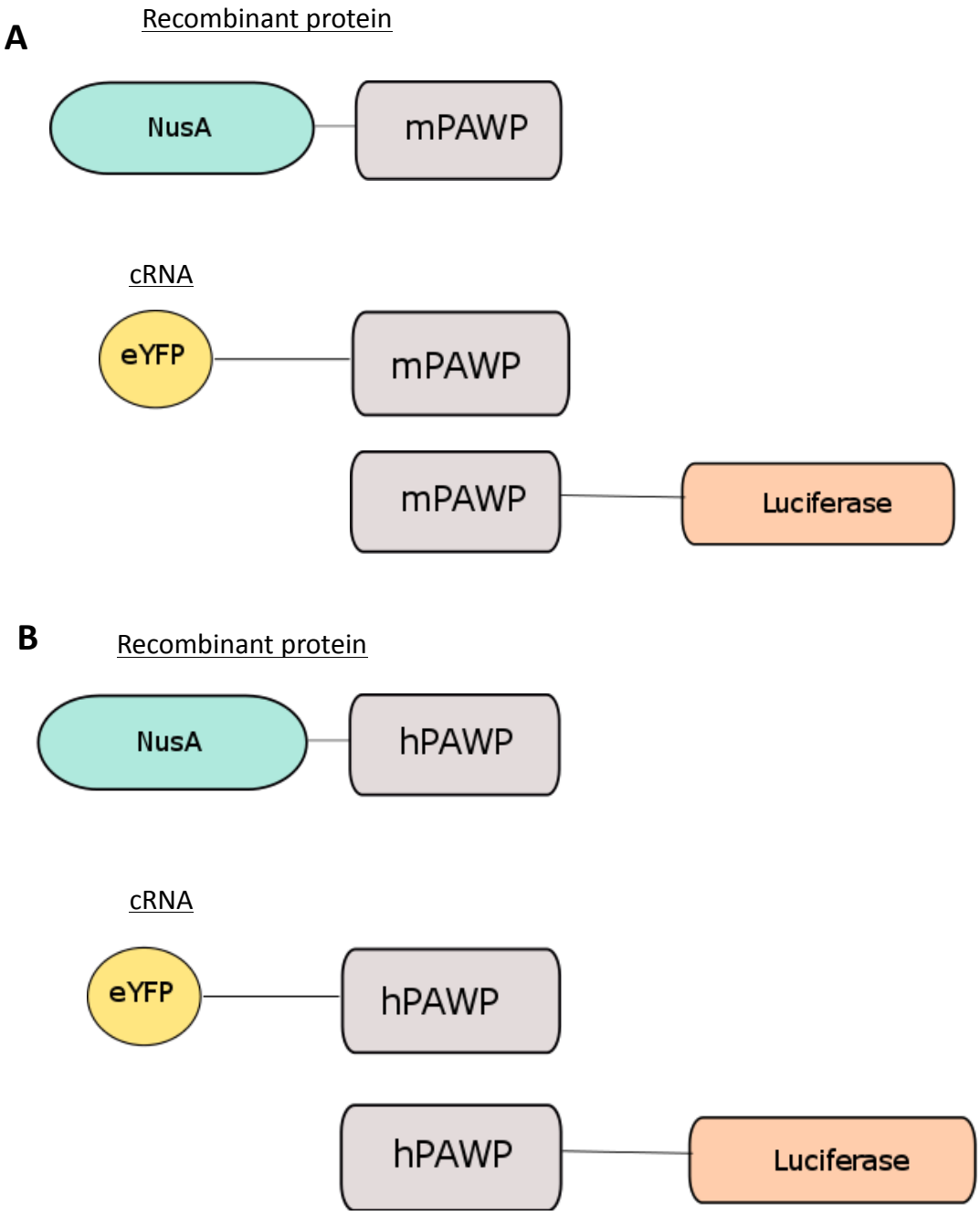


Figure 3.1 Schematic showing the different PAWP constructs that were microinjected into eggs

A) mouse derived PAWP constructs , **B)** human derived PAWP constructs

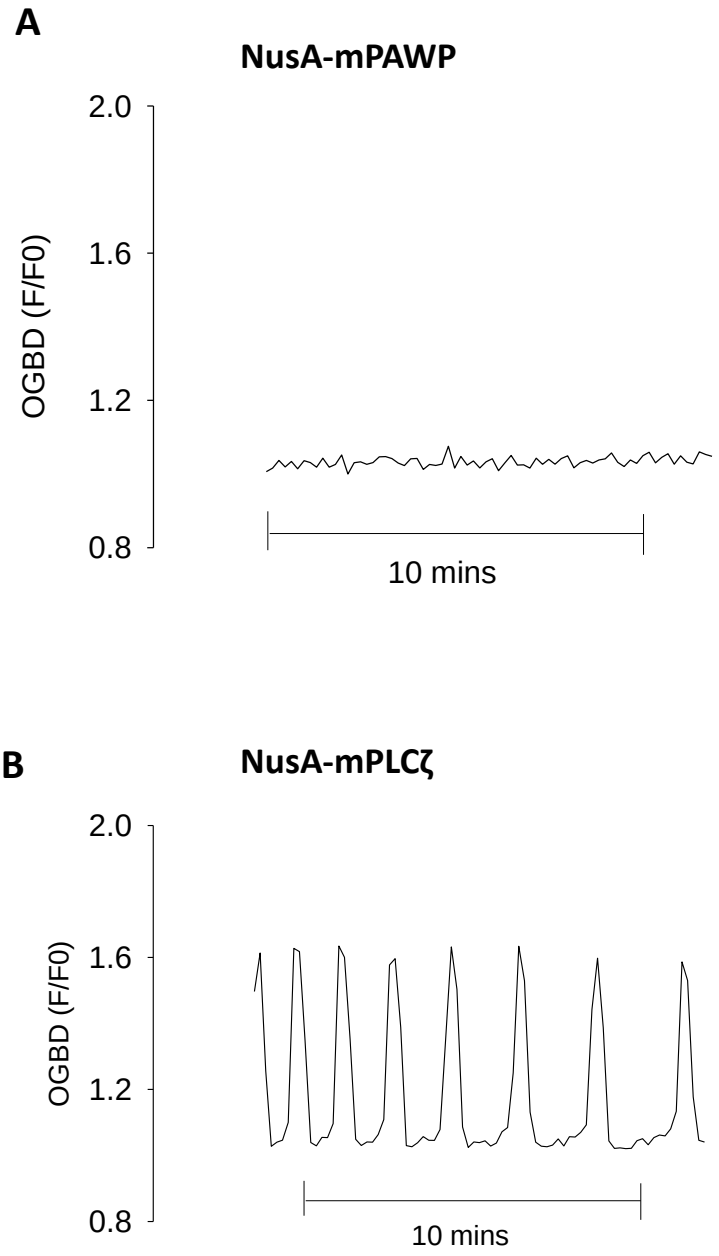


Figure 3.2 Example Ca^{2+}_i traces in eggs following injection of mPAWP and PLCζ recombinant protein

Examples of traces showing the changes in cytosolic Ca^{2+}_i in MII oocytes following microinjection of the Ca^{2+}_iindictor Oregon Green BAPTA Dextran (OGBD) mixed with; **A)** NusA-mPAWP recombinant protein (pipette concentration = 0.5 µg/µl, n= 25 oocytes), and **B)** NusA-mPLCζ(pipette concentration = 0.5 µg/µl, n= 12 oocytes)

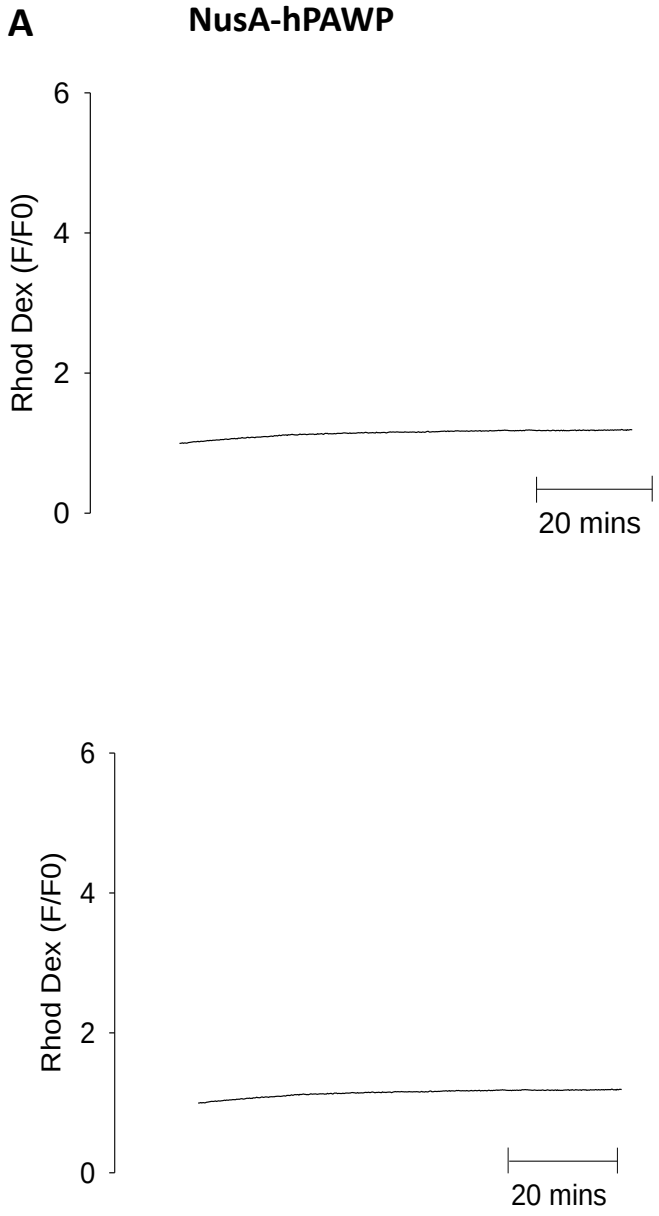


Figure 3.3 Example Ca^{2+}_i traces in eggs following injection of hPAWP recombinant protein

Sample trace showing the changes in cytosolic Ca^{2+}_i concentrations of MII eggs injected with NusA-hPAWP recombinant protein of pipette concentrations **A)** 0.05 µg/µl (n= 8) and **B)** 0.5 µg/µl (n=10) with Rhod Dextran.

PAWP untagged cRNA does not cause Ca^{2+}_i oscillations in mouse eggs

(Aarabi *et al.* 2014b) previously microinjected untagged human derived PAWP cRNA into mouse eggs and found it was able to induce Ca^{2+}_i oscillations. In light of this result, we wanted to explore whether mouse derived PAWP cRNA could elicit Ca^{2+}_i oscillations in mouse eggs. To ensure that the activity of PAWP was not affected by the addition of the NusA moiety, untagged mouse PAWP cRNA (mPAWP cRNA) synthesized by MN, at a pipette concentration of 1.25 µg/µl was microinjected into unfertilised MII mouse eggs with the Ca^{2+}_i indictor Rhod Dextran so Ca^{2+}_i changes could be recorded. The egg endogenously expresses this RNA to manufacture mPAWP protein intracellularly, as a result, the amount of PAWP protein in the egg was expected to begin at zero and increase with time. No changes in cytosolic Ca^{2+}_i concentrations were recorded in any of these eggs injected with mPAWP cRNA (fig 3.4). In steep contrast, microinjection of mouse PLCζ cRNA at a much lower pipette concentration (0.04 µg/µl) did cause Ca^{2+}_i oscillations which were comparable in frequency and pattern to those seen following fertilisation, IVF or ICSI (fig 3.4).

As with the PAWP recombinant protein, to be sure that there were no functional differences between the PAWP derived from mouse and the human derived form of PAWP used by (Aarabi *et al.* 2014b), we microinjected a different group of mouse eggs with hPAWP untagged cRNA (synthesised by MN). A range of pipette concentrations of hPAWP cRNA were injected (0.006 µg/µl, 0.06µg/µl), 0.3 µg/µl and 1 µg/µl) and in all instances no changes in cytosolic Ca^{2+}_i concentrations of the eggs were seen (fig 3.5). To verify that the cRNA injected was definitely being expressed a western blot was conducted using eggs that had been injected with 1µg/µl of hPAWP cRNA (conducted by MN). Despite no Ca^{2+}_i oscillations being recorded in eggs expressing this concentration of cRNA a huge amount of hPAWP protein was found in the eggs, indicating that the hPAWP cRNA was expressed very well (data not shown). These data suggest that mPAWP and hPAWP cRNA are not able to elicit Ca^{2+}_i oscillations at either very low nor very high concentrations and that the lack of Ca^{2+}_i oscillations elicited by NusA-mPAWP and NusA-hPAWP recombinant protein is unlikely to be due to the NusA tagging.

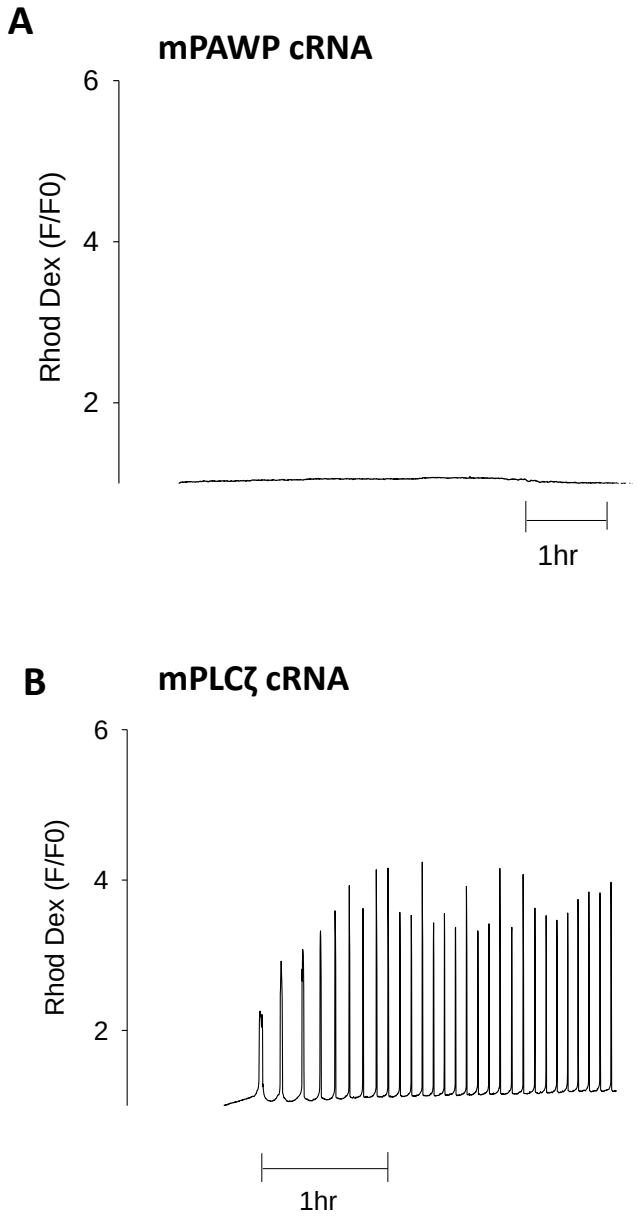


Figure 3.4 Example Ca^{2+}_i traces in eggs following injection of mPAWP and PLCζ cRNA

Examples of traces showing the changes in cytosolic Ca^{2+}_i in an MII egg following microinjection of the Ca^{2+}_i indictor Rhod Dextran mixed with; **A)** untagged mPAWP cRNA (pipette concentration = 1.25 µg/µl, n= 28 eggs), and **B)** untagged mPLCζ cRNA (pipette concentration = 0.04 µg/µl, n= 24 eggs)

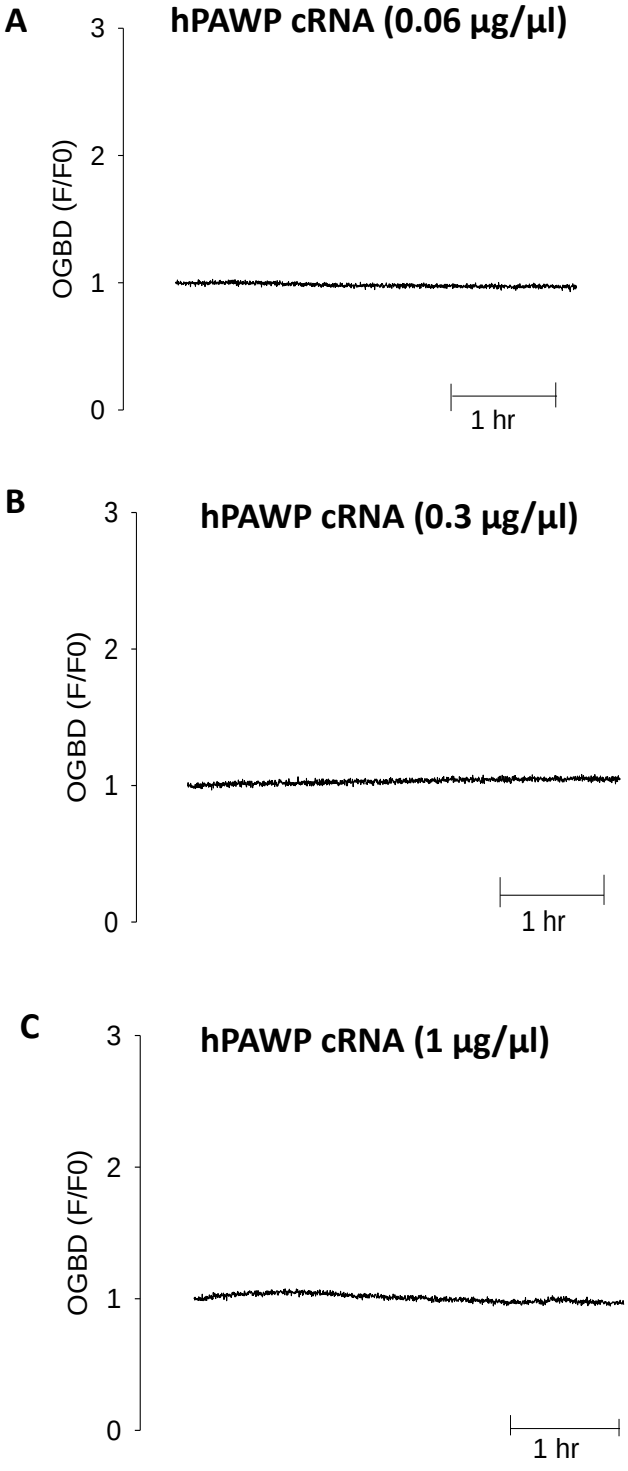


Figure 3.5 Example $Ca^{2+}i$ traces in eggs following injection of hPAWP cRNA

Sample traces showing changes in cytosolic Ca^{2+}_i concentrations of eggs microinjected with the Ca^{2+}_i indicator OGBD h-PAWP untagged cRNA of different pipette concentrations **A)** 0.06 µg/µl (n=14), **B)** 0.3 µg/µl (n=12) and **C)** 1µg/µl (n=11). A pipette concentration of 0.006 µg/µl hPAWP cRNA was also microinjected (n=23) (data not shown).

Mouse eYFP-PAWP cRNA does not cause Ca^{2+}_i oscillations in mouse eggs

In order to ensure faithful mouse PAWP cRNA expression in the eggs whilst they were being imaged a fluorescent e-YFP n -terminally tagged mPAWP cRNA (eYFP-mPAWP) was constructed (fig 3.1) by MN. eYFP-mPAWP at a pipette concentration of 1.5 µg/µl mixed with the Ca^{2+}_i indicator Rhod Dextran was microinjected into MII arrested mouse eggs prior to imaging. Fluorescence from the red channel was used to measure changes in Ca^{2+}_i whereas the fluorescence from the green channel showed changes in the amount of YFP inside the egg. As expected, YFP fluorescence increased with time showing that the RNA was successfully expressed and the amount of PAWP protein inside the egg was increasing through the duration of the experiment (fig 3.6). As the YFP signal was visible and very strong a large amount of eYFP-mPAWP must have been inside the egg, however once again no changes in cytosolic Ca^{2+}_i were seen in any eggs throughout the duration of the experiment (fig 3.6). Furthermore no sub cellular localisation of PAWP in the egg was evident as the fluorescence pattern appeared relatively uniform and therefore cytoplasmic in its distribution.

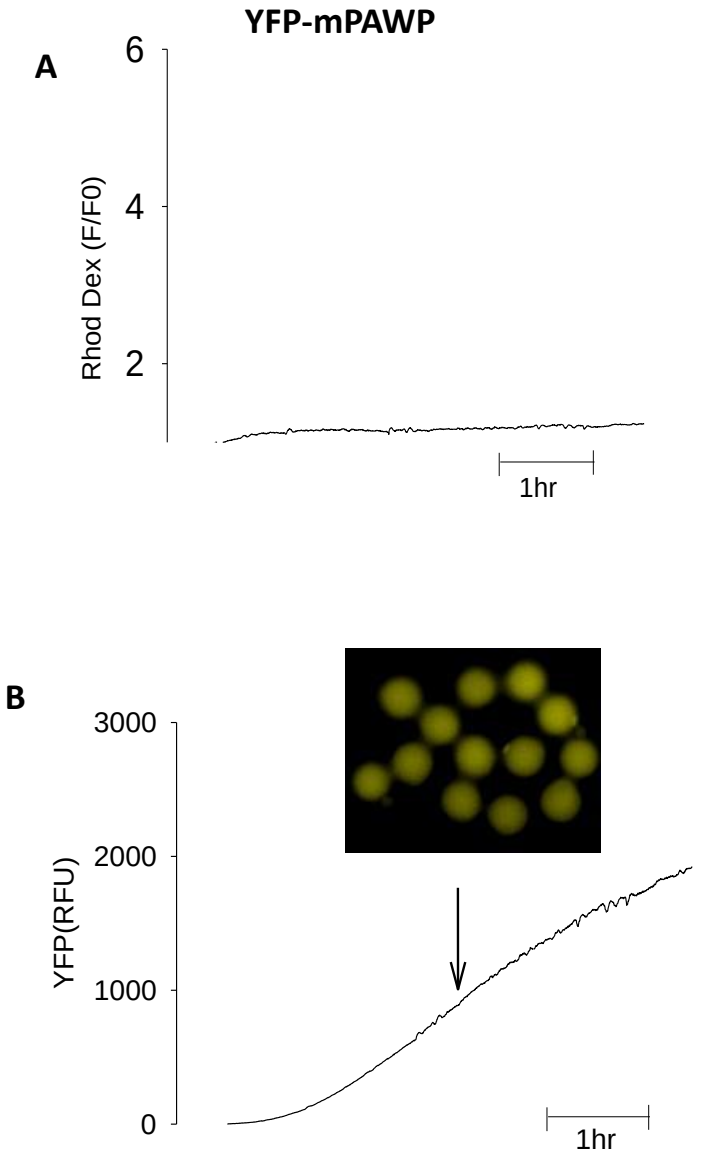


Figure 3.6 Example Ca^{2+}_i traces in eggs following injection of YFP-PAWP cRNA

Examples of traces showing, **A)** the changes in cytosolic Ca^{2+}_i in an MII egg following microinjection of the Ca^{2+}_i indictor Rhod Dextran mixed with eYFP tagged mPAWP cRNA (pipette concentration =1.5 µg/µl, n= 23 eggs) and **B)** the YFP fluorescence for the same eggs, demonstrating expressing of the cRNA and an image showing the fluorescence of 13 of these eggs injected with eYFP-mPAWP cRNA. The visible fluorescence signal indicates faithful and high expression of cRNA.

PAWP is not able to induce Ca^{2+}_ioscillations at levels present in the sperm

In order to accurately quantify the amounts of mPAWP protein being manufactured by the egg and how this related to physiological conditions, a final form of mPAWP cRNA was synthesised by MN and injected into MII eggs. mPAWP cRNA possessing a c-terminal firefly luciferase tag (mPAWP-luciferase) was constructed (fig 3.1) and mixed with the Ca^{2+}_i indicator OGBD. Using this construct it was possible to record both luminescence (which represents RNA expression) and fluorescence of the Ca^{2+}_i indicator (which represents changes in cytosolic Ca^{2+}_i) simultaneously by switching between recording luminescence and fluorescence every 10 seconds (Swann*et al.* 2009). Using the luminescence recordings it was possible to calibrate the amount of mPAWP protein inside the egg at the end of imaging. MII eggs were microinjected with mPAWP- luciferase at a range of pipette concentrations (0.04 µg/µl and 1.25 µg/µl). It has previously been reported that bovine sperm contains the equivalent of approximately 87 fg of bovine PAWP protein (Wu *et al.* 2007). However, MII eggs containing approximately 89 fg of PAWP protein following injection did not undergo any Ca^{2+}_i changes (fig. 3.7). This indicates that mPAWP is not able to cause Ca^{2+}_i oscillations in the egg at physiological levels. Furthermore, eggs containing up to 1.6 pg of mPAWP protein, which is far exceeding physiological levels, also did not show any changes in Ca^{2+}_i levels (fig. 3.8).To directly compare the activity of PAWP to PLCζ at similar levels, PLCζ- luciferase cRNA was also microinjected into MII eggs alongside OGBD at varying RNA pipette concentrations (0.06µg/µl and 1µg/µl). Measurements for luminescence and fluorescence were recorded in the same way as for mPAWP-luciferase and mPLCζ protein levels were calculated. In eggs containing approximately 59 fg of PLCζ luciferase protein robust Ca^{2+}_i oscillations like those seen at fertilisation were recorded (fig 3.7). If PLCζ-luciferase expression was increased to 360 fg Ca^{2+}_i oscillations again were recorded however this time at a higher frequency than those seen at fertilisation (fig 3.8).

To determine if similar levels of expression were seen with the human derived PAWP used by (Aarabi *et al.* 2014b) a hPAWP-luciferase cRNA construct was produced by MN (hPAWP-luciferase) (fig 3.1). A variety of pipette concentrations (0.04 µg/µl and 0.4 µg/µl) of hPAWP-luciferase cRNA mixed with OGBD were microinjected into eggs. The luminescence and fluorescence were measured in the same way as form PAWP-luciferase. At an average peak luminescence of 3.9 cps per egg which equates to approximately 59 fg of hPAWP protein inside the egg, which is comparable to the levels that should operate physiologically (Wu *et al.* 2007), no cytosolic Ca^{2+}_i changes were recorded (fig 3.9). No Ca^{2+}_i response was still seen in eggs expressing hPAWP-luciferase up to 46 cps, which far

exceeds physiological levels of PAWP found in the sperm (fig 3.9). These data suggest that human or mouse derived PAWP does not cause Ca^{2+}_i oscillations in mouse eggs at physiological levels or far exceeding them.

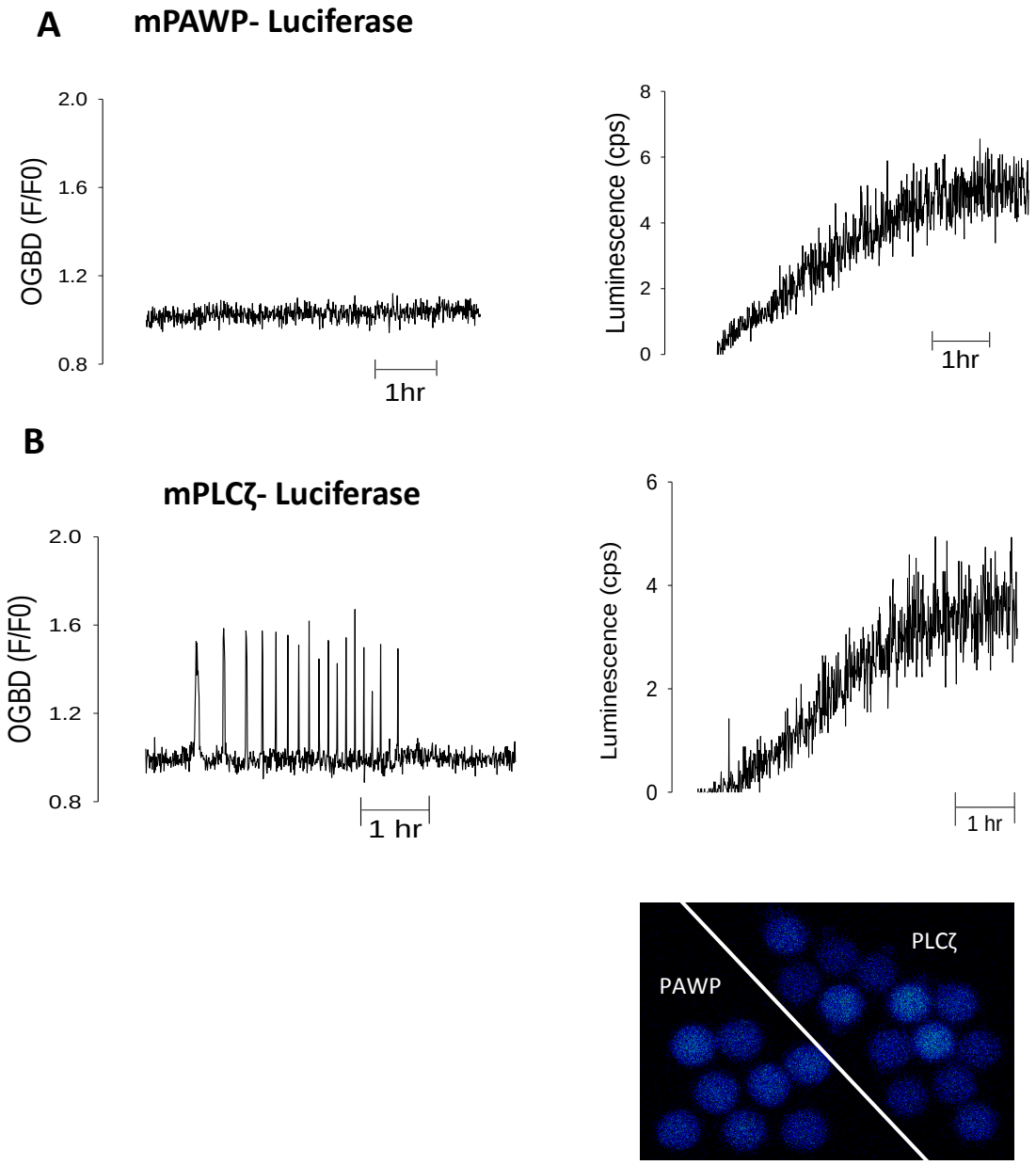


Figure 3.7 Example Ca^{2+}_i traces in eggs following injection of mPAWP-luciferase and mPLCζ-luciferase cRNA

Examples of traces showing changes in cytosolic Ca^{2+}_i in MII eggs alongside firefly luciferase expression. Oocytes were microinjected with the Ca^{2+}_i indictor OGBD mixed with: **A)** mPAWP-luciferase cRNA (pipette concentration= 0.08 µg/µl, n=18 eggs) where the left trace shows changes in cytosolic Ca^{2+}_i in one egg and the right trace shows the luciferase expression in the same egg quantifying expression of mPAWP-luciferase cRNA, average luminescence at the end of the experiment for these eggs was 5.6 cps which equates to ~ 89 fg of mPAWP-luciferase protein per egg; and **B)** mPLCζ-luciferase cRNA (pipette concentration= 0.06µg/µl, n=13 eggs) again the left trace shows changes in cytosolic Ca^{2+}_i in one egg and the right trace shows the luciferase expression in the same egg quantifying expression of mPLCζ -luciferase cRNA average luminescence for these eggs at the end of the experiment was 3.9 cps which equates to ~59 fg of mPLCζ-luciferase protein per egg. The image shows the luminescence of mPAWP-luciferase and PLCζ- luciferase injected eggs being imaged at the same time (light integrated for 30 mins starting at 4 hours post injection)

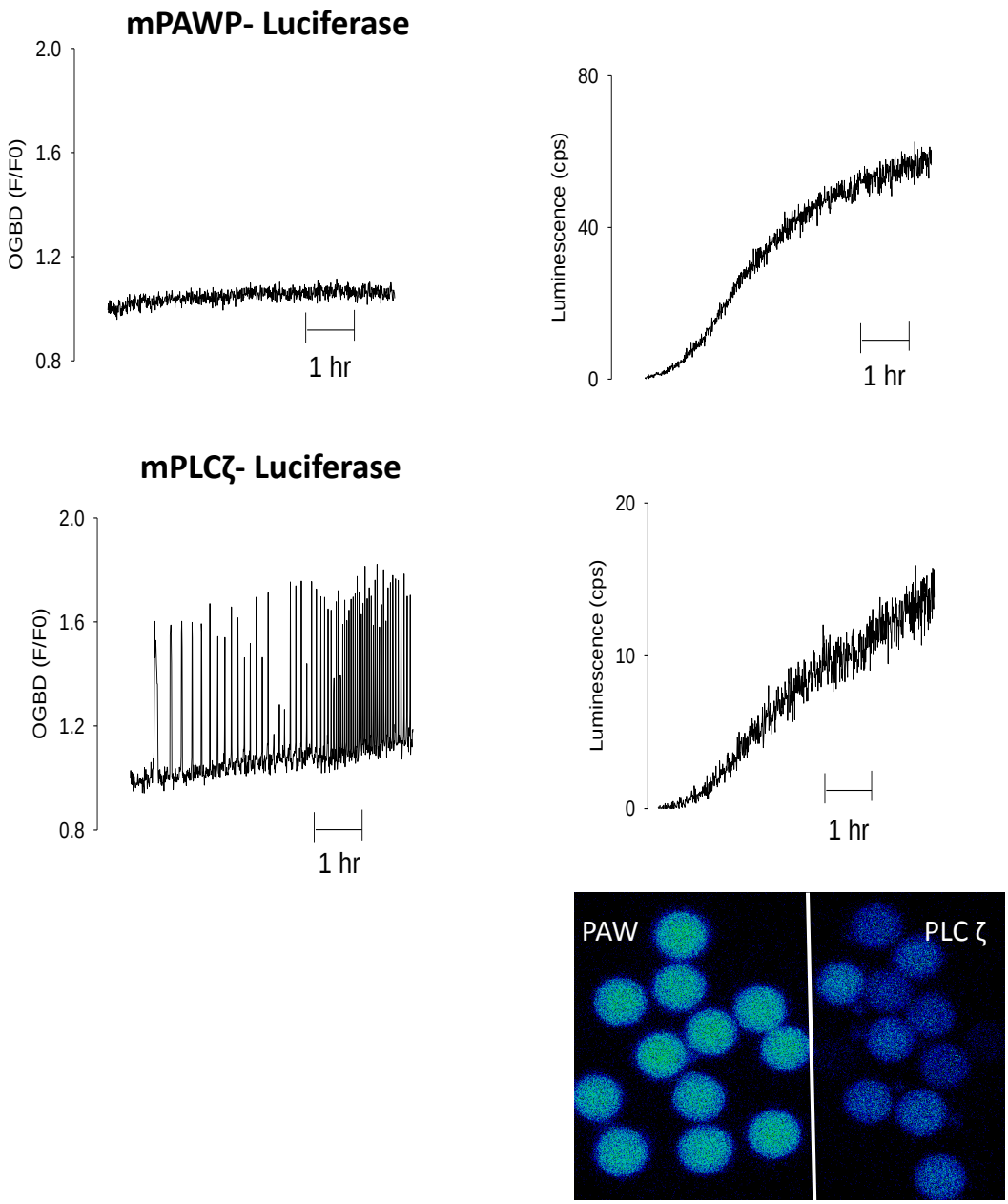


Figure 3.8 Example Ca^{2+}_i traces in eggs following injection of high dose mPAWP-luciferase and mPLCζ-luciferase cRNA

Examples of traces showing changes in cytosolic Ca^{2+}_i in MII eggs alongside firefly luciferase expression. Oocytes were microinjected with the Ca^{2+}_i indictor OGBD mixed with: **A)** mPAWP-luciferase cRNA (pipette concentration= 1.25 µg/µl, n=58 eggs) where the left trace shows changes in cytosolic Ca^{2+}_i in one egg and the right trace shows the luciferase expression in the same egg quantifying expression of mPAWP-luciferase cRNA average luminescence at the end of the experiment for these eggs was 56 cps which equates to ~ 1.6 pg of mPAWP-luciferase protein per egg; and **B)** mPLCζ-luciferase cRNA (pipette concentration= 1 µg/µl, n=57 eggs) again the left trace shows changes in cytosolic Ca^{2+}_i in one egg and the right trace shows the luciferase expression in the same egg quantifying expression of mPLCζ - luciferase cRNA average luminescence at the end of the experiment for these eggs was 18 cps which equates to ~ 0.36 pg of mPLCζ-luciferase protein per egg. The image shows the luminescence of mPAWP-luciferase and PLCζ- luciferase injected eggs being imaged at the same time (light integrated for 30 mins starting at 4 hours post injection)

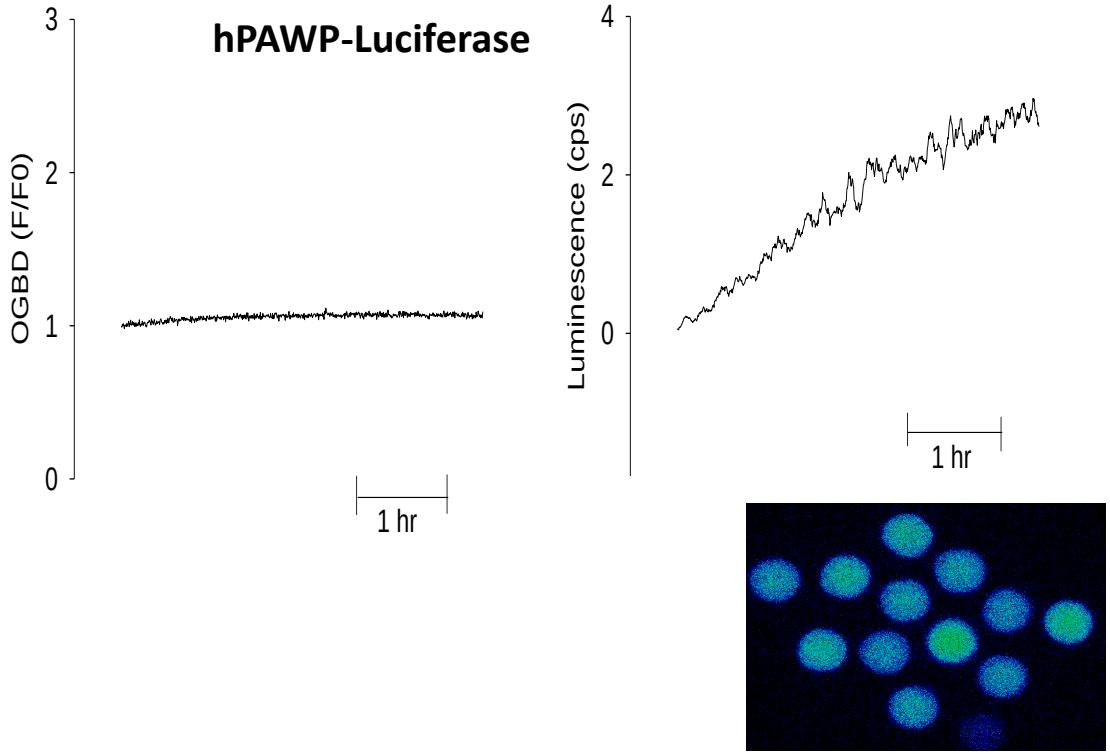


Figure 3.9 Example Ca^{2+}_i trace in an egg following injection hPAWP-luciferase cRNA

Examples of traces showing changes in cytosolic Ca^{2+}_i in an egg alongside firefly luciferase expression. Oocytes were microinjected with the Ca^{2+}_i indictor OGBD mixed with PAWP-luciferase cRNA (pipette concentration= 0.04 µg/µl, n=27 eggs) where the left trace shows changes in cytosolic Ca^{2+}_i in one egg and the right trace shows the luciferase expression in the same egg quantifying expression of PAWP-luciferase cRNA average luminescence at the end of the experiment for these eggs was 3.9 cps which equates to approximately 59 fg of hPAWP-luciferase protein in the egg. Luminescence plotted as running average over 5 minutes. A pipette concentration of 0.4 µg/µl hPAWP–luciferase cRNA was also microinjected (n=15). These eggs had an average luminescence of 46 cps at the end of the experiment (data not shown). The image shows the luminescence of hPAWP-luciferase injected eggs being imaged at the same time (light integrated for 30 mins starting at 4 hours post injection)

PAWP is able to induce increased rates of 2nd polar body formation but not pronuclear formation

The egg activating properties of PAWP were first recorded by (Wu *et al.* 2007) where bovine PAWP recombinant protein was found to induce pronuclear formation and meiotic resumption in porcine, bovine, *Xenopus* and rhesus monkey eggs. To investigate whether mouse PAWP has the ability to cause egg activation in mouse eggs following microinjection of untagged mPAWP, eYFP- mPAWP or mPAWP-Luciferase cRNA, eggs were scored for the resumption of meiosis indicated by 2nd Polar body extrusion and then pronuclear formation. These scores were then compared to those eggs injected with PLCζ- luciferase cRNA at similar levels as well as eggs injected with a control luciferase cRNA. On comparing activation levels eggs microinjected with mPAWP cRNA showed a small increase in the rate of 2nd polar body extrusion (fig 3.10).

Of the eggs microinjected with mPAWP-luciferase (pipette concentration of 0.08 μg/μl) 11% formed 2nd polar bodies compared to 5% seen with luciferase cRNA controls. Similar rates were seen in eggs injected with untagged mPAWP cRNA where 14 % formed 2nd polar bodies. A slightly higher 2nd polar body extrusion rateof 22%was found in eggs injected with eYFP mPAWP (Table 3.1). Despite this slight elevation in the amount of MII eggs resuming meiosis following PAWP cRNA introduction no pronuclear formation was seen in any of these eggs imaged. Furthermore as no Ca^{2+}_{i} oscillations were recorded in any eggs injected with PAWP the elevated rates of meiotic resumption can not a result of the physiological egg activation process which requires Ca^{2+}_{i} signalling. In contrast 100% of eggs injected with PLCζ-luciferase cRNA at similar concentrations formed 2nd polar bodies and underwent full egg activation to pronuclear formation. These results indicate that though PAWP may have the ability under some circumstances to resume meiosis, eggs microinjected with mPAWP or hPAWP do not undergo full egg activation including pronuclear formation.

cRNA injected	Pipette Concentration of cRNA (µg/µl)	n	2nd Polar body formation rate (%)
mPAWP- luciferase	0.08	18	11
PLCζ- luciferase	0.06	13	100
mPAWP untagged	1.25	28	14
YFP-PAWP	1.5	23	22
Luciferase Control	1.1	19	5

Table 3.1 Summary showing the number of eggs forming 2nd Polar bodies following the microinjection of different cRNA

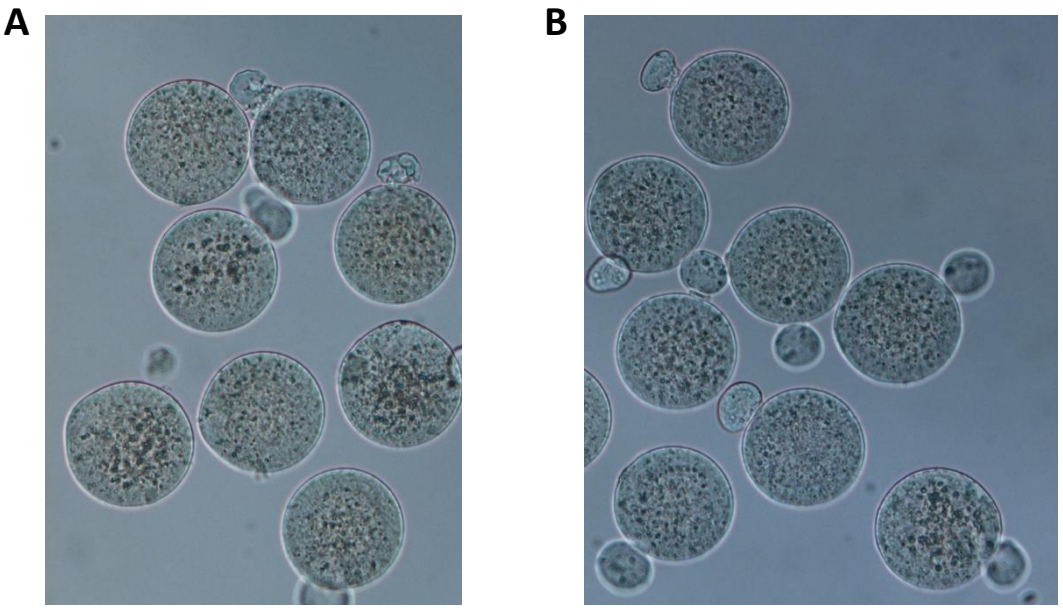


Figure 3.10 Images of mouse eggs 4 hours post injection of PAWP or PLCζ

MII eggs following microinjection of **A)** mPAWP-luciferase cRNA (pipette concentration= 1.25µg/µl, n= 18) and **B)** mPLCζ-luciferase (pipette concentration= 1µg/µl, n=13). 2nd Polar body formation is visible in both groups at the end of 4 hours imaging

PPXY motif containing peptide does not inhibit Ca^{2+}_i oscillation in mouse eggs fertilised by IVF

It has previously been suggested that the PY motif (PPXY) found within PAWP is crucial for its binding to the WWI domain of YAP, an interaction which is necessary for PAWP to stimulate the Ca^{2+}_i signalling pathway inside the egg (Wu *et al.* 2007). In order to investigate the role of this PY motif- WWI domain interaction in eliciting Ca^{2+}_i oscillations in mouse eggs a 20 amino acid synthetic peptide (PPVRYGSPPPGYEAPT) containing the PPXY motif (PPGY) derived from PAWP (PPXY peptide) was microinjected into MII eggsat pipette concentrations of either 0.5 µg/µl or 2.5 µg/µl alongside the Ca^{2+}_i indicator OGBD. This was the exact same peptide sequence used by Aarabi *at al*. and upon microinjection, this PPXY peptide should competitively bind to YAP thus inhibiting the interaction of PAWP with YAP (Wu *et al.* 2007). These eggs were then fertilised by IVF and Ca^{2+}_i changes were recorded. Eggs injected with the peptide displayed oscillations very similar to those normally seen at fertilisation and similar to control eggs not injected with the peptide (fig 3.11 and Table 3.2). Similar effects were seen in those eggs that had been fertilised by ICSI (fresh sperm injection) as there was no clear difference between amount of eggs that oscillated following injection of the PPXY peptide compared to those eggs that underwent ICSI alone (data not shown, conducted by colleague Randa Sanusi). These results imply that the PPXY peptide has no inhibitory effect on Ca^{2+}_i signalling in fertilised eggs and therefore the PY motif-WWI domain interaction is not necessary to elicit Ca^{2+}_i oscillations at fertilisation.

PPXY motif containing peptide does not inhibit PLCζ inducedCa^{2+}_i oscillations

In order to ensure that the PPXY motif didn't perturb the activity of PLCζ, the PPXY motif containing peptide (same pipette concentrations as above) was microinjected into mouse eggs prior to a secondary microinjection of PLCζ-luciferase cRNA at a pipette concentration of 0.1 µg/µl. These eggs displayed Ca^{2+}_i oscillations similar to those seen at fertilisation and those elicited by PLCζ alone (fig 3.12). This suggests that inhibiting the PY motif-WWI domain interaction does not disrupt Ca^{2+}_i oscillations elicited by PLCζ as well as IVF and ICSI.

Pipette concentration of PPXY peptide (μg/μl)	n	mean number of Ca^{2+}_i oscillations in 2 hrs	SD of mean number of oscillations/2hrs
0.5	8	27.9	6.1
2.5	9	21.9	7.7
No peptide	8	22.4	5.9

Table 3.2 Summary showing the effect of PPXY on the Ca^{2+}_i oscillations at fertilisation

Summary of the mean number of Ca^{2+}_i oscillations in 2 hours for IVF fertilised eggs that have been injected with different pipette concentrations of the PPXY peptide (peptide containing a PPGY motif) which may disrupt YAP-PAWP binding and control eggs not injected with PPXY peptide

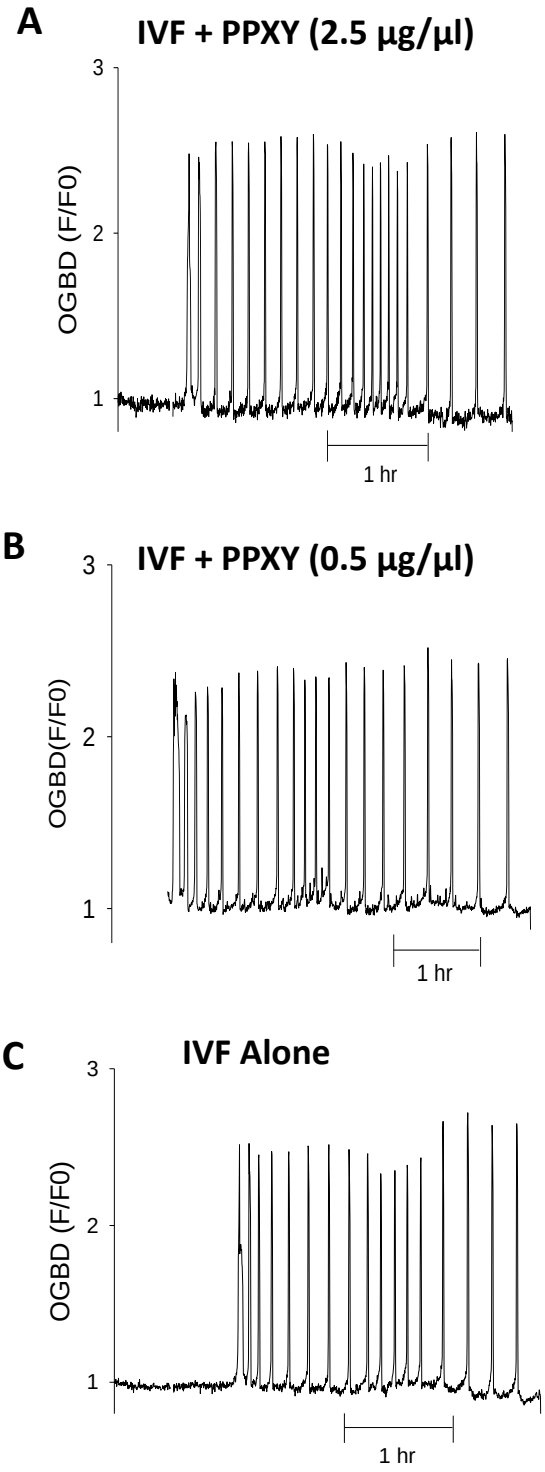


Figure 3.11 Example Ca^{2+}_i traces of fertilised mouse eggs following injection of PPXY

Sample traces showing changes in cytosolic Ca^{2+}_i in eggs that were microinjected with Ca^{2+}_i indictor OGBD and **A)** a competitive peptide containing the PPXY motif that is thought to inhibit PAWP – YAP interaction (pipette concentration 2.5 µg/µl, n=13 eggs), **B)** the same peptide but at a lower pipette concentration (pipette concentration 0.5 µg/µl, n=8 eggs) **C)** OGBD alone (n= 12). All eggs were then fertilised by IVF using fresh mouse sperm during imaging.

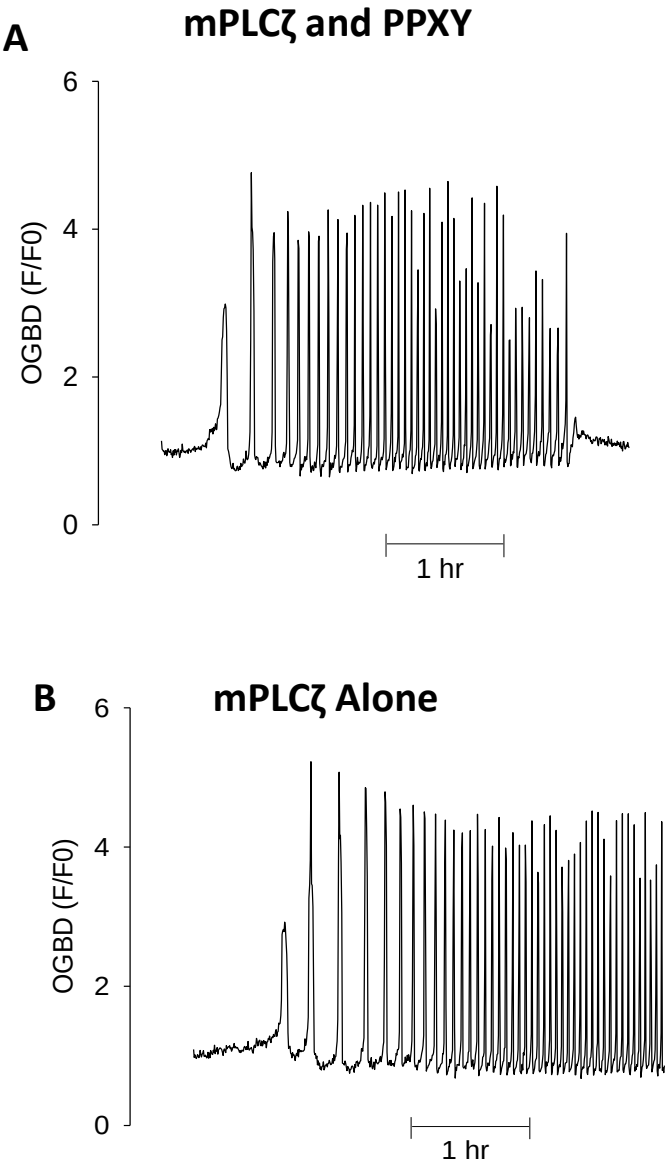


Figure 3.12 Example Ca^{2+}_i traces in mouse eggs initiated by PLCζ following injection of PPXY

Sample traces showing changes in cytosolic Ca^{2+}_i in eggs that were microinjected with Ca^{2+}_i indictor OGBD and mPLCζ-luciferase cRNA (pipette concentration 0.1 μg/μl). **A)** also injected with a competitive peptide containing the PPXY motif that is thought to inhibit PAWP – YAP interaction (pipette concentration 0.5 μg/μl, n= 16 eggs) (average luminescence for all eggs = 0.9cps); whereas **B)** was injected with mPLCζ-luciferase alone (n=8) (average luminescence for egg = 0.6 cps)

Discussion

Though the importance of Ca^{2+}_i oscillations for egg activation is undisputed there is still some debate surrounding the identity of the sperm factor which is responsible for triggering Ca^{2+}_i oscillations in the egg. The most widely accepted view is that the sperm specific PLCζ, which is deposited into the egg at fertilisation, stimulates IP_3 induced Ca^{2+}release (Saunders *et al.* 2002). However, the sperm specific structural protein PAWP has been proposed as an alternative sperm factor candidate. PAWP has been found to induce egg activation up to pronuclear formation in bovine, rhesus monkey, porcine and *Xenopus* eggs following the microinjection of bull derived PAWP recombinant protein (Wu *et al.* 2007). PAWP recombinant protein has also been reported to induce a Ca^{2+}_i change following its injection into *Xenopus* eggs (Aarabi *et al.* 2010). It is important to note however that this Ca^{2+}_i change recorded in these eggs was not comparable to the Ca^{2+}_i increase seen during fertilisation in *Xenopus*. Usually in a fertilised *Xenopus* egg a regenerative Ca^{2+}_i wave crosses the egg starting at the point of sperm entry and takes approximately 5 minutes to propagate across the egg (Bugrim *et al.* 2003). On microinjection of recombinant PAWP into *Xenopus* eggs the Ca^{2+}_i appeared to increase uniformly through the entire cytoplasm within 1 minute of imaging (Aarabi *et al.* 2010). This is not comparable to the physiological Ca^{2+}_i wave seen in *Xenopus* eggs at fertilisation. In addition a membrane permeable Ca^{2+}_i indictor was used during this study (Aarabi *et al.* 2010) which is generally not used in *Xenopus* eggs. As the eggs of these species are very large, the length of incubation time necessary with membrane permeable dyes is much longer than usually used. As a result dyes may be compartmentalised in the egg cortex and therefore not diffuse across the whole cell. Therefore changes in Ca^{2+}_i that occur deep in the cytoplasm may not be detected accurately.

Bovine recombinant protein and bovine PAWP cRNA have also been shown to elicit Ca^{2+}_i oscillations in mouse and human eggs respectively (Aarabi *et al.* 2014b) at final concentrations of 100 fg to 2.5 pg per oocytes with the equivalent of approximately 89 fg of PAWP protein calculated in a single bull sperm (Wu *et al.* 2007). Despite these data, the effects of PAWP are yet to be reproduced by another independent research group. The current study aimed to investigate the egg activating effects of PAWP and whether mouse PAWP was able to induce Ca^{2+}_i oscillations in mouse eggs as would occur physiologically and therefore verify the results previously recorded. In the study by (Aarabi *et al.* 2014b) untagged (his tag only) recombinant hPAWP protein was microinjected into the eggs however this protein was found to be insoluble so instead a NusA tagged form of mPAWP

and hPAWP recombinant protein was produced to microinject into mouse eggs. However, in these experiments neither NusA-mPAWP nor NusA-hPAWP recombinant protein were able to induce Ca^{2+}_i oscillations, unlike the results previously reported using human PAWP recombinant protein (Aarabi *et al.* 2014b). It has been suggested that the presence of a molecular tag such as NusA could affect the activity of PAWP (Aarabi *et al* 2015) however it is unlikely that this would be the case as the activity of PLCζ is unperturbed by the addition of a NusA, fluorescent or luminescence based tag. However, to be sure this was not the case untagged PAWP cRNA was also microinjected into eggs which becomes expressed endogenously by the egg. Neither mouse nor human untagged cRNA was able to cause Ca^{2+}_i oscillations in mouse eggs at a large range of concentrations. Expression of PAWP was verified by carrying out a western blot on hPAWP cRNA expressing eggs using an antibody against hPAWP (conducted by MN, Nomikos *et al* 2014a). To further quantify the effect of PAWP, mPAWP- luciferase cRNA was microinjected into mouse eggs to range from approximately 100 fg – 2 pg final concentration of protein per egg. However, even though physiologically PAWP at these levels should elicit Ca^{2+}_i oscillations (Wu *et al.* 2007) no cytosolic Ca^{2+}_i changes were recorded in any of eggs injected with mouse or human PAWP cRNA. This result is in steep contrast to the Ca^{2+}_i oscillations induced by human PAWP cRNA in human eggs by (Aarabi *et al.* 2014b). In experiments by Aarabi *et al* PAWP was found to be incredibly potent, causing Ca^{2+}_i oscillations at a pipette concentration as low as 0.006 µg/µl. However, this was not found to be the case in the current experiments as fluorescent tagged proteins must be expressed at cellular concentrations of at least 1µM to be seen. This means eggsinjected with YFP-mPAWP cRNA must have contained around 10 pg of YFP-mPAWP protein at the end of the experiment which is approximately 100 x more mPAWP in the eggs than was previously been reported to release Ca^{2+} (Wu *et al.* 2007). Despite the eggs being microinjected with final concentrations of PAWP cRNA either equivalent to, or at much higher levels than those previously reported to work, no Ca^{2+}_i oscillations were recorded in any eggs. PAWP was not able to elicitCa^{2+}_i oscillations following microinjection of either human and mouse derived untagged PAWP cRNA, YFP-PAWP cRNA, or mouse and human derived PAWP-luciferase cRNA. This would indicate that PAWP is not able to activate eggs by triggering Ca^{2+}_ioscillations under physiological conditions at PAWP concentrations found within the sperm or far exceeding. In contrast, the equivalent tagged constructs of PLCζ at the same levels and under the same conditions produced Ca^{2+}_i oscillations similar to those seen at fertilisation.

Tagging PAWP cRNA with a luciferase or fluorescent tag as described above acted as an internal verification that the cRNA was being expressed. However, in the study by (Aarabi *et al.* 2014b) no such verification or quantification of PAWP expression was performed. As a result we cannot be sure of the levels of PAWP that were being expressed in the eggs injected with PAWP cRNA during the previous study. In the paper by (Aarabi *et al.* 2014b) intracellular Ca^{2+}_i changes were measured using a membrane permeable Ca^{2+}_i indictor in which the eggs are incubated in prior to microinjection of PAWP cRNA or recombinant PAWP protein. This means that the fluorescence signal produced by the eggs was independent of microinjection. Consequently even if a fluorescence signal is seen it is unclear what quantity of PAWP cRNA, if any, was microinjected into the eggs. In the present study not only was PAWP cRNA tagged for quantification of expression but the Ca^{2+}_i indictor was co-injected with either PAWP cRNA or recombinant protein. This means that the fluorescence signal from the Ca^{2+}_i indicator ensures that both the Ca^{2+}_i dye and cRNA or recombinant protein have both been injected into the egg.

One other important factor to consider is the method of microinjection. In the study by (Aarabi *et al.* 2014b) solution was injected into the eggs using an ICSI style pipette with a wide bore. The injection solution was then sucked into the injection pipette and carried over to the egg for injection. This method may have issues with diffusion of solution out of the end of the pipette and back-flux of media into pipette prior to insertion into egg. When using a low pressure injection method such as this back-flux and diffusion of injection solution can be avoided by "capping" the end of the injection pipette with oil prior to injection (Kline 2009). However, it does not appear that (Aarabi *et al.* 2014b) took these precautions. In the paper by (Wu *et al.* 2007) control eggs were injected with BSA in the same way as PAWP using an ICSI style pipette. BSA is easy to inject and not necessarily a good control as it is not comparable to injecting protein or cRNA containing solutions. As the activation rates of BSA injected control eggs were relatively high (around 18%) this would indicate that an elevated activation rate occurred as result of the microinjection process alone (Wu *et al.* 2007). During the present study control eggs were microinjected with luciferase cRNA and activation rates of 5% were recorded. It is likely that the form for microinjection process used by (Wu *et al.* 2007) and (Aarabi *et al.* 2014b) resulted in the elevated egg activation rate reported. In the present study a slightly elevated rate of eggs undergoing meiotic resumption was recorded in eggs injected with mPAWP cRNA however this was considerably lower than the 80% activation rate previously recorded (Wu *et al.* 2007). Furthermore none of the eggs formed pronuclei. The slightly elevated rate of

meiotic resumption may be a result of a non-physiological effect of PAWP downstream of Ca^{2+} signalling.

As PAWP lacks any known catalytic or PLC hydrolytic activity it was not possible to verify its activity before injecting it into eggs. It was found that, *in vitro*, PAWP is unable to hydrolyse $PI(4,5)P_2$ or act as a generic activator of PLC (Nomikos *et al.* 2014a). However, it has previously been argued that PAWP may not directly hydrolyse $PI(4,5)P_2$ instead works by activating PLCγ indirectly through PAWP binding YAP (Aarabi *et al.* 2014b). If this is the case we would not expect PAWP to hydrolyse $PI(4,5)P_2$ *in vitro* unless YAP and PLCγ were also present. There is evidence to suggest that stimulation of PLCγ is responsible for Ca^{2+} release at fertilisation in some invertebrate species for example in ascidian eggs (Runft and Jaffe 2000), sea urchin eggs (Carroll 1999; Abassi *et al.* 2000) and starfish eggs (Giusti *et al.* 1999b). In these cases the Ca^{2+}_i increase at fertilisation could be inhibited by the injection of SH2 domains of Src family proteins which act as an inhibitor of Src family kinases. Src kinases such as Fyn and Yes undergo autophosphorylation to become activated which then allows them to phosphorylate and activate PLCγ. PLCγ may then hydrolyse $PI(4,5)P_2$ to elicit IP_3 induced Ca^{2+}release. Indeed in *Xenopus* eggs, immunoprecipitation studies have shown that PLCγis tyrosine phosphorylated and activated within minutes of fertilisation. Furthermore inhibiting the interaction of PLCγ and xyk (Src related tyrosine kinase) with a Src- related protein- tyrosine kinase (PTK) inhibitor blocks the Ca^{2+}_i increase seen at fertilisation (Sato *et al.* 2000). This evidence adds support for the role of PLCγ in stimulating Ca^{2+}release in eggs at fertilisation however the mechanism by which PLCγ is stimulated is still unknown in these species. The idea that the Ca^{2+}_i oscillations at fertilisation may be a result of PLCγ induced Ca^{2+}release has been around for some time and various sperm derived stimuli for this pathway have been proposed. For example in *Xenopus* eggs the sperm may cause phospholipase D (PLD) induced phosphatidic acid (PA) production which then activates egg derived Src to elicit PLCγ induced Ca^{2+} release (Bates *et al.* 2014). PA levels have been seen to increase soon after fertilisation and inhibitors for PA production prevent some of the events of fertilisation including gravitational rotation and delays in the Ca^{2+}_i wave normally seen at fertilisation (Bates *et al.* 2014).

In mouse eggs an alternative sperm derived stimuli has previously been proposed in addition to PAWP in the form of the tyrosine kinase receptor tr-kit which accumulates in elongating spermatids (Sette *et al.* 1997). This is a truncated form of c-kit tyrosine kinase receptor which includes the carboxy terminus needed for PLCγ interaction and the phosphotransferase portion of cytoplasmic catalytic domain (Sette *et al.* 1997). Tr-kit is

located in the equatorial region of the sperm and appears to be an indicator for sperm quality as TUNEL analysis has shown a sufficient amount of tr-kit is required to maintain sperm DNA integrity (Muciaccia *et al.* 2010). Recombinant tr-kit protein and tr-kit mRNA have been found to induce parthenogenetic egg activation including resumption of meiosis and pronuclear formation following microinjection into MII mouse eggs (Sette *et al.* 1997). Furthermore co-injection of SH domains of PLCγ fused to a glutathione-s-transferase tag (SH-GST) alongside tr-kit has the ability to competitively inhibit egg activation (Sette *et al.* 1998). Interestingly it is the SH3 region which is most effective at this inhibition as appose to SH2 domain and the effect is specific to SH3 domains derived from PLCγ. SH3 domains from Growth Receptor bound protein 2 (Grb2) do not have the same effect (Sette *et al.* 1998). It has been proposed that tr-kit becomes phosphorylated by Fyn (a Src family kinase) whichin turn mediates the phosphorylation and auto-phosphorylation of PLCγ1 (Sette *et al.* 2002).

Even though this data suggests that tr-kit may be the sperm specific factor that causes egg activation it also adds support to the idea that Ca^{2+}_i oscillations at fertilisation in mouse eggs are initiated by the stimulation of PLCγ via its SH3 domain as proposed by (Aarabi *et al.* 2014b). It's important to note however that tr-kit has never been reported to cause Ca^{2+}_i oscillations in eggs. Furthermore the role of PLCγ in stimulating IP_3 induced Ca^{2+}release in mammalian eggs has previously been challenged. The microinjection of SH2 and SH3 domains for PLCγ1 and PLCγ2 in eggs at an excess of approximately 100-400 times the endogenous levels of PLCγ did not inhibit Ca^{2+} release at fertilisationin mouse eggs. However this did inhibit the release of Ca^{2+} by PLCγ exogenously stimulated by platelet-derived growth factor (PPGF) receptor (Mehlmann *et al.* 1998). In addition microinjection of SH2 domains from Src family kinases such as Fyn and Yes had no inhibitory effect on the Ca^{2+}release at fertilisation in mouse eggs (Mehlmann and Jaffe 2005) unlike some invertebrates like sea urchins (Kinsey and Shen 2000). Furthermore incubating porcine sperm extracts with Src family kinase inhibitors had no effect on the sperm extracts ability to cause Ca^{2+}_i oscillations when injected into eggs (Kurokawa *et al.* 2004). This would suggest that SH domain mediated activation of PLCγ or Src family kinase, in contrast to some invertebrates and possibly amphibians, are not necessary for the Ca^{2+}release at fertilisation in mammalian species. As a result, PAWP is unlikely to work through this pathway. It is possible however, that Src family kinases are involved in egg activation events downstream of Ca^{2+}_i signalling and may be required for effective oocyte maturation (Levi and Shalgi 2010).

To investigate the proposal that PAWP-YAP interaction is vital for physiological Ca^{2+}_i signalling initiation in mammalian eggs a peptide including the PPXY motif was injected into eggs alongside a Ca^{2+}_i indicator and then fertilised by IVF. The PPXY peptide has previously been shown to inhibit egg activation (Wu *et al.* 2007) and Ca^{2+}_i oscillations in mouse and human eggs fertilised by ICSI or those injected with PAWP (Aarabi *et al.* 2014b). The peptide is suggested to inhibit these oscillations by disrupting PAWP binding to YAP by outcompeting its binding through the PPXY motif (Aarabi *et al.* 2014b). The present study found no disruption in the Ca^{2+}_i oscillations of eggs fertilised by IVF following injection of PPXY when compared to control IVF eggs. Both frequency and pattern of oscillations were indistinguishable in eggs injected with Ca^{2+}_i indicator alone and a range of PPXY peptide concentrations. Furthermore no difference was seen in the amount of eggs that oscillated post ICSI fertilisation in those injected with the PPXY peptide and not. If PAWP does initiate IP_3 induced Ca^{2+}release in this way there would be some delay in the Ca^{2+}_i increase recorded. Therefore it would not be possible for a uniform Ca^{2+}_i increase to be seen in a *Xenopus* egg within 1 minute as has previously been reported by (Aarabi *et al.* 2010).These data show further flaws in the pathway of PAWP signalling that has previously proposed (Aarabi *et al.* 2014b). Though PAWP may have the ability to bind to YAP and src-family tyrosine kinases expressed in the egg (Wu *et al.* 2007) it does not appear to initiate Ca^{2+}release in this way physiologically during fertilisation.

PAWP's lack of involvement in Ca^{2+}release at fertilisation has recently been further supported through the development of a PAWP null mutant mouse model (Satouh *et al.* 2015). Male mice of this strain appeared to be perfectly fertile and the sperm from these mice was able to sufficiently activate eggs through Ca^{2+}release in a manner comparable to controls. The sperm morphology of these mice was also unaltered and embryos produced via ICSI using this sperm developed at rates indistinguishable from controls (Satouh *et al.* 2015). These data suggest that PAWP plays no involvement in egg activation in mammalian eggs and does not correlate convincingly to sperm quality parameters in terms of fertilisation or early development rates. This result strongly supports the current studies that PAWP appears to play not obvious role in egg activation and is not the sperm factor causing Ca^{2+}_i oscillations at fertilisation.

4. FURTHER INVESTIGATIONS INTO STRUCTURAL PROPERTIES OF PLCZ THAT DETERMINE CA^{2+} RELEASING ACTIVITY

Introduction

PLCζ is the only sperm derived protein that has been shown without dispute to cause Ca^{2+}_i oscillations when introduced into mammalian eggs at physiological levels (Saunders *et al.* 2002). This feature is unique amongst phospholipase C isoforms and the only other PLCs that are able to initiate Ca^{2+} release in eggs do so at levels far greater than physiological (Mehlmann *et al.* 2001; Swann *et al.* 2006; Igarashi *et al.* 2007). Despite PLCζ possessing the most elementary structure of all PLCs it has a far greater sensitivity for Ca^{2+} inside the egg which allows it to be active at basal levels of Ca^{2+}_i (Kouchi *et al.* 2004; Nomikos *et al.* 2005). PLCζ has been found to be most homologous with PLCδ1 for which it shares 47% similarity and is 33% identical, however, PLCδ1 has some major structural differences when compared to PLCζ which regulates its activity (Saunders *et al.* 2002). To begin with PLCζ lacks the pleckstrin homology (PH) domain that is found in PLCδ1 and many other PLCs. This domain allows binding to the target plasma membrane which is essential in enabling PLCδ1 to interact with its substrate $PI(4,5)P_2$ (Lemmon *et al.* 1995). Interestingly PLCζ does not appear to hydrolyse $PI(4,5)P_2$ at the plasma membrane, instead seems to target an alternative intracellular source of $PI(4,5)P_2$ found on small vesicles (Yu *et al.* 2012). This raises the question of how PLCζ is able to discriminate between different pools of $PI(4,5)P_2$ or convey membrane specificity in terms of its localisation inside the egg. It appears the structure of PLCζ may have a significant role in determining these unusual properties.

It has already been shown that unique properties of the linker region between the X and Y catalytic domains of PLCζ influence its activity by modulating membrane interactions (Nomikos *et al.* 2007). The X-Y linker region of most PLCs has a regulatory role in catalytic auto-inhibition (Hicks *et al.* 2008). In most isoforms this region contains many negatively charged residues which exclude the negatively charged $PI(4,5)P_2$ from the active site through electrostatic repulsion (Hicks *et al.* 2008; Hajicek *et al.* 2013). However, unlike other PLCs PLCζ does not regulate auto inhibition. In contrast the X-Y linker of PLCζ is longer and contains more positively charged amino acid residues than, for example, PLCδ1 (Cox *et al.* 2002; Saunders *et al.* 2002). Testing the Ca^{2+}releasing activity of PLCζ following point mutations which neutralise the positively charged residues in the X-Y linker has revealed the importance of these residues in allowing PLCζ to bind to $PI(4,5)P_2$ (Nomikos *et al.*

2011b). A series of constructs were created that contained PLCζ with point mutations to neutralise the positively charged residues within the X-Y linker. These mutations were then tagged to the firefly luciferase sequence to produce fusion constructs. These constructs were synthesised into RNA and injected into eggs so their $Ca^{2+}{}_i$ releasing effects could be measured (Nomikos *et al.* 2011b). By measuring the luminescence of the eggs during the course of the experiment this allowed quantification of the expression levels of the PLCζ cRNA which acted as an internal control so allowing constructs to be compared (Swann *et al.* 2014). Neutralisation of one, two or three of these positive residues causes a sequential decrease in the frequency of $Ca^{2+}{}_i$ oscillations induced by PLCζ as a result of reduced $PI(4,5)P_2$ binding (Nomikos *et al.* 2011b). This work shows these residues create a positively charged region close to the catalytic domains that is able to electro-statically interact with the negatively charged $PI(4,5)P_2$ (Nomikos *et al.* 2011b; Nomikos *et al.* 2011c). However, even deletion of the whole X-Y linker region does not abolish all Ca^{2+} releasing ability of PLCζ (Nomikos *et al.* 2011c) which suggests that $PI(4,5)P_2$ binding and membrane interaction is also regulated by other structural domains possibly in a redundant fashion.

One possible structural feature of PLCζ that could facilitate lipid interaction is the EF hands domain. Indeed this appears to be the case for PLCδ1 as a region of hydrophobic cationic residues found in the first EF hand motif has been found to positively interact with anionic phospholipids (Cai *et al.* 2013). These anionic phospholipids are found in target membranes such as the plasma membrane and the interaction is thought to enable PLCζ access to membrane bound $PI(4,5)P_2$ (Cai *et al.* 2013). Interestingly this region of cationic residues is conserved in PLCζ, however, whether this region has a role in regulating PLCζ membrane and substrate interaction is yet to be established.

Another structural domain of PLCζ that could have a role in conferring membrane specificity is the C2 domain. In other proteins C2 domains usually mediate membrane binding in a Ca^{2+} dependent manner through direct interaction with phospholipids (Medkova and Cho 1999). For example the C2 domain of PLCδ1 associates with plasma membrane bound phosphatidylserine where it becomes sequestered therefore stimulating activity of the protein (Lomasney *et al.* 1999). PLCζ is not able to initiate Ca^{2+} release following deletion of the C2 domain from its sequence which suggests that this region is vital for PLCζ's Ca^{2+}releasing activity in eggs (Nomikos *et al.* 2005). *In vitro* the C2 domain of PLCζ has been found to bind to the phospholipids PI(3)P and PI(5)P with low affinity (Kouchi *et al.* 2005). However, neither PI(3)P or PI(5)P appear to be distributed in the same vesicular pattern as PLCζ is found to be in the egg (Yu and Swann 2012 unpublished). This

implies that PLCζ is not topologically interacting with PI(3)P or PI(5)P making them unlikely binding partners *in vivo.* Furthermore it has been suggested that *in vitro* interaction with particularly PI(3)P actually inhibits the activity of PLCζ (Kouchi *et al.* 2005). This leaves the nature of PLCζ C2 domain membrane interaction unclear. Recently however, a human homozygous mutation within the C2 domain of PLCζ has been associated with infertility in patients (Escoffier *et al.* 2015a). A clinical study was conducted of 2 infertile brothers who both had total fertilisation failure following ICSI however had completely normal sperm morphology parameters. Whole exomic sequencing was carried out and only one missense mutation was found and that was in the C2 domain of PLCζ where isoleucine (Ile) 489 is converted into a phenylalanine (F) (Escoffier *et al.* 2015a). Injection of this protein into eggs was found to elicit an abnormal $Ca^{2+}{}_i$ oscillatory pattern and poor developmental outcomes for embryos (Escoffier *et al.* 2015a). Furthermore it was suggested that these effects could be a result of reduced levels of PLCζ protein in the sperm, mislocalisation of the protein in the egg and a reduction of enzymatic activity possibly as a result of protein instability or misfolding (Escoffier *et al.* 2015a). However, there is not yet any solid evidence for these claims so exactly how this mutation within the C2 domain results in such a striking phenotype remains unknown.

It is also possible that the structure of PLCζ could modulate its activity indirectly through interactions with mediator proteins. These interactions may be particularly important forPLCζ as it lacks many of the additional specific structural regions like a PH domain found in other PLCs that help modulate activity and membrane binding specificity (Lemmon *et al.* 1995). For example PLCβ has a calmodulin (CaM) binding site within its N' terminusand *in vitro* studies have shown that PLCβ positively interacts with CaM (McCullar *et al.* 2003). In this case CaM regulates PLCβ binding to membrane associated proteins such as G coupled proteins in a Ca^{2+} dependent manner therefore stimulating the enzymes activity (McCullar *et al.* 2007). A CaM binding motif has also been found in the catalytic domain of PLCδ1 (Sidhu *et al.* 2005). However, in this case, CaM binding acts as an inhibitor for PLCδ activity, an affect that can be reversed by the activation of the small GTPase Ral (Sidhu *et al.* 2005). It has therefore been proposed that Ral and CaM act as a regulatory complex for PLCδ1 activity (Sidhu *et al.* 2005). Interestingly PLCζ also has a CaM binding motif however the role of this motif, CaM or any other potentially regulatory protein is yet to be established.

It has been suggested that proteolytic processing of the X-Y linker could modulate PLCζ activity (Kurokawa *et al.* 2007). It was previously discovered that there are two fractions of the sperm that are able to initiate $Ca^{2+}{}_i$ oscillations on injection into mouse eggs (Kurokawa

et al. 2005). The soluble cytosolic fraction which can be easily extracted contains a large amount of full length PLCζ which can be detected by immunoblotting (Kurokawa *et al.* 2005). In contrast the high pH soluble fraction contains very little full length 72kDa PLCζ however still causes Ca^{2+}_i oscillations when introduced into eggs (Kurokawa *et al.* 2005). It was later found that this high pH soluble fraction contained PLCζ that had been proteolytically cleaved in the X-Y linker (Kurokawa *et al.* 2007). In fact if recombinant full length PLCζ is cleaved using the protease V8 derived from S*taphylococcus aureus*it is still able to cause Ca^{2+}_i oscillations in eggs (Kurokawa et al. 2007). Furthermore if PLCζ cRNA comprising of the two cleaved halves is co-expressed in eggs Ca^{2+}_i oscillations can be recorded and embryos develop well (Kurokawa *et al.* 2007). However, it has not yet been established how effective this split PLCζ is at causing Ca^{2+}_i oscillations compared to full length PLCζ or how proteolytic cleavage might regulate the activity of PLCζ *in vivo*.

Aims

Though some of the structural properties of PLCζ have been linked to its Ca^{2+}_i oscillation inducing abilities in eggs there are still many unresolved issues regarding how PLCζ discriminates between different pools of $PI(4,5)P_2$ and how the structural domains of PLCζ are involved in its binding to specific membranes. Positively charged residues within the X-Y linker appear to play a role in anchoring PLCζ to its substrate $PI(4,5)P_2$ however this domain does not account for all PLCζ $PI(4,5)P_2$ binding (Nomikos et al. 2011b; Nomikos et al. 2011c). Firstly, this project aims to determine if the positively charged residues found in EF hand domain of PLCζ have a role in conveying PLCζ's Ca^{2+}releasing ability *in vivo* and if so whether this is due to specific phospholipid binding or mis-localisation of PLCζ in the egg. Secondly the role of the C2 domain in membrane binding and Ca^{2+} release will be investigated by exploiting the recent identification of a missense mutation in this region that has been correlated to male infertility. Next the role of the CaM binding motif in regulating the Ca^{2+}releasing activity of PLCζ will be investigated. Finally this project will attempt to quantify the effect of proteolytic cleavage on the activity of PLCζ RNA and whether species differences in Ca^{2+}_i oscillations elicited by this RNAcan be measured.

Results

<u>Neutralising the positively charged residues within the EF hand domain affects PLCζ's Ca^{2+}releasing ability</u>

It has previously been reported that positively charged residues within the first pair of EF hands of PLCδ1 are responsible for mediating the enzyme's interaction with membranes

through anionic phospholipid binding (Cai *et al.* 2013). This region is conserved within PLCζ and may also have a role in anchoring PLCζ to intracellular membranes. In order to investigate the role of these positively charged residues in PLCζ a series of cRNA constructs of mouse PLCζ were created with containing point mutations to neutralise positive residues within this region (courtesy of MN). Three single mutated constructs were created where the amino acids Lysine-49 (K49A), Lysine-53 (K53A) or Arginine-57 (R57A) were substituted for the neutral alanine using direct mutagenesis. In addition a double mutant construct was created with both K49A and R57A mutations (KR49,57AA), a triple mutant with all 3 mutations (KKR49,53,57AAA) and a wild type mouse PLCζ (WT). These sequences were tagged with the firefly luciferase gene so fusion cRNA constructs of PLCζ-luciferase could be produced (See fig 4.1). Tagging the PLCζ in this way allows expression of the protein *in situ* to be measured by recording the luminescence given off by the eggs and therefore direct comparisons can be made between groups.

These constructs were each tested for their Ca^{2+}releasing ability by microinjection into eggs along with the $Ca^{2+}{}_i$ indictor Orgeon Green BAPTA Dextran (OGBD) to measure $Ca^{2+}{}_i$ fluctuations. All three single mutated versions of PLCζ, PLCζ^{K49A}, PLCζ^{K53A} and PLCζ^{R57A}caused a reduction in the number of $Ca^{2+}{}_i$ oscillations recorded in the first hour of the eggs oscillating when compared to PLCζ^{WT} (see fig 4.2 and Table 4.1). Despite similar cRNA expression levels the single mutations caused a significant reduction of around 58% in the number of $Ca^{2+}{}_i$ oscillations when compared to wild type PLCζ (see fig 4.2, fig 4.6 and Table 4.1). Each of the single mutations had very similar effects on the Ca^{2+} releasing ability of PLCζ. Interestingly the double mutation, PLCζ^{KR49,57AA}, also had very similar effects on the frequency of $Ca^{2+}{}_i$ oscillations as PLCζ^{K49A}, PLCζ^{K53A} and PLCζ^{R57A} (see fig 4.2, fig 4.6 and Table 4.1). PLCζ^{KR49,57AA} caused a 62% decrease in the number of $Ca^{2+}{}_i$ oscillations in the first hour of oscillating when compared to PLCζ^{WT} at comparable expression levels and was shown to be significantly different (see Table 4.1). However, the triple mutation PLCζ^{KKR49,53,57AAA} caused a more severe reduction in the number of $Ca^{2+}{}_i$ oscillations of around 71% and again this was significantly reduced compared to PLCζ^{WT} at a similar expression levels (see Table 4.1). This suggests that neutralising even one residue in the positively charged region of the EF hand domain of PLCζ has a detrimental impact on the ability for PLCζ to cause $Ca^{2+}{}_i$ oscillations. This effect is more severe if all three of these positively charged residues are neutralised.

The effect of mutations within the EF hand domain can be rescued by over-expression

As neutralising the positive residues within this region of the first EF hand reduces the frequency of $Ca^{2+}{}_i$ oscillations it was important to see if this effect was due to reduced Ca^{2+}releasing ability of PLCζ and whether this effect could be rescued with higher levels of protein expression. To test this a higher concentration of mouse $PLC\zeta^{KKR49,53,57AAA}$–luciferase cRNA was injected into eggs and the number of $Ca^{2+}{}_i$oscillations in the first hour of oscillating was calculated the same way as previously. At expression levels 16 times the level of $PLC\zeta^{WT}$, $PLC\zeta^{KKR49,53,57AAA}$ was able to cause $Ca^{2+}{}_i$ oscillations at a frequency that were not significantly different from $PLC\zeta^{WT}$ (see fig 4.3, fig 4.6 Table 4.1). However, this data appeared much more scattered than that seen using $PLC\zeta^{WT}$ (see Table 4.1). This is probably because as the pipette concentration of cRNA is increased so does the variation of expression level within the egg. These data suggest the positive residues within the EF hand domain do have some role in determining PLCζ $Ca^{2+}{}_i$ releasing activity and mutating these residues makes the enzyme less efficient at releasing Ca^{2+}. However, this effect can be rescued with over-expression of the protein.

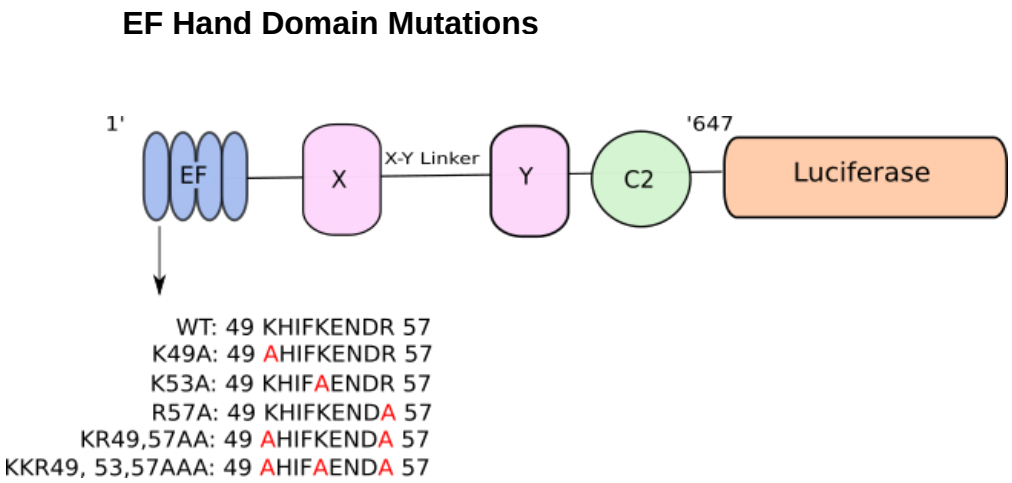


Figure 4.1 Schematic showing the mutations made the EF hand domain of mPLCζ

The structural domains of mouse PLCζ and the residue substitutions carried out by point directed mutagenesis to create mutant RNA constructs of mPLCζ- luciferase. Positively charged residues in the 1stEF hand were neutralised for the amino acid alanine. Mutations were as follows: **WT**) wild type PLCζ sequence, **K49A)** substitution of the lysine at residue 49 for alanine, **K53A)** substitution for lysine at residue 53 for alanine, **R57A)** substitution for arginine at residue 57 for alanine, **KR49,57AA)** both the K49A and R57A substitutions were made simultaneously and **KKR49,53,57AAA)** the K49A,K53A and R57A substitutions were made simultaneously

EF Hand Domain Mutations

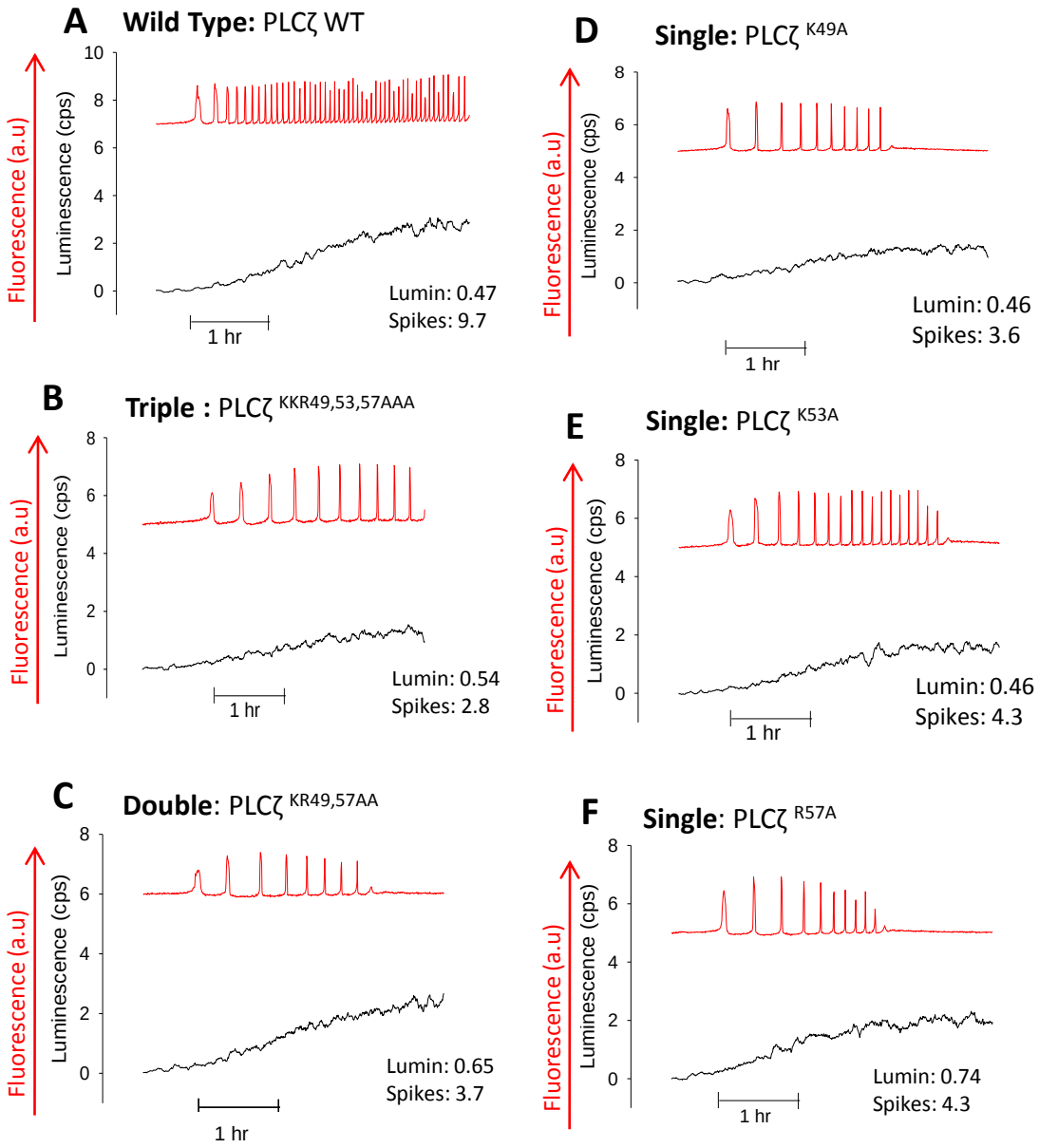


Figure 4.2 Sample $Ca^{2+}{}_i$ traces initiated by mPLCζ containing mutations in the EF hand domain

$Ca^{2+}{}_i$ and luminescence changes that occurred in individual eggs following microinjection of the $Ca^{2+}{}_i$ indicator OGBD with the one of the following mPLCζ RNA constructs: **A)** Wild Type PLCζ- Luciferase RNA, **B)** PLCζ^{KKR49,53,57AAA}- luciferase RNA (Triple EF mutant), **C)** PLCζ^{KR49,57AA} - luciferase RNA (Double EF mutant), **D)** PLCζ^{K49A}-luciferase RNA, **E)** PLCζ^{K53A}-luciferase RNA and **F)** PLCζ^{R57A}-luciferase RNA. All constructs were injected at a pipette concentration between 0.025 and 0.05 µg/µl. Fluorescence coloured in red shows relative changes in cytosolic $Ca^{2+}{}_i$ and luminescence shown in black is measured in photon counts per second and is used as an indicator of PLCζ protein expression. Lumin = mean counts per second in the first hour of oscillating across the group, Spikes= mean total number of $Ca^{2+}{}_i$ spikes in 1st hour of oscillations. See Table 4.1 for details of n values.

EF Hand Domain Mutations

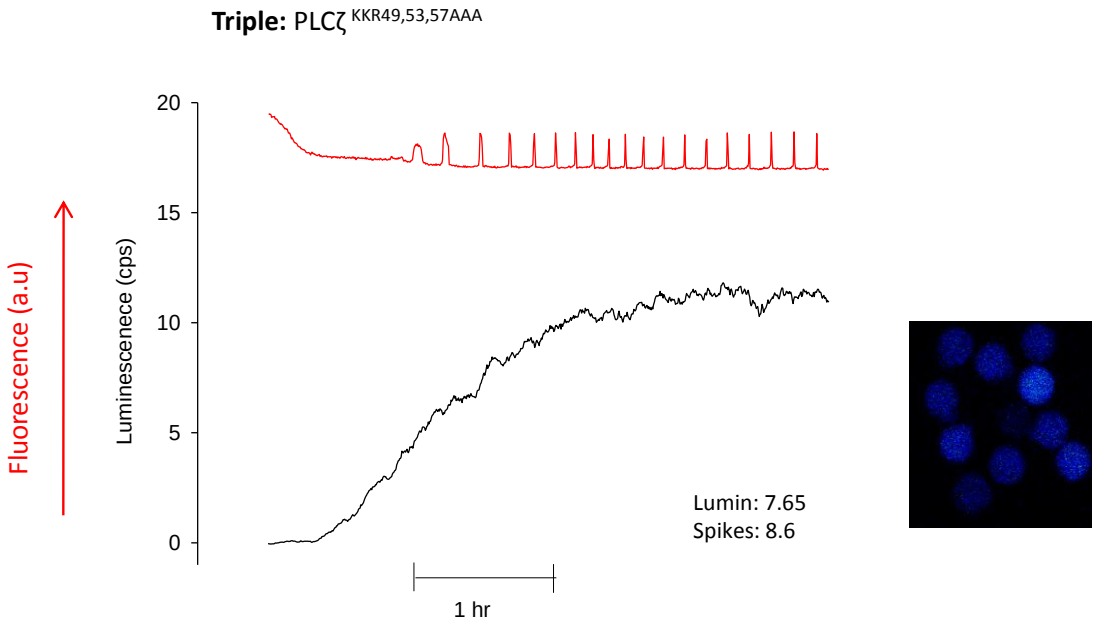


Figure 4.3 Sample Ca^{2+}_i trace initiated by mPLCζ containing triple mutation in the EF hand domain

Ca^{2+}_i and luminescence changes that occurred in individual eggs following microinjection of the Ca^{2+}_i indicator OGBD with $PLC\zeta^{KKR49,53,57AAA}$- luciferase RNA (Triple EF mutant) injected at a pipette concentration of 0.1 µg/µl. Fluorescence coloured in red shows relative changes in cytosolic Ca^{2+}_i and luminescence shown in black is measured in photon counts per second and is used as an indicator of PLCζ protein expression. Lumin = mean counts per second in the first hour of oscillating across the group, Spikes= mean total number of Ca^{2+}_i spikes in 1st hour of oscillations. See Table 4.1 for details of n values. Image insert shows the luminescence signal of a group of eggs injected with $PLC\zeta^{KKR49,53,57AAA}$- luciferase RNA (pipette concentration = 1 µg/µl) at approximately 4 hours post injection where the signal was integrated for 30 minutes.

Construct	Pipette concentration (μg/μl)	n	Number of eggs oscillated	Mean number of oscillations in first hour of spiking	Mean PLCζ expression in 1st hour of oscillations (cps)	Number of spikes significantly different from wild type?	Mean total counts/spike in 1st hour of oscillating (counts/spike)	Counts/spike significantly different from wild type?
mPLCζ^{WT}	0.025 - 0.05	31	31/31	9.7 ± 0.63	0.47 ± 0.038	N/A	14.61 ± 2.05	-
mPLCζ^{K49A}	0.025	19	19/19	3.6 ± 0.16	0.48 ± 0.024	Yes (p= <0.001)	25.92 ± 1.17	Yes (p=<0.001)
mPLCζ^{K53A}	0.025	39	39/39	4.4 ± 0.13	0.46 ± 0.014	Yes (p= <0.001)	20.78 ± 0.85	Yes (p=<0.001)
mPLCζ^{R57A}	0.025	30	30/30	4.3 ± 0.13	0.74 ± 0.032	Yes (p= <0.001)	38.21 ± 2.1	Yes (p=<0.001)
mPLCζ^{KR49,57AA}	0.05	30	30/30	3.7 ± 0.14	0.65 ± 0.030	Yes (p= <0.001)	34.96 ± 1.94	Yes (P=<0.001)
mPLCζ^{KKR49,53,57AAA}	0.025 - 0.05	43	42/43	2.8 ± 0.074	0.54 ± 0.031	Yes (p= <0.001)	37.35 ± 2.24	Yes (p=<0.001)
mPLCζ^{KKR49,53,57AAA}	1	11	11/11	8.6 ± 1.7	7.65 ± 0.92	No (p = 0.15)	-	-
mPLCζ EF^{KKR49,53,57AAA} XY Link.^{k374,5,7,AAA}	0.1	25	0/25	0 ± 0	0.53 ± 0.046	Yes (p= <0.001)	-	-
mPLCζ EF^{KKR49,53,57AAA} XY Link.^{k374,5,7,AAA}	2	20	0/20	0 ± 0	14.43 ± 0.80	Yes (p= <0.001)	-	-

Table 4.1 Summary showing the effects on the Ca^{2+} releasing activity of mouse PLCζ following mutations in the EF hands domain

All PLCζ cRNA constructs were tagged with luciferase and cytosolic $Ca^{2+}{}_i$ dynamics were measured using the fluorescent $Ca^{2+}{}_i$ indicator Oregon Green BAPTA Dextran (OGBD). Test for significant differences were conducted using a Mann-Whitney Ranked test for comparison of two groups (with a 95% confidence interval) following a Pearson's test for normality which all groups of data failed (data not shown). Due to the non-linear relationship between the expression of PLCζ protein and the frequency of Ca oscillations it was not possible to calculate "mean total counts/spike in 1st hour of oscillating" for higher PLCζ cRNA expression levels, as a result this figure was only calculated in those instances where PLCζ expression level was below 1 cps, +/- = standard error of the mean, n = number of eggs injected

EF hand domain and X-Y linker work in synergy to anchor PLCζ to PI(4,5)P_2

The X-Y linker also has a positively charged region that has been found to be involved in PI(4,5)P_2 binding (Nomikos *et al.* 2011b). Interestingly despite the fact that neutralising the residues in this region also has a negative effect on the Ca^{2+} releasing ability of PLCζ, even if the whole X-Y linker is deleted $Ca^{2+}{}_i$ oscillations can still be induced by PLCζ albeit at a much higher expression (Nomikos *et al.* 2011c). With this in mind it possible that the positively charged region of the EF hand works in synergy with the X-Y linker to bind to PI(4,5)P_2 . In order to test this a "double triple" mutant PLCζ- luciferase construct was created that had both the triple EF hand mutation (PLCζ^{KKR49,53,57AAA}) and the triple X-Y linker mutation (PLCζ^{K374,5,7,AAA}) to create a PLCζ EF ^{KKR49,53,57AAA} XY Link.^{k374,5,7,AAA} -luciferase constructs (see fig 4.4, designed and synthesised by MN). This construct was injected into eggs in the same way as above and $Ca^{2+}{}_i$ oscillations and expression were recorded. PLCζ EF ^{KKR49,53,57AAA} XY Link.^{k374,5,7,AAA} was not able to cause $Ca^{2+}{}_i$ oscillations in eggs even if expression levels reach 30x the level of PLCζ^{WT} previously used (see fig 4.5, fig 4.6 and Table 4.1). This suggests that the positively charged residues of the EF hand and the XY linker both play a role in conveying Ca^{2+}releasing of PLCζ, possibly through PI(4,5)P_2 binding.

EF hand and XY Linker mutation (Double Triple)

PLCζ EF ^{KKR49,53,57AAA} XY Link ^{K374,375,377AA}

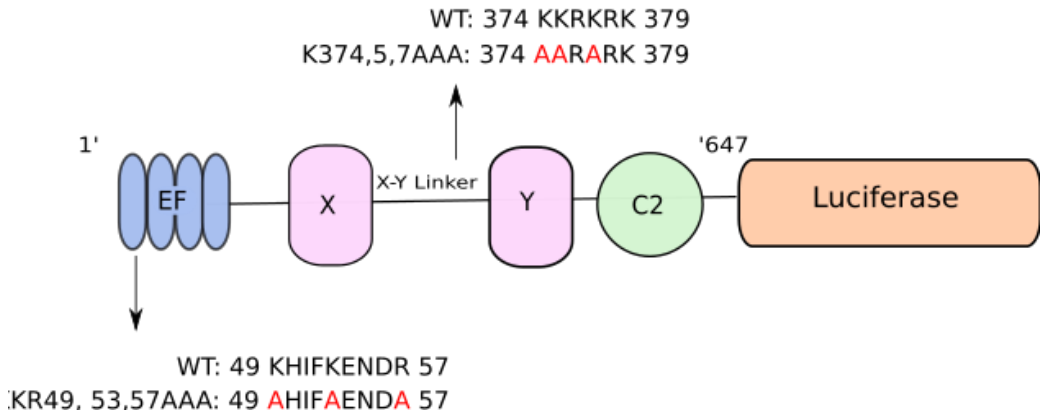


Figure 4.4 Schematic showing the mutations made in the EF hand domain and X-Y linker of mPLCζ

The structural domains of mouse PLCζ and the residue substitutions carried out by point directed mutagenesis to create the "double triple" PLCζ mutant (PLCζ EF ^{KKR49,53,57AAA}XY Link$^{.K374,5,7,AAA}$-luciferase cRNA). This mutation was made by substituting K49A,K53A and R57A in the 1st EF hand and substituting the lysine residues at the 374, 375 and 377 position for alanines in the X-Y linker.

EF hand and XY Linker mutation (Double Triple)

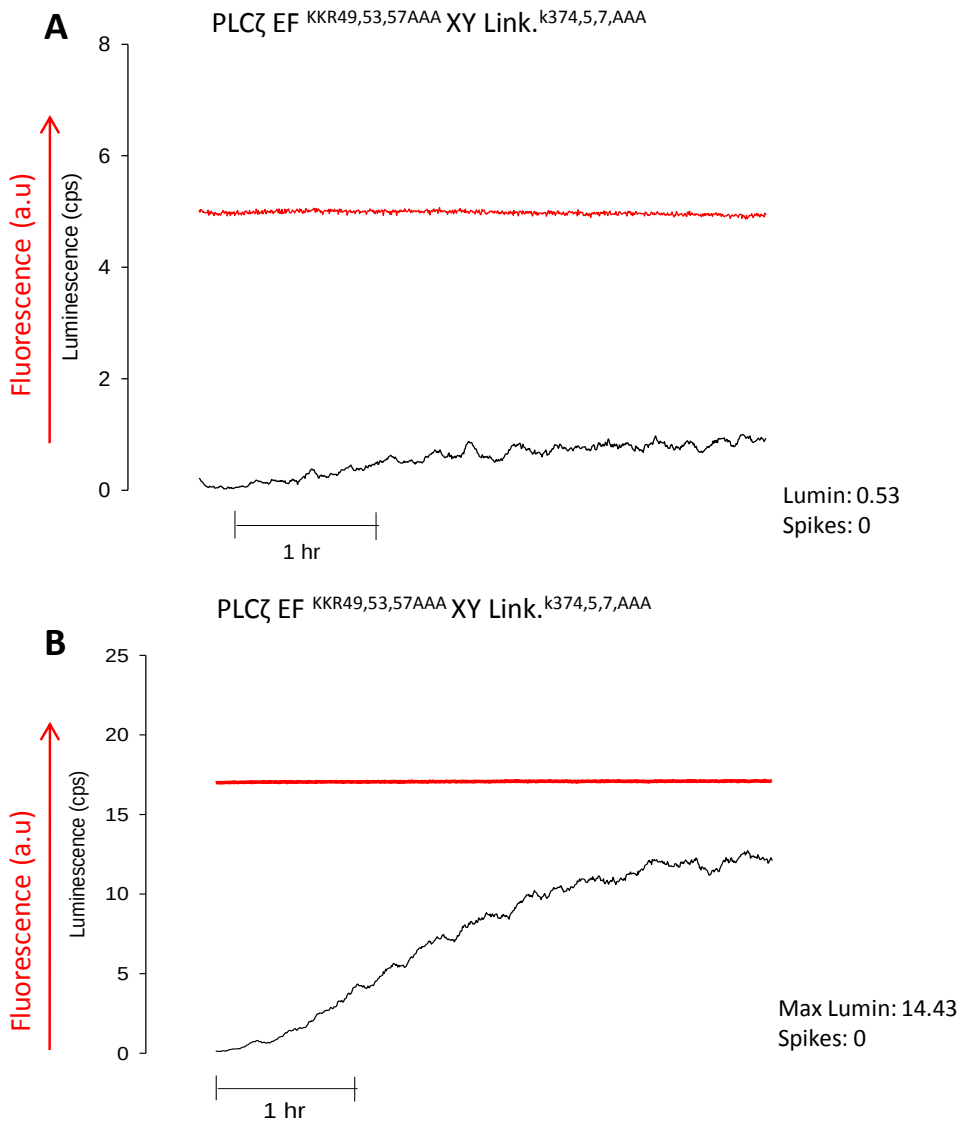


Figure 4.5 Sample Ca^{2+}_i trace initiated by mPLCζ containing triple mutation in the EF hand domain and X-Y linker

Ca^{2+}_i and luminescence changes that occurred in individual eggs following microinjection of the Ca^{2+}_i indicator OGBD with PLCζ EF ^{KKR49,53,57AAA} XY Link^{K374,5,7,AAA}-luciferase RNA at a pipette concentration of either **A)** 0.1 µg/µl or **B)** 2 µg/µl. Fluorescence trace coloured in red shows relative changes in cytosolic Ca^{2+}_i and luminescence shown in black is measured in photon counts per second and is used as an indicator of PLCζ protein expression. Lumin = mean counts per second in the first hour of oscillating across the group, Max Lumin= mean peak counts per seconds during recording averaged across the group of eggs, Spikes= mean total number of Ca^{2+}_i spikes in 1st hour of oscillations. See Table 4.1 for details of n values.

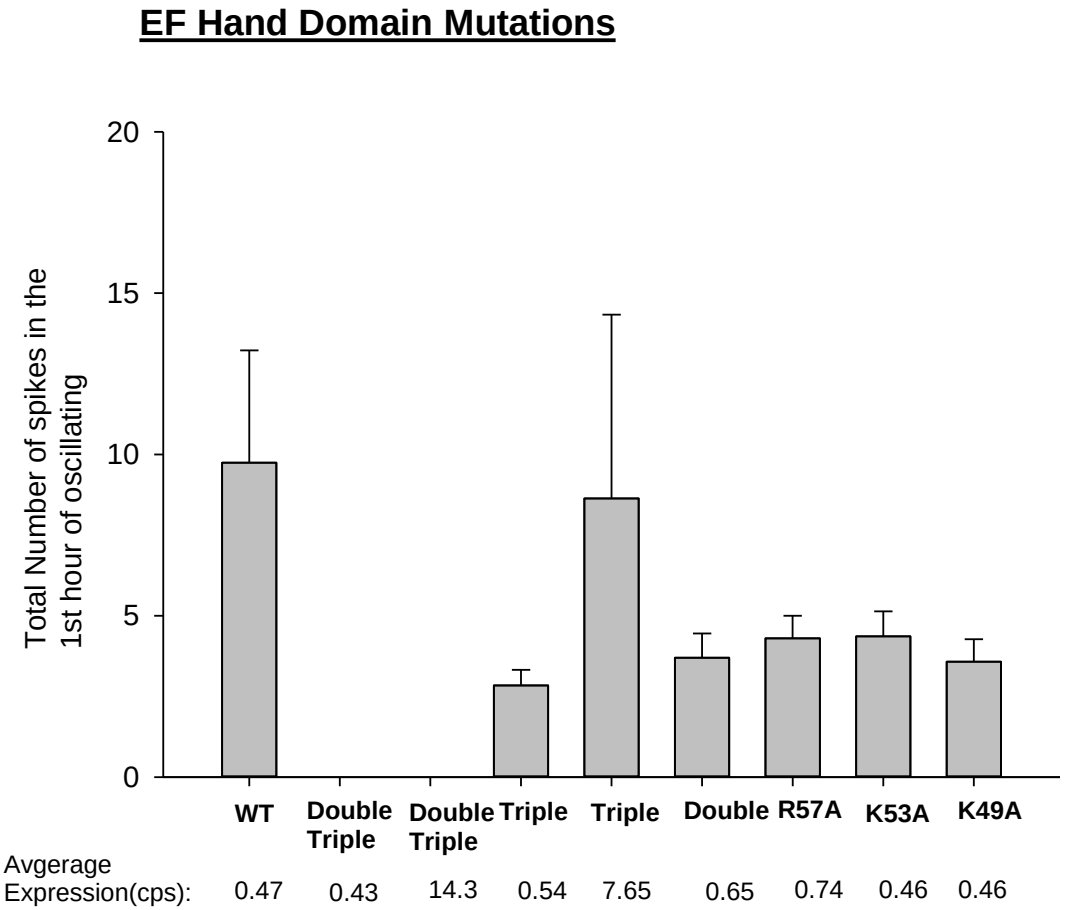


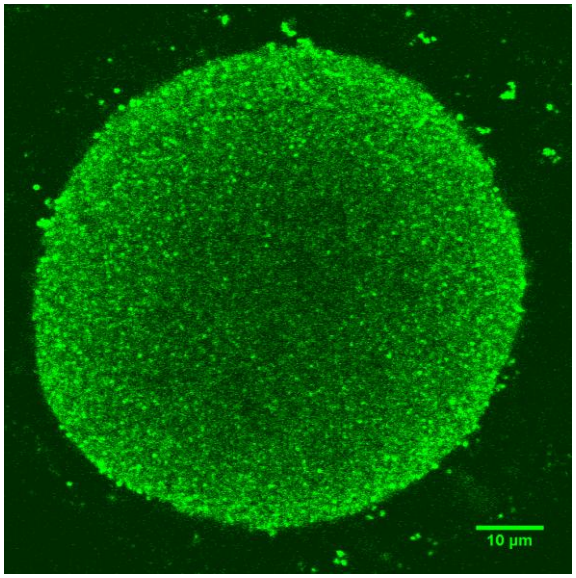
Figure 4.6 Summary showing the effects of mutations in EF hand domain and X-Y linker of PLCζ on Ca^{2+}_i oscillations

Summary bar graph showing the effect of neutralising positively charged residues in the EF hands domain of PLCζ on the frequency of Ca^{2+}_i oscillations it produced in eggs. Plot shows the mean total number of Ca^{2+}_i spikes recorded in the 1st hour of oscillating in eggs injected with a range of PLCζ-Luciferase cRNA constructs with mutations in the EF hands domain as follows: **WT)** Wild type PLCζ-Luciferase, **Double Triple)** PLCζ EF ^{KKR49,53,57AAA} XY Link.$^{K374,5,7,AAA'}$**Triple)** PLCζ^{KKR49,53,57AAA}- luciferase**, Double)** PLCζ^{KR49,57AA}- luciferase, **R57A)** PLCζ^{R57A}-luciferase, **K53A)** PLCζ ^{K53A}-luciferase and **K49A)** PLCζ ^{K49A} -luciferase. Mean Expression (cps) = mean counts per second of luminescence recorded in the 1st hour of eggs oscillating. Error bars show standard deviations. See Table 4.1 for n values and statistics.

The disruption of PI(4,5)P_2 binding following mutations in the EF hands domain of PLCζ cannot be detected by *in vivo* immunocytochemistry

In order to investigate whether the localisation of PLCζ inside the egg appeared to be disrupted in response to these mutations in the EF hands domain of PLCζ immunocytochemistry of PLCζ in eggs was carried out. Two RNA constructs were created one with wild type PLCζ sequence attached to a c-Myc tag and a EF hand domain mutated $PLC\zeta^{KKR49,53,57AAA}$ again attached to a c-Myc tag (both constructs produced by MN). These constructs were both injected into separate groups of eggs and allowed to express *in vivo* for approximately 2 hours before these cells were fixed and stained using the standard protocol (see Chapter 2). An anti c-Myc mouse monoclonal antibody was used as a primary antibody to mark PLCζ localisation and a fluorescent secondary anti-mouse AlexaFluor-488 antibody. The eggs were then imaged using confocal microscopy to look at fluorescent distribution as a measure of PLCζ localisation. No significant differences were seen in localisation of PLCζ in the eggs injected with $PLC\zeta^{KKR49,53,57AAA}$ compared to those injected with the wild type PLCζ (see fig 4.7). In both cases PLCζ was found to localise to vesicular structures inside the egg as has been previously recorded (see fig 4.7). This suggests that either the localisation of PLCζ is not affected by mutations in the EF hands domain or that any disruption in binding or localization cannot be detected using this technique.

A **Wild Type PLCζ**



Triple EF hand mutant: PLCζ ^{KKR49,53,57AAA}

B

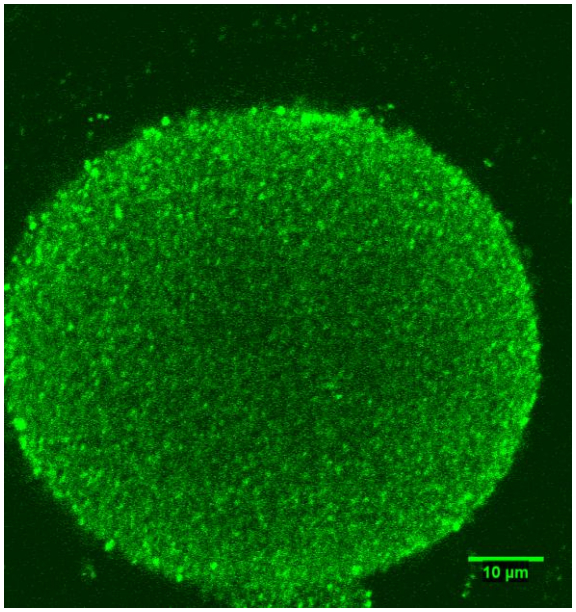


Figure 4.7 Images of WT and EF hand triple mutated mPLCζ localisation in mouse eggs

Confocal images showing examples of eggs injected with **(A)** cMyc tagged mouse PLCζ cRNA (n=10) and **(B)** cmyc tagged mouse PLCζ^{KKR49,53,57AAA} cRNA (n=8), (pipette concentration of 0.1 µg/µl). The eggs were fixed 3 hours post injection and stained using an anti-cMyc epitope mouse monoclonal primary antibody. An anti-mouse goat Fluor Alexa-488 secondary antibody was used before imaging the eggs on a confocal microscope (see Chapter 2 for full details of protocol).

The I489F missense mutation in the C2 domain affects human PLCζ's Ca^{2+} releasing ability

A mutation in the C2 domain of human PLCζ, $PLC\zeta^{I489F}$, has recently been identified that reduces PLCζ's ability to cause Ca^{2+}_i oscillations in male patients carrying this missense mutation (Escoffier *et al.* 2015a). Untagged $PLC\zeta^{I489F}$cRNA appears to cause abnormal Ca^{2+}_i oscillations when injected into eggs, an affect which it has been suggested to be due to mis-localisation of PLCζ to the target membrane or a reduction in the catalytic activity in PLCζ (Escoffier *et al.* 2015a). Exactly how this mutation affects PLCζ's Ca^{2+} releasing activity and to what degree it is affected is still unknown. To test this, a human $PLC\zeta^{I489F}$ cRNA construct tagged with the firefly luciferase sequence was synthesised alongside human $PLC\zeta^{WT}$– luciferase cRNA so the Ca^{2+}releasing activity could be compared with comparable expression levels (see fig 4.8, RNA synthesised by MN). These constructs were injected into two groups of eggs at varying pipette concentrations. Physiological levels of $hPLC\zeta^{WT}$ caused reliable low frequency oscillations that lasted for many hours (see fig 4.9). However, similar expression levels of $hPLC\zeta^{I489F}$ didn't cause any Ca^{2+}_i oscillations in 18/19 eggs (see fig 4.9, fig 4.10 and Table 4.2). $hPLC\zeta^{I489F}$ was found to be roughly 18 times worse at causing Ca^{2+}_i oscillations compared to $hPLC\zeta^{WT}$ (see fig 4.10 and Table 4.2). Interestingly if $hPLC\zeta^{I489F}$ is expressed at much higher levels (approximately 12 times the level of expression) it is able to cause Ca^{2+}_i oscillations. If $hPLC\zeta^{WT}$ is expressed even at half the amount of this level then the eggs become overstimulated, resulting in an early termination of Ca^{2+}_i oscillations. The oscillations initiated by high levels of $hPLC\zeta^{I489F}$ are still at a lower frequency than that caused by $PLC\zeta^{WT}$ at a low expression (see fig 4.9 and Table 4.2). These data clearly show that physiological levels of PLCζ containing theI489F missense mutation is not able to cause Ca^{2+}_i oscillations at the frequency usually seen at fertilisation and therefore cannot initiate egg activation.

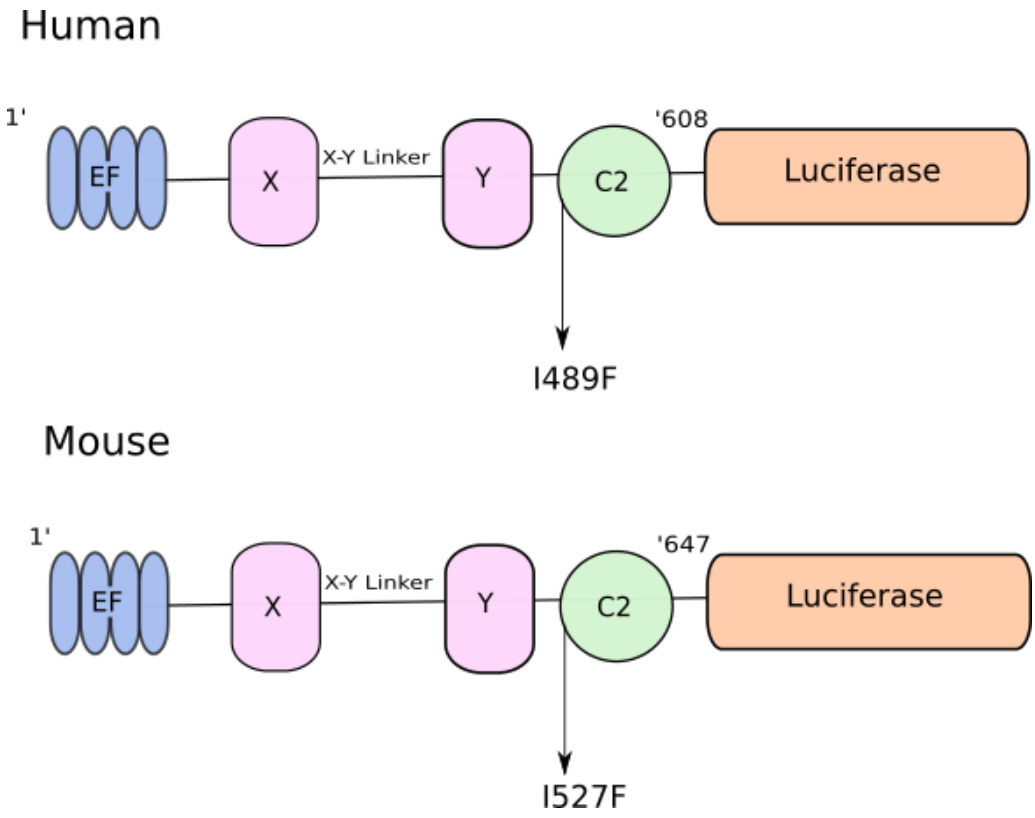


Figure 4.8 Schematic showing the mutations made in the EF hand domain and X-Y linker of PLCζ

The structural domains of mouse PLCζ and the residue substitutions carried out by point directed mutagenesis to create the "double triple" PLCζ mutant (PLCζ EF ^{KKR49,53,57AAA}XY Link^{K374,5,7,AAA}- luciferase cRNA). This mutation was made by substituting K49A,K53A and R57A in the 1st EF hand and substituting the lysine residues at the 374, 375 and 377 position for alanines in the X-Y linker

C2 Domain Mutation

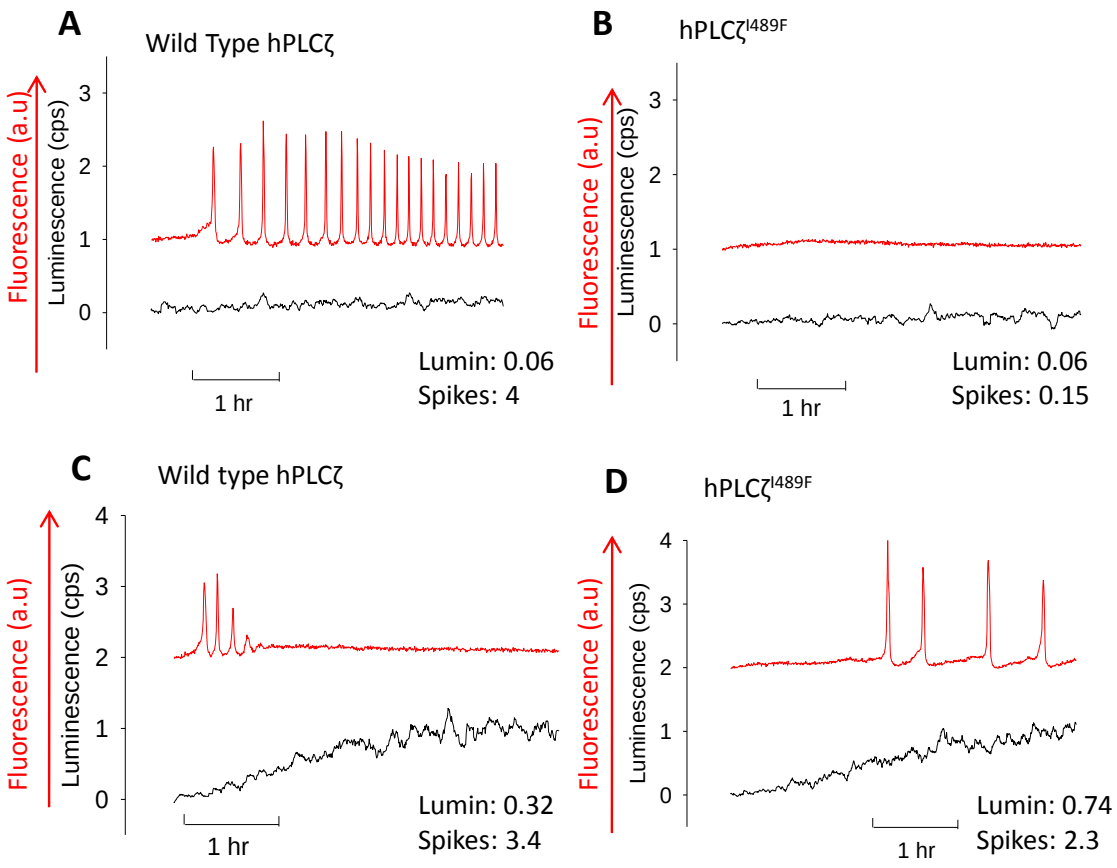


Figure 4.9 Sample Ca^{2+}_i traces initiated by human PLCζ containing a missense mutation in the C2 domain

Ca^{2+}_i and luminescence changes that occurred in individual eggs following microinjection of the ca^{2+}_i indicator OGBD with wild type hPLCζ-luciferase (**A** and **C)** and hPLCζ ^{I489F}- luciferase (**B** and **D**) injected at pipette concentrations of either 0.015 µg/µl (**A** and **B**), 0.3 µg/µl (**C**) or 0.1 µg/µl (**D**). Fluorescence traces are coloured in red shows relative changes in cytosolic Ca^{2+}_i and luminescence shown in black is measured in photon counts per second and is used as an indicator of PLCζ protein expression. Lumin = mean counts per second in the first hour of oscillating across the group, Spikes= mean total number of Ca^{2+}_i spikes in 1sthour of oscillations. See Table 4.2 for details of n values.

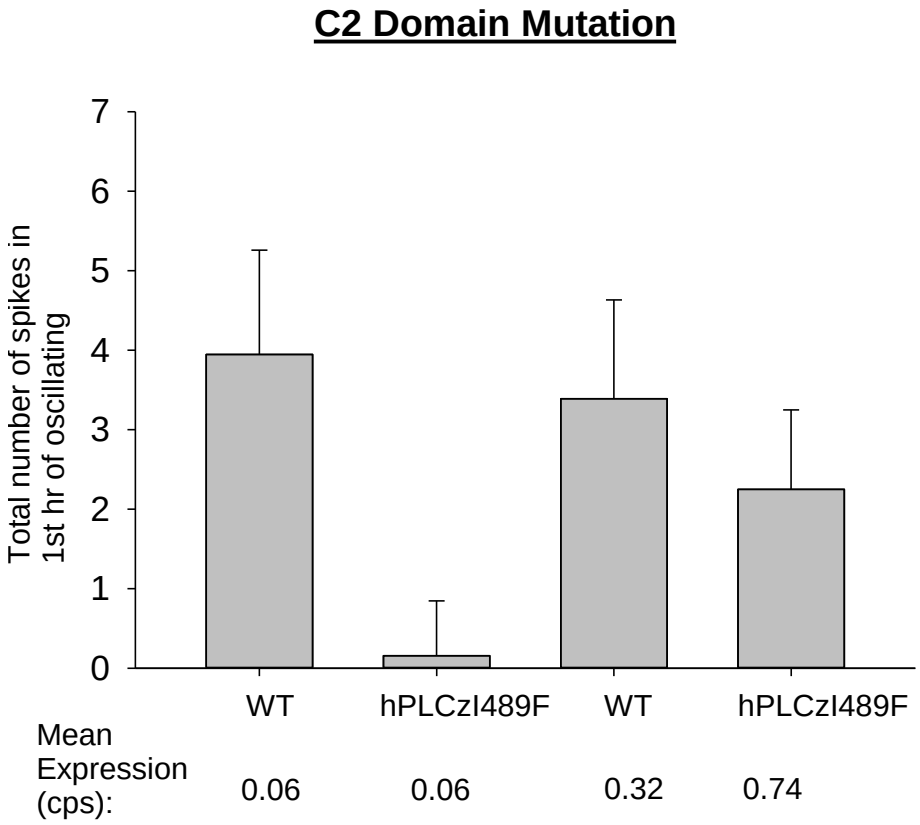


Figure 4.10 Summary showing the effects of missense mutation in C2 of hPLCζ on Ca^{2+}_i oscillations

Bar graph showing the effect of the 1489F mutation in the C2 domain of human PLCζ on the frequency of Ca^{2+}_i oscillations it produced in eggs. Plot shows the mean total number of Ca^{2+}_i spikes recorded in the 1st hour of oscillating in eggs injected with both wild type human PLCζ-luciferase cRNA (WT) and human $PLC\zeta^{1489F}$-luciferase cRNA ($hPLC\zeta^{I489F}$) at different cRNA expression levels. Mean Expression (cps) = mean counts per second of luminescence recorded in the 1st hour of eggs oscillating. Error bars show standard deviations. See Table 4.2 for n values and SEMs. Following a Mann-Whitney Rank Sum test a significant difference (P=<0.001, at a 95% confidence interval) was seen between the number of Ca^{2+}_i oscillations elicited by a low level of WT human PLCζ- luciferase and human $PLC\zeta^{1489F}$-luciferase cRNA at an average of 0.06 cps. A significant reduction in the number of Ca^{2+}_i oscilllations initiated by a higher level of $PLC\zeta^{1489F}$-luciferase (0.074 cps) and the lower level of WT PLCζ- luciferase (0.06 cps) was also recorded following the same test (P=0.001, at a 95% confidence interval).

Construct	Pipette concentration (µg/µl)	n	Number of eggs oscillating	Number of eggs that stopped oscillating before end of 1st hr	Mean number of spikes in 1st hr of oscillating	Mean expression PLCζ in 1st hr of oscillating (cps)
hPLCζ^{WT}	0.015	20	19/20	0/20	4 +/- 0.29	0.06 +/- 0.007
hPLCζ^{I489F}	0.015	19	1/19	0/19	0.15 +/- 0.16	0.06 +/- 0.04
hPLCζ^{WT}	0.3	18	18/18	18/18	3.4 +/- 0.29	0.32 +/- 0.015
hPLCζ^{I489F}	0.1	16	15/16	2/16	2.3 +/- 0.25	0.74 +/- 0.038

Table 4.2 Summary showing the effect of missense mutation in the C2 domain of human PLCζ on Ca^{2+}_i oscillations

The effects on the Ca^{2+} releasing activity of human PLCζ as a result of the I489F C2 domain mutation at different pipette concentrations. All PLCζ cRNA constructs were tagged with luciferase and cytosolic Ca^{2+}_i dynamics were measured using the fluorescent Ca^{2+}_i indictor OGBD. +/- = standard error of the mean, n= number of eggs injected

Construct	Pipette concentration (µg/µl)	n	Number of eggs oscillating	Number of eggs that stopped oscillating within 1 hr	Mean number of spikes 1st hr of spiking	Mean expression PLCζ in 1st hr of oscillating (cps)
mPLCζ^{WT}	0.025 - 0.05	31	31/31	0/31	9.7 ± 0.63	0.47 ± 0.04
mPLCζ^{I527F}	0.02 - 0.03	13	13/13	0/13	3.2 +/- 0.09	0.4 +/- 0.04
mPLCζ^{WT}	0.06	6	6/6	6/6	2.8 +/- 0.40	1.5 +/- 0.13
mPLCζ^{I1527F}	0.06	7	7/7	0/7	3.3 +/- 0.68	0.9 +/- 0.08

Table 4.3 Summary showing the effect of missense mutation in the C2 domain of mouse PLCζ on Ca^{2+}_i oscillations

The effects on the Ca^{2+} releasing activity of mouse PLCζ as a result of the I527F C2 domain mutation at different pipette concentrations. All PLCζ cRNA constructs were tagged with luciferase and cytosolic Ca^{2+}_i dynamics were measured using the fluorescent Ca^{2+} indictor OGBD. +/- = standard error of the mean, n= number of eggs injected

The homologous I527F missense mutation in the C2 domain also affects the Ca^{2+}releasing ability of mouse PLCζ

To see if the I489F mutation in the C2 domain has similar effects on the Ca^{2+}releasing ability of mouse PLCζ, a mouse PLCζ luciferase tagged cRNA construct containing the homologous I527F mutation was created (see fig 4.8, constructs courtesy of MN). The activity of this $mPLC\zeta^{I527F}$-luciferase cRNA was tested for Ca^{2+} releasing activity and compared to that of wild type mouse PLCζ-luciferase by microinjection into mouse eggs following the protocol previously used. Like the human $PLC\zeta^{I489F,}$ mouse $PLC\zeta^{I527F}$showed reduced Ca^{2+} releasing ability (see fig 4.11 and Table 4.3). Though all the eggs expressing a physiological level of this $mPLC\zeta^{I527F}$ were able to elicit $Ca^{2+}{}_i$ oscillations these oscillations occurred at a frequency approximately 3 times lower than that of wild type mouse PLCζ of similar protein expression levels (see fig 4.11 and Table 4.3). If the expression levels were approximately doubled no significant increase in the frequency of $Ca^{2+}{}_i$ oscillations was recorded however these oscillations began sooner therefore continued for longer (see fig 4.11 and Table 4.3). If wild type PLCζ was expressed at these higher non-physiological levels an overdose effect was seen where the oscillations are high frequency and terminated early (see fig 4.11 and Table 4.3). This would suggest that the I527F missense mutation also affects the Ca^{2+} releasing ability of mouse PLCζ however in a manner that is not as extreme as the I489F mutation in human PLCζ. It is important to note however human PLCζ has a much increased potency compared to mouse PLCζ and therefore is able to release Ca^{2+} at much lower expression levels.

C2 Domain Mutation

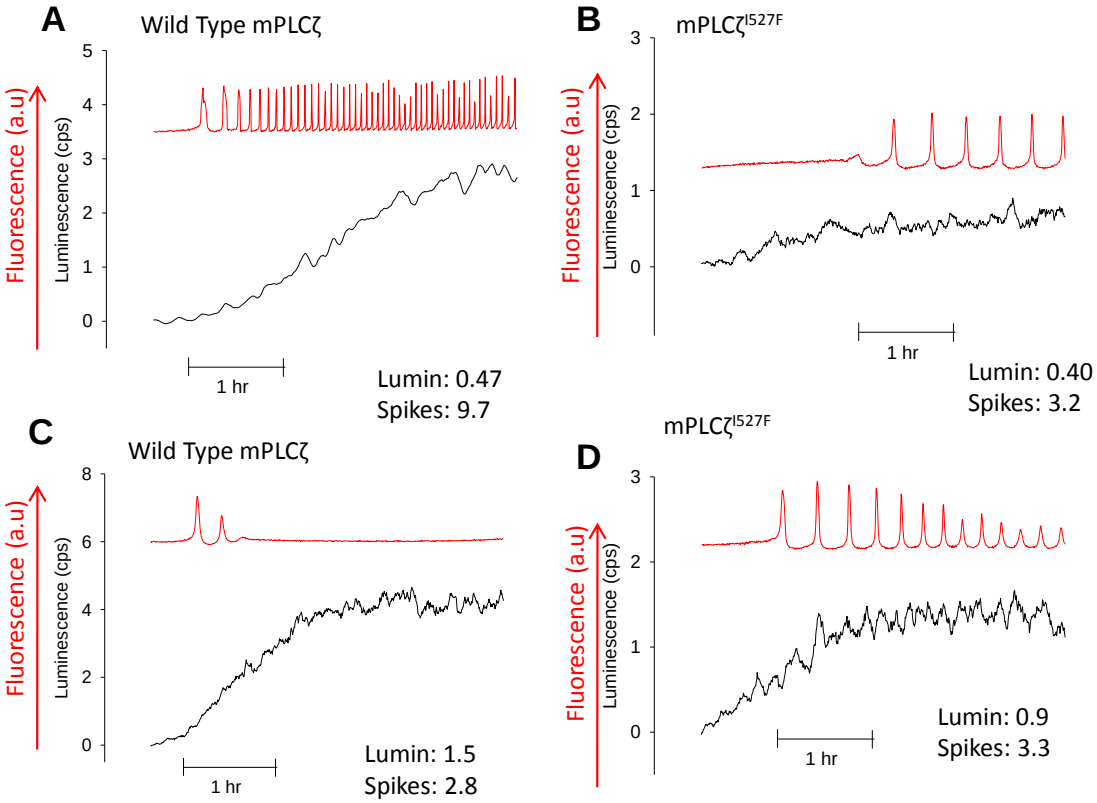


Figure 4.11 Sample Ca^{2+}_i traces initiated by mouse PLCζ containing a missense mutation in the C2 domain

Ca^{2+}_i and luminescence changes that occurred in individual eggs following microinjection of the Ca^{2+}_i indicator OGBD with wild type mPLCζ-luciferase (**A** and **C**) and mPLCζ^{I527F}- luciferase (**B** and **D**) injected at pipette concentrations of either 0.025, 0.02 µg/µl (**A** and **B**) or 0.0.06 µg/µl (**C**and **D**). Fluorescence traces are coloured in red shows relative changes in cytosolic Ca^{2+}_i and luminescence shown in black is measured in photon counts per second and is used as an indicator of PLCζ protein expression. Lumin = mean counts per second in the first hour of oscillating across the group, Spikes= mean total number of Ca^{2+}_i spikes in 1st hour of oscillations. See Table 4.3 for details of n values. Following a Mann-Whitney Rank Sum test a significant difference (P=<0.001, at 95% confidence interval) was seen between the number of Ca^{2+}_i oscillations elicited by a low level of WT mouse PLCζ- luciferase and human PLCζ^{1527F}-luciferase cRNA at an average of 0.47 cps and 0.4 cps respectively. A significant reduction in the number of Ca^{2+}_i oscilllations initiated by a higher level of PLCζ^{1527F}-luciferase (0.9 cps) and the lower level of WT PLCζ- luciferase (0.47 cps) was also recorded following the same test (P=0.001, at 95% conficence interval).

Mutations within the CaM binding motif of PLCζ do not affect its Ca^{2+}releasing ability

Other PLCs use binding to CaM in order to regulate their activity, for example PLCδ1 is inhibited when CaM is bound to its CaM binding domain, a domain which is conserved in PLCζ (McCullar *et al.* 2003; Sidhu *et al.* 2005). To investigate whether the CaM binding motif of PLCζ has a role in regulating its activity a series of point mutations were created within this region of human PLCζ. CaM binds to a 16 residue region in the X-Y linker (KKTRKLKIALALSDVL), however the positively charged residues in this region also contribute to the interaction of PLCζ with its substrate $PI(4,5)P_2$ so these residues could not be altered (Nomikos *et al.* 2011b; Nomikos *et al.* 2011c). Therefore more subtle mutations were made where some of the neutrally charged residues (alanine and leucine) in this region were substituted glycine. A series of these mutations in human PLCζ were created by point mutagenesis with single ($hPLC\zeta^{A346G}$), double ($hPLC\zeta^{AA346G,348GG}$) and triple ($hPLC\zeta^{LAA343,346348GGG}$) mutations made, attached to a luciferase tag and synthesised into cRNA alongside wild type human $PLC\zeta^{WT}$(see fig 4.12, designed and synthesised by MN). The constructs were tested for Ca^{2+}releasing ability by microinjection into mouse eggs and the changes in Ca^{2+}_i were recorded using a fluorescent Ca^{2+}_i indicator. Injection of all constructs of PLCζ caused Ca^{2+}_i oscillations at low levels of RNA expression (see fig 4.13 and Table 4.4). Similar levels of cRNA expression of both $hPLC\zeta^{WT}$and triple mutant ($hPLC\zeta^{LAA343,346348GGG}$) produced a very similar number of Ca^{2+}_i oscillations in the first hour of oscillating indicating similar Ca^{2+} releasing activity (see fig 4.13, fig 4.14 and Table 4.4). However, those eggs injected with the triple mutant had a greater number of eggs where the oscillations terminated early (before the end of the 1st hour) (see fig 4.13, fig 4.14 and Table 4.4). This value was relatively low at 7/26 eggs stopping before the end of the 1st hour of oscillating however this could be an indicator of increased PLCζ activity in these eggs, like that seen if PLCζ protein levels are overexpressed past physiological levels. Very similar Ca^{2+}_i responses were recorded in eggs injected with the single mutant $hPLC\zeta^{A346G}$ when compared to those injected with $hPLC\zeta^{WT}$ however eggs injected with the double mutant ($hPLC\zeta^{AA346G,348GG}$) had Ca^{2+}_i oscillations at a slightly lower frequency than $hPLC\zeta^{WT}$(see fig 4.13, fig 4.14 and Table 4.4). These variations could be accounted for by the small variations in expression levels of the cRNA between the groups. These data therefore show that no significant differences can be recorded in the Ca^{2+}releasing ability of PLCζ following these mutations in the CaM binding motif of PLCζ.

CaM Binding Domain Mutations

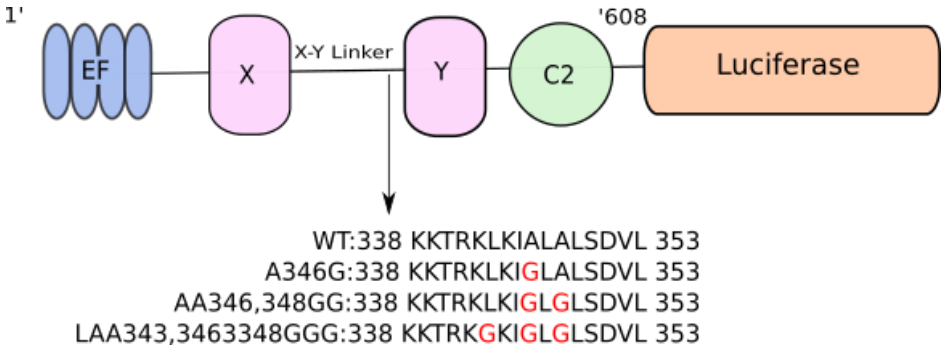


Figure 4.12 Schematic showing mutations made in the CaM binding domain of hPLCζ

The structural domains of human PLCζ and the residue substitutions carried out by point directed mutagenesis to create RNA constructs of PLCζ- luciferase with mutations in the CaM binding motif in the X-Y linker. Mutations were as follows: WT) wild type PLCζ sequence, A346G) substitution of the alanine at residue 346 for glycine (single), AA346,348GG) substitution of the alanines at residue 346 and 348 for glycines (double) and LAA343,346,348GGG) substitution of the alanines at residue 343, 346 and 348 for glycines (triple).

Construct	Pipette concentration (µg/µl)	n	Number of eggs oscillating	Number of eggs that stopped oscillating within 1 hr	Mean number of spikes 1st hr of oscillating	Mean expression in 1st hr of oscillating (cps)
hPLCζ^{WT}	0.02	24	24/24	0/24	6.0 +/- 0.36	0.06 +/- 0.004
hPLCζ^{A346G} (Single)	0.02	12	12/12	1/12	7.2 +/- 0.62	0.08 +/- 0.004
hPLCζ^{AA346G,348GG} (Double)	0.02	11	11/11	0/11	3.4 +/- 0.40	0.04+/- 0.005
hPLCζ^{LAA343,346348GGG} (Triple)	0.02	26	26/26	7/26	6.1 +/- 0.38	0.06 +/ 0.004

Table 4.4 Summary showing the effects of mutations in the CaM binding domain of human PLCζ on Ca^{2+}_i oscillations

The effects on the Ca^{2+} releasing activity of human PLCζ as a result of different mutations in the CaM binding motif of this protein. All PLCζ RNA constructs were tagged with luciferase and cytosolic Ca^{2+}_i dynamics were measured using the fluorescent Ca^{2+}_i indictor OGBD. +/- = standard error of the mean, n= number of eggs injected

CaM Binding Domain Mutations

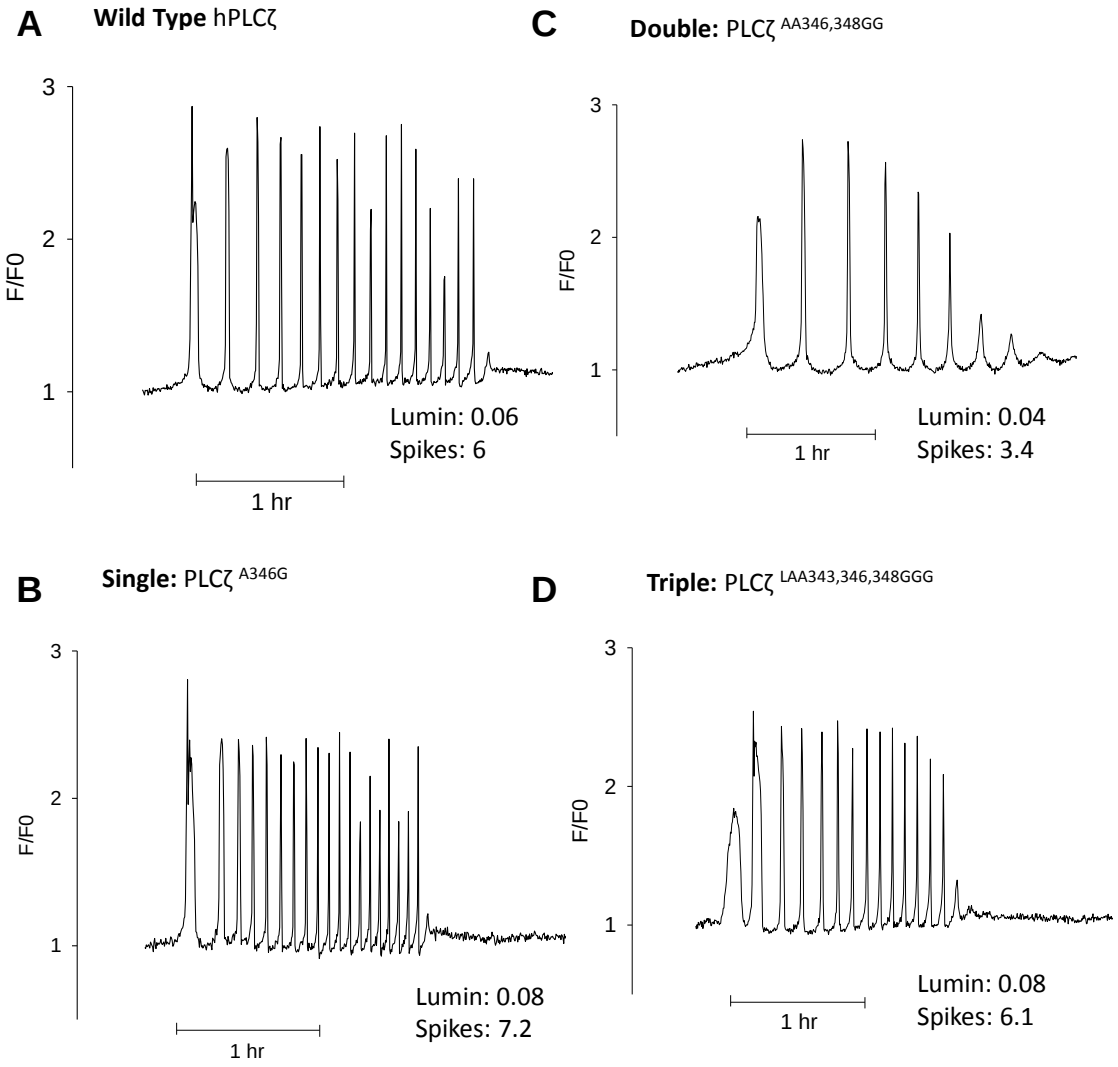


Figure 4.13 Sample Ca^{2+}_i traces initiated by hPLCζ containing mutations in the CaM binding domain

Ca^{2+}_i changes that occurred in individual eggs following microinjection of the Ca^{2+}_i indicator OGBD with the one of the following PLCζ RNA constructs: **A)** Wild Type human PLCζ- Luciferase cRNA, **B)** PLCζ ^{A346G}- luciferase RNA (single mutant), **C)** PLCζ ^{AA346,348GG}-luciferase cRNA (double mutant) and **D)** PLCζ ^{LAA343,346,348GGG}-luciferase cRNA (triple mutant). All constructs were injected at a pipette concentration of 0.02 µg/µl. Relative fluorescence plotted as F/F0, Lumin = mean counts per second in the first hour of oscillating across the group, Spikes= mean total number of Ca^{2+}_i spikes in 1st hour of oscillations. See Table 4.4 for details of n values.

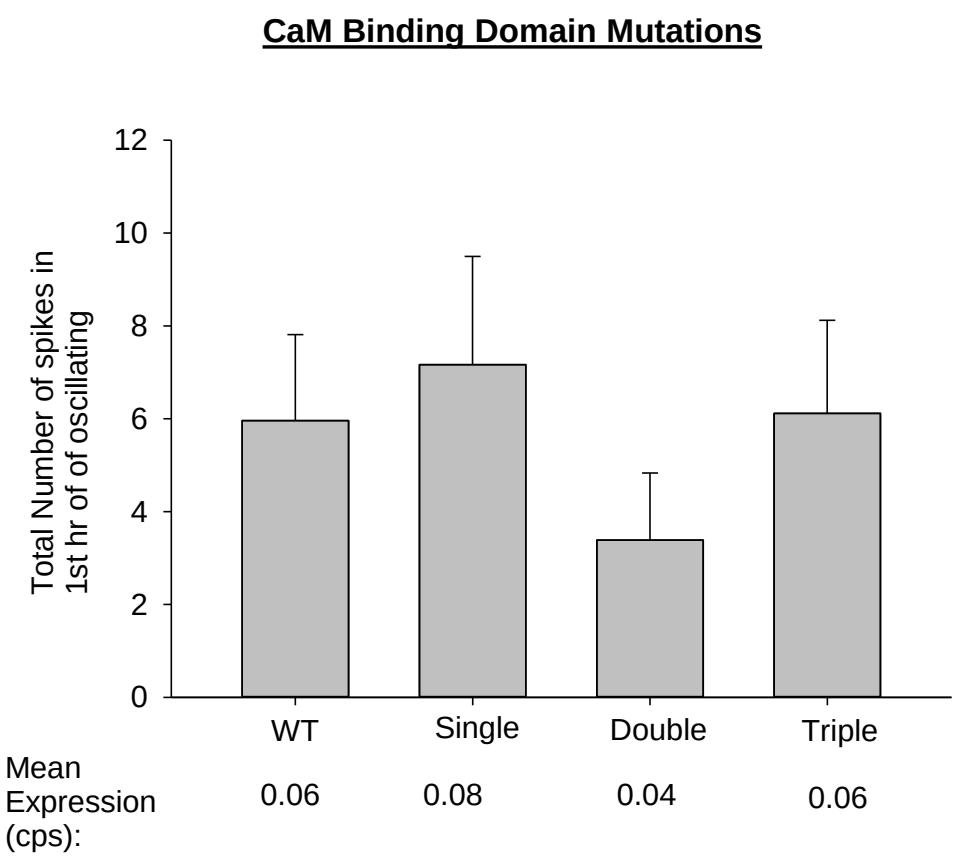


Figure 4.14 Summary showing the effects of mutations in CaM binding domain of hPLCζ on Ca^{2+}_i oscillations

Bar graph showing the effect of point mutations in the CaM binding motif of human PLCζ on the frequency of Ca^{2+}_i oscillations it produced in eggs. Plot shows the mean total number of Ca^{2+}_i spikes recorded in the 1st hour of oscillating in eggs injected with 0.02 µg/µl of wild type human PLCζ-luciferase cRNA (WT), PLCζ $^{A346G-}$ luciferase cRNA (**single**), PLCζ ^{AA346,348GG}-luciferase cRNA (**double**) and PLCζ ^{LAA343,346,348GGG}-luciferase cRNA (**triple**). Mean Expression (cps) = mean counts per second of luminescence recorded in the 1st hour of eggs oscillating. Error bars show standard deviations. See Table 4.4 for n values and SEMs

Split PLCζ is able to cause Ca^{2+}_i oscillations when injected into mouse eggs however with reduced efficiency

It has previously been shown that a fraction of PLCζ in the sperm of pigs is proteolytically cleaved at the X-Y linker however is still able to hydrolyse $PI(4,5)P_2$ and Ca^{2+}_ioscillations (Kurokawa *et al.* 2007). In order to investigate how effective this "split" PLCζ is, at releasing Ca^{2+} constructs were created with two halves of mouse PLCζ (residues 1-361 and residues 362-647) and each half was tagged with a different half of luciferase sequence (see fig 4.15, constructs created by MN). This way if the two halves of PLCζ come together the two halves of luciferase will also join, become enzymatically active and produce luminescent light. The corresponding human PLCζ constructs were also created (residues 1-328 and residues 329-608). The two halves of the split PLCζ were co-microinjected into eggs alongside the Ca^{2+}_i indictor OGBD and both the Ca^{2+}_i and luminescence were measured simultaneously. Both split mouse PLCζ and split human PLCζ were able to cause Ca^{2+}_i oscillations in mouse eggs however with less efficiency (See fig 4.16, fig 4.17 and Table 4.5). Split mPLCζ was required approximately 5-10 times the pipette concentration that would usually be required of full length mPLCζ in order to cause reliable Ca^{2+}_i oscillations and these oscillations were much lower frequency with a delay in the timing of the first spike (See fig 4.16 and Table 4.5). Despite a considerably higher pipette concentration of cRNAthe luminescence values were a lot lower than is seen with full length mPLCζ (see Table 4.5). In fact at expression levels at one fifth of that seen for full length mPLCζ, split mPLCζ caused 7/16 eggs to oscillate however 6 of these stopped oscillating within one hour which could indicate an overdose effect (See Table 4.5). Interestingly split hPLCζ was able to cause Ca^{2+}_i oscillations at a much lower pipette concentration at levels that were very similar to full length hPLCζ causing 10/11 eggs to oscillate (see fig 4.17 and Table 4.5). However, at this level no luminescence was seen, the frequency of oscillations were reduced compared to full length hPLCζ and there was a delay in the timing of the first spike (see Table 4.5). If the pipette concentration was increased luminescence could still not be recorded even though all eggs oscillated and 8/11 appeared to stop early in response to an overdose effect (see fig 4.17 and Table 4.5). These data suggest that proteolytically cleaved PLCζ is able to cause Ca^{2+}_i oscillations in eggs and this may occur at fertilisation however it is not as efficient as full length PLCζ. Furthermore as with human PLCζ there are dramatic differences between species regarding the potency of PLCζ. Interestingly the luminescence values were much lower than for wild type full length PLCζ.

Cleaved PLCζ Chimeras

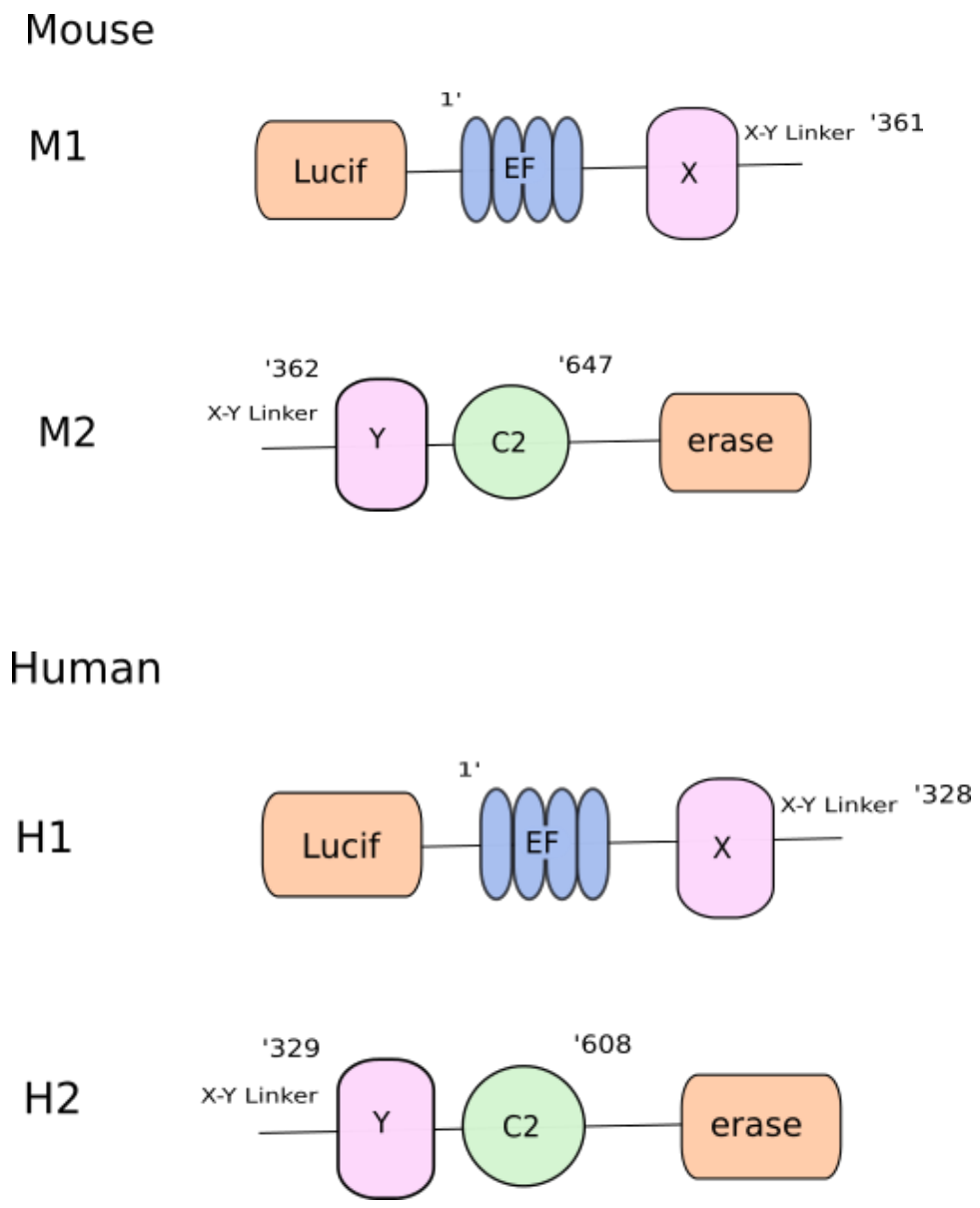


Figure 4.15 Schematic showing the structural domains of proteolytcially cleaved (split) mouse and human PLCζ-luciferase constructs

cRNA of mouse PLCζ was synthesized in two halves, residues 1- 361 (M1) and 362- 647 (M2) each of these was then tagged with opposite halves of the luciferase sequence. cRNA of human PLCζ was synthesized in two halves, residues 1- 328 (H1) and 329-608 (H2) with each of these also attached to opposite halves of the luciferase sequence.

Cleaved Mouse PLCζ

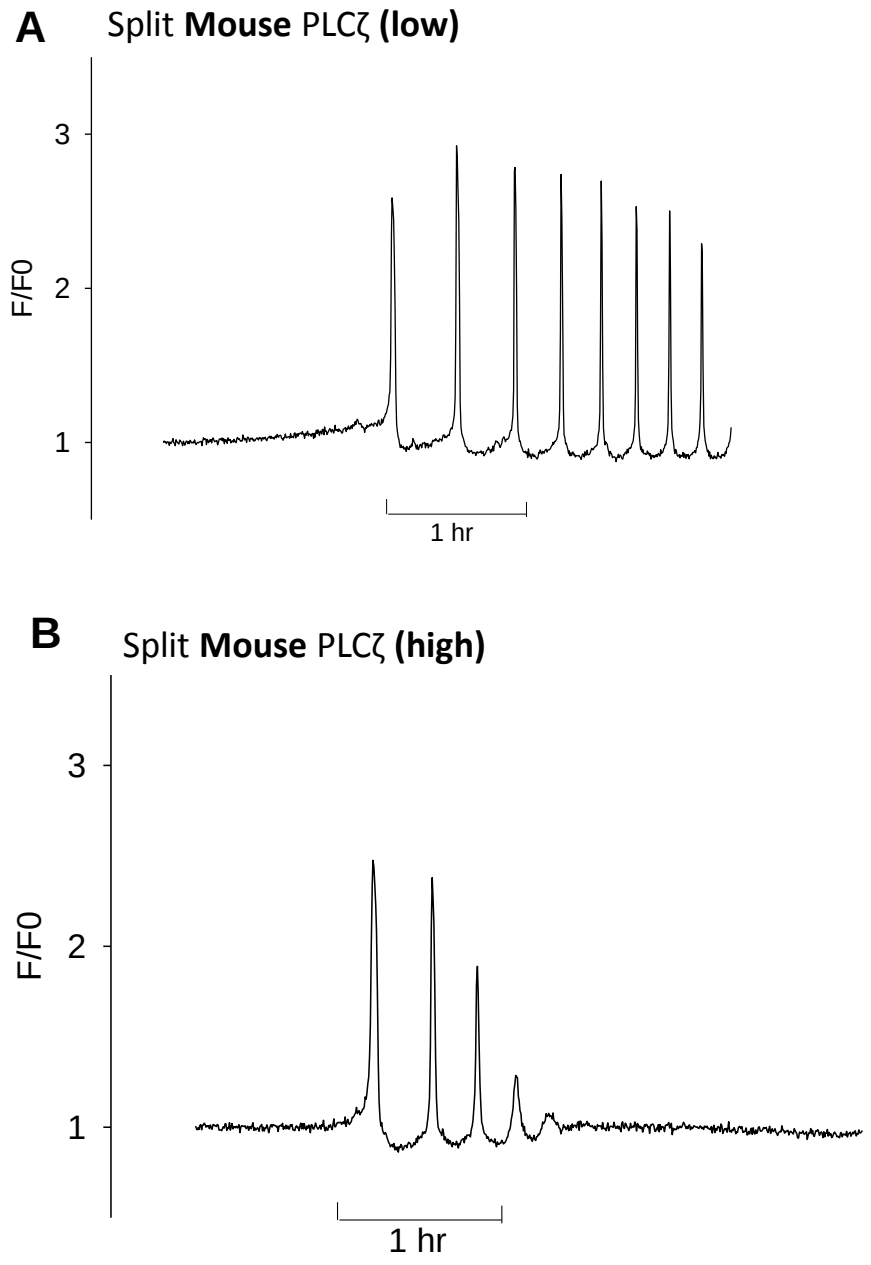


Figure 4.16 Sample Ca^{2+}_i traces initiated by split mouse PLCζ

Ca^{2+}_i changes that occurred in individual eggs following microinjection of the Ca^{2+}_i indicator OGBD with proteolytically cleaved mouse PLCζ. M1 (mPLCζ 1-361) and M2 (mPLCζ 362-647) cRNA were co-injected into eggs at a pipette concentration of either 0.25 µg/µl (**A**) or 0.5 µg/µl (**B**). Relative fluorescence plotted as F/F0. See Table 4.5 for full details and n values.

Cleaved Human PLCζ

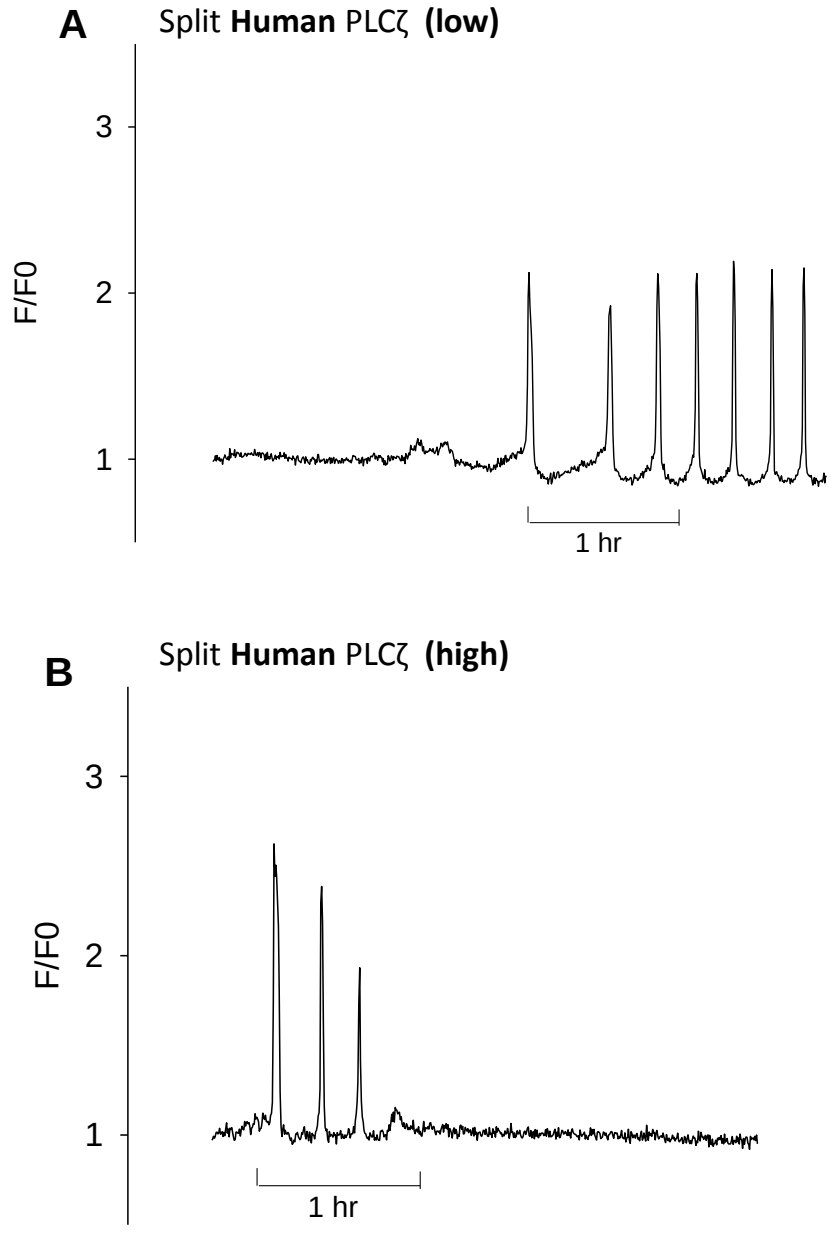


Figure 4.17Sample Ca^{2+}_i traces initiated by split human PLCζ

Ca^{2+}_i changes that occurred in individual eggs following microinjection of the Ca^{2+}_i indicator OGBD with proteolytically cleaved human PLCζ. H1 (hPLCζ 1-328) and H2 (hPLCζ 329-608) cRNA were co-injected into eggs at a pipette concentration of either 0.02 µg/µl (**A**) or 0.1 µg/µl (**B**). Fluorescence plotted as F/F0. See Table 4.5 for full details and n values.

Construct	Pipette concentration (μg/μl)	N	Number of eggs oscillated	Number of eggs that stopped oscillating within 1 hr	Mean number of spikes 1st hr of oscillating	Mean expression in 1st hr of oscillating (cps)	Mean time of 1st spike (s)
Full length mPLCζ	0.025 - 0.05	31	31/31	0	9.7 ± 0.63	0.47 ± 0.038	1921 +/- 41.6
Split mPLCζ (M1 & M2)	0.25	14	11/14	1/11	3.07 +/- 0.47	0.045 +/- 0.006	5437 +/- 266.6
Split mPLCζ (M1 & M2)	0.5	16	7/16	6/7	1.5 +/- 0.45	0.165 +/-0.012	3896 +/-118.6
Full length hPLCζ	0.015 - 0.02	43	42/43	0	5.07 +/- 0.29	0.058 +/- 0.003	2160 +/- 182.5
Split hPLCζ (H1 & H2)	0.02	11	10/11	0	2.46 +/- 0.28	-	6141 +/-558.2
Split hPLCζ (H1 & H2)	0.1	11	11/11	8/11	3.64 +/- 0.53	-	845+/- 113.1
Chimeras							
1: M1 & H2	0.025	19	15/19	0	2.0 +/- 0.29	-	11209 +/- 869.2
1: M1 & H2	0.1	20	20/20	5/20	3.7 +/- 0.15	-	2968.5 +/- 161.6
2: H1 & M2	0.025	11	11/11	8/11	3.4 +/- 0.47	-	2461 +/- 422.4
2: H1 & M2	0.1	16	16/16	0	2.3 +/- 0.11	-	6766 +/- 462.1

Table 4.5 Summary of Ca^{2+} releasing ability of mouse and human split PLCζ constructs

Differences in Ca^{2+} releasing ability of proteolytically cleaved mouse and human PLCζ compared to full length cRNA and combinations of these 2 species. All PLCζ RNA constructs were tagged with luciferase and in the case of cleaved PLCζ constructs the only half of the luciferase tag was attached to each half of the PLCζ construct. $Ca^{2+}{}_i$ dynamics were measured using the fluorescent $Ca^{2+}{}_i$ indictor OGBD. +/- = standard error of the mean, n= number of eggs injected

Chimeras of split human and mouse PLCζ are able to cause Ca^{2+}_i oscillations in mouse eggs with variations in potency

As it was established that both mouse and human cleaved PLCζ were able cause Ca^{2+}_i oscillations in mouse eggs the next question raised was whether chimeras of human and mouse split PLCζ could cause Ca^{2+}_i oscillations and whether there were any potency differences depending on the combination of these halves. To test this instead of co-injecting the two cleaved halves of mouse PLCζ the N' terminal half of mouse PLCζ was co-injected alongside the C' terminal half of human PLCζ and vice versa. A higher and a lower pipette concentration of the two chimeras were injected in the same way as described above. The first chimera which consisted of co-injection of the N' terminal half of mouse PLCζ (M1) and the C' terminal half of human PLCζ (H2) caused low frequency at a similar concentration (0.025 g/l) to that of split human PLCζ (H1 and H2) and at a similar frequency (see fig 4.18 and Table 4.5). There was however a significant delay in the time of the first spike compared to split human PLCζ (see Table 4.5). If the pipette concentration was increased to (0.1 g/l) the time of the first spike was sooner however not as soon as that split human PLCζ of the same concentration (See Table 4.5). The frequency of the oscillations was slightly higher than that of the lower concentration with 5/20 of these eggs stopping within the first hour (See Table 4.5).This rate was lower than human split PLCζ of the same concentration. This implies that the M1- H2 chimera is slightly reduced in potency compared split human H1 and H2 however is not as reduced as mouse split PLCζ and has an activity that is much more like human split PLCζ. Interestingly if the inverse chimera was used (H1-M2) the opposite appeared to be true (See fig 4.19 and Table 4.5). The lower pipette concentration of 0.025 g/l caused Ca^{2+}_i oscillations that were similar in frequency to the response elicited by the same pipette concentration of M1-H2 and human split PLCζ, however these oscillations began much earlier (See fig 4.19 and Table 4.5). This suggests that H1-M2 is more potent than mouse split PLCζ and M1-H2 chimera. In contrast a higher pipette concentration (0.1 g/l) of H1-M2 produced oscillations that were lower in frequency and delayed in initiation compared to the lower concentration H1-M2 (see fig 4.19 and Table 4.5). The pattern of these Ca^{2+}_i oscillations most closely resembled those produced by a low concentration (0.02 g/l) human split PLCζ and would therefore suggest that H1 and M2 is reduced in potency compared to human split PLCζ and M1-H2 however still more potent than split mouse PLCζ (see Table 4.5). These data indicate that various structural regions of PLCζ must convey potency differences between species however particular regions, possibly related to the 'N-terminal or 'C terminal halves of the protein,

have a more of an influence on the enzyme's potency than others. In summary it appears that human split (H1-H2) PLCζ is the most potent at releasing Ca^{2+}, followed by the chimera M1 and H2, then the chimera H1 and M2 and finally split mouse PLCζ M1- M2 is least potent at releasing Ca^{2+}.

Cleaved Mouse and Human PLCζ Chimeras

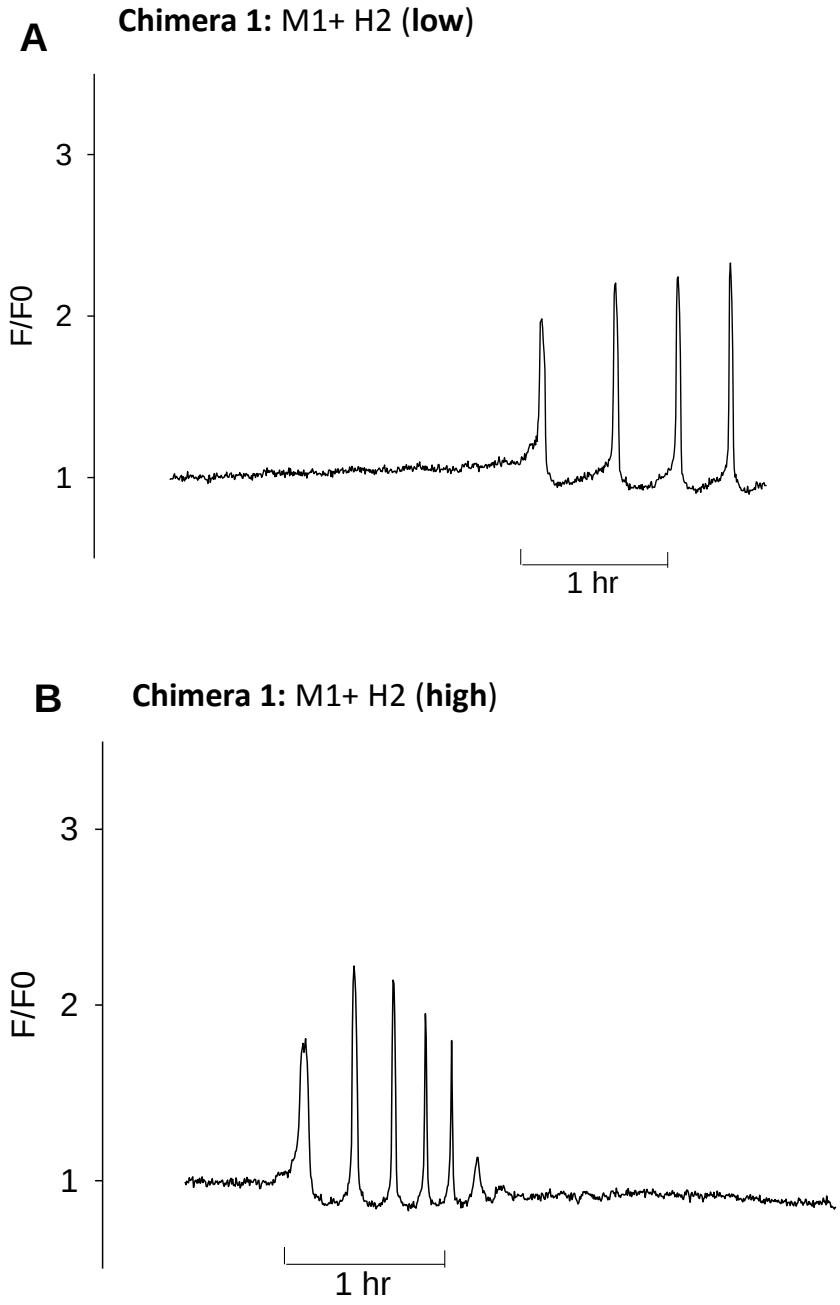


Figure 4.18 Sample Ca^{2+}_i traces initiated by split PLCζ chimera 1

Ca^{2+}_i changes that occurred in individual eggs following microinjection of the Ca^{2+}_i indicator OGBD with proteolytically cleaved combined mouse and human PLCζ M1 (mPLCζ 1-361) and H2 (hPLCζ 329-608) cRNA were co-injected into eggs at a pipette concentration of either 0.02 µg/µl (**A**) or 0.1 µg/µl (**B**). Fluorescence plotted as F/F0. See Table 4.5 for full details and n values.

Cleaved Mouse and Human PLCζ Chimeras

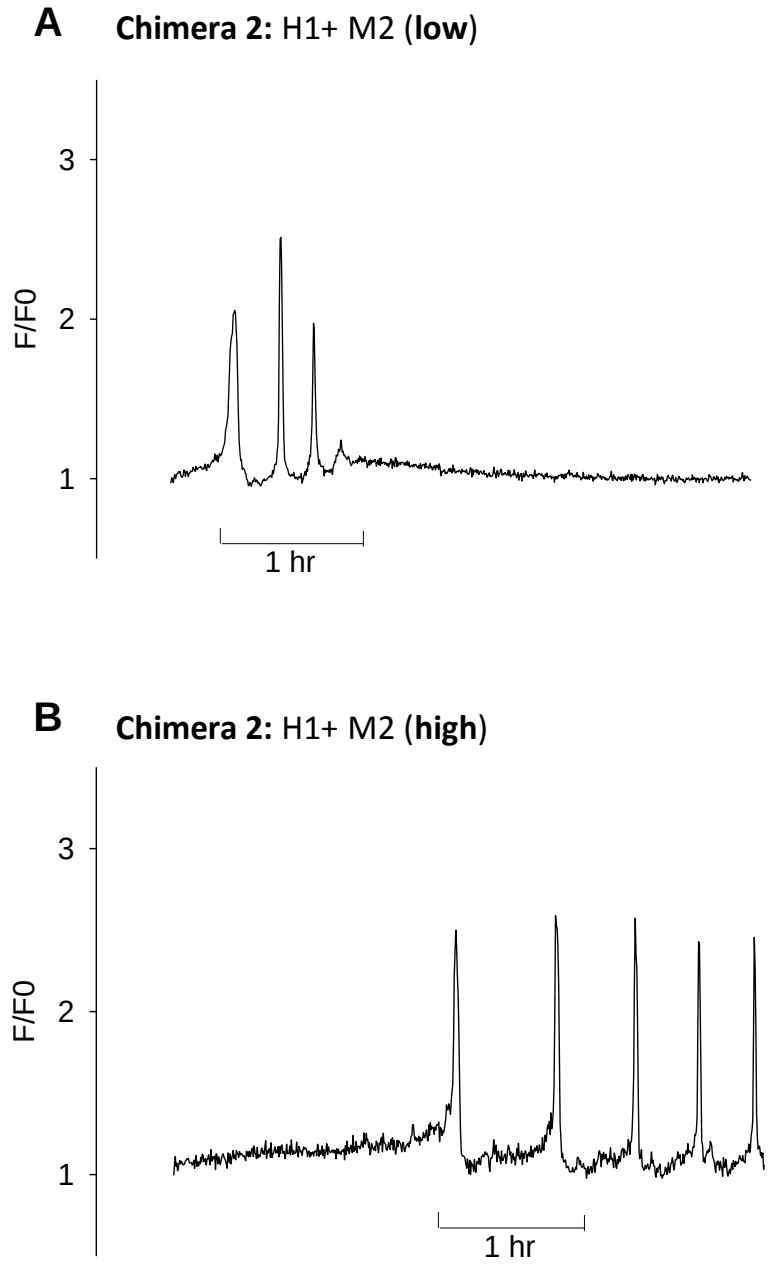


Figure 4.19 Sample Ca^{2+}_i traces initiated by split PLCζ chimera 2

Ca^{2+}_i changes that occurred in individual eggs following microinjection of the Ca^{2+}_i indicator OGBD with proteolytically cleaved combined mouse and human PLCζ. H1 (hPLCζ 1- 328) and M2 (mPLCζ 361-647) cRNA were co-injected into eggs at a pipette concentration of either 0.02 µg/µl (**A**) or 0.1 µg/µl (**B**). Fluorescence plotted as F/F0. See Table 4.5 for full details and n values.

Discussion

It is well established that the structural domains of PLCs convey their activity and allow them to work in different ways. PLCζ is no exception to this and it is already known that omission of particular functional domains either impairs or completely abolishes the ability for PLCζ to elicit Ca^{2+}release (Nomikos *et al.* 2005; Nomikos *et al.* 2011c; Theodoridou *et al.* 2013). As the substrate for PLCζ, $PI(4,5)P_2$, is membrane bound it is particularly important that PLCζ is able to localise and bind to these membranes. However, unlike other PLCs PLCζ lacks a pleckstrin homology domain (PH domain) that targets other PLCs to the target plasma membrane. PH domains are found on most PLCs however target the enzyme to the membrane in different ways which all ultimately allow it to access its phospholipid substrate (Lemmon *et al.* 1995). For example PLCδ1 binds directly to $PI(4,5)P_2$ in the target membrane via its PH domain (Pawelczyk and Lowenstein 1993). Despite PLCζ sharing a great amount of similarity with PLCδ1 this is one of the big differences between the two isoforms. This would suggest that PLCζ uses a novel mechanism to target membranes that may account for both the difference in $PI(4,5)P_2$ targeting and the ability of PLCζ to release Ca^{2+} at egg basal Ca^{2+}_i levels however the mechanism for this remain to be fully explained.

There are a variety of ways binding to a membrane may be ensured,for example either by biochemical interactions conveyed by particular structural properties of PLCζ or by interactions with additional proteins and lipids. Previous data has shown that positively charged residues within the linker region of PLCζ between the X and Y catalytic domains bind to negatively charged $PI(4,5)P_2$ (Nomikos *et al.* 2011b). Neutralising these residues impairs the ability for PLCζ to elicit Ca^{2+}release when injected into eggs (Nomikos *et al.* 2011b). Interestingly however, even removal of the whole X-Y linker does not completely ablate the Ca^{2+}_i oscillation inducing response of PLCζ or its binding to $PI(4,5)P_2$ (Nomikos *et al.* 2011c) . This suggests even though the X-Y linker does play a role in binding to $PI(4,5)P_2$ and conveying PLCζ's activity other structural features of PLCζ must also be involved. The two most likely domains to be involved in this interaction are the C2 domain and The EF hands domain.

In PLCδ1 the first N terminal EF hand has been found to bind with moderate affinity to vesicles containing anionic phospholipids (Cai *et al.* 2013). This domain contains positively charged residues which are thought to help anchor PLCδ1 to its substrate through electrostatic interactions. Direct mutagenesis of these positive residues compromises PLC

activity (Cai *et al.* 2013). This region within the 1st EF hand domain is conserved in PLCζ however the role of this in membrane targeting has not yet been established.

The current study shows that in PLCζ these positively charged residues within the first EF hand do appear to play a role in determining PLCζ's Ca^{2+}releasing ability. Neutralisation of even one positively charged residue by direct mutagenesis was shown to have an effect on the ability of PLCζ to cause Ca^{2+} release. A greater effect was seen if this was increased to neutralisation of 3 positively charged residues suggesting that the role for these residues is a cumulative one.Interestingly this effect can be rescued if the mutated PLCζ ($PLC\zeta^{KKR49,53,57AAA}$) is expressed in the egg at approximately 16 times the physiological level. This indicates that the positively charged residues in EF hand are not essential for PLCζ activity, however, they do play a role in determining the enzyme's potency and are required for maximal activity. As is the case for PLCδ1, it appears that this decrease in Ca^{2+} releasing ability is due to a reduction in binding to phospholipids. Other data by fellow colleagues has found that neutralising these positive residues reduces binding of PLCζ to $PI(4,5)P_2$ containing liposomes with an increase in Km of around 5 fold, however, *in vitro* $PI(4,5)P_2$ hydrolysis by PLCζ is not affected (Nomikos *et al.* 2015). This supports the notion that, as with PLCδ1, positively charged residues within the EF hand domain of PLCζ contribute to its Ca^{2+} releasing ability by assisting its anchoring to phospholipids on target membranes. This occurs by electrostatic interactions in a manner that is independent of enzymatic activity (Cai *et al.* 2013). This theory is supported by mathematical modelling data which shows that simulated Ca^{2+}_i oscillations created based on this theoretical relationship between PLCζ and $PI(4,5)P_2$ match extremely well with that seen physiologically in eggs as a result of cRNA injection of the different EF hand mutants (Nomikos *et al.* 2015). As a result,the difference in Ca^{2+}_i oscillation frequency seen as a result of these mutations can be accounted for by a deficiency in $PI(4,5)P_2$ binding (Nomikos *et al.* 2015). This provides a novel role for the EF hand domain of PLCζ that is distinct from its role in conveying Ca^{2+} sensitivity (Nomikos *et al.* 2005). It was previously shown that deletion of one or all the EF hands of PLCζ abolished its Ca^{2+} releasing ability (Nomikos *et al.* 2005). This effect could now be considered as a result both a loss of Ca^{2+} sensitivity and a reduction in $PI(4,5)P_2$ binding.

Mutations solely within the EF hands domain of PLCζ do not appear to be significant enough to disrupt PLCζ localisation as immunostaining shows no obvious difference between intracellular localisation of $PLC\zeta^{KKR49,53,57AAA}$ compared to wild type PLCζ. There could be several reasons for this, the first could be that PLCζ localisation simply isn't

disrupted enough to be seen by these methods and a significant amount of PLCζ is still binding to the target membrane. This idea is supported by the findings that overexpression of PLCζ protein is able to rescue the effects of this mutation. Furthermore in order to get an adequate fluorescent signal using this method it is often necessary to introduce a slightly higher concentration of RNA into the eggs than would be physiological. This provides an excess of RNA that is also stained and can provide a localisation pattern that isn't physiological. Another possibility is that the cMyc antibody underwent some nonspecific binding which would, following secondary antibody treatment, show fluorescence signal in other areas of the cell in addition to where PLCζ was localised. Finally it is possible that PLCζ is still localised to the correct membranes due to reduced $PI(4,5)P_2$ binding the enzyme isn't able to hydrolyse its substrate effectively. As a result much of the correctly localised PLCζ protein is not able to elicit Ca^{2+}release.

The electrostatic interactions between the positive residues of the EF hand and negatively charged $PI(4,5)P_2$ appear to serve a very similar purpose to those between the positively charged residues in the X-Y linker and $PI(4,5)P_2$ (Nomikos *et al.* 2011b) . For this reason it was necessary to establish whether the X-Y linker and the EF hand domain had redundant roles in terms of $PI(4,5)P_2$ binding or whether these two domains work together to sufficiently anchor PLCζ to membrane bound $PI(4,5)P_2$. The current study tested this using a double mutated form of RNA with triple neutralisations of positive residues in both the X-Y linker (previously used by (Nomikos *et al.* 2011b) and the EF hand domain. It was found that if the positively charged residues in the X-Y linker and the EF hand domain were neutralised simultaneously PLCζ is not able to elicit Ca^{2+}_i oscillations. This remained the case even if the expression of this RNA is increased to 30 times the level the physiological level. The fact that a good luciferase signal was seen in the eggs following injection of this mutation confirms that the RNA was being expressed in the eggs and this was confirmed by other colleagues (credit Michail Nomikos) using an immunoblot which showed clear faithful expression of the un-fragmented double mutant PLCζ in eggs (Nomikos *et al.* 2015). Further work by (Nomikos *et al.* 2015) showed that despite the PLCζ EF ^{KKR49,53,57AAA} XY Link.^{k374,5,7,AAA} losing all ability to release Ca^{2+} in eggs *in vivo*, biochemical assays show this mutant still retains 80% of its enzymatic activity and has unchanged Ca^{2+} sensitivity (Nomikos *et al.* 2015). However, this mutant does display significantly reduced binding to $PI(4,5)P_2$ containing vesicles (Nomikos *et al.* 2015). This suggests that the X-Y linker and the EF hand may work in synergy so PLCζ can bind electro-statically to $PI(4,5)P_2$. It is possible that PLCζ EF ^{KKR49,53,57AAA} XY Link.^{k374,5,7,AAA} doesn't cause Ca^{2+}release in eggs because this

mutant is not able to bind sufficiently to $PI(4,5)P_2$ at the target membrane and therefore may become mislocalised. In order to investigate this fully it would be advantageous to repeat the immunocytochemistry experiments using the PLCζ EF ^{KKR49,53,57AAA}XY Link.^{k374,5,7,AAA} construct to look at the localisation of this protein in the egg. It is more likely that an effect on PLCζ localisation would be seen with this construct as it causes a more severe effect on $PI(4,5)P_2$ binding that cannot be rescued by overexpression of the protein. It appears that the positively charged regions of both the X-Y linker and the EF hands domain are required for physiological levels of PLCζ to sufficiently bind to $PI(4,5)P_2$ in order to elicit Ca^{2+} release and positive residues in either are necessary to enable PLCζ to release Ca^{2+} in eggs.

As the positively charged residues in the EF hand domain appear to be required for maximal Ca^{2+}releasing ability of both PLCζ and PLCδ1 this interactions does not provide much insight into either the differences in potency between PLCζ and PLCδ1 or how PLCζ discriminates between different sources of $PI(4,5)P_2$. In other words if PLCζ is targeted to its membrane of interest purely through electrostatic binding to $PI(4,5)P_2$ by its EF hand domain and X-Y linker there is no reason why it shouldn't bind and hydrolyse all sources of $PI(4,5)P_2$ available in the cell. However, this is not the case as PLCζ is able to discriminate between different sources of $PI(4,5)P_2$ and subsequently only hydrolyses vesicular $PI(4,5)P_2$ not plasma membrane $PI(4,5)P_2$ (Yu *et al.* 2012) . This difference could be accounted for by PLCζ's lack of a PH domain, however it has previously been shown that the addition of a PH domain onto the N' terminus of PLCζ does not give it the ability to hydrolyse plasma membrane $PI(4,5)P_2$ (Theodoridou *et al.* 2013). In addition this does not alter theCa^{2+}releasing abilities of PLCζ at all (Theodoridou *et al.* 2013). This would suggest that other structural properties of PLCζ other than the EF hand domain and the lack of a PH domain enable vesicular $PI(4,5)P_2$ hydrolysis or prevent plasma membrane $PI(4,5)P_2$ hydrolysis.

One structural domain that could be involved in this is the C2 domain. Despite the C2 domain of PLCζ being necessary for it to cause Ca^{2+} release it is not yet clear what the role of this domain is (Nomikos *et al.* 2005). C2 domains are characteristically considered as membrane targeting domains with a wide range of selectivity for membrane lipids (Medkova and Cho 1999). In PLCδ1 for example the C2 domain binds to phosphatidylserine in a Ca^{2+} dependent manner to allow substrate access to the active site (Lomasney *et al.* 1999). In PLCζ however there is currently no evidence for this. Furthermore liposome binding assays and protein lipid overlay experiments have shown the C2 domain of PLCζ

does not to bind to PI(4,5)P_2 (Nomikos *et al.* 2011b). In fact *in vitro* studies have shown the C2 domain of PLCζ will only bind to mono phospholipids specifically PI(3)P and PI(5)P however significantly has not been shown to bind not PI(4)P (Kouchi *et al.* 2005). However, the significance of this binding remains unknown. However, a mutation in the C2 domain of human PLCζ that has been shown to result in human infertility has recently been characterised (Escoffier *et al.* 2015a). This point mutation is the result of isoleucine (I) 489 being switched for a phenylalanine (F) and results in the production of PLCζ that has a reduced ability to cause Ca^{2+} release when injected into eggs (Escoffier *et al.* 2015a). Though it has been suggested that this effect could be due to mislocalisation of PLCζ in the egg, misfolding or reduced stability of the PLCζ protein the biochemical interactions of this mutated PLCζ with potential target lipids has yet to be established nor has the difference in Ca^{2+}releasing ability been sufficiently quantified (Escoffier *et al.* 2015a).

The current study aimed to accurately quantify the reduction in Ca^{2+}releasing ability of this mutation and try and establish why theCa^{2+}releasing ability of PLCζ is impaired. To do this, luciferase tagged human PLCζ RNA containing this point mutation was tested for Ca^{2+}releasing ability against wild type human PLCζ- luciferase RNA. It was found that at physiological levels of expression the mutated PLCζ was not able to cause Ca^{2+}_i oscillations however this effect could be partially rescued by overexpression of this PLCζ protein by up to 10 fold. This effect was supported by other data from colleagues showing that MBP tagged human PLCζ recombinant protein containing this mutation was not able to cause Ca^{2+}_i oscillations in eggs at physiological levels however if the concentration of this protein was doubled it was able to cause Ca^{2+}_i oscillations (Nomikos *et al.* 2017a). A similar effect on Ca^{2+}_i oscillations was seen following injection of mouse PLCζ cRNA containing this mutation however the impairment on Ca^{2+} release was not as extreme as that seen for human PLCζ suggesting there may be some species differences in the role or structure of the C2 domain of mouse compared to human.

It appears that the effect of this I469F mutation on Ca^{2+} release is not due to a reduction in PLCζ's ability to hydrolyse PI(4,5)P_2, a reductioninCa^{2+}sensitivity nor PI(4,5)P_2 binding (Nomikos *et al.* 2017a). However, liposome binding assays and pull downs have shown that PLCζ binding to mono- phospholipids, specifically PI(3)P and PI(5)P is reduced by up to 50% (Nomikos *et al.* 2017a). It could therefore be suggested that the C2 domain conveys Ca^{2+}releasing activity on PLCζ by localising it to specific membranes by binding to these phospholipids. By discrete localisation of PLCζ to these membranes via its C2 domain, the enzyme is then able to bind to PI(4,5)P_2 localised there by electrostatic interactions of the

X-Y linker and the EF hands domain. This theory would suggest that PLCζ C2 domain binding to the mono phospholipids PI(3)P and PI(5)P is necessary for optimal Ca^{2+}releasing ability of PLCζ and could even explain why PLCζ does not localise to the plasma membrane. This idea is interesting because even though PLCζ has been found to bind to both PI(3)P and PI(5)P *in vitro*, PI(3)P particularly has actually been found to reduce the hydrolytic activity of PLCζ suggesting that this lipid is likely to have a regulatory role on the enzyme's activity(Kouchi *et al.* 2005). It could therefore be considered unlikely that binding to an inhibitory lipid such as PI(3)P would be required for optimal activity of PLCζ. Furthermore, it is unlikely that PI(3)P and PI(5)P are involved in localising PLCζ to specific membranes mainly because topologically inside the egg these molecules are not found in the same compartments. PI(3)P is found almost solely in the membranes of early endosomes which is supported by both immunostaining overlap with early endosome markers and the fluorescently tagged FYVE domain localisation which binds very specifically to PI(3)P (Gillooly *et al.* 2003). The vesicular source of $PI(4,5)P_2$ found within the egg which overlaps strongly with PLCζ localisation does not overlap with early endosome markers such as the small GTPase Rab 5 or with YFP-FYVE domain RNA expressed in the egg (Yu, Moon and Swann 2012 unpublished). Instead the most likely origin for the intracellular vesicles that PLCζ localises to is the Golgi apparatus. This idea is supported by co-localisation of immunocytochemistry of Golgi markers such as Giantin and Rab 6 with PLCζ and $PI(4,5)P_2$ (Yu *et al.* 2012). However, there is no evidence to suggest that PI(3)P is located on Golgi membranes, instead the mono-phospholipid located here is PI(4)P which has not been found to bind to the C2 domain of PLCζ *in vitro* (Lorente-Rodríguez and Barlowe 2011). Furthermore the inhibition of PI(3)P production by the drug Wortamannin, which inhibits the enzyme PI3K, does not seem to affect the Ca^{2+}releasing ability of PLCζ in the egg in fact appears to increase the frequency of these $Ca^{2+}{}_i$ oscillations (Moon and Swann 2012 unpublished). This would suggest that it is unlikely that PI(3)P binding by the C2 domain is required for PLCζ's ability to release Ca^{2+}. Infact it is possible that the localisation of PI(3)P on other membranes could regulate PLCζ activity and prevent it hydrolysing $PI(4,5)P_2$ there. Little is known about the cellular localisation of PI(5)P however most data suggests that it is mainly localised to the nucleus however its' localisation and role in other cellular membranes remains to be established (Bulley *et al.* 2015).

One other way that localisation and activity of PLCζ could be controlled is indirectly by its interaction with accessory proteins through biochemical interactions with the structural domains of PLCζ. One example of this is Calmodulin (CaM). Like other PLCs PLCζ has a CaM

binding site within its structure and in PLCζ this sequence is found within the X-Y linker. In other PLCs interaction with CaM can have both negative and positive effects. For example PLCβ has a positive association with CaM (McCullar *et al.* 2003; McCullar *et al.* 2007). There is evidence that CaM may help localisation of PLCβ to the plasma membrane in a Ca^{2+}_i dependent manner and therefore stimulate it's activity (McCullar *et al.* 2007). In contrast CaM appears to have an inhibitory effect on PLCδ1 in a manner that is reversible by the small GTPase Ral (Sidhu *et al.* 2005). Even though PLCζ possesses a binding motif for CaM the significance of this motif and the role of CaM interaction on PLCζ activity is still unknown. Recent results have shown that CaM reduces PLCζ hydrolytic activity at high Ca^{2+}_i concentrations by binding to it (Nomikos *et al.* 2017b).

To investigate the role of the CaM binding motif of PLCζ in the current study 3 mutated versions of human PLCζ RNA were created containing point mutations within selected residues in the CaM binding motif. Each were tested for their Ca^{2+} releasing ability in mouse eggs. It was found that none of these mutations had a significant effect on the ability for PLCζ to release Ca^{2+}. A slight increase was recorded in the number of eggs that had Ca^{2+}_i oscillations terminate early in those eggs injected with the triple mutant ($PLC\zeta^{LAA343,346,348GGG}$) which can be an indicator of high Ca^{2+}releasing activity for example like that seen when wild type PLCζ is expressed at high levels. However, this effect is not significant enough to allow any strong conclusions to be drawn. This is particularly relevant as human PLCζ was used in these experiments. Due to the high potency of human PLCζ (higher than that of mouse PLCζ) expression levels of the protein required to give a physiological response are often very low. As a result the luminescence signal recorded per egg is much lower and therefore contains more noise in that signal, which ultimately gives a less accurate measure of expression. As human PLCζ is so potent very small differences in expression levels, particularly between different eggs, can have a significant effect the Ca^{2+}_i response recorded. This makes measuring subtle changes in the Ca^{2+}releasing activity of PLCζ is response to subtle mutations difficult to measure.

In this experiment it was difficult to make significant alterations to the residues in the X-Y linker without altering $PI(4,5)P_2$ binding therefore the mutations made within the binding domain may have been too subtle to have an effect on CaM interaction. It is unclear precisely how CaM would interact with PLCζ and whether it would have a positive or negative effect on its activity. Unlike PLCδ1 there is no evidence that PLCζ is stimulated by Ral activity so it is unlikely that PLCζ is regulated by a CaM –Ral complex in the same way as PLCδ1. Furthermore unlike PLCβ, PLCζ does not require high cytosolic Ca^{2+}_i levels in order

to hydrolyse $PI(4,5)P_2$ (Nomikos *et al.* 2005). Instead, PLCζ is a lot more sensitiveto Ca^{2+} and is able to operate at basal cytosolic concentrations of Ca^{2+}_i making it unlikely that PLCζ is stimulated by CaM in a Ca^{2+} dependent manner the way PLCβ is (McCullar *et al.* 2007). It is possible however that CaM is able to regulate PLCζ activity through the involvement of other accessory proteins. There may be proteins other than Ral, particularly other small GTPases that could help regulate PLCζ activity. Most other PLC isoforms interact with small GTPases in some way (Gresset *et al.* 2012) so it is quite possible that PLCζ also does however the identity of this protein and the nature ofthis potential interaction remains unknown. Interestingly, small GTPases are often localised to specific intracellular membranes and can act as markers for these organelles (Simons and Zerial 1993). It is not yet understood whether a small GTPase is involved in regulating PLCζ activity by playing a role in membrane targeting or by forming a regulatory complex with CaM in the way Ral does for PLCδ.

Though the structure of PLCζ's functional domains undoubtedly play a significant role in regulating and determining its Ca^{2+}_i releasing activity it is possible that post translational modifications of PLCζ may also play a role. One such modification that has previously been identified is proteolytic cleavage of the PLCζ protein. It has previously been shown that the sperm extract from some species, porcine and mouse in particular, contains not only a large percentage of freely extractable full length PLCζ but also a significant amount of cleaved non-soluble PLCζ that may only be extracted under alkaline conditions (Kurokawa *et al.* 2005). Interestingly, following extraction, this sperm extract fraction containing cleaved PLCζ is able to cause Ca^{2+}_i oscillations when injected into eggs (Kurokawa *et al.* 2007). Furthermore if cleaved PLCζ RNA is synthesised and then injected into eggs it is also able to cause Ca^{2+}_i oscillations (Kurokawa *et al.* 2007). These active PLCζ fragments always appear to be cleaved at a similar point in the X-Y linker region suggesting that proteolytic cleavage may occur as a post translational modification therefore could play a role in regulating PLCζ activity (Kurokawa *et al.* 2007). Neither of these halves of PLCζ are able to cause Ca^{2+} release alone because all structural domains of PLCζ have been found to be necessary in enabling PLCζ to release Ca^{2+} (Kurokawa *et al.* 2007). When these two halves of PLCζ are co-injected they form a functional complex in the egg that co-localises enabling hydrolysis of $PI(4,5)P_2$ (Kurokawa *et al.* 2007). It still remains unclear exactly how cleaved PLCζ is able to do this or how functional this split PLCζ is compared to full length PLCζ.

The current study aimed to investigate the differences between the functional activities of cleaved human and mouse PLCζ compared to their full length counterparts. In order to

quantify these differences a colleague synthesised the two halves of cleaved PLCζ RNA and attached two separate halves of a luciferase tag so expression of the protein could be measured. These two halves were co-injected into eggs for both the human and the mouse PLCζ so that the Ca^{2+} releasing activity could be compared to full length PLCζ –luciferase RNA. It appears that for both species there is a reduction in the functional activity of cleaved PLCζ compared to full length PLCζ however at even relatively low RNA pipette concentrations cleaved PLCζ was still able to cause $Ca^{2+}{}_i$ oscillations. It is most likely that the reduction in functional activity of split PLCζ is due to a reduced efficiency in these two halves of PLCζ coming together and co-localising in the egg. The main evidence for this is for a given concentration of RNA the luminescence signal was much lower for split PLCζ RNA compared to full length PLCζ. This would suggest that despite a reasonable expression of protein the efficiency of these two halves of PLCζ protein coming together in the right location in the egg is somewhat reduced. As a result the pipette concentration of RNA and protein expression would need to be higher in order to get the same amount of functionally active PLCζ protein compared to full length PLCζ RNA. This data is supported by the time delay in the beginning of $Ca^{2+}{}_i$ oscillations seen in eggs injected with split PLCζ RNA compared to wild type PLCζ. However, as it is necessary for the two ends of luciferase to come together in order for the luciferase enzyme to work, it is also possible that a reduction in luminescence signal is due to a reduced efficiency in the two halves of luciferase joining. If this is the case it may be possible that despite an equivalent level of functional, available PLCζ protein the luciferase signal is still reduced. It is also possible that the differences in luciferase signal could be a result of differences in protein expression of the split PLCζ RNA compared to full length wild type PLCζ or differences in protein stability. Nevertheless the differences in luminescence signal between the eggs injected with split PLCζ RNA and full length PLCζ make it harder to accurately compare the functional activity of these two groups.

The proteolytic cleavage of PLCζ occurs within the X-Y linker region which may be significant because despite having an important role in $PI(4,5)P_2$ binding this region can be considered the most structurally variable between species (Nomikos *et al.* 2011c). The X-Y linker of other PLC isoforms has been found susceptible to *in vitro* proteolysis including PLCγ (Fernald *et al.* 1994) , PLCβ (Schnabel and Camps 1998) and PLCδ1 (Ellis *et al.* 1993). It is thought these fragments come together to form a functional complex which in some cases has been found to stimulate activity (Jones and Wu 2000). This is because cleavage in the X-Y linker can lead to a conformational change which allows $PI(4,5)P_2$ to gain better

access to the X and Y catalytic domain (Jones and Wu 2000). The X-Y linker of PLCζ is different from other isoform in that it is so variable and though PLCζ is universal at releasing Ca^{2+}in mammalian eggs there are strong differences in the potency of the protein in terms of Ca^{2+} releasing ability between species (Nomikos *et al.* 2014b). It is also possible that some species possess more proteolytically cleaved PLCζ compared to others. With both full length and split PLCζ marked differences in potency between mouse and human PLCζ have been recorded during all the current experiments. One explanation for this could be due to differences in the length and structure of the X-Y linker. To investigate this further the mouse and human split PLCζ RNA previously used was combined so N' terminal half of one species was co-injected with C' terminal half of the other species. Interestingly the potency of PLCζ appears to be dependent on which half of the split PLCζ originates from which species. For example, it appears that the N' terminal half of PLCζ before the proteolytic split has more influence on the potency of PLCζ than the C' terminal half. This may be because the majority of the X-Y linker is found in this half of PLCζ and in fact varies more in length between mouse and human derived PLCζ when compared to the C'terminal half. Human PLCζ is shorter in length than mouse PLCζ with a shorter X-Y linker so it is possible that this structural difference may contribute to the increased potency of human PLCζ compared to mouse (Cox *et al.* 2002; Saunders *et al.* 2002). However, when the N' terminal half of human PLCζ is co-injected with the C' terminal half of mouse PLCζ the $Ca^{2+}{}_{i}$ response recorded in the eggs is not as strong as that seen when both halves of PLCζ are human derived. This suggests that it is not just the N-terminal half of PLCζ that confers potency differences between species. Furthermore it remains to be established whether it is structural differences the X-Y linker of PLCζ that are responsible for species differences in potency or whether it is because of other differences in other structural domains of PLCζ such as the EF hand domains or the catalytic domains. Right now it remains uncertain exactly which structural features of PLCζ determine the potency of the enzyme between species. More experiments need to be conducted to establish how the functional domains of PLCζ vary between different species and what role post translational modifications, such as proteolytic cleavage, play in regulating its activity.

It is clear that the structural properties of PLCζ play a significant role in regulating its activity and all functional domains appear to be necessary in order for PLCζ to be maximally functionally active. These functional domains play independent distinct roles in conferring PLCζ's Ca^{2+}releasing ability and some domains have multiple roles in this regard. For example these experiments demonstrated a novel role for the EF hands domain separate to

conferring Ca^{2+} sensitivity. This region has been found to work in conjunction with the X-Y linker in binding to the substrate $PI(4,5)P_2$ therefore enabling the anchoring of PLCζ to this membrane bound substrate. However, it has not been established how PLCζ becomes targeted to specific pools of intracellular $PI(4,5)P_2$ and which structural properties of the enzyme are involved in this process. The current studies suggest the possibility that the C2 domain of PLCζ binding to specific membrane markers for example other phospholipids. However, it remains unclear what the significance of this domain is in terms of membrane localisation. It is likely that other protein interactions are involved in regulating the activity of PLCζ but it has still not been established what these interactions are. Likewise though proteolytic cleavage may play a role in regulating PLCζ activity the significance of this and other post translational modifications of PLCζ still need to be explored in more detail. As a result the question to why PLCζ is uniquely able to cause Ca^{2+}_i oscillations in eggs remains to be fully answered, though it is highly likely this process involves both egg cellular factors aswell as particularly structural properties of the PLCζ enzyme.

5. CHARACTERISTICS OF EGGS WHICH ENABLE CA^{2+} RELEASE BY PLCZ

Introduction

At fertilisation the ability for eggs to release Ca^{2+}in response to PLCζ is essential to ensure egg activation occurs effectively (Saunders *et al.* 2002). Though PLCs release Ca^{2+} via IP_3 in a large array of somatic cells, eggs appear to be the only cell type in which physiological levels of PLCζ are able to cause Ca^{2+} release. If PLCζ is expressed in CHO cells no Ca^{2+} release is seen, however, if these transfected cells are then injected into eggs $Ca^{2+}{}_i$ oscillations are triggered in these eggs (Phillips *et al.* 2011). This implies there is something unique about the cell biology of eggs that enables PLCζ to release Ca^{2+}. Indeed, this is a feature that seems to develop during oocyte maturation. Immature oocytes, specifically GV oocytes, do not release Ca^{2+}aseffectively as mature MII arrested eggs. Original evidence for this comes from experiments by fertilising immature hamsteroocytes. It was found that GV oocytes did not release Ca^{2+}as well as MII eggs in response to sperm. Specifically there was a reduction in frequency, duration and amplitude of these oscillations (Fujiwara *et al.* 1993). This effect has also been recorded in immature mouse oocytes (Jones *et al.* 1995b). Similar results were seen in mouse oocytes where $Ca^{2+}{}_i$ oscillations were induced using hamster derived sperm extracts. There was a 2 fold increase in size and duration of Ca^{2+} release in MII eggs compared to immature oocytes (Carroll *et al.* 1994). Furthermore the Ca^{2+} release in immature oocytes did not cross the oocyte as a wave in the same way as that seen in MII eggs (Swann *et al.* 1994). This idea was further supported by experiments using PLCζ RNA that found $Ca^{2+}{}_i$ oscillations initiated by PLCζ in GV oocytes were lower in frequency and amplitude compared to those initiated in mature eggs with the most marked difference occurring after GVBD (Wakai *et al.* 2012). These differences in $Ca^{2+}{}_i$ releasing ability have been attributed to an increase in IP_3 receptor sensitivity during oocyte maturation (Mehlmann and Kline 1994). Many factors may influence the sensitivity of the IP_3 receptor including its phosphorylation status, the amount of Ca^{2+} in the ER and the localisation of those receptors. Experiments using thapsigargin and ionomycin have shown that immature mouse oocytes contain significantly less Ca^{2+} in their stores compared to mature eggs (Jones *et al.* 1995b). In fact there is an 11 fold increase in the area under the curve for Ca^{2+} release in response to thapsigargin in mature eggs compared to immature oocytes (Tombes *et al.* 1992; Jones *et al.* 1995b). This change in luminal ER Ca^{2+} concentration directly affects the IP_3R sensitivity (Missiaen *et al.* 1992). In addition to an

increase in ER Ca^{2+} levels, oocyte maturation is also correlated with an increase in the number of IP_3R of approximately 2 fold (Mehlmann *et al.* 1996). In addition to this the IP_3R appear to become phosphorylated during oocyte maturation (Wakai *et al.* 2012). Collectively these changes are believed to increase the sensitivity of the IP_3R and therefore increase Ca^{2+} release. However, it is not yet known what role components upstream of IP_3R play in enabling Ca^{2+} release during oocyte maturation, for example the availability of $PI(4,5)P_2$.

One significant difference between mature eggs, immature oocytes and somatic cells is their point in the cell cycle. Somatic cells spend the majority of the time in interphase and only enter mitosis when they divide. Oocytes begin the process of oogenesis as oogonia which are very much like somatic cells however as they differentiate into oocytes these cells enter meiosis. GV oocytes become arrested in interphase of the first round of meiosis however during oocyte maturation they resume meiosis I and eventually become arrested in metaphase of meiosis II as mature eggs. This means that GV oocytes, maturing oocytes and eggs are at different phases of the meiotic cell cycle and Ca^{2+}_i releasing ability seems to be dependent on what point of the cell cycle thes oocutes are in. When fertilised eggs form pronuclei and enter interphase sperm induced Ca^{2+}_i oscillations cease however if these eggs are prevented from exiting metaphase Ca^{2+}_i oscillations persist for many hours (Jones *et al.* 1995a). Furthermore if MI oocytes are induced to enter interphase they fail to oscillate. However, if these oocytes then undergo nuclear envelope breakdown and renter metaphase Ca^{2+}_i oscillations return (Jones *et al.* 1995a; Kono *et al.* 1996). Interestingly when zygotes enter their first mitotic division and the nuclear envelope breaks down Ca^{2+}_i transients return but this effect is not seen in embryos activated by strontium chloride (Kono *et al.* 1996). If karyoplast are taken from these fertilised eggs or 2 cell embryos and microinjected into unfertilised eggs Ca^{2+}_i oscillations can be recorded in these eggs (Kono *et al.* 1995). This effect can be seen using karyoplasts from embryos produced by fertilisation and those parthenogenetically activated with sperm extract suggesting that a Ca^{2+}releasing factor becomes associated with the nucleus at interphase (Kono *et al.* 1995). It has been shown that fluorescent tagged PLCζ becomes localised to the pronucleus when Ca^{2+}_i oscillations cease (Larman *et al.* 2004; Yoda *et al.* 2004). This suggests that PLCζ is not able to cause Ca^{2+}release during interphase due to nuclear localisation, however when this nuclear envelope breaks down PLCζ is free to cause Ca^{2+}release (Larman *et al.* 2004).

One reason why PLCζ is unable to cause Ca^{2+}_i oscillations whilst sequestered in the nucleus could be the inaccessibility to its substrate $PI(4,5)P_2$. All other PLCs translocate to the

plasma membrane on activation (Suh *et al.* 2008). This is because in somatic cells the majority of $PI(4,5)P_2$ in the cell is found at the plasma membrane (Watt *et al.* 2002). However, PLCζ does not appear to hydrolyse this source of $PI(4,5)P_2$ under physiological conditions (Yu *et al.* 2012). Fluorescently tagged versions of PLCζ do not localize in the plasma membrane (Yoda *et al.* 2004; Ito *et al.* 2008; Phillips *et al.* 2011). In addition PLCζ does not cause DAG production at the plasma membrane during Ca^{2+}_i oscillations however PLCδ1 does (Yu *et al.* 2008). Furthermore no change in plasma membrane $PI(4,5)P_2$ are recorded during PLCζ activity and depleting plasma membrane $PI(4,5)P_2$ has no effect on Ca^{2+}_i recorded at fertilisation (Yu *et al.* 2012). However, the opposite is true for PLCδ1 induced Ca^{2+}_i oscillations (Yu *et al.* 2012). This suggests that PLCζ is hydrolysing an intracellular source of $PI(4,5)P_2$ different to other PLCs. There is evidence using immunostaining that this $PI(4,5)P_2$ is found on small vesicles in the cytoplasm in mature mouse (Yu *et al.* 2012). This is significant as the availability of a vesicular source of $PI(4,5)P_2$ and sufficient targeting of PLCζ to it may be necessary for effective Ca^{2+} release in mature eggs. Previous evidence using immunocytochemistry has shown that significantly less vesicular $PI(4,5)P_2$ is present in GV oocytes and instead most $PI(4,5)P_2$ is found to be associated with the plasma membrane and the nucleus (Yu *et al.* 2012).

All previous measures of intracellular $PI(4,5)P_2$ in mouse eggs have been carried out on fixed cells using immunostaining. One reason for this is the lack of specific probes to measure $PI(4,5)P_2$ in live cells. Though $PI(4,5)P_2$ and its parent derivative PI(4)P are the most prevalent phosphoinositides in cells, phosphoinositides collectively only make up approximately 5% of all phospholipids in the cell (Di Paolo and De Camilli 2006). There is therefore a challenge in distinguishing between small quantities of specific phosphoinositides in the cell. PH domain based probes have routinely been used to measure $PI(4,5)P_2$ at the plasma membrane (Balla 2007; Yoon *et al.* 2011). The PH domain of PLCδ1 binds to both $PI(4,5)P_2$ and IP_3 so if this domain is tagged with a fluorescent protein such as GFP, a fluorescent $PI(4,5)P_2$ probe can be developed (Lemmon *et al.* 1995; Holz *et al.* 2000). In somatic cells these probes have shown that in somatic cells the majority of $PI(4,5)P_2$ is present in the plasma membrane (Watt *et al.* 2002). PH domain probes have also successfully been used to detect changes in $PI(4,5)P_2$ levels at the plasma membrane in response to agonist stimulation (Szentpetery *et al.* 2009). Interestingly GFP-PH domain was also found to localise to the plasma membrane in eggs (Halet *et al.* 2002; Chun *et al.* 2010). Despite this no change in signal was seen in response to PLCζ or fertilisation (Yu *et al.* 2012). However, PH probes are not cell permeable so even if they are

transfected or injected into cells it may be difficult for the probes to access the $PI(4,5)P_2$ located on the inner leaflet of intracellular organelles (Mondal *et al.* 2016).

Recently some alternative probes have been developed that allow $PI(4,5)P_2$ to be measured in live cells dynamically using a radiometric method. These probes are based on the actin regulating protein gelsolin (Yin and Stossel 1979). Gelsolin has two $PI(4,5)P_2$ binding domains located on short polypeptides (Janmey *et al.* 1987; Janmey and Stossel 1987). The binding properties of gelsolin peptides have been examined *in vitro* and a 20 amino acid sequence of gelsolin (residues 150-169) has been shown to bind both PI(4)P and $PI(4,5)P_2$ however with an increased selectivity for $PI(4,5)P_2$ (Janmey *et al.* 1992). Residues 160-169 contain multiple cationic side chains which bind to the negatively charged headgroup of $PI(4,5)P_2$ by electrostatic interactions (Liepina *et al.* 2003). This 20 amino acid sequence was tagged with the polarity sensitive fluorophore 2-dimethylamino-6-acyl-naphthalene (DAN) (Mondal *et al.* 2016). DAN undergoes a significant 60-70 nm shift from a green emission to a blue emission signal following binding to membrane bound phosphoinositides (Cicchetti *et al.* 2004). This can then be conjugated to proteins by attaching it to a cysteine (Prendergast *et al.* 1983). To create a DAN tagged gelsolin based peptide the Val 159 residue of this 20 amino acid gelsolin peptide was replaced with a cysteine to allow attachment of DAN (Mondal *et al.* 2016). This produced a $PI(4,5)P_2$ probe called DAN20 that responds in a ratiometic way to dynamic changes in $PI(4,5)P_2$ (Mondal *et al.* 2016). DAN20 has been shown to bind SUVs containing PC, $PI(4,5)P_2$ and PI(4)P with a shift in blue signal following an increase in the amount of $PI(4,5)P_2$ (Mondal *et al.* 2016). This probe is sensitive enough to detect low level $PI(4,5)P_2$ in vesicles down to 2% of membrane constituents. DAN20 has also been able used to measure $PI(4,5)P_2$ in live HEK293T cells. As to be expected the greatest intensity in blue signal was detected at the plasma membrane indicating the greatest amount of $PI(4,5)P_2$ present here (Mondal *et al.* 2016). A significant blue signal was also detected in the perinuclear region suggesting that there is also an alternative pool of intracellular $PI(4,5)P_2$ (Mondal *et al.* 2016). Other shorter peptides of gelsolin have also been used as probes for measuring $PI(4,5)P_2$ for example the 10 residue peptide PBP10 is labelled with the fluorophore rhodamine-B which produces a bright and stable red signal (Bucki *et al.* 2001; Cunningham *et al.* 2001). As this peptide is shorter it more readily permeates cell membranes.PBP10 has been shown to successfully bind $PI(4,5)P_2$ *in vitro* and somatic cells (Bucki *et al.* 2001; Cunningham *et al.* 2001). None of these gelsolin based probes have been used to measure the distribution or amount of

$PI(4,5)P_2$ in the eggs or oocytes. Furthermore the availability and role of vesicular $PI(4,5)P_2$ in Ca^{2+} release at fertilisation is yet to resolved in detail.

It also remains unclear what the origin of these $PI(4,5)P_2$ containing vesicles are. Usually particular phosphoinositides are associated with particular membrane compartments and help convey the specificity of that membrane (De Matteis and Godi 2004). For example PI(3)P is usually associated with endocytic membranes such as endosomes (Mills *et al.* 2001; Dale 2012). Fluorescent FYVE domain that bind to PI(3)P has been in expressed in eggs and shows binding to large vesicular structures that are most concentrated in the cortex (Stenmark *et al.* 2002; Dale 2012)(Moon and Swann 2013 unpublished). However, marking for these vesicles using immunostaining against Rab 5 and immunostaining for PLCζ do not overlap (Yu and Swann 2013 unpublished)

Though $PI(4,5)P_2$ is usually associated with the plasma membrane it also found in small quantities in the Golgi apparatus (Godi *et al.* 1999; Tan *et al.* 2015). The Golgi also contains PI(4)P which is the precursor for $PI(4,5)P_2$ (Godi *et al.* 1999; De Matteis and D'Angelo 2007). Previous data has shown that there is a strong evidence that PLCζ could hydrolyse $PI(4,5)P_2$ localised to the Golgi apparatus. Immunocytochemistry shows that the vesicles that PLCζ is bound to overlap with staining for Golgi marker giantin and $PI(4,5)P_2$ (Yu and Swann 2012 unpublished).

In somatic cells the Golgi takes the form of stack like structures. However, there is evidence that the Golgi apparatus is restructured during oocyte maturation and takes a different form in eggs. In GV oocytes the Golgi appears as small stacks termed “mini-Golgi” (Payne and Schatten 2003). During germinal vesicle breakdown these structures get fragmented into vesicles which spread outwards from the centre of the egg during meiosis I (Moreno *et al.* 2002; Payne and Schatten 2003). This feature appears to be cell cycle dependent because a similar process is seen during mitosis of somatic cells. The Golgi fragments into very small vesicles called the “Golgi haze” which is a very transient condition (Altan-Bonnet *et al.* 2003; Axelsson and Warren 2004). Unlike somatic cells, eggs are maintained in the M phase of the meiotic cell cycle and therefore these Golgi fragments persist much longer than would be seen during mitosis. It remains unclear what role Golgi fragmentation plays on enabling mature eggs to release Ca^{2+} in response to sperm.

Another possible origin of $PI(4,5)P_2$ containing vesicles is the ER. In fact fluorescent Venus tagged human derived PLCζ cRNA is found to overlap with the ER marker DsRed-ER cRNA in mouse eggs (Escoffier *et al.* 2015a). The ER also contains the parent phospholipid PI which

is required for the synthesis of if all phosphoinositides including $PI(4,5)P_2$ (van Golde *et al.* 1974). Like the Golgi apparatus the ER alters significantly in distribution during oocyte maturation. In a GV oocytes the ER is continuous with the nuclear envelope however after GVBD the ER forms a ring around the spindle apparatus (FitzHarris *et al.* 2007). In MII eggs the ER appears as cortical clusters which are packed close to mitochondria (FitzHarris *et al.* 2007). As the ER acts as the Ca^{2+} store in eggs it has been suggested that this re-distribution may be involved in enabling effective Ca^{2+} release in eggs**.**

It is likely that the cytoplasmic features of eggs play a role enabling PLCζ to release Ca^{2+}at fertilisation. Furthermore these features are likely to be acquired during oocyte maturation in a cell cycle dependent manner. It still remains very unclear however precisely what these features are and specifically what impact they have on PLCζ induced $Ca^{2+}{}_i$ oscillations.

Aims

The aim of the current study is to investigate the particular cytoplasmic features of eggs that may enable PLCζ induced $Ca^{2+}{}_i$ oscillations. Specifically this involves determining the localisation of PLCζ, and the potential sources of intracellular $PI(4,5)P_2$. The identity of these vesicles will then be explored with particular focus on the distribution of the Golgi apparatus and ER in eggs. The functional effect of disrupting $PI(4,5)P_2$ metabolism or availability will also be examined. Finally the differences in the PLCζ induced $Ca^{2+}{}_i$ releasing ability at different stages of oocyte maturation will be quantified with the distribution of Golgi apparatus and intracellular $PI(4,5)P_2$ being compared to that of mature eggs.

Results

PLCζ localises to intracellular vesicles in eggs

Immuno-staining confirms PLCζ localises to intracellular vesicles in the egg

There is evidence that PLCζ hydrolyses an intracellular vesicular source of $PI(4,5)P_2$ instead of plasma membrane derived $PI(4,5)P_2$ (Yu *et al.* 2008; Yu *et al.* 2012).
Immunocytochemistry of cMyc tagged PLCζ has previously shown that PLCζ localises to small vesicles in mature eggs (Yu *et al.* 2012). However, this data is yet to be reproduced so in order to verify this result the experiment was repeated. As few specific antibodies against PLCζ exist, mouse PLCζ DNA was tagged with a cMyc sequence and synthesised into cRNA that could be microinjected into eggs and endogenously expressed. This allows antibodies against cmyc to be used to look at the localisation of PLCζ. This cMyc-mPLCζ cRNA was microinjected into eggs at a concentration of 0.01 μg/μl and left to express for 3

hours. Injected eggs were then fixed using 4% paraformaldehyde and permeabilised using 0.1 % triton prior to incubation in the mouse Ig blocking reagent then 10% BSA as a blocking agent. The eggs were then incubated in mouse derived anti- cMyc (epitope) antibody for 1 hour followed by a secondary anti-mouse fluorescent Alexa (488) antibody for 45 minutes. The eggs were washed through clean media and imaged on a confocal microscope. Fluorescent staining appeared concentrated on small vesicles distributed throughout the cytoplasm, particularly concentrated in the cortical region however with no significant fluorescence signal at the plasma membrane (see fig 5.1). This would suggest that PLCζ localises on intracellular vesicles and does not bind to the plasma membrane.

It has previously been shown that mPLCζ contains a nuclear targeting sequence which localises PLCζ to the nucleus following pronuclei formation possibly leading to the cessation of the $Ca^{2+}{}_{i}$ oscillations (Yoda *et al.* 2004). To establish whether this effect could be verified using immunostaining the protocol above was repeated however the eggs were left to express the cMyc-mPLCζ cRNA for 5 hours prior to fixation. This ensured the eggs had become fully parthenogenetically activated including the formation of pronuclei. When these activated eggs were imaged on the confocal microscope some fluorescence was concentrated on small vesicles throughout the cytoplasm however the strongest fluorescent signal came from the pronucleus which appeared to contain a lot of cMyc-mPLCζ (see fig 5.1). This suggests as previously described cMyc-mPLCζ becomes localised to the nucleus following pronuclear formation at egg activation. However, the fact that some PLCζ is still present in the cytoplasm suggests that not all the PLCζ is sequestered in the nucleus.

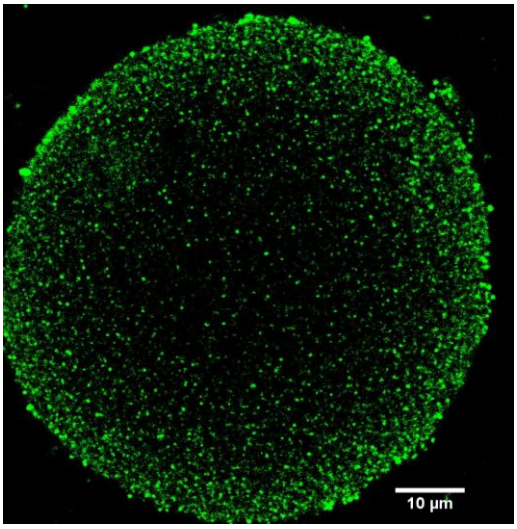
Fluorescently tagged PLCζ does not localise specifically in eggs

In order to carry out immunostaining it is necessary to first fix the cells. Therefore, to look at the localisation of PLCζ in live cells it is necessary to develop a genetically encoded probe that may be expressed in eggs. However, as PLCζ hydrolyses $PI(4,5)P_2$ and releases $Ca^{2+}$the localisation of PLCζ may alter. To avoid this a probe was developed based on a mutated catalytically inactive version of the mouse PLCζ gene ($mPLC\zeta^{D210}$). This sequence was then tagged with fluorescent mCherry to produce a $mPLC\zeta^{D210}$- mCherry (by MN). This fusion protein was then synthesised into cRNA so it could expressed in live eggs and fluorescently imaged to determine the localisation of this PLCζ. This RNA was injected into live eggs at a pipette concentration of 0.5 μg/μl and left to be expressed into protein for approximately 3 hours. The eggs were then imaged on a confocal microscope to look at localisation of the fluorescent probe. The eggs emitted a red fluorescence indicating that the $PLC\zeta^{D210}$-

mCherry RNA was expressed by the eggs. However the localisation of the mPLCζ^{D210}-mCherry was uniform throughout the cytoplasm and did not appear to localise to any particular membrane. The fluorescent signal emitted by PLCζ^{D210}- mCherry was very weak despite the high concentrations of cRNA injected (see fig 5.2). To try and increase the fluorescent signal emitted the mPLCζ^{D210}gene was tagged with an alternative fluorescent tag called tdTomato which is known to produce a very bright signal (Morris *et al.* 2010). mPLCζ^{D210}– tdTomato cRNA was synthesised and microinjected into eggs at a pipette concentration of 1.7 µg/µl and left to translate into protein for approximately 3 hours. The eggs were then imaged on a confocal imaging system to determine the distribution of mPLCζ^{D210}– tdTomato. The fluorescent signal emitted by the eggs was extremely low and not visible using the confocal microscope and therefore it was not possible to determine the localisation of the protein. This suggests thatmPLCζ^{D210}– tdTomato construct was not successfully translated into protein by the eggs.

c-Myc PLCζ immuno-staining

MII egg



PN

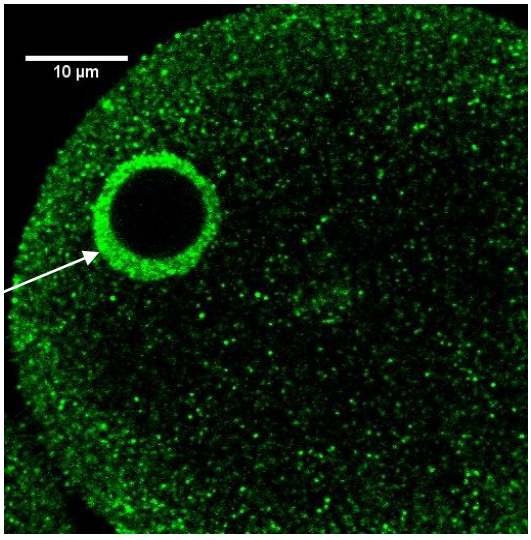


Figure 5.1 Confocal image showing the immunostaining of cMyc-PLCζ in an MII egg

3 hours post injection of c-Myc-PLCζ cRNA (pipette concentration of 0.01 µg/µl, n=12) and a fully activated egg (PN) 5 hours post-injection of c-Myc-PLCζ cRNA (pipette concentration of 0.01 µg/µl, n=6)

PLCζ^{D210}- mCherry

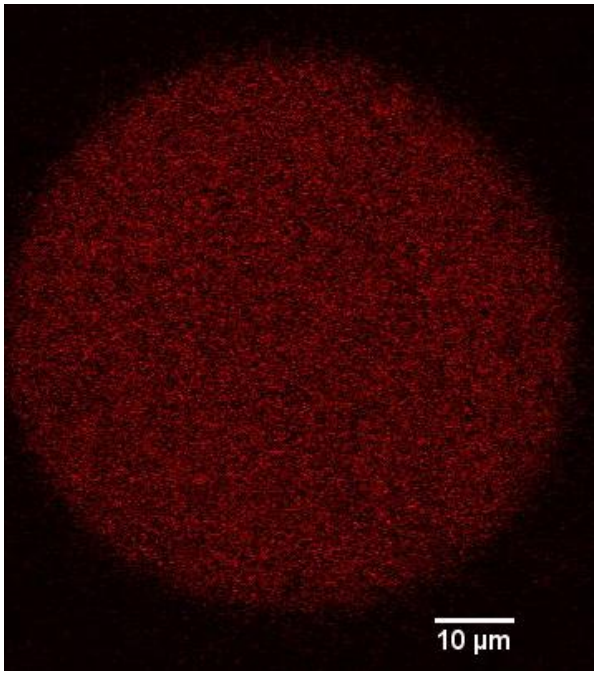


Figure 5.2 Confocal image showing distribution of PLCζ ^{D210}- mCherry in an egg

MII egg 3 hours post injection of PLCζ^{D210} - mCherry cRNA (pipette concentration of 0.5 µg/µl, n=8)

Intracellular vesicles in eggs contain $PI(4,5)P_2$

Immuno-staining shows that $PI(4,5)P_2$ is found on intracellular vesicles

Given the evidence that PLCζ does not hydrolyse plasma membrane $PI(4,5)P_2$ and that PLCζ localises to intracellular membranes it is likely that these vesicles contain $PI(4,5)P_2$ (Yu *et al.* 2012). A vesicular, intracellular source of $PI(4,5)P_2$ has previously been identified in eggs by immunostaining using antibodies raised against $PI(4,5)P_2$ (Yu *et al.* 2012). Furthermore, these $PI(4,5)P_2$ positive vesicles appeared to co-localise with cMyc tagged PLCζ (Yu and Swann 2012, unpublished). To verify the endogenous $PI(4,5)P_2$ localisation in eggs immuno-staining was performed using antibodies raised against $PI(4,5)P_2$. The same immuno-staining protocol was used as that for cMyc-PLCζ but mouse monoclonal anti-$PI(4,5)P_2$ was used as a primary antibody instead of the anti-cMyc antibody. These eggs were then imaged using the confocal microscope to determine the localisation of these antibodies. Some fluorescent vesicles were visible however there was also a very large amount of diffuse fluorescence throughout the cytoplasm (fig 5.3). A greater amount of vesicles were found in the cortical region of the egg however it was difficult to ascertain whether specific plasma membrane staining was apparent. This suggests there is some endogenous vesicular $PI(4,5)P_2$ in eggs however there was a large amount of background fluorescent staining which made it difficult to resolve these vesicles accurately. Furthermore, there was a large degree of variability in staining between eggs and across repeats.

PI(4,5)P_2 immunostaining

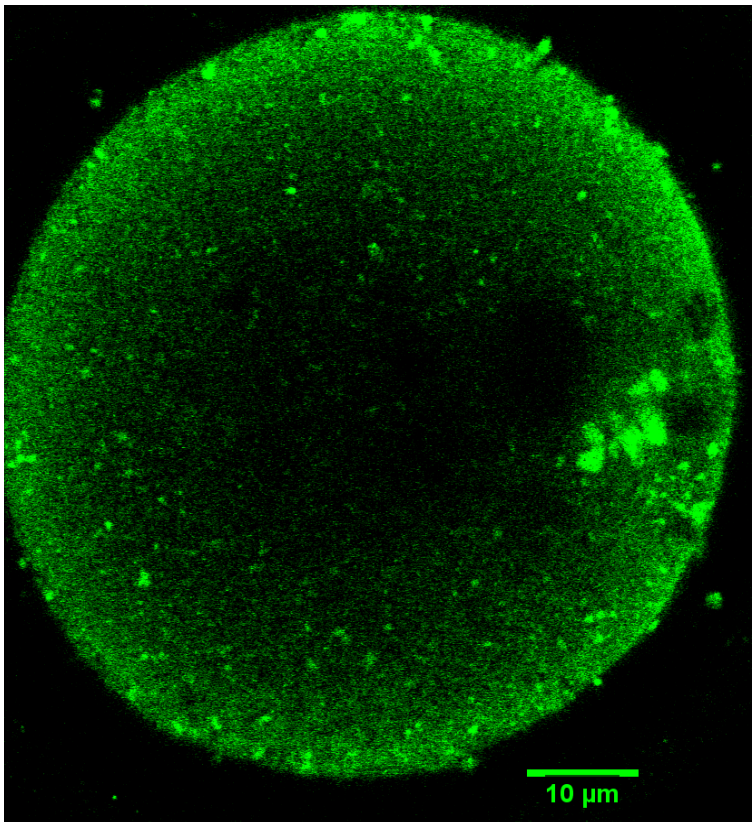


Figure 5.3 Confocal image showing immunostaining of PI(4,5)P_2 in an MII egg

Immunostaining against endogenous PI(4,5)P_2 in an MII egg (n=10)

Fluorescent PI(4,5)P_2 localises to intracellular vesicles in eggs

As the immunostaining was not able to quantitatively measure PI(4,5)P_2 in live cells and because of the difficulties in obtaining consistent results free of background signal, an alternative way to verify whether a vesicular source of PI(4,5)P_2 is present in eggs was required. For this a fluorescent BODIPY-FL tagged version of PI(4,5)P_2 (FL-PI(4,5)P_2) was microinjected into eggs at a pipette concentration of 1 mM in KCL HEPES and the localisation was observed on a confocal microscope (see fig 5.4). This fluorescent tagged PI(4,5)P_2 has previously been used to measure PI(4,5)P_2 distribution in somatic cells (Golebiewska *et al.* 2008). In FL-PI(4,5)P_2 the BODIPY fluorophore is located on one of the acyl chains within the synthetic PI(4,5)P_2. Within the first 30 minutes post injection FL-PI(4,5)P_2 this staining appeared to localize within multiple internal membranes but mainly within reticular structures. With time the fluorescence reduces significantly in these compartments and small fluorescent vesicles began to become visible. Between 45 minutes and 1.5 hours post injection virtually all reticular staining disappears and the FL-PI(4,5)P_2 appears only to be bound to small vesicles. These vesicles were diffuse throughout the cytoplasm but very little fluorescent staining of the plasma membrane was evident. This supports the notion that a vesicular intracellular source of PI(4,5)P_2 is present in eggs. Furthermore as the FL-PI(4,5)P_2 partitions with time this suggests that PI(4,5)P_2 becomes anchored to small intracellular vesicles and is excluded from other membranes and plasma membrane (see fig 5.5).

To determine if the distribution of FL-PI(4,5)P_2 was disrupted by PLCζ activity a group eggs were injected with mPLCζ cRNA(pipette concentration of 0.02 µg/µl) and left for half an hour to allow for expression of PLCζ protein. These eggs were then injected with FL-PI(4,5)P_2 in the same manner as described previously alongside control eggs that were not pre-injected with mPLCζ cRNA. After 45 minutes these eggs were imaged on the confocal microscope so the distribution of FL-PI(4,5)P_2 could be visualised. Those eggs injected with mPLCζ cRNA showed a more diffuse and less vesicular distribution of fluorescence compared to PLCζ-free control eggs. All the eggs were then left for a further 45 minutes before being imaged again on the confocal microscope. In the eggs injected with PLCζ there was an even further reduction in fluorescence signal and by this stage no vesicular staining was seen in these eggs. However, after this time the control eggs that had not been injected with PLCζ still showed strong vesicular fluorescence staining (fig 5.6). This shows that FL- PI(4,5)P_2 distribution is disrupted and reduced by PLCζ activity suggesting that PLCζ is affecting the metabolism of the vesicular FL-PI(4,5)P_2 .

BODIPY-FL PI(4,5)P_2 Structure

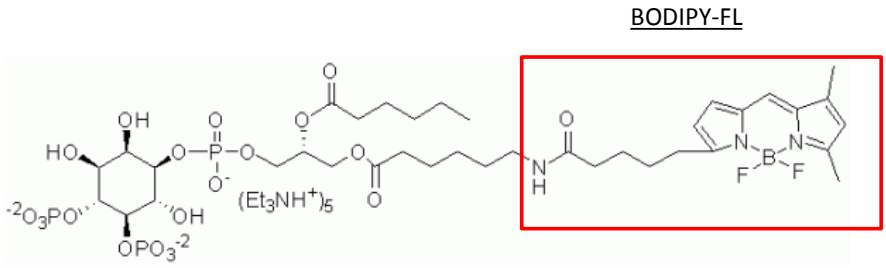


Figure 5.4 Schematic showing the chemical structure of BODIPY-FL PI(4,5)P_2

(taken from echelon-inc.com)

Time course of **BODIPY-FL PI(4,5)P_2 in MII eggs**

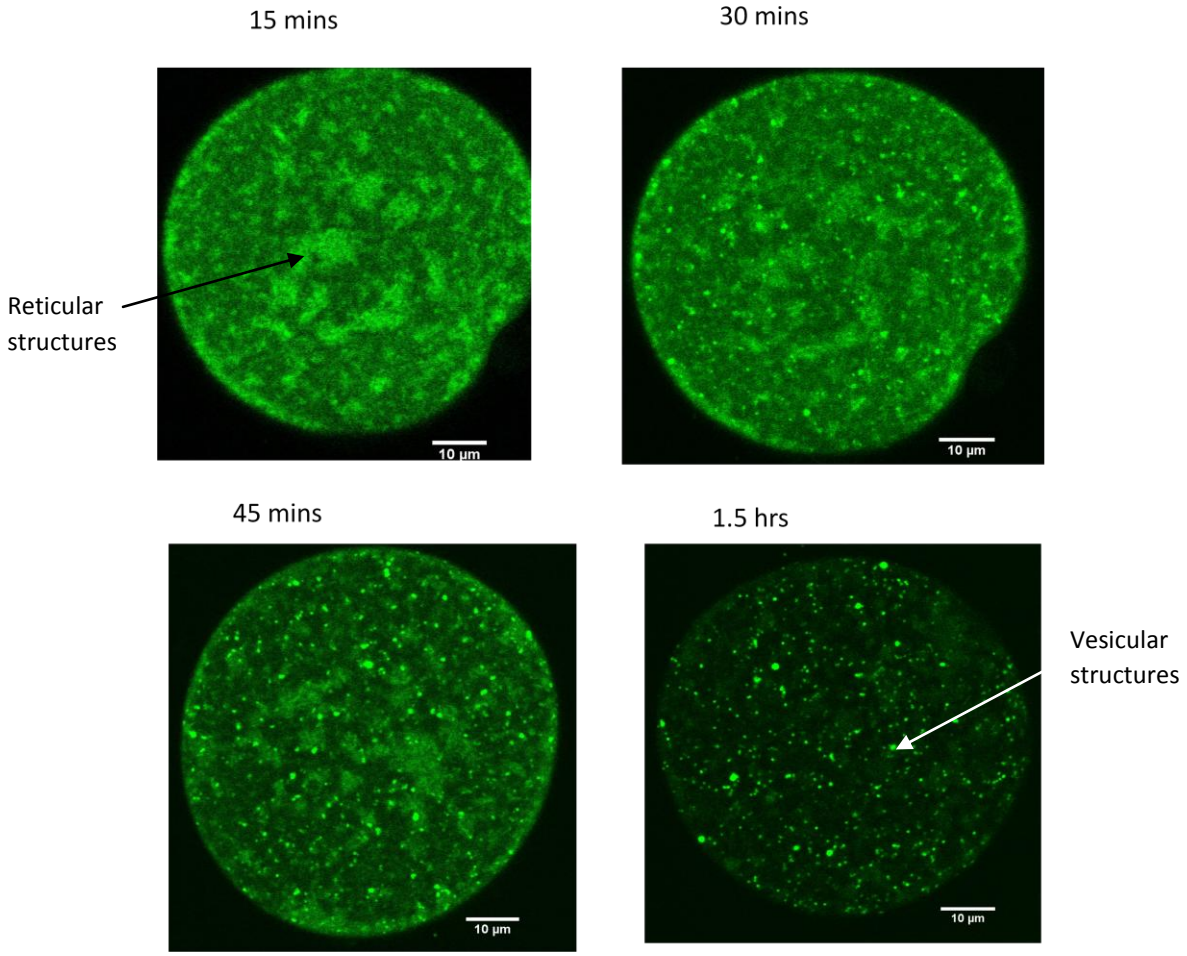


Figure 5.5: Confocal images showing the distribution of BODIPY-FL PI(4,5)P_2 in MII eggs

BODIPY-FL PI(4,5)P_2 (pipette concentration=1mM) in MII eggs at different time points post injection (n=7)

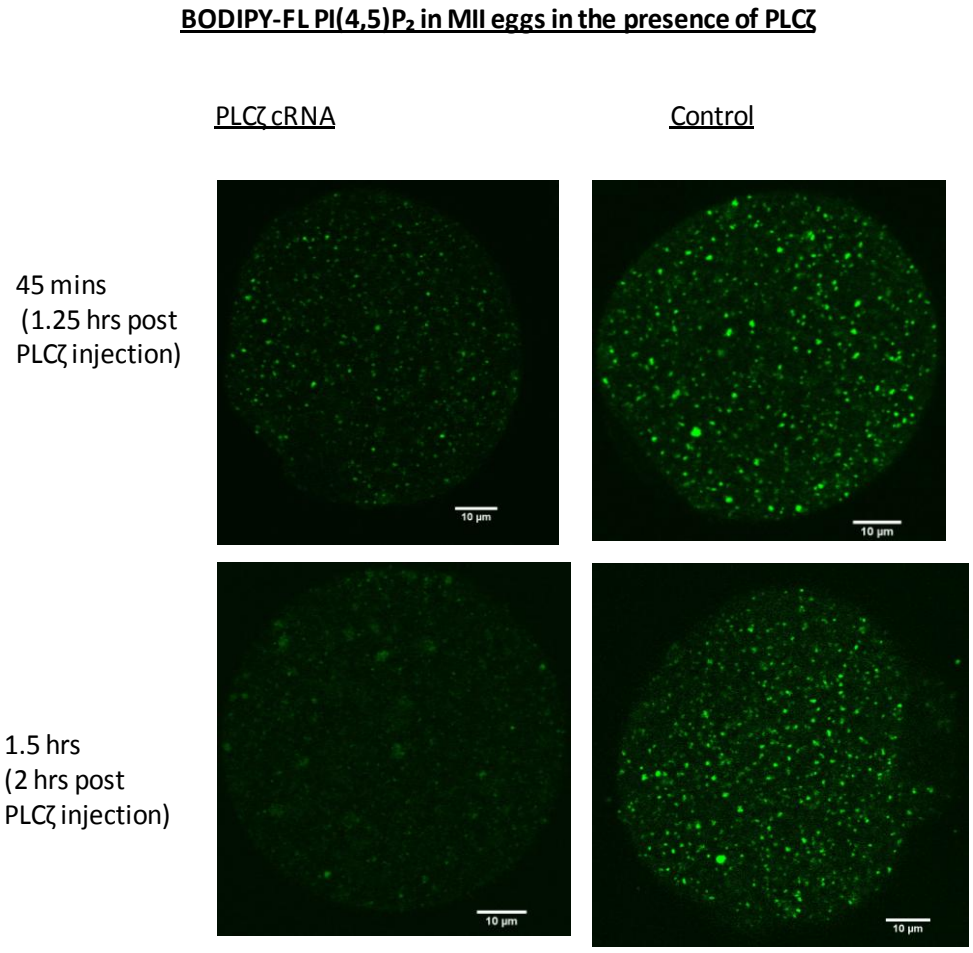


Figure 5.6 Confocal images showing how the distribution of BODIPY-FL PI(4,5)P_2 is affected by PLCζ

The distribution of BODIPY-FL PI(4,5)P_2 in MII eggs either injected with PLCζ cRNA (pipette concentration 0.04 µg/µl, n=7) or not injected with PLCζ cRNA (n=6) Images show eggs 45 minutes and 1.5 hours post BODIPY-FL PI(4,5)P_2 injection

The DAN20 PI(4,5)P_2 probe shows that PI(4,5)P_2 is localised to intracellular vesicles in eggs

Though fluorescent PI(4,5)P_2 provided a reliable and simple method to determining where exogenous PI(4,5)P_2 becomes localised in live eggs it does not allow endogenous PI(4,5)P_2 to be measured. As a result a live cell probe that bound to endogenous PI(4,5)P_2 was required to verify this vesicular localisation of PI(4,5)P_2 . The ratiometric fluorescent DAN20 probe has previously been used to measure PI(4,5)P_2 in live somatic cells (Mondal *et al.* 2016) (see fig 5.7). This probe is based on a gelsolin peptide that binds to PI(4,5)P_2 and therefore is able to measure PI(4,5)P_2 in live cells dynamically. To determine whether DAN20 could be used to effectively measure PI(4,5)P_2 distribution in eggs, DAN20 was injected into eggs at a concentration of 270 μM diluted in KCL HEPES (experiments conducted with the help of Sophie Davies). Approximately 1 hour after injection these eggs were imaged on the confocal microscope. Light emitted from the blue and green channels were measured simultaneously. In both channels however DAN20 appeared to bind small vesicles diffuse throughout the cytoplasm. These vesicles looked similar in size and distribution to that FL-PI(4,5)P_2 bound. Some larger vesicular structures were also apparent which looked different to that seen with the other PI(4,5)P_2 markers (see fig 5.8). No obvious plasma membrane staining in eggs was detected using DAN 20. This suggests that DAN20 is an effective probe to measure intracellular pools of PI(4,5)P_2 in live eggs. Unfortunately, the DAN20 signal was found to bleach quickly and after approximately 1 and a half hours post injection only a weak signal was recorded.

DAN20 Structure

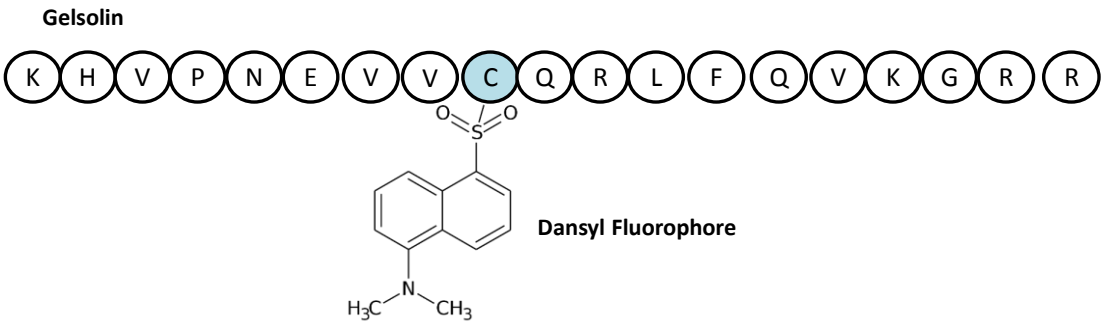


Figure 5.7 Schematic showing the structure of the PI(4,5)P₂ probe DAN20

DAN20 (PI(4,5)P₂ probe) in MII egg

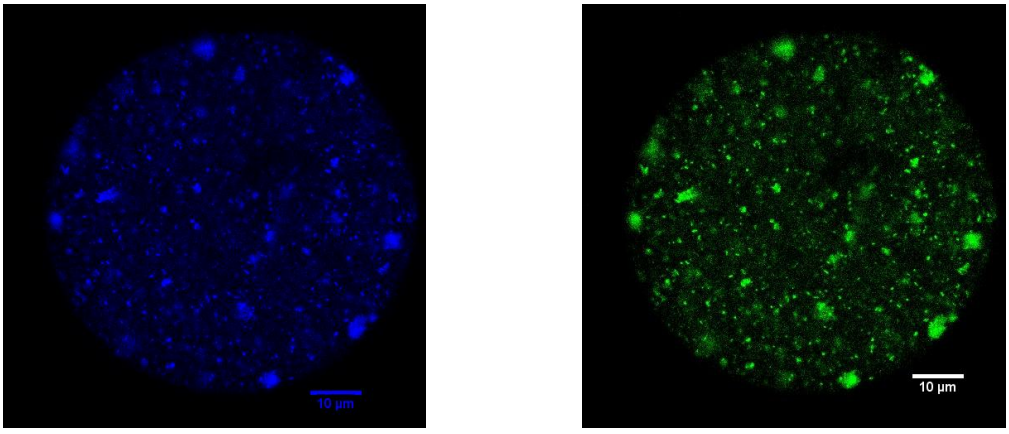


Figure 5.8 Confocal image showing the distribution of DAN20 in an MII egg

DAN20 1 hour post injection in an MII egg. Signal emitted from the green and blue channel shown (n=17)

The PI(4,5)P_2 probe PBP10 shows that PI(4,5)P_2 is localised to intracellular vesicles in eggs

PBP10 is another live cell PI(4,5)P_2 peptide that is based on the polyphosphoinositide binding site of gelsolin however in this case is conjugated to fluorescent rhodamine B (Bucki *et al.* 2001; Cunningham *et al.* 2001). This means that this probe emits red fluorescent light and could be considered slightly more stable and less environmentally sensitive than DAN20. As a result the localisation of PBP10 was used as an alternative marker for intracellular PI(4,5)P_2. PBP10 at a concentration of 1 mM mixed with KCL HEPES was injected into eggs and approximately 1 hour post injection the distribution of PBP10 was examined using a confocal microscope (experiments conducted with the help of Sophie Davies). PBP10 was once again found to localise primarily to small vesicles throughout the cytoplasm similar to that seen by DAN20 and FL-PI(4,5)P_2 . Unlike DAN20, PBP10 did not localise to larger vesicles or any other membrane structures (see fig 5.9). Furthermore PBP10 did not appear to bind to the plasma membrane. This provides further support to the idea that an intracellular vesicular source of PI(4,5)P_2 is present in eggs and has the potential to provide the alternative pool of PI(4,5)P_2 hydrolysed by PLCζ.

PBP10 (PI(4,5)P_2 probe) in MII egg

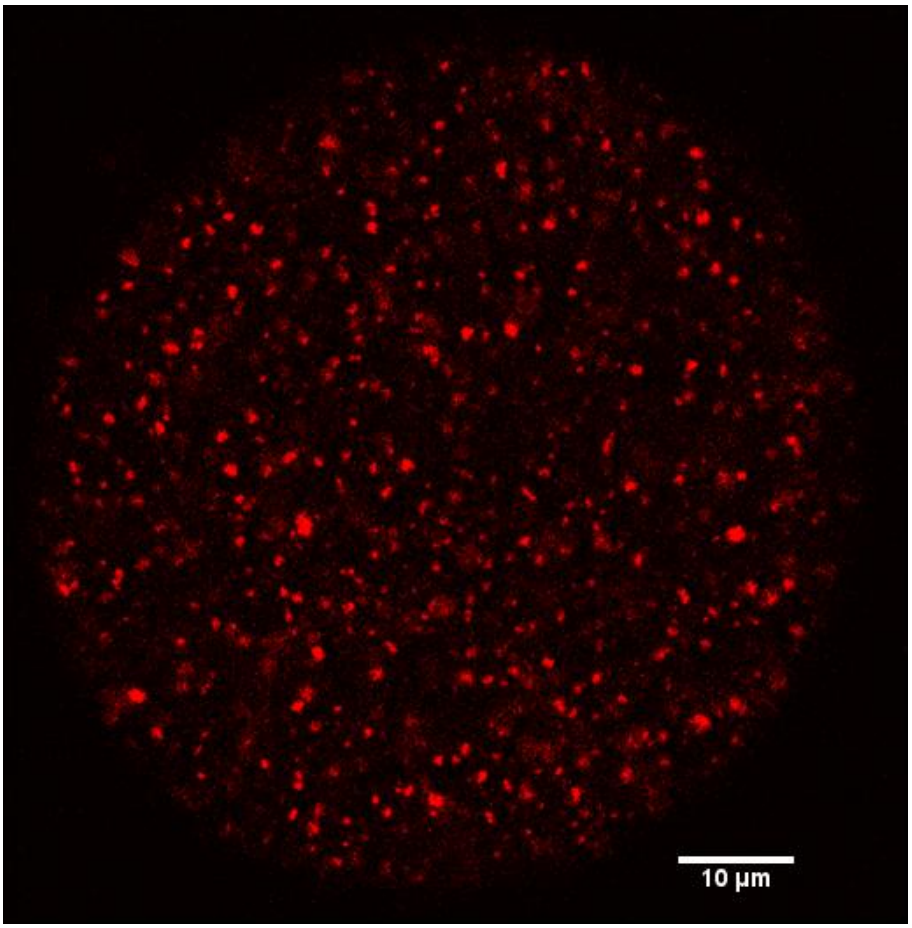


Figure 5.9 Confocal image showing the distribution of PBP10 in an MII egg

PBP10 1 hour post injection in an MII egg (n=9)

The distribution of the Golgi apparatus and endoplasmic reticulum in eggs

The dye $DiIC_{18}(3)$ labels the endoplasmic reticulum in live eggs and does not localise to intracellular vesicles

Through there is evidence indicating that PLCζ localises to intracellular vesicles in the egg the origin or identity of these vesicles remains to be established. These vesicles are very likely to contain the vesicular source of $PI(4,5)P_2$ that is hydrolysed by PLCζ to initiate Ca^{2+} release. Given studies on somatic cells, it is most likely that these $PI(4,5)P_2$ containing vesicles originate either from the ER or the Golgi. It has previously been suggested that PLCζ could bind to or associate with the endoplasmic reticulum in eggs. Venus tagged PLCζ was found to co-localise with the ER marker DsRed-ER (Escoffier *et al.* 2015a). Furthermore in HEK293T cell lines PLCζ appears to bind to cytoplasmic structures associated with the reticular network (Kashir *et al.* 2011) . It was therefore important to determine the localisation and structural appearance of the ER so this could be compared to the distribution of PLCζ and $PI(4,5)P_2$. The ER dye $DiIC_{18}(3)$ is a well-established marker for the ER (see fig 5.10) and has previously been microinjected into eggs (FitzHarris *et al.* 2007). In order to micro-inject $DiIC_{18}(3)$ it must first be dissolved in soybean oil. This $DiIC_{18}(3)$ in soybean oil is then microinjected direct into eggs using a very high pressure of around 40 psi. After approximately 30 minutes post injection the $DiIC_{18}(3)$ became partitioned into the ER and was imaged on a confocal microscope. $DiIC_{18}(3)$marked reticular structures throughout the egg cytoplasm as seen previously and therefore is likely to specifically mark the ER (Fitzharris *et al* 2007) (see fig 5.11). This data confirms that staining of the endoplasmic reticulum is very different the vesicular distribution of PLCζ and $PI(4,5)P_2$.

DiIC$_{18}$(3)

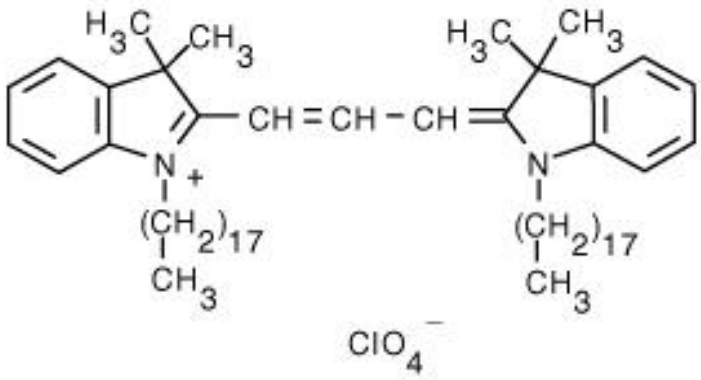


Figure 5.10 Schematic showing the chemical structure of DiIC$_{18}$(3)

(taken from thermofisher.com)

DiIC$_{18}$(3) (ER marker) in MII eggs

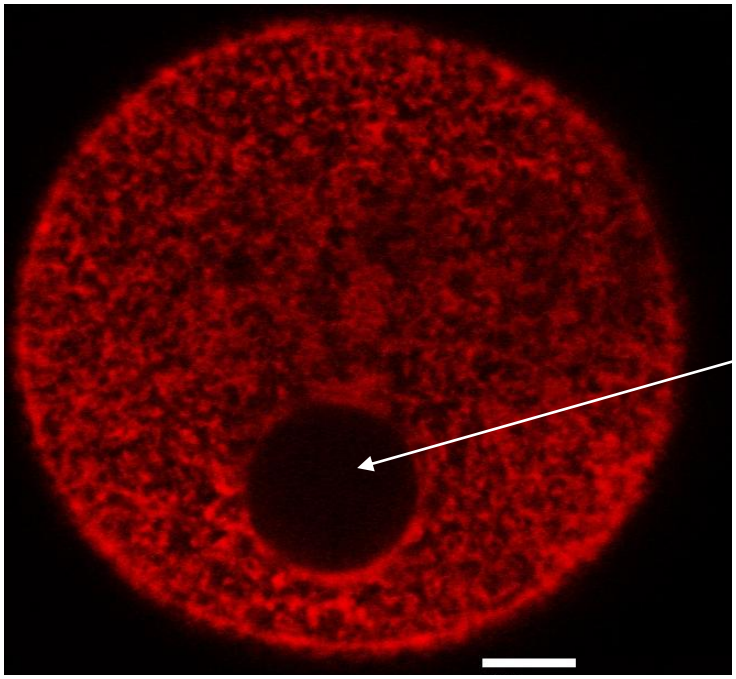


Figure 5.11 Confocal image of DiIC$_{18}$(3) in MII eggs

DiIC$_{18}$(3) in MII eggs approximately 30 minutes post injection (n=8). The excluded area shows the soybean oil drop that was injected into the egg

Immuno-staining of the Golgi marker giantin shows the Golgi apparatus forms vesicles in eggs

There is some evidence that the Golgi apparatus takes a fragmented form in eggs that appear as vesicles (Moreno *et al.* 2002; Payne and Schatten 2003). Immunostaining against the Golgi marker giantin has previously shown that the Golgi appears as small vesicles throughout the cytoplasm which are particularly concentrated in the cortex (Yu and Swann 2012, unpublished). Furthermore these giantin containing vesicles co-localise significantly with immunostaining of $PI(4,5)P_2$ (Yu and Swann 2012, unpublished).

To verify the structure and nature of the Golgi in mature eggs immunostaining was repeated against the endogenous Golgi specific marker, giantin. Eggs were fixed and permeabilised as stated previously before being bathed in 5% goat's serum as a blocking agent. The eggs were then incubated in rabbit derived polyclonal anti-giantin antibody followed by anti-rabbit Alexa (488) conjugated secondary antibody. These immuostained eggs were then imaged on a confocal microscope to determine the localisation of giantin. Giantin was found to be located in small vesicles throughout the cytoplasm however these vesicles are more concentrated in the cortical region of the egg fig 5.12). This data confirms and reproduces previous results and indicates that the Golgi is fragmented into small vesicles in mature eggs (Payne and Schatten 2003 .

The Golgi apparatus marker fluorescent AC-lectin localises to intracellular vesicles

Immunostaining can provide inconsistent results as it is multi-step process with many parameters that are often difficult to optimise. To avoid this issue and validate the Golgi distribution indicated by giantin staining the localisation of another Golgi specific marker was investigated. *Amarathus caudatus* lectin (AC-lectin) binds oligosaccharide containing galactosyl-beta 1,3-N-acetylglucosamine (Galβ3GalNAC) and is very specific to the Golgi apparatus (Rinderle *et al.* 1990). If this is conjugated to a fluorescent fluorescein tag then fluorescent AC-lectin can be used as a marker of the Golgi. As this AC-lectin is fluorescently tagged it is not necessary to use fluorescent antibodies so the cells only need to be fixed and permeabilised as previously described prior to incubation in AC-lectin (diluted 1:100) for 1 hour. After being washed through in clean media these eggs were then placed on a confocal imaging system so the localisation could be determined. As with giantin immunostaining, AC-lectin appeared to label small vesicles diffuse throughout the cytoplasm however particularly concentrated in the cortical region (see fig 5.13). This data strongly suggests that the Golgi apparatus is structured as small vesicles in eggs.

Giantin immunostaining in MII eggs

Equatorial Section

Cortical Section

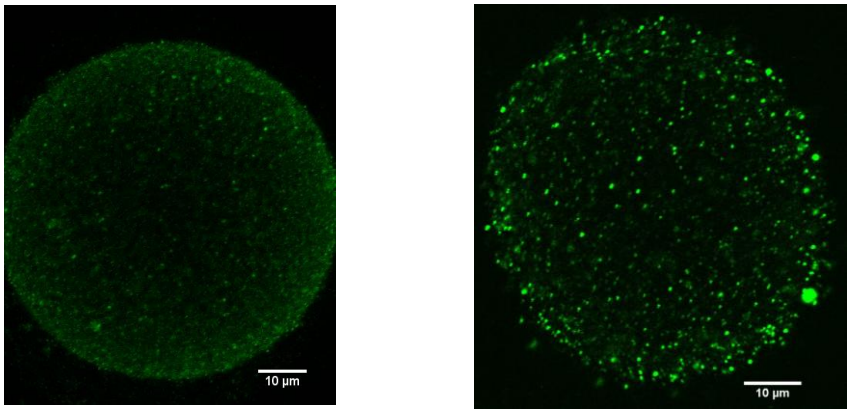


Figure 5.12 Confocal images showing immunostaining of giantin in MII eggs

Images show an equatorial and a cortical section of the whole egg (n=8)

AC-Lectin (Golgi marker) in MII eggs

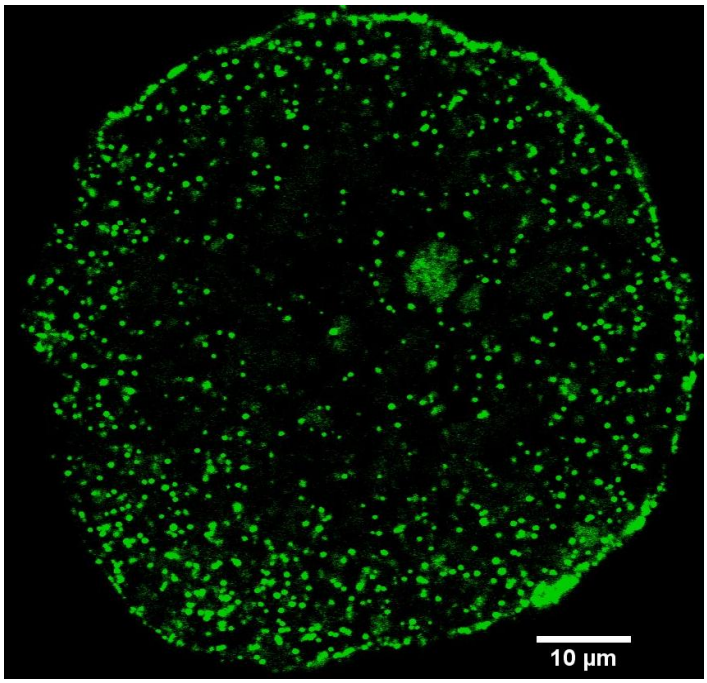


Figure 5.13 Confocal image showing AC-Lectin staining in an MII egg

(n=9)

The Golgi apparatus marker BODIPY-FL ceramide does not localise specifically to intracellular vesicles

Though these markers for the Golgi apparatus appear to be specific neither of them is able to measure Golgi distribution in live cells or dynamically. To do this the distribution of the Golgi marker BODIPY-FL ceramide was used (see fig 5.14). This is a fluorescently tagged version of the lipid ceramide which is taken into the Golgi. Though BODIPY FL-ceramide is cell permeable it relies on CERT expression to get into the Golgi compartment (Hanada 2010). Little is known about the extent of membrane trafficking or lipid transport in eggs so the BODIPY ceramide was directly injected into eggs instead. BODIPY-FL ceramide was diluted in KCL HEPES prior to microinjection at a pipette concentration of 500 μM. These eggs were then imaged on a confocal microscope after approximately 1 hour to examine the localisation of the marker. Unlike the other Golgi markers used, BODIPY-FL ceramide did not appear to localise to small intracellular vesicles but instead appeared to mark membranes that were more reticular in structure which appeared dense in distribution (see fig 5.15). This could suggest that BODIPY-FL ceramide does not specifically mark the Golgi apparatus but may also be binding to other membranes such as the endoplasmic reticulum.

The Golgi apparatus marker GOLGI-ID® localises to intracellular vesicles

As BODIPY FL ceramide does not act as a specific marker for Golgi apparatus, the distribution of another live cell marker for the Golgi was tested. GOLGI-ID® is a commercially available assay kit that contains a green fluorescent dye for staining the Golgi apparatus. This dye is promoted as being very specific at targeting the Golgi apparatus. Egg were incubated in the GOLGI-ID® dye for one hour in the fridge before undergoing an extensive wash out in media containing 10% serum for an hour and a half. These eggs were then imaged on the confocal microscope to determine the distribution of the GOLGI-ID® probe. GOLGI-ID® did appear to localise to some vesicles in the cytoplasm (see fig 5.16). There was also some fluorescence seen emitted from other intracellular membranes that appeared more reticular, however this staining was much weaker than the vesicular staining. This suggests that GOLGI-ID® binds to Golgi derived intracellular vesicles throughout the cytoplasm. However it also binds in a less specific fashion to other membranes albeit with a reduced affinity.

BODIPY-FL ceramide

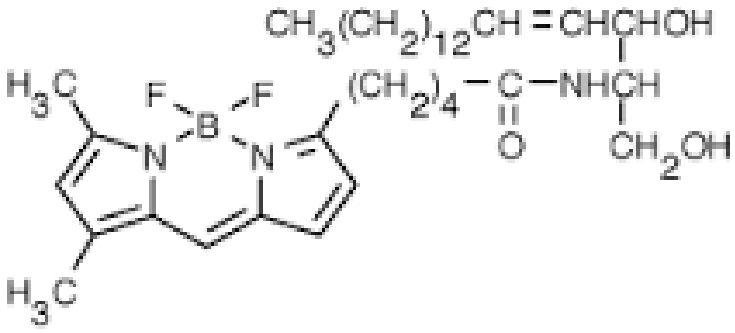


Figure 5.14 Schematic showing the chemical structure of the BODIPY-FL ceramide

(taken from thermofisher.com)

BODIPY-FL ceramide (Golgi marker) in MII eggs

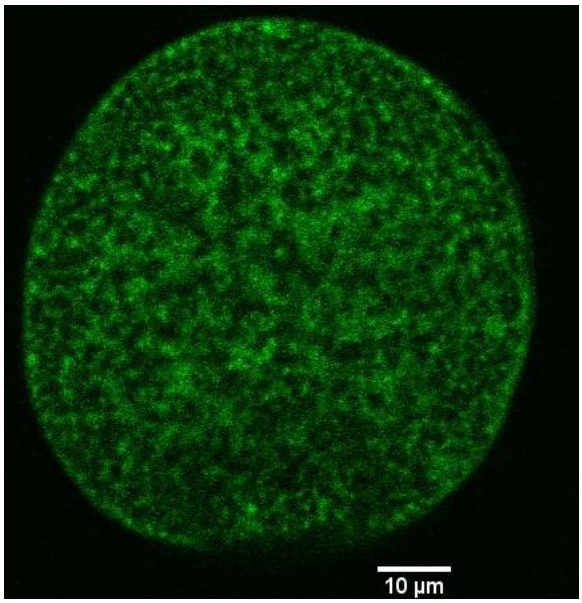


Figure 5.15 Confocal image showing BODIPY-FL ceramide staining in an MII egg

Approximately 30 minutes post injection of BODIPY-FL cermamide (pipette concentration 500 µM, n=10)

GOLGI- ID (Golgi marker) in MII eggs

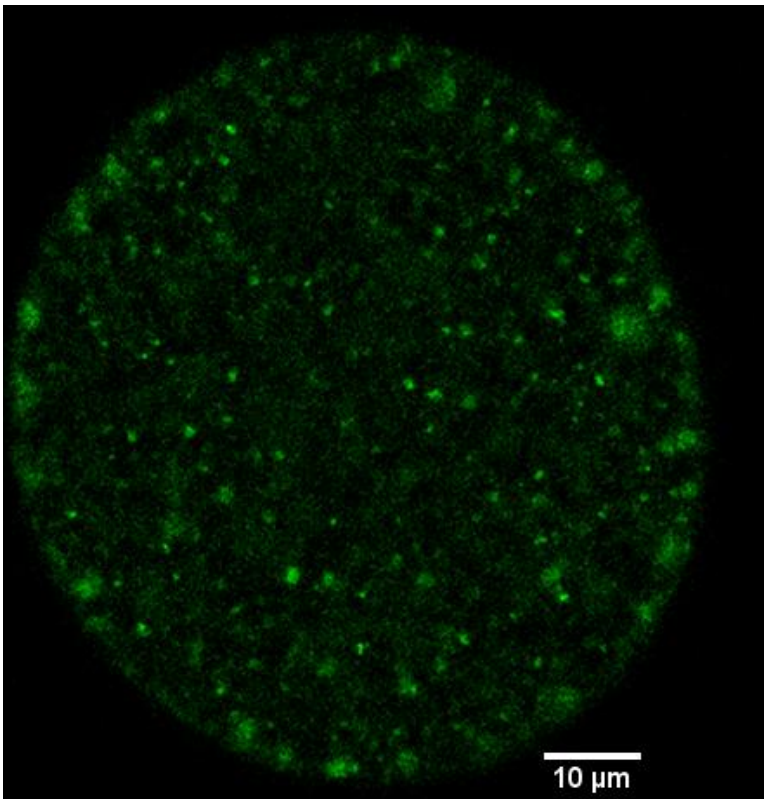


Figure 5.16 Confocal image showing GOLGI-ID staining in an MII egg

(n=15)

The Golgi apparatus dye BODIPY FL- ceramide overlaps strongly with the ER marker $DiIC_{18}(3)$

As BODIPY-FL ceramide appears to mark reticular structures in the egg cytoplasm that looked very similar to the distribution of the ER, it is possible that BODIPY FL- ceramide is acting as a marker for the ER rather than the Golgi. To determine if this was the case eggs were injected with both $DiIC_{18}(3)$ and BODIPY FL- ceramide (at a pipette concentration of 500 μM) during 2 separate injections. Eggs were first injected with BODIPY FL- ceramide and then $DiIC_{18}(3)$ half an hour later. Approximately 30 minutes following the $DiIC_{18}(3)$ injection the eggs were imaged on a confocal microscope to determine whether these two probes co-localised. Images were captured of the fluorescent signals emitted by both BODIPY FL-ceramide and $DiIC_{18}(3)$, these images were then overlaid to determine where the signals overlapped. The co-localisation of these two markers was extremely strong suggesting that rather than acting as a marker for the Golgi apparatus BODIPY FL- ceramide is actually acting as a marker for the endoplasmic reticulum in eggs (see fig 5.17).

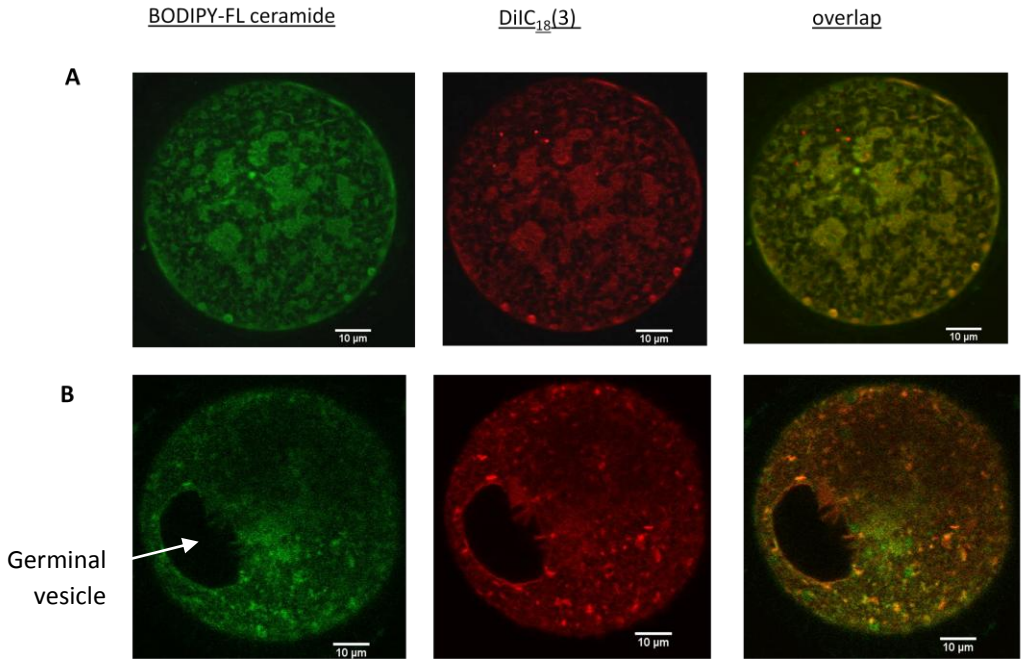


Figure 5.17 Confocal images showing the co-localisation between BODIPY-FL ceramide and $DiIC_{18}(3)$

A) in MII eggs (n=6) **B)**, GV oocytes (n=8). Oocytes/eggs were injected first with BODIPY FL-ceramide, then 30 minutes later injected with $DiIC_{18}(3)$. Eggs were then imaged 30 minutes post injection of $DiIC_{18}(3)$. Both dyes were excluded from the germinal vesicle

The Golgi associated protein Rab 6 may localise to intracellular vesicles in eggs

Rab proteins are a large family of membrane bound GTPases that play a key role vesicle trafficking. Different members of the Rab family are specifically associated with different membranes and therefore can be used as markers for specific membrane compartments (Simons and Zerial 1993). Rab 6 is known to be associated with the Golgi and the trans-Golgi network (Goud *et al.* 1990). Therefore Rab 6 distribution can be used as a marker for the Golgi apparatus. Immunostaining of Rab 6 in eggs has previously shown that it is localised to small vesicles diffused throughout the cytoplasm (Yu and Swann 2012, unpublished). Furthermore this immunostaining of Rab 6 has been found to co-localise with cMyc-PLCζ suggesting that Rab 6 positive vesicles may be the target for PLCζ in the egg (Yu and Swann 2012, unpublished). To investigate the distribution of Rab 6 in live eggs, fluorescently labelled Rab 6 cRNA was microinjected into eggs and left to be translated for approximately 3 hours prior to confocal imaging. A series of 3 different Rab 6 cRNA constructs were created, first of these was the Rab 6 sequence tagged with fluorescent GFP sequence. Rab 6- GFP cRNA was synthesised by MN and microinjected into mouse eggs at a concentration of 1 µg/µl. When imaged on the confocal microscope Rab 6-GFP appeared to localise to intracellular vesicles however there was also fluorescence signal emitted from other intracellular membranes that appear more aggregated in structure (see fig 5.18).

This additional membrane staining could be Rab 6 –GFP localising to the ER because Rab 6 has been shown to be involved in anterograde and retrograde membrane trafficking, shuttling proteins between the Golgi and the ER (Martinez *et al.* 1994). Overexpression of the GTP bound form of Rab 6 inhibits anterograde trafficking through the Golgi and promotes retrograde transport. As a result Golgi resident proteins can end up in the ER (Martinez *et al.* 1994; Martinez *et al.* 1997). In contrast if a mutated GDP bound form of Rab 6 is over-expressed, Rab6^{T27N}, this retrograde transport is inhibited (White *et al.* 1999) To try and minimise ER localisation this Rab6^{T27N}sequence was used to produce a fluorescent construct that was incapable of moving between the ER and Golgi compartments. The Rab6^{T27N} sequence was tagged with either YFP or mCherry fluorescent tags and synthesised into cRNA by MN. These constructs were then injected into eggs in the same manner as Rab 6 –GFP and left to express for approximately 3 hours. Confocal imaging showed the fluorescence emitted by Rab6^{T27N}-YFP was very low which made it too difficult to resolve specific localisation. A similar result was seen in those eggs microinjected with Rab6^{T27N}-mcherry.

Immuno-staining shows Rab 6 is localised to intracellular vesicles in the egg

Due to variable results obtained using exogenous fluorescent Rab 6 it was important to verify that endogenous Rab 6 is localised to vesicles in eggs. To do this immunostaining was performed using the same protocol as for giantin however using rabbit polyclonal antibodies against Rab 6 instead. When these eggs were imaged on the confocal microscope Rab 6 was seen to localise to small vesicles throughout the cytoplasm (see fig 5.19). Some fluorescence was emitted from other intracellular membranes that did not appear vesicular in structure however this fluorescence was much weaker than that emitted from intracellular vesicles.

Rab 6- GFP

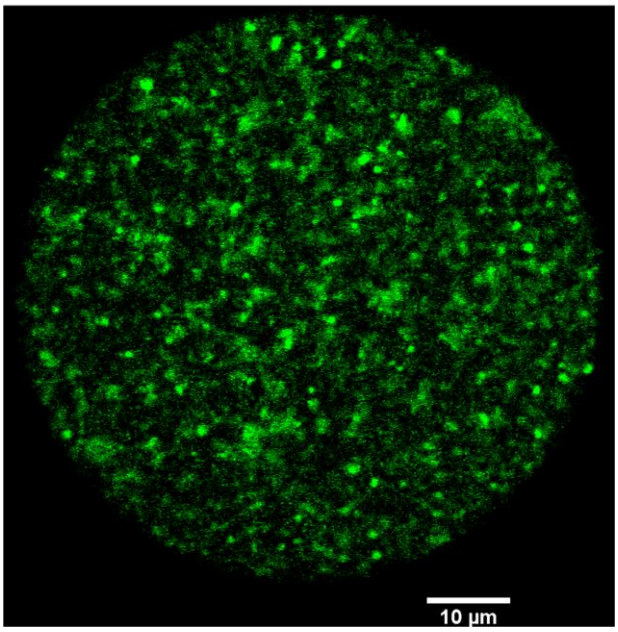


Figure 5.18 Confocal image of Rab 6-GFP distribution in an MII egg

3 hours post injection of Rab 6-GFP cRNA (pipette concentration of 1 µg/µl) (n=5)

Rab 6A immunostaining in MII eggs

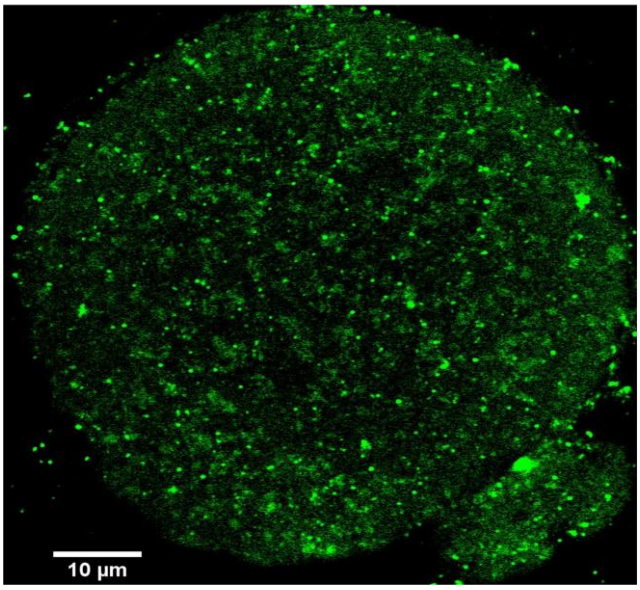


Figure 5.19 Confocal image showing the immunostaining of Rab 6A in an MII egg

(n= 15)

Functional effects of treatments designed to disrupt PI(4,5)P_2 availability and PI cycling on the Ca^{2+}_i releasing ability of eggs in response to PLCζ

Expressing the phosphatase OCRL1 in eggs does not affect PLCζ induced Ca^{2+}_i oscillations

As it is the substrate of PLCζ the availability of PI(4,5)P_2 is likely to play a role in the Ca^{2+}_i releasing ability of PLCζ in eggs. Therefore to investigate whether the availability of PI(4,5)P_2 is a limiting factor for PLCζ activity several experiments were designed to specifically reduce PI(4,5)P_2 levels in live eggs and then analyse Ca^{2+} oscillations initiated by PLCζ.

OCRL1 is a gene that is endogenously expressed in cells that encodes for inositol polyphosphate 5-phosphatase (OCRL1) (Zhang *et al.* 1995). OCRL1 protein is localised primarily to the Golgi apparatus (Olivos-Glander *et al.* 1995). As it is a phosphatase OCRL1 catalyses the conversion of PI(4,5)P_2 to PI(4)P and therefore regulates specific pools of PI(4,5)P_2 levels (Zhang *et al.* 1995). To determine whether the Ca^{2+} releasing activity of PLCζ was affected by the cellular levels of PI(4,5)P_2, OCRL1 cRNA (concentration of (1 μg/μl) was microinjected into eggs in order to try and deplete the levels of endogenous PI(4,5)P_2 . These eggs were then left to express this cRNA for approximately 3 hours before being injected with mPLCζ-luciferase cRNA (0.02 μg/μl) and the Ca^{2+}_i indicator OGBD. The Ca^{2+}_i oscillations in these eggs were then compared to eggs that had only been injected with mPLCζ-luciferase and not OCRL1. OCRL1 did cause a small reduction in the frequency of Ca^{2+}_i oscillations compared to controls not injected with OCRL1 however this reduction is not significant (see fig 5.20).

It is possible that much of the PI(4,5)P_2 is synthesised during oocyte maturation so to try and reduce the levels of PI(4,5)P_2 during this time OCRL1 cRNA was microinjected into GV oocytes that were left to mature to MII *in vitro*. These *in vitro* matured eggs were then injected with mPLCζ-luciferase cRNA (0.02 μg/μl) mixed with OGBD so the dynamics of Ca^{2+}_i oscillations could be measured. Again there was a slight reduction in the frequency of Ca^{2+}_i oscillations compared to those eggs not injected with OCRL1 however this difference was not significant. This suggests that OCRL1 over expression does not affect PLCζ's Ca^{2+}releasing ability significantly (see fig 5.20).

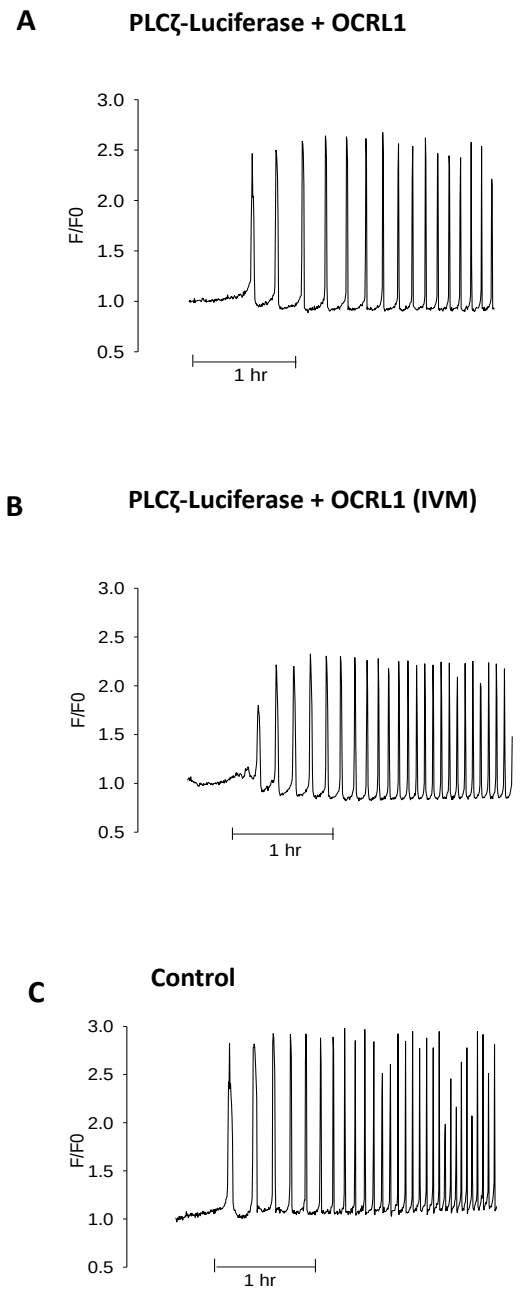


Figure 5.20 Ca^{2+}_i traces showing the effect of OCRL1 on oscillations induced by PLCζ

Ca^{2+}_i oscillations induced by PLCζ –luciferase cRNA (pipette concentration of 0.5 µg/µl) **A)** eggs were injected with untagged OCRL1 cRNA (pipette concentration of 1.5 µg/µl and left to express for approximately 3 hours before injection of PLCζ –luciferase cRNA (n= 9, mean number of Ca^{2+}_i oscillations in the first hour of spiking = 5.3, mean luminescence in the first hour of spiking =0.5 cps) **B)** GV oocytes were injected with untagged OCRL1 cRNA (pipette concentration of 1.5 µg/µl and left to express for approximately 18 hours whilst maturing before injection of PLCζ –luciferase cRNA (n= 6, mean number of Ca^{2+}_i oscillations in the first hour of spiking = 7.8, mean luminescence in the first hour of spiking =0.8 cps **C)** eggs were injected with only PLCζ –luciferase cRNA (n= 13, mean number of Ca^{2}_i oscillations in the first hour of spiking = 7.2, mean luminescence in the first hour of spiking =0.4 cps)

Attempts to deplete Golgi located PI(4,5)P_2 using phosphatase tagged Golgi markers does not affect PLCζ induced Ca^{2+}_i oscillations

As it is was possible to determine which pools of PI(4,5)P_2 are being metabolised by OCRL1 a series of other constructs were developed by MN in attempt to deplete specific pools of PI(4,5)P_2 notably that found at the Golgi apparatus. Firstly the C1b domain of PKC was selected to target the Golgi as it binds to both DAG and PI(4)P, both of which should be localised to the Golgi apparatus (De Matteis and Godi 2004). This domain was tagged to a 5' phosphatase and a mCherry fluorescent tag to createC1b- mCherry-phosphatase fusion construct that was then synthesised into cRNA (by MN). This cRNA was then microinjected into eggs at a concentration of 1µg/µl. Approximately 3 hours after microinjection these eggs were then microinjected with mPLCζ-luciferase cRNA (0.02 µg/µl) mixed with the Ca^{2+}_i indictor OGBD so the Ca^{2+}_i dynamics could be recorded and analysed. These results were then compared to the Ca^{2+}_i oscillations of control eggs that had been injected with mCherry cRNA (1µg/µl) instead of C1b- mCherry-phosphatase. No difference in the frequency or pattern of Ca^{2+}_i oscillations were seen between the two groups which suggests that the expression of C1b- mCherry-phosphatase cRNA does not affect PLCζ induced Ca^{2+}_i oscillations (fig 5.21).

The second targeted construct that was developed contained the PH domain of oxy-sterol binding protein (OSBP) which should also target the Golgi apparatus through binding to PI(4)P (Levine and Munro 1998). This OSBP(PH) was tagged again to a 5'phosphatase and synthesised into cRNA (by MN). The OSBP(PH)-phosphatase cRNA was microinjected into eggs at a pipette concentration of 1µg/µl. Approximately 3 hours after injection these eggs were then microinjected a second time with mPLCζ-luciferase cRNA and OGBD as previously described. The Ca^{2+}_i oscillations in these eggs were analysed and compared to those eggs that had been injected with mPLCζ-luciferase cRNA and OGBD alone. The addition of OSBP (PH)-phosphatase did not appear to affect the Ca^{2+}_i oscillations initiated by PLCζ when compared to those that had not been microinjected with OSBP(PH)-phosphatase cRNA (see fig 5.21). This would suggest that either targeting 5'phophatase to the Golgi apparatus does not affect Ca^{2+}_i oscillations induced by PLCζ or these constructs are not targeting the Golgi.

It was important to establish if the C1b domain and OSBP(PH) domain were correctly targeting the construct to the Golgi apparatus. To identify where C1b domain and

OSBP(PH) were localising in the egg, fluorescent mCherry tagged constructs were created of each domain. Firstly the C1b domain was tagged with mCherry and synthesised into cRNA (by MN). This cRNA was microinjected into eggs at a concentration of 1 µg/µl and left to express for approximately 3 hours before being imaged on the confocal microscope to determine localisation of the C1b-mCherry construct. Unlike other markers for the Golgi apparatus C1b-mcherry did not appear to localise to small intracellular vesicles but instead was more reticular in appearance and more similar to the distribution of markers of the ER (see fig 5.22). The same experiment was conducted using the OSBP(PH) domain tagged with mCherry that was synthesised into OSBP(PH)-mCherry cRNA (by MN). Eggs were microinjected with OSBP(PH)-mCherry cRNA (1.5 µg/µl) and left to express this RNA for approximately 3 hours. These eggs were then imaged on the confocal microscope to analyse the distribution of the construct. Like C1b-mcherry, OSBP(PH)-mCherry didn't appear to be vesicular in distribution but instead appeared as a reticular pattern like markers of the ER however this signal was extremely low (see fig 5.22). This would suggest that neither C1b nor OSBP (PH) domain localise to a vesicular form of the Golgi or at least the same compartment of the Golgi that intracellular vesicular $PI(4,5)P_2$ resides. This could explain why these constructs did not have any effect on Ca^{2+}_i oscillations.

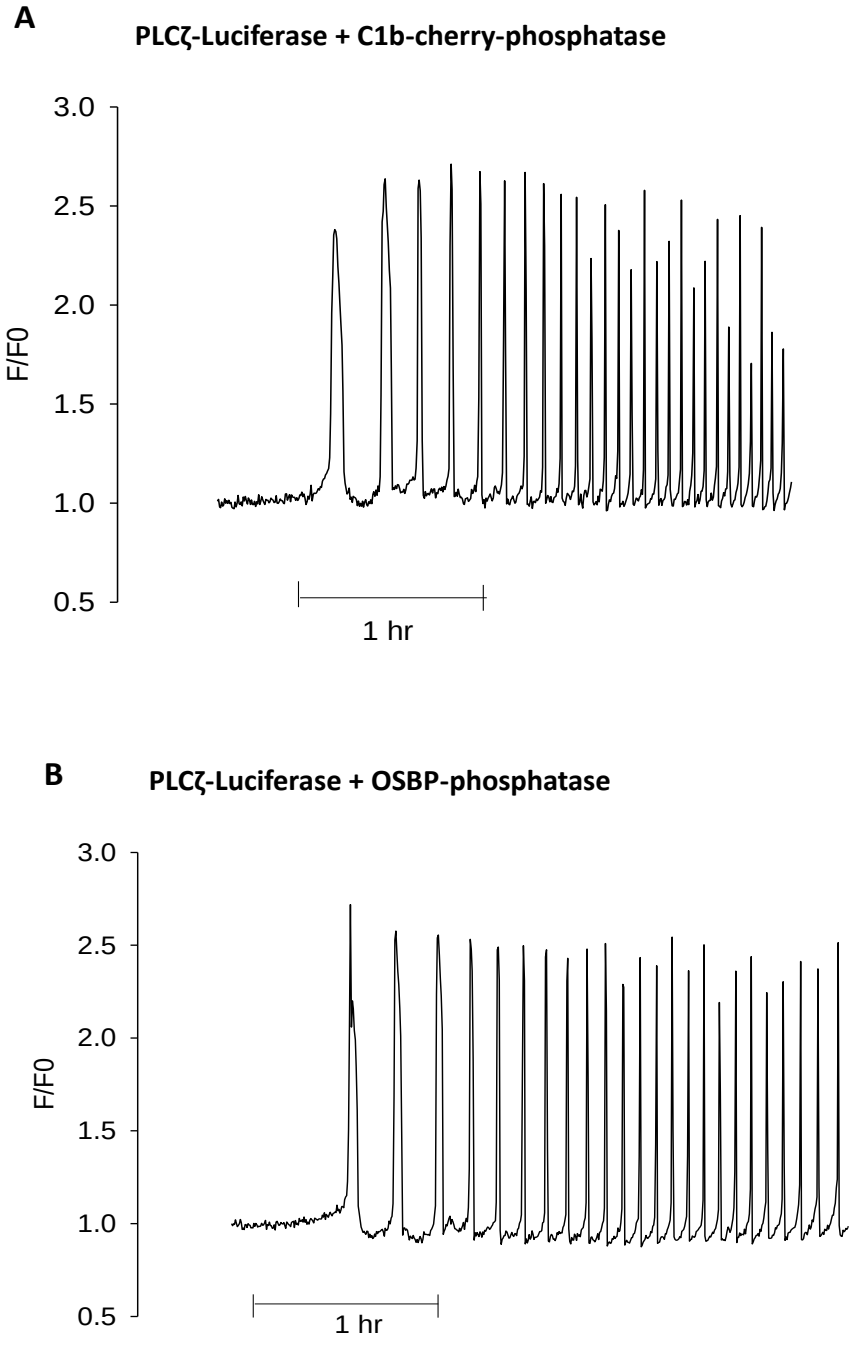


Figure 5.21 Ca^{2+}_i traces showing the effect of phosphatase tagged OSBP(PH) and C1b domain on oscillations induced by PLCζ

Ca^{2+}_i oscillations induced by PLCζ –luciferase cRNA (pipette concentration of 0.5 µg/µl) **A)** eggs were injected with c1b-cherry-phosphatase cRNA (pipette concentration of 1 µg/µl and left to express for approximately 3 hours before injection of PLCζ –luciferase cRNA (n= 8, mean number of Ca^{2+}_i oscillations in the first hour of spiking = 10.3, mean luminescence in the first hour of spiking =1.3 cps) **B)** eggs were injected with OSBP-phosphatase cRNA (pipette concentration of 1 µg/µl and left to express for approximately 3 hours before injection of PLCζ –luciferase cRNA (n= 10, mean number of Ca^{2+}_i oscillations in the first hour of spiking = 6.4, mean luminescence in the first hour of spiking =0.5 cps)

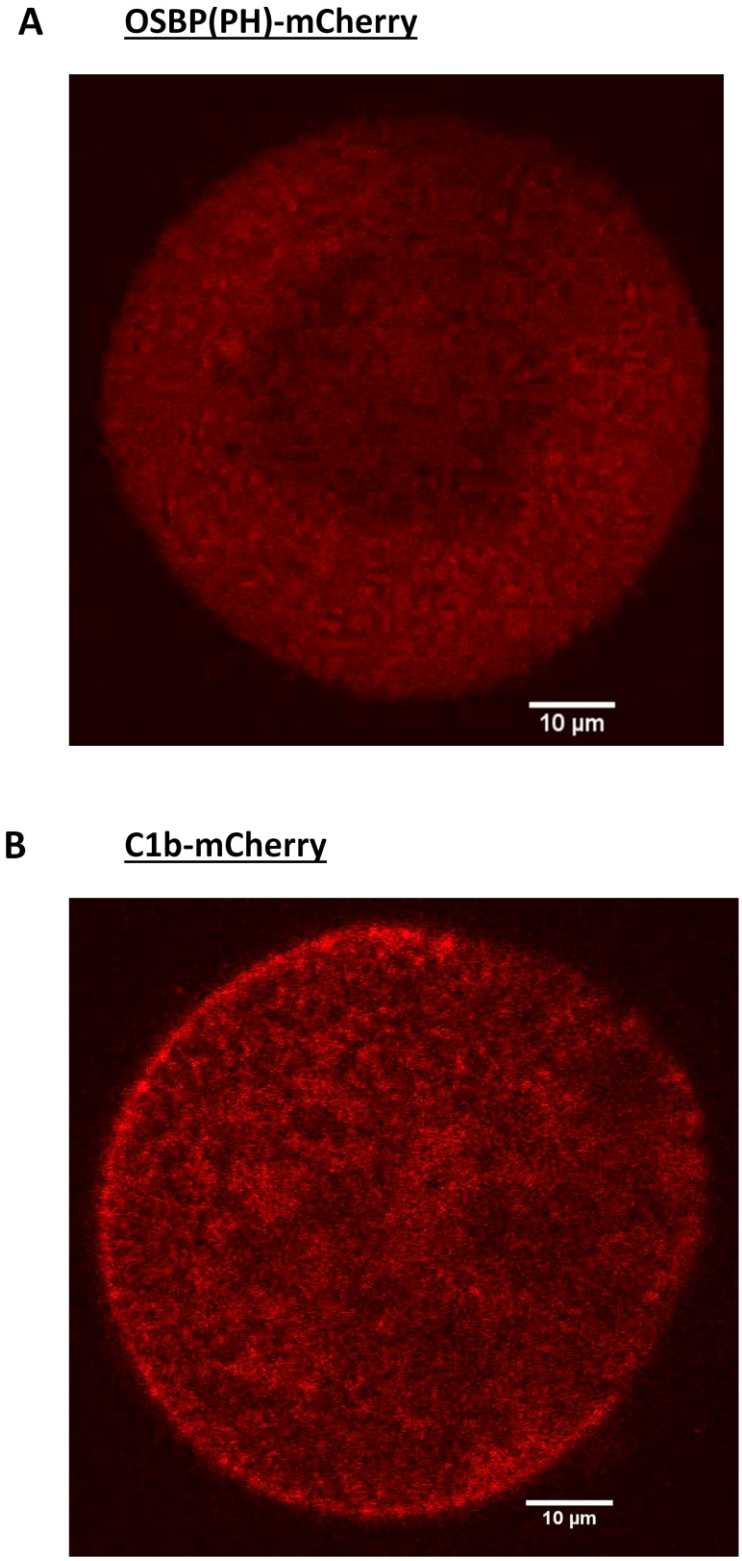


Figure 5.22 Confocal images showing the distribution of OSBP(PH)-mCherry and C1b-mCherry in MII eggs

A) mCherry tagged PH domain of OSBP cRNA pipette concentration 1.5 µg/µl) ((n=5) and **B)** mCherry tagged C1b domain cRNA (pipette concentration 1 µg/µl) (n=5)

Disrupting PI cycling through inhibition of DAG kinase prevents PLCζ induced Ca^{2+}_i oscillations

As opposed to depleting already synthesised PI(4,5)P_2 using 5' phosphatases another method for reducing the levels of PI(4,5)P_2 is to prevent its synthesis from other phosphoinositides. The enzyme DAG kinase (DGK) is responsible for catalysing the production of phosphatidic acid (PA) from DAG (Call and Rubert 1973). This PA feeds into the PI cycle by interacting with CTP to produce CTP-DAG which is the precursor for phosphoinositol (PI)(Carter and Kennedy 1966). As a result, preventing the synthesis of PA by inhibiting DGK could prevent all further PI cycling and synthesis (see fig 5.23). To investigate the impact of disrupting PA production on PLCζ induced Ca^{2+}_i oscillations, the effect of DAG kinase inhibitor on the pattern of PLCζ induced Ca^{2+}_i oscillations was analysed. Eggs were first microinjected with mPLCζ cRNA mixed with the Ca^{2+}_i indicator Rhod Dextran and the Ca^{2+}_i oscillations initiated were recorded using an epifluorescence imaging system. After approximately 30 minutes of Ca^{2+}_i oscillations 10 µM DGK inhibitor was added to the media surrounding the eggs and imaging was continued. Due to the chemical structure of DGK inhibitor this drug was likely to be hydrophobic and therefore may partition into the mineral oil covering the media containing the eggs therefore the drug was added to media alone that was not covered with oil instead evaporation was prevented using a fitteddish lid. The addition of DGK inhibitor inhibited the PLCζ induced Ca^{2+}_i oscillations (see fig 5.24). Furthermore the basal level of Ca^{2+}_i remained fairly constant after the addition of DGK inhibitor. This would suggest that inhibiting PA synthesis by DGK inhibitor disrupts Ca^{2+}_i oscillations induced by PLCζ.

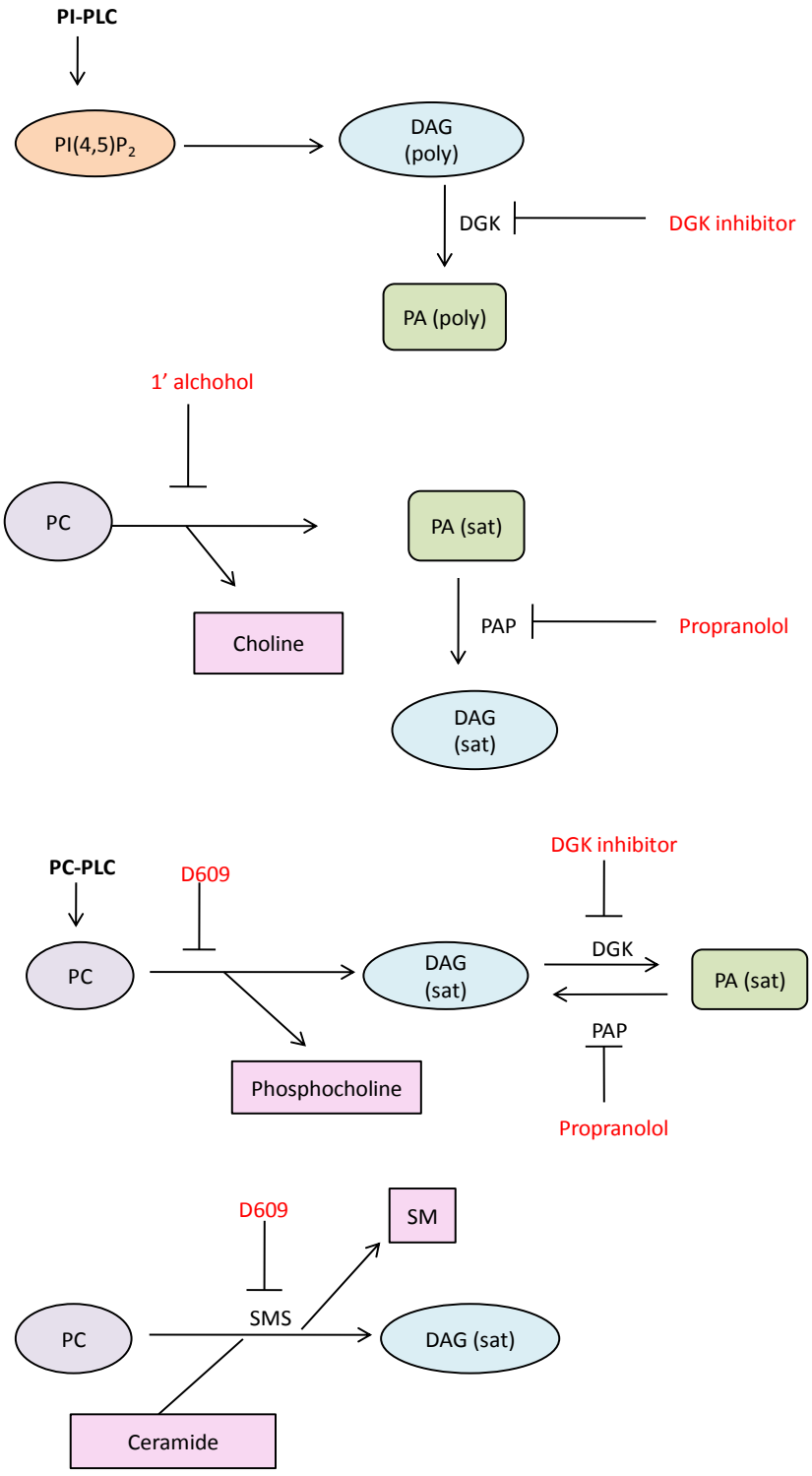


Figure 5.23 Schematic showing the different pathways of DAG and PA production

The inhibitors of these pathways are shown in red. Two species of DAG and PA are shown those with poly unsaturated acyl chains (poly) and those with saturated acyl chains (poly).

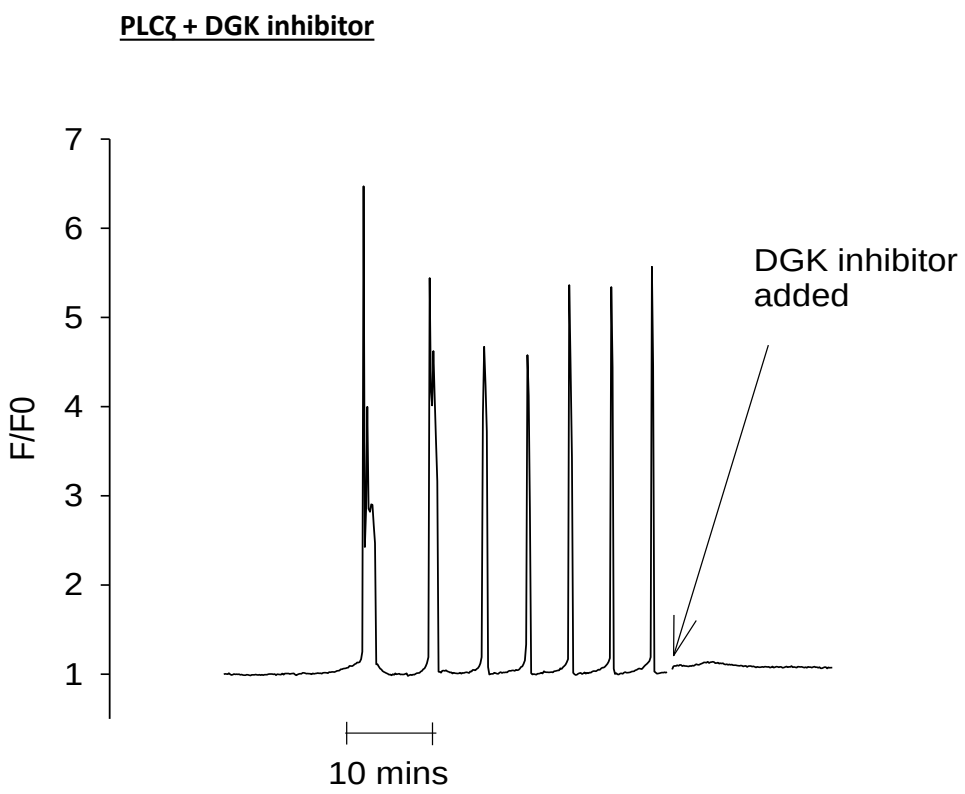


Figure 5.24 Ca^{2+}_i trace showing the effect of DGK inhibitor on oscillations in MII eggs

The effect of DAGK inhibitor (10μM) on Ca^{2+}_i oscillations induced by PLCζ cRNA (pipette concentration of 0.2 μg/μl) mixed with the Ca^{2+}_i indicator Rhod dex (n=13)

The effect of preventing PA synthesis by other pathways on PLCζ induced Ca^{2+}_i oscillations

PA is synthesised by multiple pathways and not all pathways are connected to the PI cycle. An example of one such pathway is Phospholipase D (PLD) stimulated PA synthesis. PLD hydrolyses phosphatidylcholine (PC) to produce choline and PA (Liscovitch *et al.* 1993). The production of PA via this pathway can be prevented by the addition of primary alcohol which prevents PLD hydrolysing PC instead it undergoes a transphosphatidylation reaction to produce phosphatidyl alcohols instead of PA (Kotter and Klein 1999) (see fig 5.23).In *Xenopus* frog eggs there is a suggestion that a sperm factor stimulates PLD mediated PA synthesis, which the triggers src family PLCγ activation in order to release Ca^{2+}(Bates *et al.* 2014). To investigate whether PA synthesised by this pathway is also essential to maintain PLCζ induced Ca^{2+}_i oscillations the primary alcohol 1-butanolol was used to inhibit PA synthesis. Egg were first microinjected with PLCζ cRNA alongside the Ca^{2+}_i indicator OGBD, these eggs were then imaged for approximately 1 hour and all eggs were reported to have relatively low frequency Ca^{2+}_i oscillations. 1-butanol was then added to the media containing the eggs. 1-butanolol was found to raise the baseline Ca^{2+}_i of the eggs and dramatically increase the frequency of the Ca^{2+}_i oscillations (see fig 5.25). To determine whether this was a specific effect of primary alcohols a control experiment was conducted where the secondary alcohol 2-butanol was added instead of the 1-butanol. However, 2-butanol had a very similar effect as 1-butanolol which suggests that this effect was not a specific response to primary alcohol (fig 5.25).

DAG and PA are also produced as a result of phosphatidylcholine (PC) specific PLCs. This pathways operates distinctively from DAG and PA produced by phosphoinositol specific PLCs such as PLCζ. The anti-viral tricyclodecan-9-yl-xanthogenate (D609) is an inhibitor of PC-PLCs and therefore inhibits the synthesis of DAG and PA by this pathway (Adibhatla *et al.* 2012) (see fig 5.23). It is important to note it also inhibits the production of metabolic DAG produced from PC by SMS (see fig 5.23). To determine whether D609 has an impact on Ca^{2+}_i oscillations induced by PLCζ, eggs were injected with PLCζ cRNA mixed with the Ca^{2+}_i indicator OGBD and the Ca^{2+}_i changes were measured using an epifluorescence system. After approximately 1 hour of imaging D609 was added to the media surrounding the eggs. Once D609 had been added the eggs continued to oscillate for several minutes before the oscillations stopped (see fig 5.26). When the oscillations stopped the baseline Ca^{2+}_i increased slightly and reached a plateau.

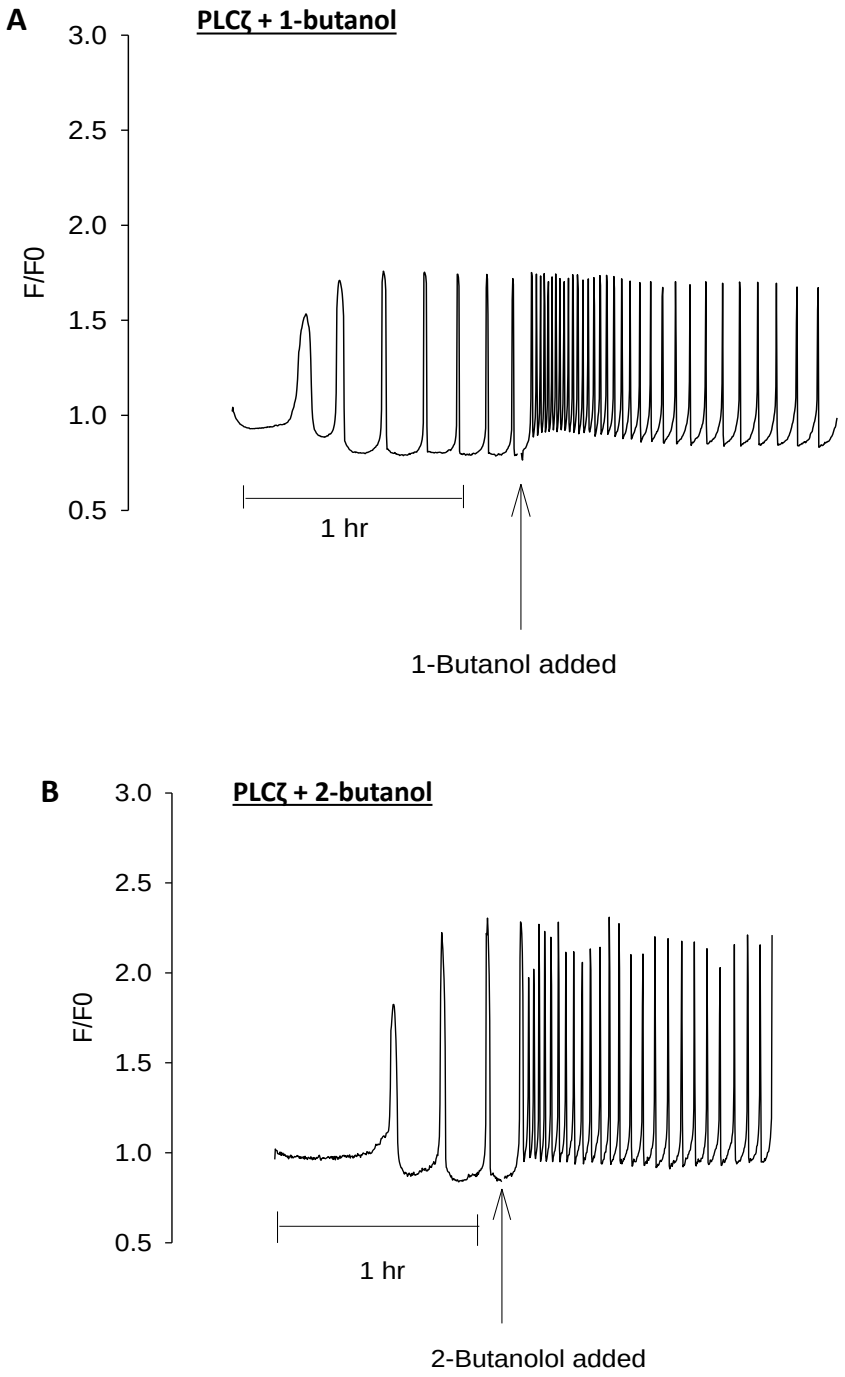


Figure 5.25 Ca^{2+}_i traces showing the effect of primary and secondary alcohols on oscillations in MII eggs

Traces showing the effect of **A)** the primary alcohol 1-butanolol on Ca^{2+}_i oscillations induced by PLCζ cRNA (pipette concentration of 0.2 µg/µl) n=13 **B)** the secondary alcohol 2-butanolol on Ca^{2+}_i oscillations induced by PLCζ cRNA (pipette concentration of 0.2 µg/µl) n=9

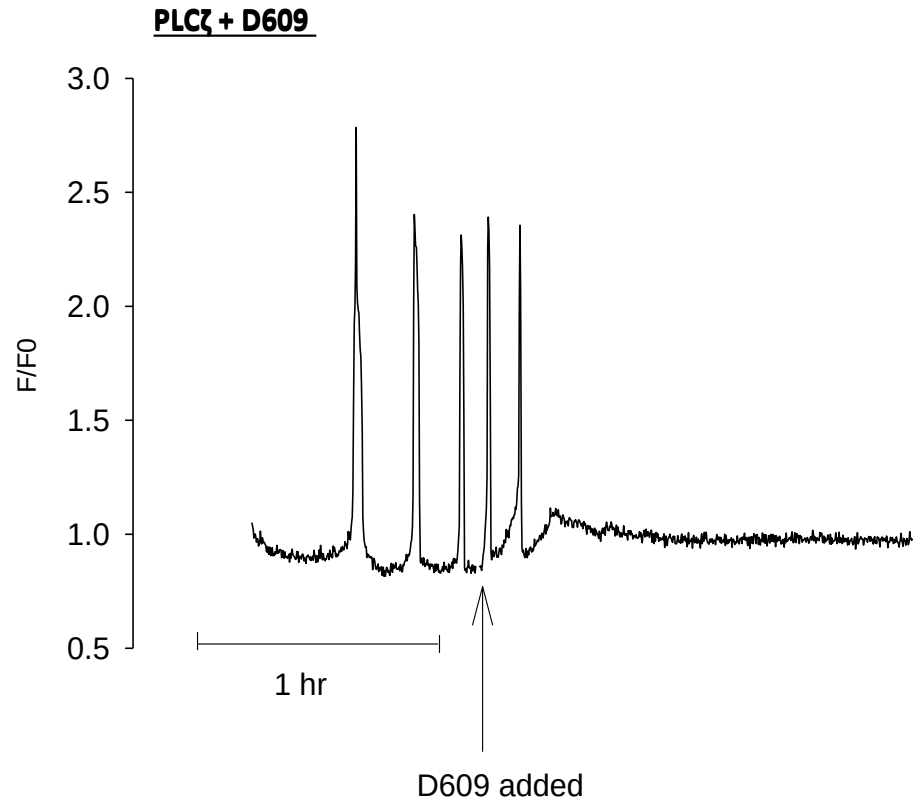


Figure 5.26 Ca^{2+}_i trace showing the effect of D609 on oscillations in MII eggs

Showing the effect of the drug D609 (100 μM) Ca^{2+}_i oscillations induced by PLCζ cRNA (pipette concentration of 0.2 μg/μl) n=11

Propranolol, a drug that disrupts the localisation of Golgi structural proteins, inhibits Ca^{2+}_i oscillations

The structure of the intracellular membrane bound organelles like the Golgi is maintained by charged lipids such as DAG. Propranolol is an inhibitor of the enzyme phosphatidate phosphohydrolase (PAP) which produces DAG by the phosphatidylcholine (PC) pathway (Asp *et al.* 2009) (see fig 5.23). As this metabolic DAG is required for maintaining the structure the Golgi, propranolol disrupts the structure of the Golgi and results in the mis-localisation of Golgi associated proteins (Baron and Malhotra 2002). It is possible that Golgi structure and proteins may be involved in determining PLCζ localisation and activity therefore it was interesting to determine what effect propranolol has on the Ca^{2+}_i oscillations induced by PLCζ. Ca^{2+}_i oscillations were induced in eggs following microinjection of untagged mouse PLCζ cRNA (0.02 μg/μl) mixed with Rhod dextran and allowed to oscillate for approximately 2 hours. Propranolol was then added to the media containing the eggs at a concentration of 300 μM. Within minutes of adding propranolol the Ca^{2+}_i oscillations in the eggs ceased. Furthermore the basal level Ca^{2+}_i remained low after addition of propranolol for the remaining of the recording (see fig 5.27).

To determine whether this effect was reversible the same experiment was repeated however approximately 1 hour after addition of propranolol clean media was perfused into the dish surrounding the eggs to wash out the propranolol. Once the propranolol had been removed the Ca^{2+}_i oscillations restarted. These Ca^{2+}_i oscillations were higher in amplitude and increased in frequency in the 1 hour after propranolol removal compared to the Ca^{2+}_i oscillations initiated before propranolol addition (see fig 5.27. The basal Ca^{2+}_i levels were also found to increase following propranolol removal and in some cases resulted in a very large increase in Ca^{2+}_i after approximately 1 hour post propranolol removal.

PLCδ1 causes Ca^{2+}_i oscillations in eggs at very high expression levels. Unlike PLCζ, PLCδ1 appears to produce IP_3 and release Ca^{2+} by hydrolysing $PI(4,5)P_2$ at the plasma membrane (Yu *et al.* 2012). In order to establish what affect propranolol has on Ca^{2+}_i oscillations induced by PLCδ1, eggs were microinjected with untagged mouse PLCδ1 cRNA mixed with Rhod dextran and left to oscillate for approximately 2 hours before adding 300 μM of propranolol in the same was as described previously. As for PLCζ, Ca^{2+}_i oscillations initiated by PLCδ1 were inhibited very soon after the addition of propranolol. Once again the basal Ca^{2+}_i levels remained low following the addition of propranolol (see fig 5.28). These data suggest that propranolol can inhibit Ca^{2+}_i oscillations associated with PI turnover in mouse eggs but the effect is not specific to PLCζ.

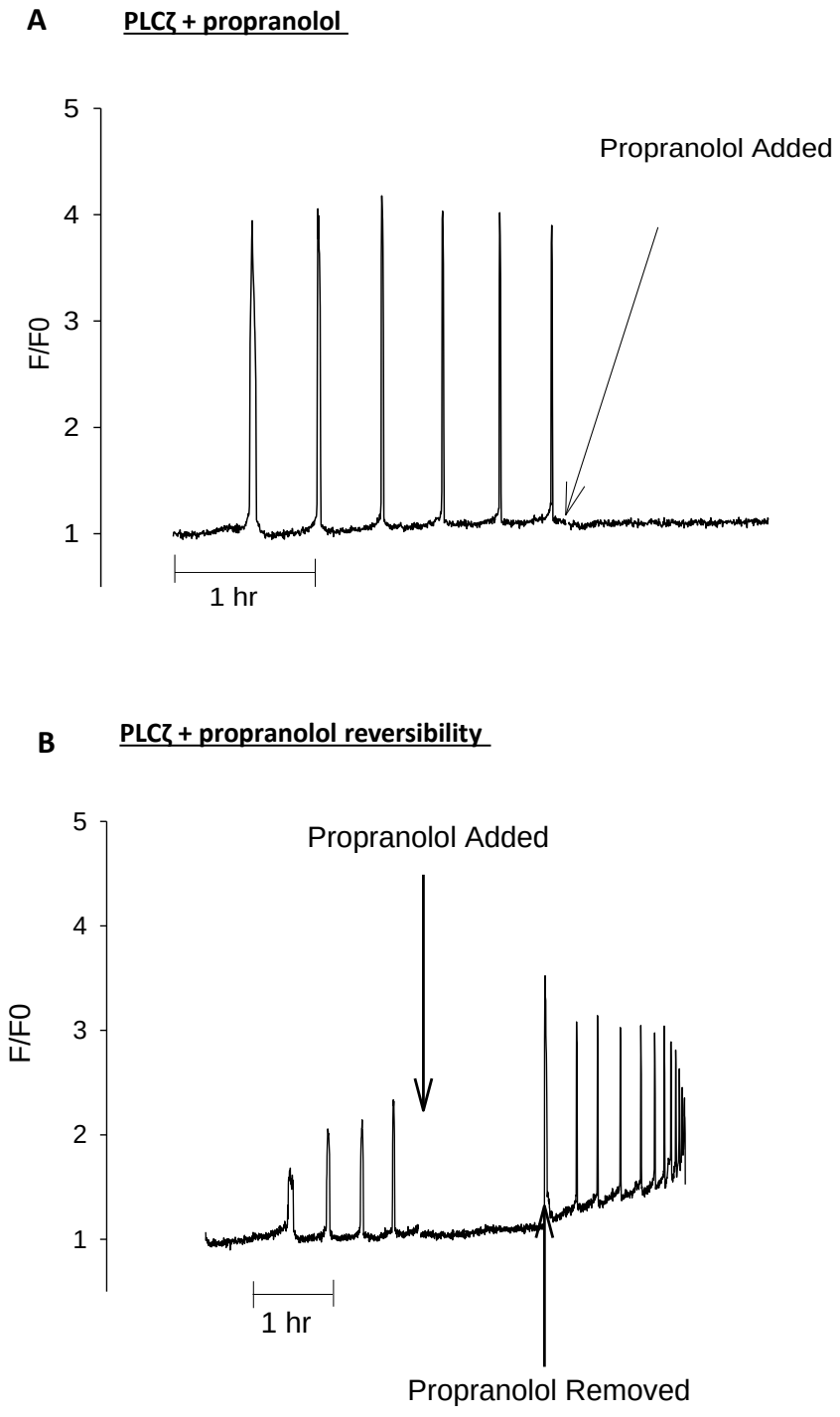


Figure 5.27 Ca^{2+}_i traces showing the effect of propranolol on PLCζ induced oscillations in MII eggs

A) The effect of propranolol (300 μM) on Ca^{2+}_i oscillations induced by PLCζ cRNA (pipette concentration of 0.025 μg/μl) n=36, **B)** the effect of propranolol (300 μM) on Ca^{2+}_i oscillations induced by PLCζ cRNA (pipette concentration of 0.025 μg/μl) and effect of the removal of this propranolol (n=8)

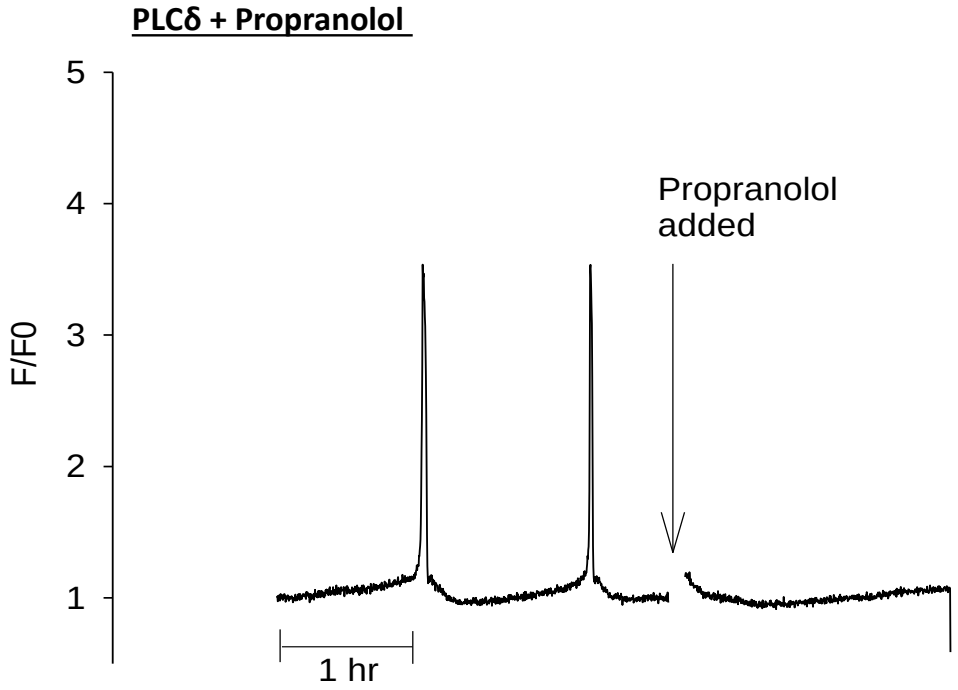


Figure 5.28 Ca^{2+}_i trace showing the effect of propranolol on PLCδ1 induced oscillations in MII eggs

The effect of propranolol (300 μM) on Ca^{2+}_i oscillations induced by PLCδ1 (pipette concentration of 1μg/μl) n=36

Brefeldin A, a drug that disrupts retrograde membrane trafficking, inhibits Ca^{2+}_i oscillations

As well as disrupting the localisation of Golgi resident proteins, propranolol also inhibits the formation of vesicular buds at the Golgi which play a role in retrograde membrane transport from the Golgi to the ER (Asp *et al.* 2009). Brefeldin A (BFA) is an antiviral antibiotic that inhibits protein transport from the ER to the Golgi (Fujiwara *et al.* 1988). Furthermore BFA induces retrograde transport leading to the accumulation of Golgi derived proteins in the ER (Doms *et al.* 1989). To examine the effects of BFA on the Ca^{2+}_i oscillations induced by PLCζ, eggs were injected with mouse PLCζ-luciferase cRNA at a pipette concentration of (0.05µg/µl) mixed with OGBD. These eggs were left to express the cRNA for approximately 30 minutes before being incubated and imaged in media containing luciferin and 5µM BFA. Ca^{2+}_i oscillations were recorded for approximately 1 hour before these oscillations ceased with a slight elevation in basal level Ca^{2+}_i. After approximately 1 hour many of these eggs experienced a large increase in Ca^{2+}_i and died (see fig 5.29). This suggests that BFA does inhibit Ca^{2+}_i oscillations however this does not occur quickly and eventually has a toxic effect on these eggs.

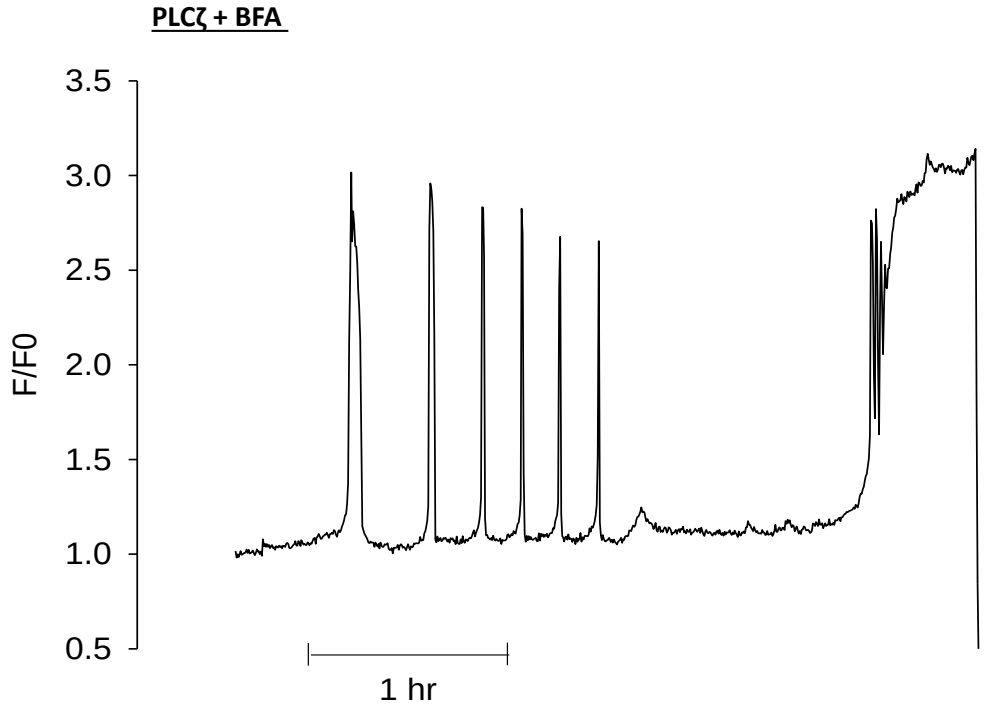


Figure 5.29 Ca^{2+}_i trace showing the effect of BFA on PLCζ induced oscillations in MII eggs

The effect of Brefeldin A (BFA) 5μM Ca^{2+}_i oscillations induced by PLCζ cRNA (pipette concentration of 0.2 μg/μl) (n=10)

Eggs acquire the ability to release Ca^{2+} in response to PLCζ during oocyte maturation

Immature oocytes have a reduced sensitivity to PLCζ

It has previously been established that eggs acquire the ability to release Ca^{2+}in response to sperm during oocyte maturation (Carroll *et al.* 1994; Jones *et al.* 1995b). This effect has also been mirrored in Ca^{2+}_i oscillations induced by PLCζ cRNA however these effects have not been quantified effectively (Wakai *et al.* 2012). To directly compare the PLCζ sensitivity of oocytes at different stages of oocyte maturation oocytes were microinjected with mPLCζ-luciferase cRNA so that expression levels could be accurately quantified. GV oocytes, MI oocytes (characterised as approximately 5 hours post collection from the ovary) and MII eggs were all injected with the same concentration of mPLCζ-luciferase cRNA (0.02 μg/μl) mixed with OGBD (conducted with the help of Ethan Bates). The Ca^{2+}_i oscillations initiated in these eggs were recorded and analysed. The luminescence signal emitted was also recorded so the amount of protein expression could be correlated with the Ca^{2+}_i pattern. Of 21 GV oocytes microinjected with mPLCζ-luciferase cRNA only 3 oocytes were recorded to have oscillations in Ca^{2+}_i at a luminescence value of 0.26 counts per second (in the first hour of Ca^{2+}_i oscillations) (see fig 5.30, fig 5.31). In contrast 20 out of 26 MI oocytes emitting very similar luminescence value experienced Ca^{2+}_i oscillations (see fig 5.30, 5.31). Ca^{2+}_i oscillations were recorded in all MII eggs (31 out of 31) microinjected with mPLCζ-luciferase cRNA (se fig 5.30, 5.31). This data suggests that GV oocytes do not release Ca^{2+}as well as MI or MII oocytes in response to PLCζ. Consequently it appears oocytes undergo a dramatic increase in PLCζ sensitivity after GVBD.

The amplitude of Ca^{2+}_i oscillations increases during oocyte maturation

It has previously been suggested that both the amplitude and frequency of Ca^{2+}_i oscillations increase in response to PLCζ during oocyte maturation (Carroll *et al.* 1994; Jones *et al.* 1995b; Wakai *et al.* 2012). To quantify the differences in the amplitude of Ca^{2+}_i oscillations between GV oocytes, MI oocytes and MII eggs the mean Ca^{2+}_i oscillation amplitude of each egg and oocyte used in the experiment described above was calculated. In the case of GV oocytes this was only calculated for the 3 oocytes that experienced Ca^{2+}_i oscillations. Mean Ca^{2+}_i oscillation amplitudes were then taken across the group of GV oocytes, MI oocytes and MII eggs and compared. MII eggs have significantly larger amplitude of Ca^{2+}_i oscillations compared to GV and MI oocytes expressing mPLCζ-luciferase cRNA at similar levels (see fig 5.31). There was also a reduction in the amplitude of the Ca^{2+}_i oscillations recorded in GV oocytes compared to MI oocytes however this was much smaller than the difference in

amplitude recorded between MI oocytes and MII eggs (see fig 5.31). This data indicates that the amplitude of PLCζ induced $Ca^{2+}{}_i$ oscillations increases in the later stages of oocyte maturation between metaphase I and metaphase II.

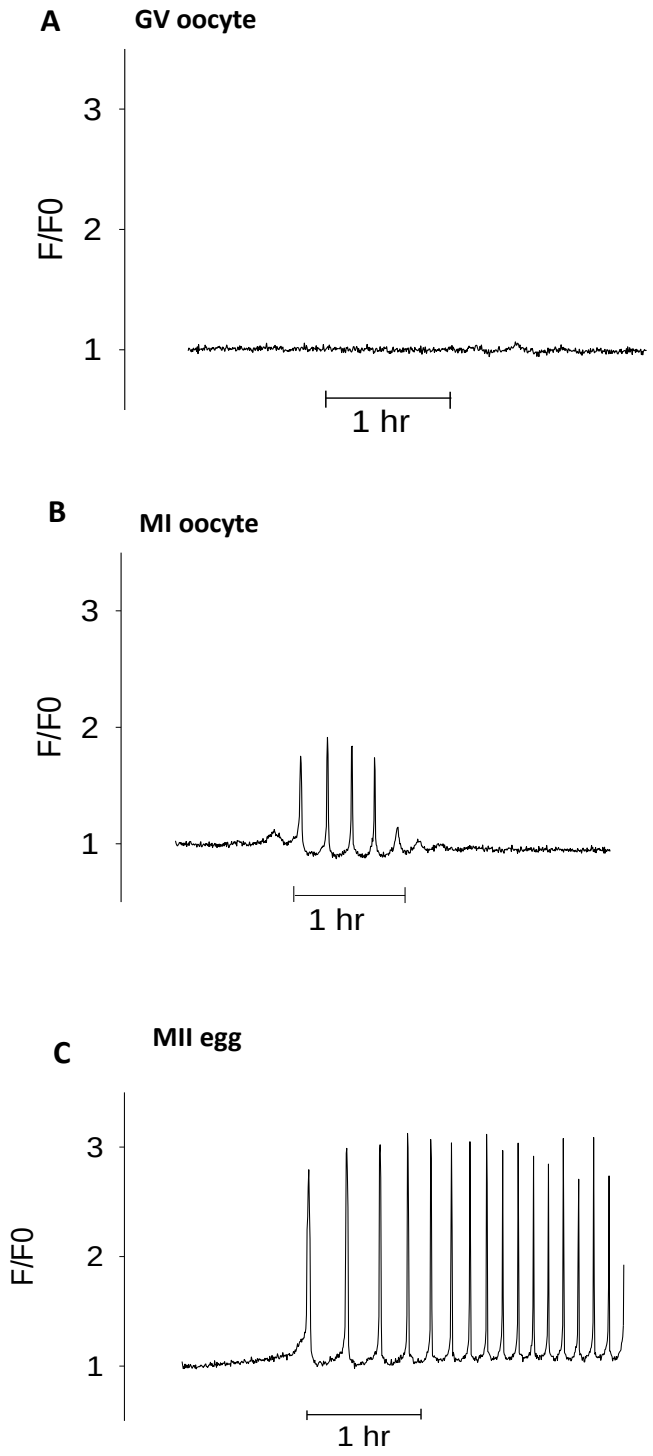


Figure 5.30 Ca^{2+}_i traces showing oscillations induced by PLCζ in oocytes at different stages of maturation

Ca^{2+}_i oscillations induced by PLCζ-luciferase cRNA (pipette concentration of 0.02 µg/µl) **A)** in GV oocytes (n=21) 3/21 oocytes oscillated at a mean luminescence value of 0.26 cps, **B)** in MI oocytes (n=26) 20/26 oocytes oscillated at a mean luminescence value of 0.24 cps, **C**) in MII eggs (n=31) 31/31 oocytes oscillated at a mean luminescence value of 0.18 cps

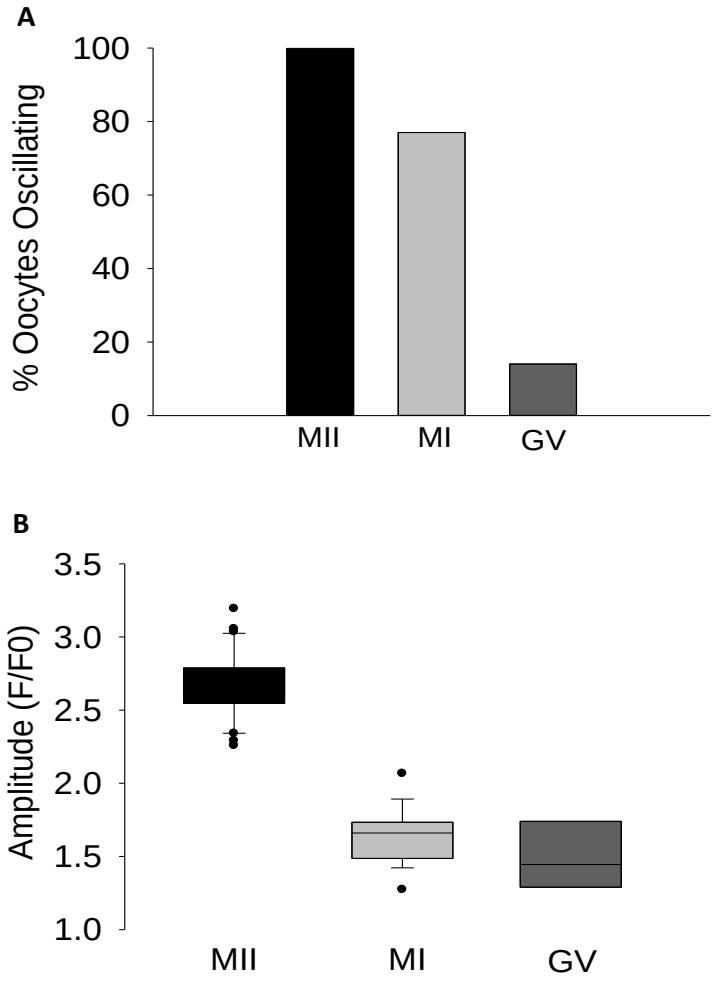


Figure 5.31 Summary of the $Ca^{2+}{}_i$ releasing ability of oocytes in response to PLCζ at different stages of maturation

A) Bar graph showing the percentage of oocytes experiencing $Ca^{2+}{}_i$ oscillations induced by PLCζ-luciferase cRNA (pipette concentration of 0.02 μg/μl) in GV oocytes (n=21), in MI oocytes n=26 oocytes and MII eggs n=31, **B)** box plot showing the amplitude of the $Ca^{2+}{}_i$ oscillations recorded in these oocytes

Deficiency of Ca^{2+}_i oscillations in GV oocytes cannot be rescued by increased levels of PLCζ

To investigate whether the decrease in sensitivity to PLCζ in GV and MI oocytes could be overcome by increasing the amount of PLCζ protein expressed by the cells the previous experiments were repeated using GV and MI oocyte however using higher concentrations of mPLCζ-luciferase cRNA (conducted with the help of Ethan Bates). Once again the number of oocytes that had oscillations in Ca^{2+}_i were recorded as well as the frequency of those oscillations. When the protein expression levels of mPLCζ-luciferase cRNA were increased to a mean of 1.7 cps, approximately 7 times the protein expression used previously, Ca^{2+}_i oscillations were recorded in all 11 MI oocytes with a slightly increased frequency (see fig 5.32, fig 5.33). However, in GV oocytes if PLCζ-luciferase cRNA expression was increased to 5.5 cps (up to 30 times the PLCζ expression used previously) only 6 out of the 25 oocytes oscillated (see fig 5.32, fig 5.33). This indicates that increasing the amount of PLCζ protein expressed in GV oocytes does not overcome the effects of the reduction in sensitivity to PLCζ. In contrast MI oocytes do have a slightly decreased sensitivity to PLCζ compared to MII eggs that can be partially overcome by an increase in PLCζ protein expression levels.

IP_3 response increases during oocyte maturation

It has previously been suggested that the differences in the Ca^{2+} releasing ability of oocytes at different stages of maturation could be attributed to differences in the sensitivity of the IP_3 receptor (Wakai *et al.* 2012). It is believed that IP_3 receptor sensitivity increases during oocyte maturation possibly due to an increase in ER Ca^{2+} concentrations and phosphorylation of the IP_3R. However, this is considered as a gradual process during oocyte maturation. To determine the difference in sensitivity to IP_3 between oocytes at different stages of maturation, the Ca^{2+} release in response to 'caged-IP_3' was investigated. Caged-IP_3 remains in-active in cells until it is exposed to UV light which photolyses the bonds that attach the protective groups to the phosphate groups of IP_3 which releases active IP_3. As a result the longer the exposure to UV light the more IP_3 is active to release Ca^{2+}. Caged IP_3 (pipette concentration of 1mM) mixed with the Ca^{2+}_i indicator OGBD was microinjected into GV oocytes, MI oocytes and MII eggs and then transferred onto the epifluorescence imaging system. The fluorescence signals from the eggs and oocytes were recorded as a measure of Ca^{2+}_i whilst they were exposed short pulses of UV light of 50, 100, 500, 1000 and 3000 ms in duration. The amplitude of the Ca^{2+}_i increase during each of these pulses was recorded and the mean Ca^{2+}_i amplitudes were calculated for GV oocytes, MI oocytes and MII oocytes (see fig 5.34). All eggs and oocytes experienced an increase in Ca^{2+}_i following every UV pulse including the 50 ms pulse. The amplitude of Ca^{2+}release increased

most significantly between 50 ms and 500 ms UV pulses in all eggs and oocytes before reaching a plateau (see fig 5.34).All Ca^{2+}_{i} increases recorded in GV oocytes regardless of duration of the UV pulse were reduced in amplitude compared to MI oocytes and MII eggs (see fig 5.34). Furthermore the Ca^{2+}_{i} increases seen in MI oocytes were also reduced in amplitude compared to MII eggs (see fig 5.34). The greatest difference in amplitude was recorded between MII eggs and MI oocytes suggesting that these differences in response to IP_3 are most significant later in oocyte maturation (see fig 5.34). The reduction in amplitude of Ca^{2+}release in GV and MI oocytes in response to IP_3 is suggestive of a reduction in the levels of Ca^{2+}_{i} available in the ER and not of a reduced sensitivity to IP_3.

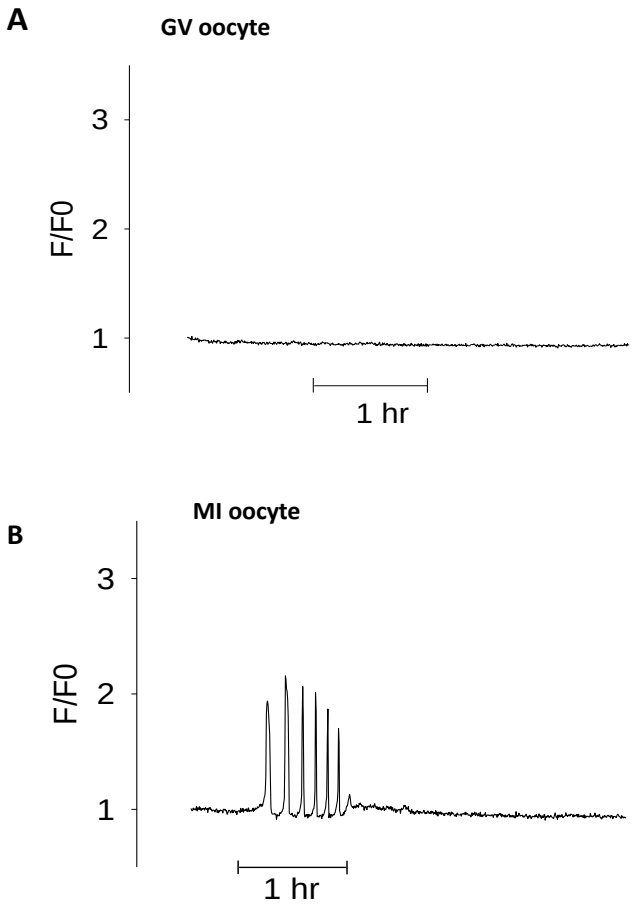


Figure 5.32 Ca^{2+}_i traces showing oscillations induced by high levels of PLCζ in GV and MI oocytes

Ca^{2+}_i oscillations induced by PLCζ-luciferase cRNA (pipette concentration of 0.25 µg/µl) **A)** in GV oocytes (n=25) 6/25 oocytes oscillated at a mean luminescence value of 5.5 cps, **B)** in MI oocytes (n=11) 11/11 oocytes oscillated at a mean luminescence value of 1.7 cps

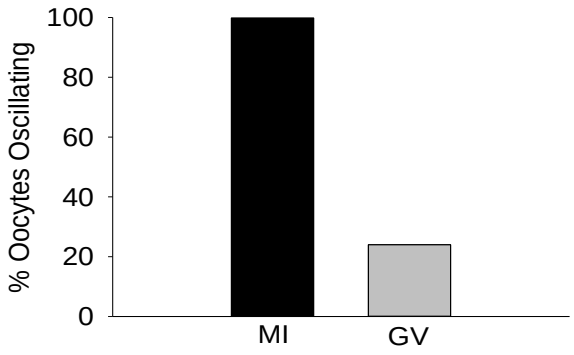


Figure 5.33 The percentage of MI and GV oocytes oscillating in response to high levels of PLCζ

Bar graph showing the percentage of oocytes experiencing Ca^{2+}_i oscillations induced by PLCζ-luciferase cRNA (pipette concentration of 0.25 µg/µl) in GV oocytes (n=25) and in MI oocytes (n=11)

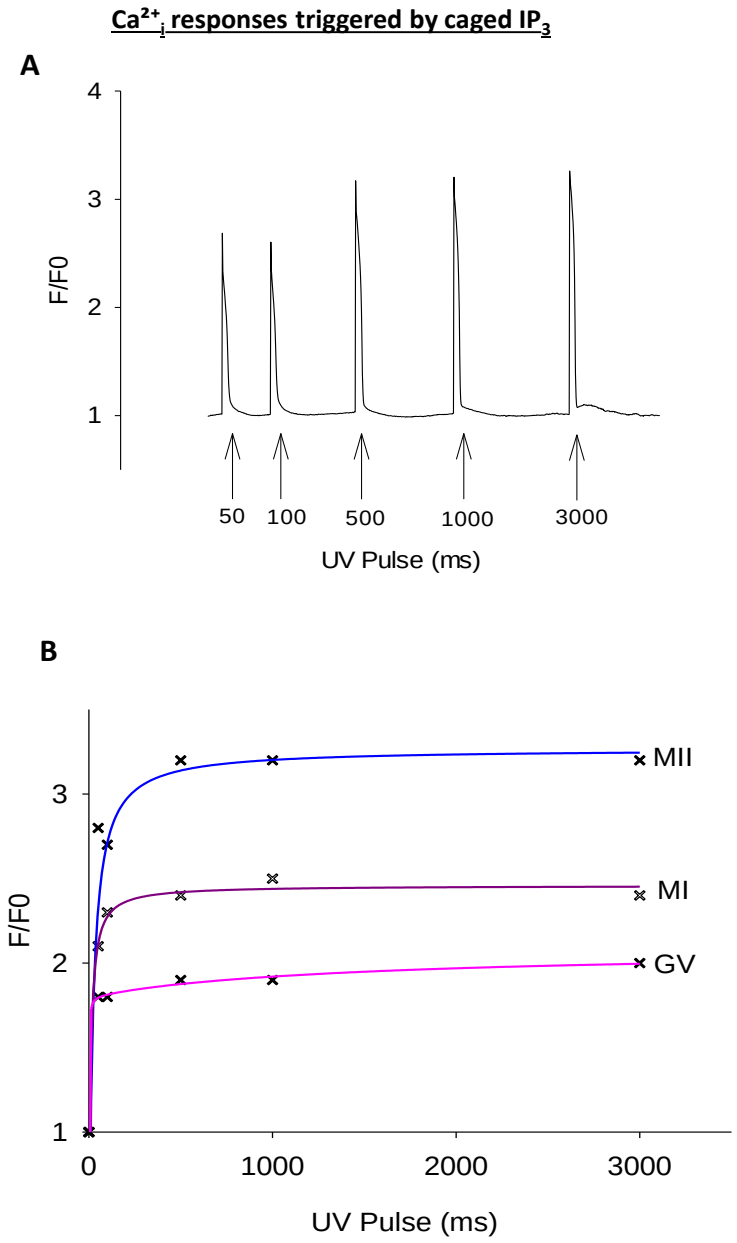


Figure 5.34 Ca^{2+}_i responses triggered by caged IP_3 in oocytes at different stages of maturation

A) Example of Ca^{2+}_i increases in an MII egg in response to the photo-release of caged IP_3 by different duration of UV pulses (in ms) (n=5), **B)** line graph showing the mean amplitude of Ca^{2+}_i increase in response to the photo-release of caged IP_3 by different duration of UV pulses (in ms), in MII eggs (n= 5), MI oocyte (n=5) and GV oocytes (n=5)

The lack of Ca^{2+}_i response in GV oocytes cannot be accounted for by nuclear localisation of PLCζ

Mouse derived PLCζ contains a nuclear localisation sequence (NLS) in its structure that targets PLCζ to the nucleus when the pronuclei form during fertilisation (Larman *et al.* 2004). This process occurs many hours after sperm egg fusion and coincides with the termination of Ca^{2+}_i oscillations (Larman *et al.* 2004; Ito *et al.* 2008). During the first mitotic cycle the nuclear envelope breaks down and PLCζ is released into the cytosol once again (Larman *et al.* 2004). This results in Ca^{2+}_i oscillations that appear to play no significant role in embryo development. This suggests that PLCζ becomes sequestered in the nucleus where it is not able to hydrolyse $PI(4,5)P_2$ and release Ca^{2+}. It is therefore possible that the lack of Ca^{2+}release initiated by PLCζ in GV oocytes could be a result of nuclear localisation of PLCζ. To determine if this is the case the localisation of mPLCζ-luciferase protein was examined as this is a fusion protein concentration of luminescence value equates to concentration of PLCζ protein. Images of the luminescence signals were captured of GV oocytes expressing high levels of mPLCζ-luciferase cRNA where the luminescence signal was integrated for 30 minutes 1.5 hours post microinjection. The luminescence signal appeared distributed throughout the cytoplasm in these oocytes suggesting that mPLCζ-luciferase protein is not concentrated to the nucleus (see fig 5.35). However, as these images were of a low resolution it was important to establish if luciferase protein contained within the nucleus of a GV oocyte would be evident using this type of imaging. To test this luciferase protein was microinjected directly into the germinal vesicle of GV oocytes. The size of this luciferase protein meant that is was too big to be exported out of the nucleus through the nuclear pores as a result the protein was retained in the nucleus. When these GV oocytes were imaged in the same way as those expressing mPLCζ-luciferase cRNA, it was clear there was a strong luminescence signal emitting from the nucleus compared to the cytoplasm (see fig 5.35). This suggests that if mPLCζ-luciferase was located in the nucleus in GV oocytes this would be visible by an increase in luminescence signal emitted from the nucleus. As this was not the case it appears the lack of Ca^{2+}_i oscillations in response to PLCζ is not due to the nuclear localisation of the mPLCζ-luciferase.

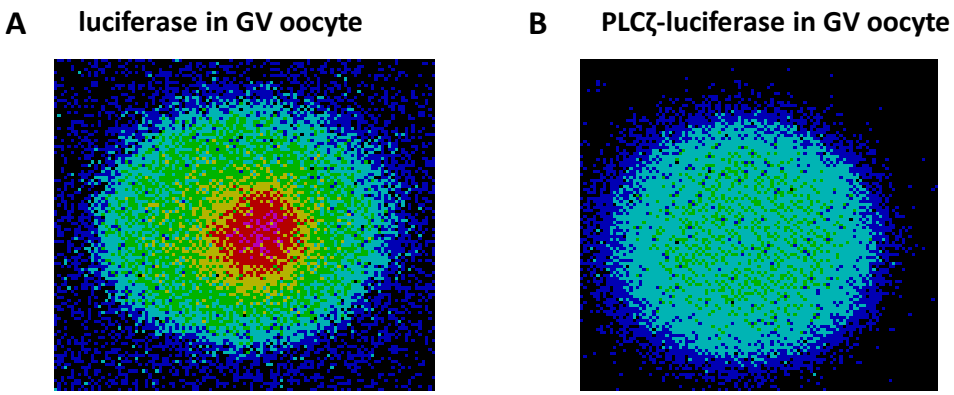


Figure 5.35 Luminescence images of GV oocytes injected with luciferase protein and PLCζ-luciferase

A) Luminescence image of a GV oocyte injected with luciferase (70 kda) protein at a pipette concentration of 1 µg/l directly into the GV, light integrated over 30 minutes (n= 4), **B)** Luminescence image of a GV oocyte injected with PLCζ-luciferase cRNA (pipette concentration of 0.25 µg/µl) Image taken 1.5 hours post injection and light integrated over 30 minutes (n= 7)

Changes in $PI(4,5)P_2$ availability and organelle distribution during oocyte maturation may enable PLCζ induced $Ca^{2+}{}_i$ oscillations to occur in eggs

Immuno-staining shows $PI(4,5)P_2$ localisation alters during oocyte maturation

As previously mentioned PLCζ is believed to hydrolyse a specific vesicular source of $PI(4,5)P_2$ in eggs in order to produce IP_3 and release Ca^{2+} (Yu *et al.* 2012). It has previously been suggested that this vesicular source of $PI(4,5)P_2$ becomes available during oocyte maturation (Yu *et al.* 2012). Immunostaining of endogenous $PI(4,5)P_2$ shows that in GV oocytes $PI(4,5)P_2$ is localised primarily to the plasma membrane and the germinal vesicle (Yu *et al.* 2012). In MI oocytes and MII eggs $PI(4,5)P_2$ can be seen in small vesicles throughout the cytoplasm however the number and density of the vesicles is much reduced in MI oocytes (Yu *et al.* 2012). This result was verified in the current study using immunostaining with antibodies raised against $PI(4,5)P_2$. Immunostaining against $PI(4,5)P_2$ was carried out as described previously on both MII eggs and GV oocytes. Once again GV oocytes were found to have very little vesicular $PI(4,5)P_2$ instead $PI(4,5)P_2$ was concentrated to nucleus however very little $PI(4,5)P_2$ was localised to the plasma membrane unlike that seen previously (see fig 5.36).

$PI(4,5)P_2$ localisation during oocyte maturation in live cells

To investigate the distribution of $PI(4,5)P_2$ in oocytes at different stages of maturation in live cells the localisation of exogenous BODIPY FL-$PI(4,5)P_2$ was investigated in GV oocytes and MII eggs. FL-$PI(4,5)P_2$ was microinjected into GV oocytes and MII eggs in the same manner as described previously. These oocytes were then imaged on the confocal microscope to determine the distribution of FL-$PI(4,5)P_2$. As recorded previously FL-$PI(4,5)P_2$ bound to small vesicles throughout the cytoplasm in MII eggs. These small $PI(4,5)P_2$ positive vesicles were also recorded in GV oocytes however the number of vesicles appeared reduced in amount and density compared to those seen in MII eggs (see fig 5.37).

In order to investigate the distribution of endogenous $PI(4,5)P_2$ in immature oocytes compared to MII eggs DAN20 was once again used as a live cell probe for measuring $PI(4,5)P_2$ distribution. DAN20 was injected into GV oocytes in exactly the same manner as it had been in MII eggs. These oocytes were then imaged using the same imaging settings on the confocal microscope as that for the MII eggs. As with MII eggs DAN20 appeared to localise small vesicles throughout the cytoplasm in GV oocytes. This suggests that a

vesicular source of $PI(4,5)P_2$ that is accessible by DAN20 is present in GV oocytes as well as MII eggs (see fig 5.38).

Finally the distribution of PBP10 was examined in GV oocytes and compared to that distribution in MII eggs. PBP10 was microinjected into GV oocytes in the same way it was injected into MII eggs and imaged using the exact same parameters. PBP10 was found to localise to small vesicles that were similar in distribution and pattern to that seen in MII eggs (see fig 5.39). Once again these results suggest that GV oocytes do contain some intracellular source of $PI(4,5)P_2$.

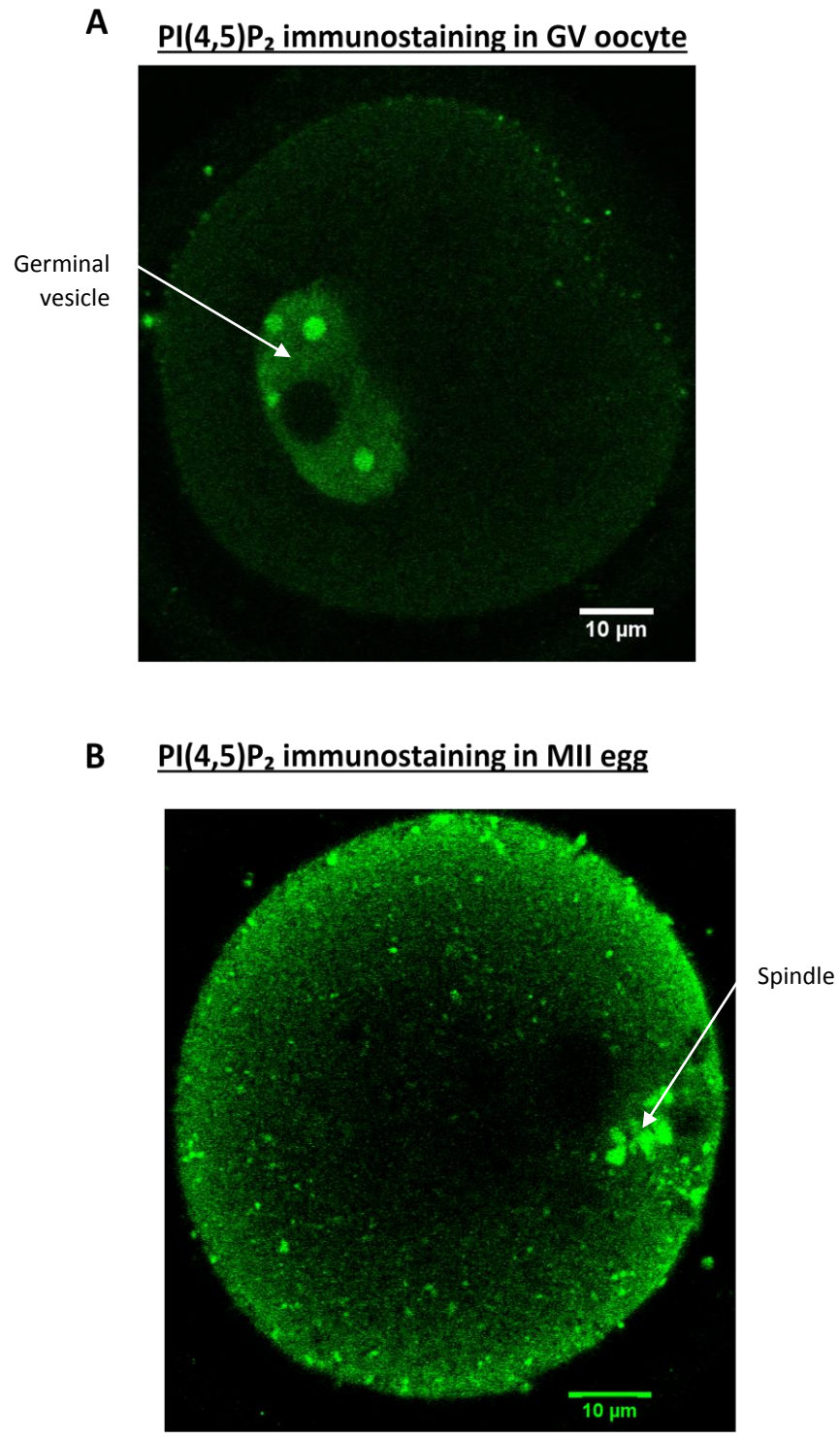


Figure 5.36 Confocal images of PI(4,5)P_2 immunostaining in GV oocyte and MII egg

A) GV oocyte (n=5), **B)** MII egg (n= 10)

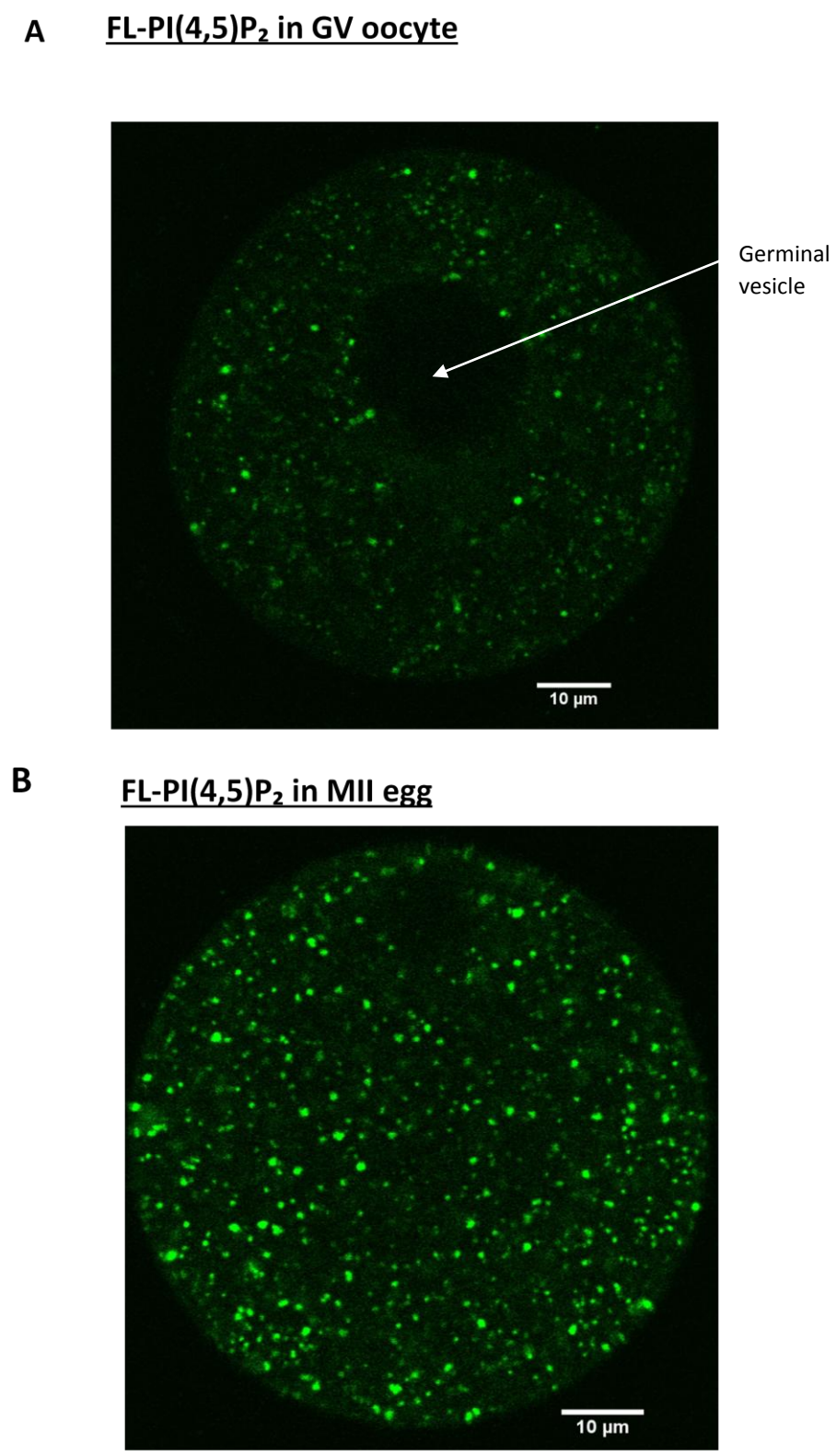


Figure 5.37 Confocal images showing the distribution of BODIPY-FL PI(4,5)P in GV oocyte and MII egg

1.5 hours post BODIPY-FL PI(4,5)P_2 injection in **A)** GV oocyte (n=5), and **B)** MII egg (n=7)

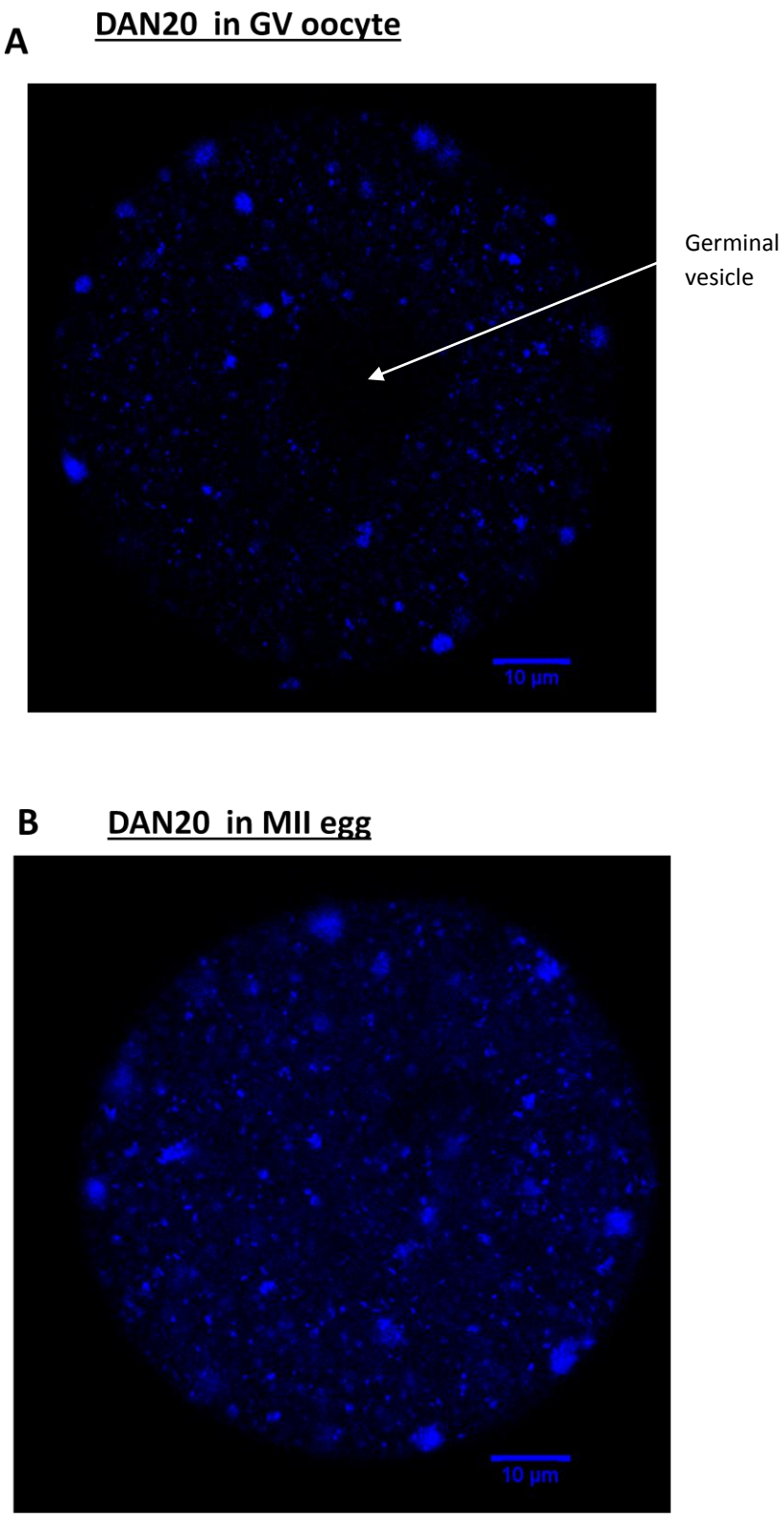


Figure 5.38 Confocal images showing the distribution of DAN20 in GV oocyte and MII egg

1 hr post DAN20 injection **A)** GV oocyte (n=6), and **B)** MII egg (n=17)

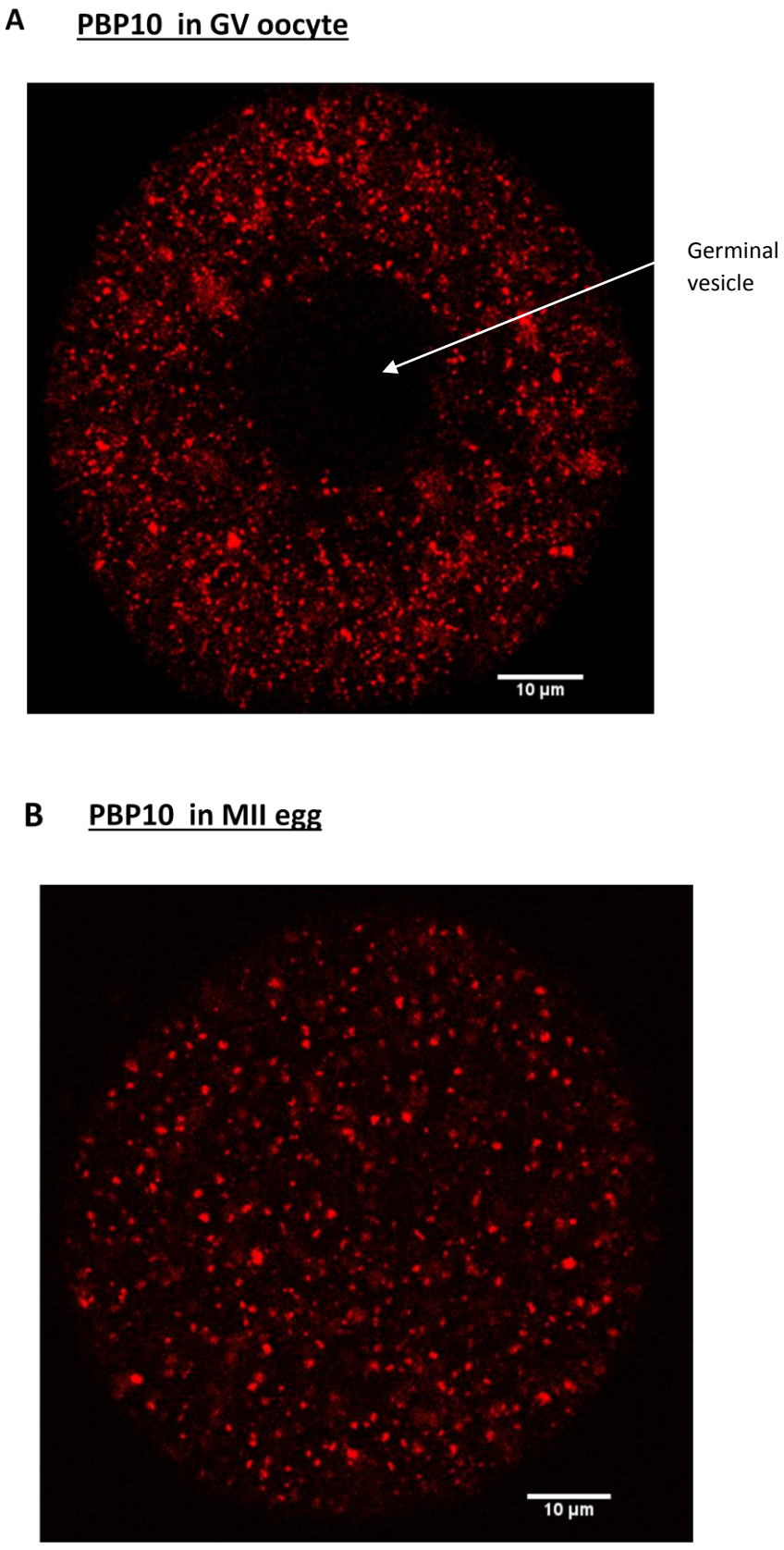


Figure 5.39 Confocal images showing the distribution of PBP10 in GV oocyte and MII egg

1.5 hr post PBP10 injection **A**) GV oocyte (n=12), and **B)** MII egg (n=15)

Golgi apparatus distribution during oocyte maturation using the Golgi marker AC-lectin in fixed cells

Experiments conducted during this study have shown that the Golgi marker AC-lectin localises to small vesicles throughout the cytoplasm of MII eggs suggesting that the Golgi is fragmented into small vesicles in mature eggs. This idea is supported by previous immunocytochemistry experiments showing that Golgi markers localise to small vesicles in mature eggs (Moreno *et al.* 2002; Payne and Schatten 2003) (Yu et al 2012 unpublished). Interestingly this Golgi fragmentation seems to occur during oocyte maturation (Moreno *et al.* 2002). GV oocytes are found to have Golgi stacks which break down and fragment during GVBD (Moreno *et al.* 2002). As it is possible that the vesicular source of $PI(4,5)P_2$ required for PLCζ activity is associated with the Golgi apparatus a change in Golgi structure during oocyte maturation may enable vesicular $PI(4,5)P_2$ to become available. The structure and cellular localisation of the Golgi apparatus during oocyte maturation was investigated further using the Golgi marker AC-lectin. GV oocytes were collected and maintained in IBMX whilst another group of GV oocytes were allowed to spontaneously mature for approximately 5 hours until GVBD had clearly occurred, these oocytes were referred to as MI oocytes. The GV oocytes, MI oocytes and MII eggs were all fixed and permeabilised before being incubated in fluorescent AC-lectin as described previously. In all oocytes and eggs the AC-lectin appeared to localise to small vesicles throughout the cytoplasm. This staining did not appear significantly different between GV, MI, and MII oocytes (see fig 5.40). This suggests that the Golgi apparatus is already fragmented in GV oocytes and this does not alter significantly during oocyte maturation.

Golgi apparatus distribution during oocyte maturation in live cells

The live cell Golgi marker GOLGI-ID® was used to determine the localisation of the Golgi in unfixed MII eggs and showed binding to small intracellular vesicles. It was therefore necessary to use this probe to establish distribution of the Golgi in live GV oocytes. GV oocytes were treated with GOLGI-ID® assay kit in the same way as described previously however with the addition of IBMX to ensure the oocytes didn't spontaneously mature. A selection of MII eggs were also treated in this way to ensure that the presence of IBMX did not alter the staining pattern of the GOLGI-ID®. The eggs and oocytes were also imaged on the confocal microscope using the same settings. As was described previously GOLGI-ID® bound mainly to small vesicles in MII eggs with some weaker nonspecific staining also (see fig 5.41). However the GOLGI-ID® did not appear to bind small vesicles in GV oocytes but instead larger more aggregated structures (see fig 5.41). This suggests that in the GV

oocytes the Golgi is not fully fragmented and during oocyte maturation it gets broken down into small intracellular vesicles.

BODIPY FL ceramide and $DiIC_{18}$(3) co-localisation does not significantly alter during oocyte maturation

BODIPY FL ceramide and $DiIC_{18}$(3) both appear to act as fluorescent markers for the endoplasmic reticulum in MII eggs. However, BODIPY FL ceramide is an established marker for the Golgi apparatus in somatic cells. The distribution of endoplasmic reticulum is reported to alter during oocyte maturation as does the Golgi apparatus. It is possible therefore that BODIPY-FL ceramide could act as a marker for the Golgi apparatus in immature oocytes. To determine whether BODIPY- FL ceramide distribution overlaps with that of $DiIC_{18}$(3) in GV oocytes these were both microinjected into GV oocytes as described previously. The distribution of both these probes was examined in GV oocytes by imaging on a confocal microscope. As in MII eggs, BODIPY-FL ceramide and $DiIC_{18}$(3) very strongly co-localised in the GV oocytes. This would suggest that as in MII oocytes BODIPY-FL ceramide is acting as a marker for the ER in GV oocytes. The distribution of both of these probes was different in GV oocytes compared to MII eggs. For example in GV oocytes the pattern of staining appeared less reticular and more fragmented when compared to the staining in MII eggs indicating that the structure and distribution of the ER alters during oocyte maturation (see fig 5.17).

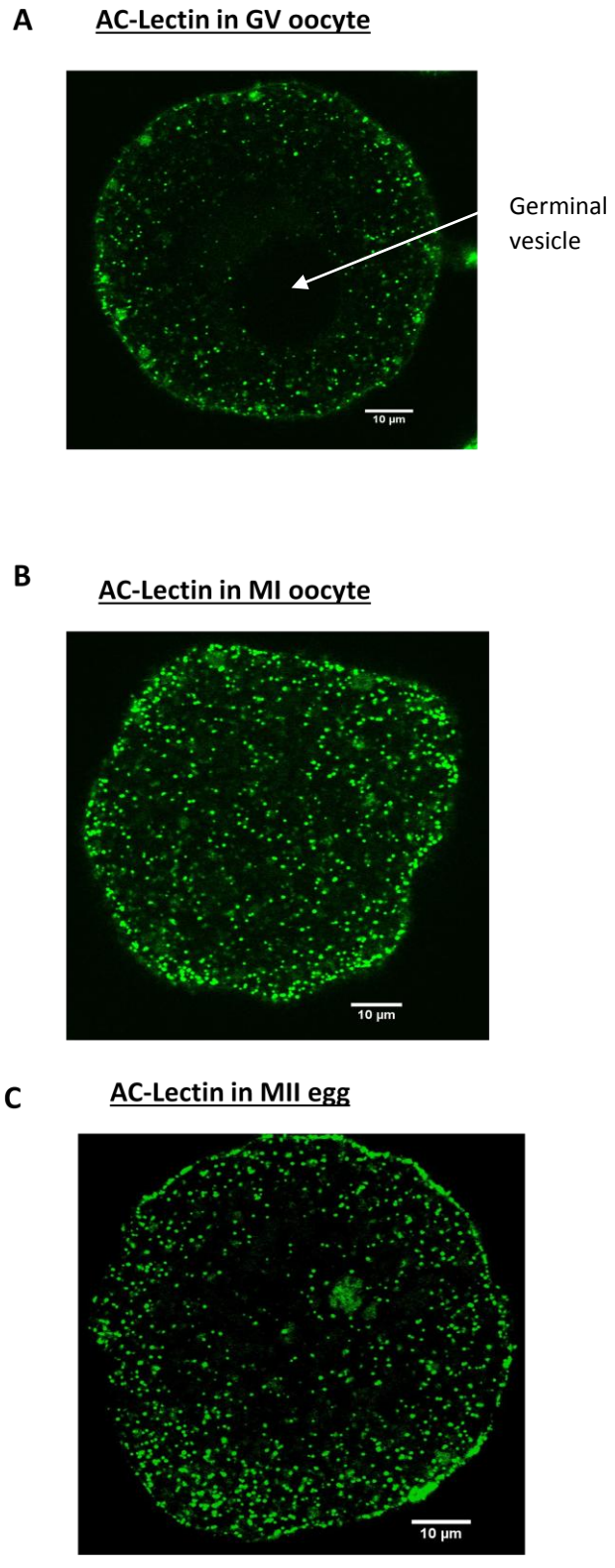


Figure 5.40 Confocal images showing AC-Lectin staining in oocytes at different stages of maturation

A) GV oocyte (n=5), **B)** MI oocyte (n=5) and C) MII egg (n=9)

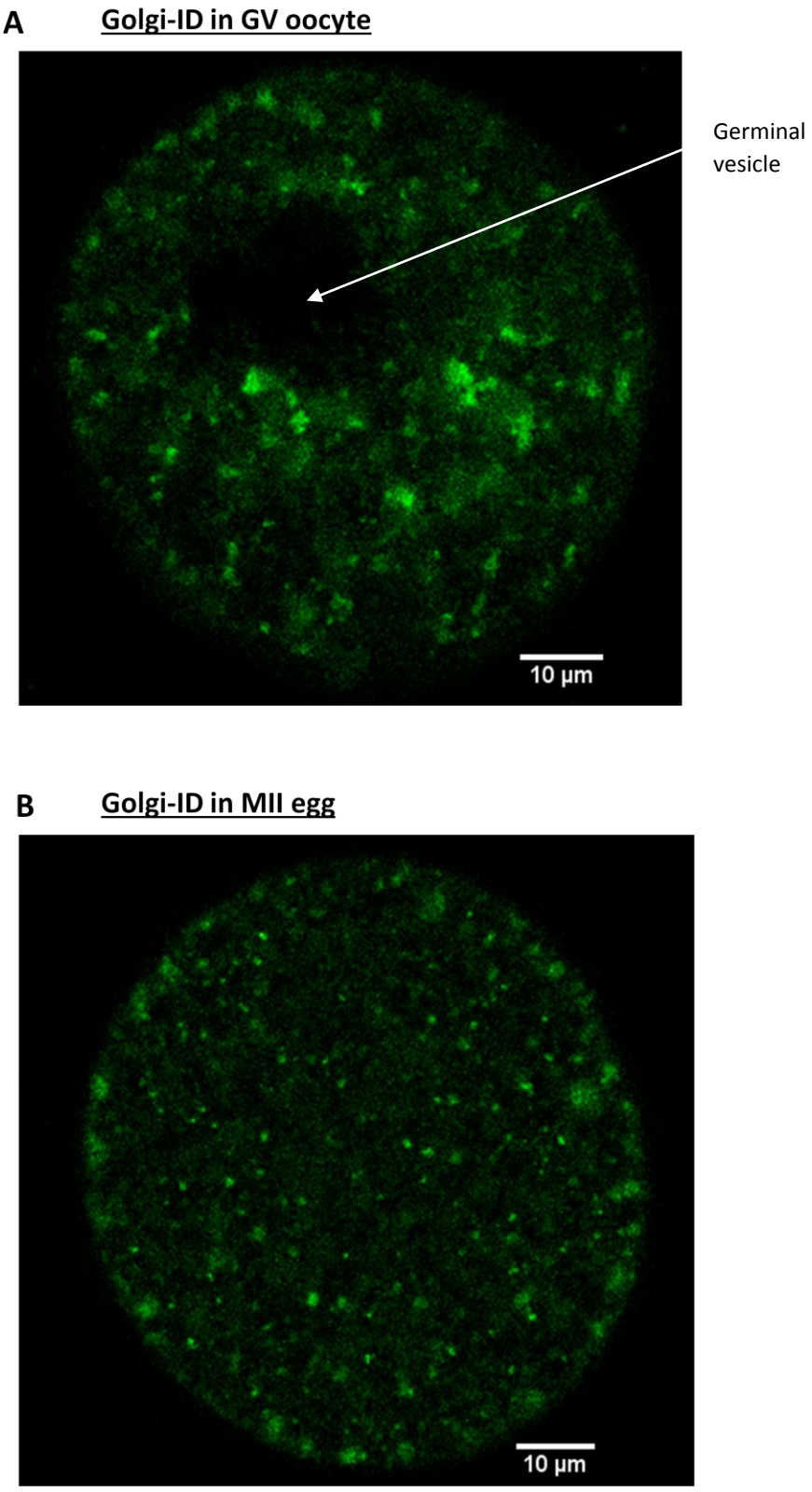


Figure 5.41 Confocal images showing the distribution of GOLGI-ID in GV oocytes and MII egg

A) GV oocyte (n=5), **B)** MII egg (n=15)

Discussion

PLCζ has a unique ability to cause Ca^{2+}_i oscillations in mature eggs at basal cellular Ca^{2+}_i levels (Saunders *et al.* 2002). This ability is not only a result of particular features of PLCζ structure but also a collection of specific cellular characteristics of the egg. This is most clearly demonstrated by the lack of Ca^{2+}_i releasing activity elicited by PLCζ in other cell types. There are many features that make mature eggs different to other cell types however exactly how these features directly impact PLCζ activity remains fully understood. Though PLCζ hydrolyses $PI(4,5)P_2$ like all other PLC isoforms, it has strongly been suggested that this $PI(4,5)P_2$ is not located on the plasma membrane. Instead PLCζ is thought to target and hydrolyse and alternative source of $PI(4,5)P_2$ (Yu *et al.* 2012). This idea is supported by immunostaining results that have shown PLCζ localises to vesicular structures in eggs and does not appear to target the plasma membrane (Yu *et al.* 2008; Yu *et al.* 2012). The current experiments aimed to determine where PLCζ is localises, what potential organelles it targets, whether there is an alternative source of $PI(4,5)P_2$ in the egg and how these features may alter during oocyte maturation.

First of all it was important to verify where PLCζ localised in mature eggs. Immunocytochemisty analysing the distribution of cMyc tagged PLCζ reproduced results seen previously that PLCζ targets small vesicles throughout the cytoplasm which are concentrated in the cortical region of the egg (Yu *et al.* 2012). Furthermore, this staining does not show PLCζ targeting to the plasma membrane which provides further support that PLCζ does not localise to the plasma membrane in eggs (Yu *et al.* 2012). However, this staining alters following pronuclei formation. Four hours post PLCζ injection the same protocol shows that PLCζ is still localised to small vesicles throughout the cytoplasm but also became concentrated in the newly formed pronuclei. This supports previous data showing that fluorescently tagged PLCζ becomes localised to the pronucleus following egg activation (Larman *et al.* 2004; Yoda *et al.* 2004).

Tagging PLCζ with cMyc enabled antibodies raised against cMyc to be used to determine cMyc-PLCζ localisation in the egg. This allows the immunostaining for PLCζ to be more specific than would be achieved using the current antibodies raised against PLCζ. However, as the cMyc antibodies used were mouse derived monoclonal antibodies and the eggs used also derived from mouse extra blocking steps were required in order to ensure specific staining. Optimising the correct blocking conditions and antibody parameters in order to get optimal staining is a challenge. This also makes it difficult to get consistent results

between groups of eggs. For example as previously recorded a greater amount of this vesicular bound PLCζ was concentrated in the cortical region of the egg (Yu *et al.* 2012). However, it is possible that this increase in fluorescent signal was a result of increased permeability of those membranes closer to the egg plasma membrane. This is because once the cells have been they must be permeablised to allow the antibodies to enter the cell. The eggs are incubated in the permeabilsation media so the membranes become permeablised from the outside of the cell inwards. Eggs are larger in size than most other cells as a result if the membranes towards the centre of the egg do not become sufficiently permeablised then the antibodies may not penetrate the egg sufficiently. In addition immunostaining may only be performed on fixed cells, therefore, this technique cannot be used to investigate PLCζ localisation in live eggs.

To try and address this issue two fluorescently tagged genetically encoded PLCζ constructs were developed that could be injected into live cells. Both of constructs were based on a mutated catalytically dead form of PLCζ, PLCζ^{D210}. The two fluorescent tags chosen are both known to be stable fluorophores that produce a bright signal. However, the fluorescent signal produced by PLCζ^{D210}-cherry expressed in eggs was low and appeared uniform throughout the cytoplasm with no specific intracellular localisation. This suggests that PLCζ does not target specific membranes in the egg. However, it is important to note that in order to see a fluorescent signal a protein must be expressed at relatively high levels of well over 100 nM whereas PLCζ's operational range is between 1 and 10 nM in mouse eggs (Niswender *et al.* 1995; Saunders *et al.* 2002). It can therefore be assumed that the detectable amount of PLCζ^{D210}-cherry far exceeds the physiological levels of PLCζ usually in the egg. This could mean the binding sites for PLCζ^{D210}-cherry may already be saturated at levels much lower than is detectable which could result in a large amount of mis-localised unbound PLCζ^{D210}-cherry in the egg cytoplasm. Once again however there was no binding of PLCζ specifically to the plasma membrane. Fluorescent Venus-tagged PLCζ has also shown previously that PLCζ remains in the cytoplasm and does not target the plasma membrane (Yoda *et al.* 2004; Ito *et al.* 2008; Phillips *et al.* 2011).

This lack of plasma membrane bound PLCζ and its binding to intracellular vesicles supports the notion PLCζ targets an alternative pool of $PI(4,5)P_2$. This is because PLCζ must bind to a $PI(4,5)P_2$ source directly in order to hydrolyse it. It was therefore important to determine whether an alternative source of $PI(4,5)P_2$ was available in eggs. The immunostaining data by (Yu *et al.* 2012)showing that $PI(4,5)P_2$ localises in intracellular vesicles was reproduced. This suggests that an alternative vesicular source of $PI(4,5)P_2$ is present in eggs however

these results were variable and the staining appeared inconsistent. This may be because $PI(4,5)P_2$ is located on the inner leaflet of membranes. Small variations in the permeablisation of intracellular membranes could result in dramatic changes in the accessibility antibodies to the $PI(4,5)P_2$. Alternatively, over permeablisation of these membranes could cause the $PI(4,5)P_2$ to be dislodged from its resident membrane and therefore immunostaining may not give a clear representation of $PI(4,5)P_2$ in live cells.

It was therefore helpful to find an alternative way to measure $PI(4,5)P_2$ localisation in live eggs. One simple way to investigate the localisation of $PI(4,5)P_2$ is to inject exogenous fluorescently tagged $PI(4,5)P_2$ into eggs and see where it localises. It is very likely that this $PI(4,5)P_2$ will be anchored to membranes that contain endogenous $PI(4,5)P_2$ (Golebiewska *et al.* 2008). In somatic cells fluorescently tagged $PI(4,5)P_2$ is shown to localise primarily to the plasma membrane (Golebiewska *et al.* 2008) When injected into eggs, FL-$PI(4,5)P_2$ appeared to bind to small vesicles throughout the cytoplasm and did not bind significantly to the plasma membrane. However, it took approximately 1 and a half hours for this $PI(4,5)P_2$ to become partitioned into these vesicles. This suggests that when first injected the $PI(4,5)P_2$ will bind to any intracellular membrane however with time $PI(4,5)P_2$ is excluded from these membranes and instead becomes concentrated on vesicles. It is therefore possible that rather than being synthesised on vesicles, endogenous $PI(4,5)P_2$ could be made on other cellular membranes and transported to these small vesicles where it becomes sequestered. PI transfer proteins (PITP) for example may be involved in this process(Cockcroft *et al.* 1985). In somatic cells for example plasma membrane localised $PI(4,5)P_2$ levels are dependent on the availability of its precursor, PI(4)P at both the plasma membrane and Golgi apparatus (Dickson *et al.* 2014).

It was next necessary to establish whether PLCζ was able to hydrolyse this vesicular source of $PI(4,5)P_2$. Indeed the FL-$PI(4,5)P_2$ localisation and signal was seen to disperse following PLCζcRNA addition. After approximately 1 hour and a half the fluorescent signal became much more diffuse in those eggs expressing PLCζ and virtually all vesicular signal had gone. This may be because as the FL-$PI(4,5)P_2$ is hydrolysed fluorescent DAG is produced which may be transported to other membranes involved in DAG metabolism and therefore this could lead to a loss of fluorescent signal from the vesicles. This is yet to be explored however. In contrast the control eggs that had not been injected with PLCζ still showed a strong fluorescent signal emitting from vesicles in the egg. This suggests the PLCζ is capable of hydrolysing vesicular localised fluorescent $PI(4,5)P_2$.

However, the localisation of exogenous PI(4,5)P_2 does not necessarily give a true representation of endogenous PI(4,5)P_2 distribution in eggs. In order to investigate this a PI(4,5)P_2 binding probe was required that was capable of binding all sources of PI(4,5)P_2 in the egg. Recently one such probe has been developed. DAN20 is a gelsolin peptide based probe that is cell permeable and produces a ratiometric fluorescent signal (Mondal *et al.* 2016). It has been shown to bind both PI(4)P and PI(4,5)P_2 *in vitro* with an increased specificity for PI(4,5)P_2 (Mondal *et al.* 2016). DAN 20 has also been used to successfully measure PI(4,5)P_2 levels and distribution in live somatic cells (Mondal *et al.* 2016). As expected DAN20 was found to localise primarily to the plasma membrane of HEK cells however staining was also seen in the perinuclear region of the cell indicating an intracellular pool of PI(4,5)P_2 (Mondal *et al.* 2016). When injected into eggs DAN20 was found to localise to intracellular vesicles throughout the cytoplasm. These vesicles appeared similar in nature to the ones FL-PI(4,5)P_2 bound to. This suggests that DAN20 is able to measure an intracellular source of PI(4,5)P_2 in eggs.

In addition to binding the small vesicular structures previously seen, DAN20 was also found to bind larger vesicular structures closer to the cortex. These structures appeared similar in size and structure to those associated with the endocytic pathway such as early endosomes (Moon and Swann 2013, unpublished). Early endosomes are rich in the phosphoinostide PI(3)P so it is possible that as well as binding PI(4,5)P_2 , DAN20 is also binding other phosphoinostides such as PI(3)P. Some binding to the precursor of PI(4,5)P_2 , PI(4)P was identified *in vitro* and though this was much lower than PI(4,5)P_2 DAN20 cannot be considered solely specific for PI(4,5)P_2 (Mondal *et al.* 2016) . However, unlike the PH domain DAN20 was not found to bind IP_3so a reduced amount of non-specific cytosolic staining would be expected (Mondal *et al.* 2016).PH domain binding to PI(4,5)P_2 is also suggested to be dependent on the levels of cholesterol present in the membrane(Rissanen *et al.* 2017). Due to ratiometric nature of the DAN tag DAN20 allows quantitative and dynamic measurements of PI(4,5)P_2 to be made. However, this tag is environmentally sensitive and was found to be susceptible to bleaching. This means that a stable signal did not last long in eggs injected with DAN20 and made designing dynamic experiments or repeat imaging difficult. As a result another gelsolin peptide based PI(4,5)P_2 probe was tested. PBP10 is based on a 10 amino acid sequence that is conjugated to fluorescent rhodamine B (Bucki *et al.* 2001; Cunningham *et al.* 2001). Rhodamine B produces a stable red signal and is much less susceptible to bleaching than DAN20 so made it easier to image. The shorter nature of PBP10 also makes it more cell permeable than DAN20

(Cunningham*et al.* 2001; Mondal *et al.* 2016). This could make access through intracellular membranes easier. However, shorter gelsolin peptides are thought to be less specific to $PI(4,5)P_2$ and more likely to bind other phosphoinositides. For example a 13 amino acid version of DAN20 (DAN13) is binds to PI(4)P with a greater affinity than DAN20 (Mondal *et al.* 2016). When injected into eggs however PBP10 appeared to bind strongly to small vesicles throughout the cytoplasm in eggs. These vesicles looked very similar in nature to that seen with DAN20 and FL-$PI(4,5)P_2$.

No clear plasma membrane staining with DAN20 or PBP10 was apparent in eggs. Though this suggests that there is not any plasma membrane $PI(4,5)P_2$ present in eggs it is possible that the $PI(4,5)P_2$ levels in the plasma membrane are simply lower than that seen on vesicles. As a result the fluorescent signal from these compartments would be much greater than that of the plasma membrane. This would make the plasma membrane signal difficult to detect. It is also possible that DAN20 and PBP10 do not actually bind to $PI(4,5)P_2$ on the plasma membrane in eggs. Though DAN20 and PH domains both show strong plasma membrane staining in somatic cells these two probes do not appear to bind to the same pools of $PI(4,5)P_2$ (Mondal *et al.* 2016). This is significant as GFP PH domains have previous shown to bind to plasma membrane resident $PI(4,5)P_2$ in eggs (Halet *et al.* 2002). However, this source of $PI(4,5)P_2$ does not seem to be hydrolysed by PLCζ (Yu et al 2012). It may be possible therefore that PLCζ discriminates between different pools of $PI(4,5)P_2$. PLCζ lacks the $PI(4,5)P_2$ binding PH domain located on other PLCs which ensures sufficient localisation and targeting to plasma membrane $PI(4,5)P_2$ (Lemmon *et al.* 1995). It is now known that other functional domains of PLCζ fulfil this role, with both the XY linker region and the EF hand domain being shown to play a role in $PI(4,5)P_2$ binding thus enabling effective Ca^{2+}release (Nomikos *et al.* 2011b), (see Chapter 4). It is possible that PLCζ targets a specific pool of $PI(4,5)P_2$ either by discrimination of $PI(4,5)P_2$ binding or binding to a target protein on a specific membrane. Unfortunately it was difficult to conduct colocalisation experiments with either PBP10 or DAN20 and other markers because neither of these probes were fixable and were environmentally sensitive.

This data shows consistently that there is a vesicular source of $PI(4,5)P_2$ present in mature eggs. It is therefore very possible that PLCζ directly binds to these $PI(4,5)P_2$ containing vesicles as has previously been suggested (Yu *et al.* 2012)(Yu and Swann 2013, unpublished). It is this accessibility to a vesicular source of $PI(4,5)P_2$ that may enable PLCζ to release Ca^{2+}in eggs. For example CHO cells do not contain a vesicular source of $PI(4,5)P_2$ instead they localise all their $PI(4,5)P_2$ to the plasma membrane and these cells are not

able to release Ca^{2+}response to PLCζ (Phillips *et al.* 2011). Therefore it is possible that PLCζ is not able to hydrolyse any $PI(4,5)P_2$ generated in cells but instead requires $PI(4,5)P_2$ present in intracellular vesicles.

It still remains unclear where these $PI(4,5)P_2$ positive vesicles come from and how they are formed. Understanding the identity of these vesicles could provide clues to how PLCζ is localised there. There are two main candidates for the origin of these vesicles based on previous evidence. First of these is he endoplasmic reticulum. The ER marker DsRed-ER has been found to co-localise with Venus tagged PLCζ in eggs (Escoffier *et al.* 2015a). mCherry tagged PLCζ was also found to bind cytoplasmic organelles associated with the reticular network in somatic cell lines (Kashir *et al.* 2011) . However, the ER is not known to take a fragmented form in eggs, instead remains reticular in structure and becomes clustered in the cortex (FitzHarris *et al.* 2007). This result was verified in the current study using the ER dye $DiIC_{18}$(3)which showed consistently that the ER formed a reticular pattern in the mature eggs. This staining pattern was very different to the small vesicles detected by both PLCζ and $PI(4,5)P_2$ markers. It is therefore unlikely that these vesicles are derived from the ER. However, during membrane trafficking portions of the ER are syphoned off to produce vesicles. During this process some proteins retain the ER proper whilst other proteins or lipids bind to the vesicles. It is therefore possible that these PLCζ binding vesicles could be vesicular fragments of the ER. However, the ER is not known to contain $PI(4,5)P_2$ or its precursor PI(4)P. Furthermore it is likely that the co-localisation seen by (Escoffier *et al.* 2015a) was an artefact due to the overexpression of fluorescent proteins therefore true localisation of these markers at physiological levels would have been difficult to ascertain.

The other potential origin for the $PI(4,5)P_2$ containing vesicles is the Golgi apparatus. There are numerous reasons why these vesicles are likely to originate from the Golgi. Firstly in eggs the Golgi is thought to be fragmented into vesicles. This is a cell cycle dependent process that sees the Golgi become fragmented into what is known as a “Golgi haze” during metaphase of mitosis (Altan-Bonnet *et al.* 2003; Axelsson and Warren 2004). A similar process is thought to occur during meiosis in eggs (Moreno *et al.* 2002; Payne and Schatten 2003). Indeed immunostaining against the Golgi marker giantin has previously shown that this protein is located in small vesicles throughout the cytoplasm and particularly concentrated in the cortex (Yu and Swann 2013 unpublished). This result is a result that was reproduced in the current study. The Golgi is also thought to contain $PI(4,5)P_2$ and its precursor PI(4)P (Godi *et al.* 1999; De Matteis and D'Angelo 2007). This would enable efficient and quick resynthesis of $PI(4,5)P_2$ following hydrolysis by PLCζ.

Finally immunostaining has shown that markers for the Golgi such as giantin co-localise significantly with PLCζ and PI(4,5)P_2 in eggs (Yu and Swann 2012 unpublished).

Given that the Golgi was a likely candidate for the origin of these PI(4,5)P_2 containing vesicles it was necessary to determine the localisation of other Golgi markers in eggs. The *Amarathus caudate* lectin acts as a specific marker for the Golgi by binding soley galactosyl-beta 1,3-N- acetylglucosamine (Galβ3GalNAC) containing oligosaccharides (Rinderle *et al.* 1990) . AC-lectin can be conjugated to a flurophore, for example fluorescein, to produce a fluorescent marker for the Golgi (AC-lectin). As this probe is already fluorescent it avoids the need to use secondary antibodies, as a result non-specific binding of the secondary antibody is eliminated. In eggs AC-lectin consistently bound to small vesicles throughout the cytoplasm. These fluorescent vesicles were similar in distribution and pattern to those seen following giantin immunostaining (Yu and Swann 2012 unpublished). These vesicles also appeared very similar to the PI(4,5)P_2 and PLCζ containing vesicles identified previously (Yu *et al.* 2012). The fluorescein tag produced a bright and stable signal however AC-lectin cannot be used in live cells as it requires the cells to be fixed and permeablised in order to get into the cell. Therefore it was necessary to explore the distribution of live cell probes for the Golgi as well.

One commonly used live cell marker for the Golgi is BODIPY- FL ceramide, a fluorescently labelled version of the lipid ceramide which is taken into to the Golgi. The advantage of BODIPY FL-ceramide is it allows dynamic changes in Golgi structure to be measured. Due to its lipid nature BODIPY-ceramide is usually considered cell permeable but depends on the expression of the lipid transfer protein CERT for it to be transported into the Golgi (Hanada 2010). It is not known whether CERT is expressed or active in eggs, so instead BODIPY FL-ceramide was microinjected into eggs to determine its localisation in eggs. However, BODIPY FL-ceramide showed a very different distribution to previous Golgi markers used. Rather than binding to small intracellular vesicles BODIPY-FL ceramide bound reticular structures that looked similar in distribution to the ER. In fact the ER marker DiIC_{18}(3) overlapped very strongly with the signal emitted from the BODIPY ceramide. It is therefore very likely that BODIPY-FL ceramide is not acting as a marker for the Golgi at all but in fact for the ER. Due to the fragmented nature of the Golgi it is possible that many proteins and lipids that usually reside the Golgi are found in the ER instead. During the formation of the "Golgi haze" in somatic cells many Golgi resident markers are temporarily absorbed into the ER (Axelsson and Warren 2004). During these experiments it was discovered that a fluorescent version of the DAG binding domain C1b localised to similar reticular structures

to that seen by BODIPY ceramide and $DiIC_{18}$(3). This suggests that a lot of DAG is present in the ER so it is possible that other lipids such as ceramide are also located here. However, ceramide is usally made in ER and rapidly transported to the Golgi(Tidhar and Futerman 2013). Alternatively the BODIPY-FL ceramide injected could contain a lot unconjugated BODIPY-FL that has become dissociated from the ceramide. This free BODIPY-FL could be binding to any membrane available in the cell non-specifically rather than marking the Golgi. The distribution of BODIPY-FL ceramide seen in these experiments could also be because it was injected directly into the cytoplasm rather than incubated from the outsideit has been in other cell types.

As there is some uncertainty surrounding the specificity of BODIPY-FL ceramide targeting an alternative live cell Golgi marker was tested in eggs. GOGLI-ID® is a commercially available Golgi localisation kit. The company claim that this dye is much more specific to Golgi membranes than other markers available and as it is cell permeable does not require microinjection. In eggs GOLGI-ID® appeared to bind to small intracellular vesicles similar in nature to that seen by AC-lectin. Some staining of other internal membranes was also seen using GOLGI- ID® however the fluorescent signal emitted from these membranes was far weaker than that emitted from the vesicles. This would suggest that GOLGI-ID® either binds to other intracellular membranes as well as the Golgi albeit with a reduced affinity or the binding target of GOLGI-ID® is also present in small quantities in other organelles in the egg. As GOLGI–ID® is commercially developed it is not possible to find out how and what it binds to and therefore makes it difficult to explain why non-specific staining may occur. The fluorescent signal emitted by GOLGI-ID® is also generally weak in comparison to that of AC-lectin. However, GOLGI-ID® shows that a live cell marker for the Golgi may successfully be used in eggs.

These Golgi derived vesicles appear very similar in nature and distribution to that bound by PLCζ. However, if PLCζ is infact binding to these Golgi derived vesicles it is unclear exactly how it becomes localised here. Though it is very likely that these vesicles contain $PI(4,5)P_2$, which PLCζ is able to bind directly to, this is unlikely to be the targeting factor for PLCζ. This is because $PI(4,5)P_2$ is also believed to reside in the plasma membrane which PLCζ doesn't bind, therefore it is much more likely that PLCζ binds an alternative partner that resides only in these intracellular vesicles. Alternatively proteins or lipids in this target membrane could regulate PLCζ activity ensuring it is only active in specific membranes. Many other PLC isoforms are regulated by small GTPases. For example PLCδ1 activity is regulated by interacting with the small GTPase Ral in the plasma membrane (Sidhu *et al.* 2005). Different

small GTPases are found in different membranes and play a role in conveying specificity of these membranes. Rab 6 is a Golgi resident small GTPase which has been identified as playing a role in membrane trafficking between the Golgi and the ER (Goud *et al.* 1990). Rab 6 is also often used as a marker for the Golgi as it appears to localise only to the Golgi and the trans-Golgi network (Goud *et al.* 1990). Given this it was interesting to determine where Rab 6 was localised in eggs and whether it was found on small intracellular vesicles. Indeed exogenous Rab 6-GFP expressed in eggs was found to localise to some small vesicles throughout the cytoplasm however this staining was not specific to vesicular structures and some Rab 6-GFP was found to bind other membrane structures made it difficult to resolve its specific localisation. As Rab 6 is involved in shuttling proteins between the ER and Golgi it is possible that some Rab 6 is also residing in the ER (Goud *et al.* 1990). Rab 6-GFP was found to bind some reticular structures that looked similar in appearance to those marked by the ER dye $DiIC_{18}(3)$. It is also unclear exactly what form the trans-Golgi network takes in eggs. Though the exogenous fluorescently tagged Rab 6 allows Rab 6 distribution to be examined in live eggs, this does not act as a direct measure of endogenous Rab 6 in the cell. It was therefore necessary to verify previous immunostaining data which shows Rab 6 is localised to small vesicles in eggs (Yu and Swann 2012 unpublished). This result was reproduced and once again many small Rab 6 positive vesicles were found distributed throughout the cytoplasm. The vesicles identified appeared similar to those seen using other markers for the Golgi, suggesting that Rab 6 distribution could be another way of determining the structure of the Golgi. These Rab 6 containing vesicles have previously been shown to co-localise with PLCζ in eggs following immunostaining (Yu and Swan 2012 unpublished). This provides further evidence that PLCζ is binding to vesicles which derive from the Golgi. However, it remains to be determined whether Rab 6 interacts with PLCζ or regulates its activity in any way.

Multiple markers for the Golgi in both live and fixed cells suggest that it takes a fragmented vesicular form in eggs which is supported by previous data (Moreno *et al.* 2002), (Yu and Swann 2012, unpublished). It is also likely that these vesicles contain $PI(4,5)P_2$ and undergo PLCζ binding at fertilisation. Some co-localisation between the Golgi markers giantin and Rab 6 with both $PI(4,5)P_2$ and PLCζ have previously been recorded with immunostaining in eggs (Yu et al 2012 unpublished). However, these cells were fixed and immunostaining can produce inconsistent results. It is therefore necessary to do co-localisation studies between PLCζ or $PI(4,5)P_2$ with the Golgi in live cells. This can be a challenge however as live cell markers can be susceptible to bleaching or may have optimum maturation times. This can

make optimising the protocol for more than one live cell probe very difficult. However, it remains very unlikely that PLCζ would be targeting $PI(4,5)P_2$ on either the ER or endocytic vesicles as the staining pattern of markers for makers for these organelles looks much different to that of PLCζ or $PI(4,5)P_2$.

It is unclear how PLCζ can discriminate between different pools of $PI(4,5)P_2$ or bind to Golgi-derived vesicles. It is possible that a target protein or lipid is present in this membrane which acts to anchor PLCζ so it may access its substrate $PI(4,5)P_2$. The C2 domain of PLCζ has been found to play a role in enabling Ca^{2+}release in eggs (Chapter 4). However, it is unclear exactly what role this domain plays in this process as it does not convey any catalytic activity, $Ca^{2+}{}_i$ sensitivity properties or binding to $PI(4,5)P_2$ (Chapter 4) . It is therefore possible that the C2 domain of PLCζ may be responsible for binding and anchoring it to its target membrane. However, the only known binding partners for the C2 domain of PLCζ are the phospholipids PI(3)P which resides in early endosomes and PI(5)P which is mainly contained in the nuclear envelope (Mills *et al.* 2001; Kouchi *et al.* 2005; Reynoird and Gozani 2014; Nomikos *et al.* 2017a).

The process of Golgi fragmentation and therefore the accumulation of $PI(4,5)P_2$ on vesicular structures may be an essential feature in ensuring eggs can respond to PLCζ. For example CHO cells transfected with PLCζ do not release Ca^{2+} (Phillips *et al.* 2011). These cells do not have a vesicular source of $PI(4,5)P_2$ or a fragmented Golgi apparatus (Phillips *et al.* 2011). It is therefore intriguing to ask how eggs would respond to PLCζ if this vesicular source of $PI(4,5)P_2$ was depleted. It has already been shown that unlike PLCδ1 PLCζ does not hydrolyse plasma membrane $PI(4,5)P_2$ (Yu *et al.* 2012). GFP-PH domain, which targets plasma membrane $PI(4,5)P_2$, has shown no decrease in plasma membrane $PI(4,5)P_2$ in response to PLCζ or sperm induced $Ca^{2+}{}_i$ oscillation (Yu *et al.* 2012). However, a large decrease in this $PI(4,5)P_2$ source is seen during PLCδ1 induced $Ca^{2+}{}_i$ oscillations (Yu *et al.* 2012). Furthermore direct depletion of plasma membrane $PI(4,5)P_2$ using a plasma membrane targeted, lyn tagged inositol polyphosphate 5'phosphatse (Lyn-Ins5P) did not affect $Ca^{2+}{}_i$ oscillations initiated by PLCζ or sperm however completely inhibited oscillations initiated by PLCδ1 (Yu *et al.* 2012). This technique was adapted in attempt to deplete intracellular $PI(4,5)P_2$. Insp54P was tagged with a catalytically dead form of PLCζ ($PLC\zeta^{D210}$) and a GFP to produce a $PLC\zeta^{D210}$-Insp5P- GFP construct. Confocal imaging showed that this construct remained in the cytoplasm and did not bind to the plasma membrane (Yu *et al.* 2012). Depleting $PI(4,5)P_2$ in this way did not affect those $Ca^{2+}{}_i$ oscillations initiated by PLCδ1, however did inhibit or greatly reduced the $Ca^{2+}{}_i$ response to PLCζ or sperm (Yu *et*

*al.*2012). Given this interesting result functional effects of trying to deplete vesicular $PI(4,5)P_2$ were examined further.

OCRL1 is an endogenously expressed inositol 5'phosphastase which converts $PI(4,5)P_2$ to PI(4)P by de-phosphorylation (Zhang *et al.* 1995). It resides in the trans-Golgi network of cells ensuring that the balance of phosphoinostides is maintained. As a result OCRL1 should target the Golgi without the need for any additional tags. OCRL1 was overexpressed in eggs in attempt to deplete Golgi localised $PI(4,5)P_2$. However, this did not appear to have any significant effect on $Ca^{2+}{}_i$ oscillations initiated by PLCζ. This was the case even if OCRL1 was expressed for over 18 hours whilst oocytes were maturing. This result is in contrast to the disruption of $Ca^{2+}{}_i$ oscillations seen following $PLC\zeta^{D210}$-Insp54P- GFP expression (Yu *et al.* 2012). Unlike $PLC\zeta^{D210}$-Insp5P- GFP, OCRL1 does not contain a targeting sequence for the Golgi. Previous evidence suggests that OCRL1 resides in the trans Golgi network of cells, however there is also data indicating that it might target $PI(4,5)P_2$ in lysosomes also to mediate membrane trafficking here (Ungewickell and Majerus 1999). As the cellular localisation of the OCRL1 expressed in mouse eggs could not be determined it's unclear whether the correct pool of $PI(4,5)P_2$ was being depleted to affect PLCζ activity.

With this in mind, to try and deplete $PI(4,5)P_2$ in Golgi alone, the Insp54P was tagged with markers which usually reliably target the Golgi. Two different targeting proteins were used, the C1b domain which is known to bind DAG in the Golgi and the PH domain of Oxy sterol binding protein (OSBP) which binds to PI(4)P in the Golgi (Burns and Bell 1991; Levine and Munro 1998). The effects of overexpressing both C1b-Ins5P and OSBP(PH)-Ins5P on PLCζ induced $Ca^{2+}{}_i$ oscillations were tested in eggs. However, no impact on $Ca^{2+}{}_i$ oscillations were recorded following expression of either of these constructs.

As these probes contained a targeting protein for the Golgi it was important to determine whether these constructs actually targeted the vesicular structures identified by other Golgi markers. However, when fluorescently tagged versions of these domains were expressed in eggs they were not found to localise to small vesicles. In contrast they appeared to bind reticular structures that looked similar to markers for the ER and BODIPY-FL ceramide staining. It is possible therefore that in eggs these domains do not target the Golgi or target a part of the Golgi that is not fragmented. It is important to note that PI(4)P is also found in other intracellular membranes thereofore OSBP(PH) may not have targeted just the Golgi (D'Angelo *et al.* 2008)hese probes could have been expressed at such high levels that the usual binding sites for these proteins became saturated. As a result excess

OSBP(PH) and C1b could have bound other membranes that they would not usually bind under physiological conditions. Nevertheless, as neither OSBP(PH) nor C1b appeared to bind these small vesicles previously identified it is unlikely that the Ins5P was being localised to the correct membrane. As a result it is unlikely that these probes targeted the correct source of $PI(4,5)P_2$. Expressing untagged Ins5P which remains in the cytoplasm has previously shown to have no impact on PLCζ or sperm induced $Ca^{2+}{}_i$ oscillations in eggs (Yu *et al.* 2012).

It is possible that even if the OCRL1 and Ins5P are dephosphorylating some $PI(4,5)P_2$ there is so much vesicular $PI(4,5)P_2$ available in the egg that it is difficult to deplete significant levels (Snow *et al.* 1996). Furthermore as PLCζ is incredibly potent at releasing $Ca^{2+}{}_i$ it may only require a relatively small amount of vesicular $PI(4,5)P_2$ in order to elicit $Ca^{2+}{}_i$ oscillations (Saunders *et al.* 2002). It has also been shown that during fertilisation in sea urchin eggs there is a very fast turnover of phosphoinositides (Ciapa *et al.* 1992). Though PI turnover has not been successfully measured in mammalian eggs this could also be the case in mouse eggs. If so the re-synthesis of $PI(4,5)P_2$ could be so fast that overexpressing phosphatases has no effect on the availability of $PI(4,5)P_2$ to PLCζ. Phosphatases act as part of a tightly regulated pathway regulating the levels of all phosphoinositides keeping everything in fine balance. As a result it may be difficult to see any functional effects of overexpressing enzymes in this pathway. The possibility cannot be ruled out that OCRL1, OSBP(PH)-Ins5P and C1b-Ins5P are depleting Golgi derived $PI(4,5)P_2$ however this pool of $PI(4,5)P_2$ is not that targeted by PLCζ and therefore does not play a role $Ca^{2+}{}_i$ oscillations at fertilisation. However, as it was not possible to quantify the specific levels of $PI(4,5)P_2$ before and after expression of these probes it was impossible to determine whether the phosphatases were actually effective at dephosphorylating $PI(4,5)P_2$.

One alternative method of depleting $PI(4,5)P_2$ is by disrupting the production if its precursors such as the parent phosphoinostide molecule, PI. PI is synthesised in the ER by the enzyme PI synthase from CDP-DAG (Tanaka *et al.* 1996). CDP-DAG is generated from signalling PA and CTP (Carter and Kennedy 1966). Therefore the formation of all other phosphoinostides can be prevented by inhibiting the generation of PI and its precursors. The PA required to make CDP-DAG comes from the conversion of DAG to PA by the enzyme DAG kinase (Call and Rubert 1973). Therefore by inhibiting DAG kinase it may be possible to prevent the synthesis of PI. Indeed an inhibitor for DAG kinase has been found to ablate $Ca^{2+}{}_i$ oscillations stimulated by PLCζ in eggs. Furthermore the basal level of $Ca^{2+}{}_i$ remains low following addition of this drug suggesting that it is unlikely to be having a toxic effect

on these eggs. Inhibiting DAG kinases in pancreatic islet and β cell lines has been shown to reduce Ca^{2+}release in these cells (Kaneko and Ishikawa 2016) Whereas in platelets DAG kinase inhibitors reduce thrombin induced Ca^{2+}influx but not Ca^{2+} release induced by thapsigargin (Marumo *et al.* 2012). However, in this cell type DAG kinase inhibitor does not appear to effect PI turnover (de Chaffoy de Courcelles *et al.* 1985).

To determine whether preventing PA synthesis by other means effected PLCζ induced Ca^{2+}_i oscillations two other inhibitors of PA synthesis were used both of which disrupt different PA synthesis by different pathways. One of the main pathways for PA production is via the PLD pathway which involves the hydrolysis of PS to produce choline and PA (Liscovitch *et al.* 1993).This process can be inhibited by the addition of primary alcohols (Kotter and Klein 1999). Therefore the primary alcohol 1-butanol (1:1000) was added to eggs oscillating in response to PLCζ to determine how this affected Ca^{2+}release. Interestingly after the addition of 1-butanol the Ca^{2+}_i oscillations increase in frequency quite dramatically which was paired with an increase in basal level Ca^{2+}_i. To be sure this affect was specific the same experiment was conducted with the secondary alcohol 2-butanol which should not affect PA synthesis by the pathway. However, a very similar effect was seen after the addition of 2-butanol (1:1000). This suggests that this could be a non-specific effect seen as a result of alcohol addition. The increase in basal level Ca^{2+}_i indicates there could be an increase in Ca^{2+} infux or Ca^{2+} ER leakage both of which would be expected to increase the frequency of Ca^{2+}_i oscillations initiated by PLCζ (see Chapter 7). This rise in basal Ca^{2+}_i could be indicative of non-specific effects on egg metabolism. Alcohols are also thought to stimulate the IP_3R which could therefore increase Ca^{2+}release by PLCζ (Mizuno *et al.* 2013).

Another pathway by which PA is generated is by the activity of PC- specific PLCs which operate in a distinct pathway to PI specific PLCs like PLCζ. The toxin D609 acts as inhibitor of PC- specific PLCs (Adibhatla *et al.* 2012). As a result the effect of D609 on Ca^{2+}_i oscillations stimulated by PLCζ was investigated. D609 was found to inhibit these Ca^{2+}_i oscillations shortly after addition however a small increase in basal level Ca^{2+}_i oscillations was seen. This suggests that though it would appear inhibiting PA production via this pathway impacted negatively on PLCζ ability to release Ca^{2+} this effect is likely to be due to non-specific toxic effects on the egg. If D609 is disrupting the metabolism of the egg for example this could prevent effective Ca^{2+} release.

It is therefore unclear whether the production of PA as a whole play in the cell plays a significant role in Ca^{2+}releasing ability of eggs or whether PA specifically generated as a

result of DAG kinase activity is vital for the production of $PI(4,5)P_2$ and therefore PLCζ activity. Preventing $PI(4,5)P_2$ or PI synthesis by inhibiting PA production is not very specific. The DAG/ PA pathway plays a significant homeostatic role in cells and maintaining correct levels of these molecules is vital to ensure any signalling in the cell occurs effectively across multiple pathways. The effects of these drugs could therefore, rather than being specific on $PI(4,5)P_2$ levels, be a result of a more general disruption of signalling in the egg.

The effect of a final drug was tested on Ca^{2+} releasing ability of eggs. Rather than disrupting the production of PI or $PI(4,5)P_2$ propranolol is thought to disrupt the structure of the Golgi apparatus, membrane trafficking and leads to mis-localisation of proteins in this region (Asp *et al.* 2009). It is possible that PLCζ binds to $PI(4,5)P_2$ positive vesicles originating from the Golgi. As a result disrupting the structure and localisation of potential target proteins here could affect PLCζ induced $Ca^{2+}{}_i$ oscillations in eggs. Indeed addition of propranolol did inhibit $Ca^{2+}{}_i$ oscillations stimulated by PLCζ and no elevation in basal level $Ca^{2+}{}_i$ was recorded. Furthermore the effects of this drug could be reversed by removal. Interestingly these $Ca^{2+}{}_i$ returned with a higher frequency which appeared unsustainable leading to an increase in basal $Ca^{2+}{}_i$ levels and in most cases lead to the eggs eventually dying after approximately 1 and a half hours. Besides disrupting the Golgi, propranolol also prevents COPI coated vesicle membrane trafficking (Asp *et al.* 2009). It has previously been shown that this effect is reversible and if propranolol is removed there is a sudden burst of trafficking vesicles formed at the ER (Asp *et al.* 2009). Propranolol may be preventing the trafficking of phosphoinostides from the ER to the Golgi for example $PI(4,5)P_2$ or PI(4)P which could be essential for PLCζ activity. When this drug is removed it could lead to a surge in trafficking vesicles, containing these molecules, to arrive at the Golgi and stimulate PLCζ activity. Alternatively propranolol may disrupt Golgi derived binding partners for PLCζ which then become relocalised following propranolol removal. Propranolol has been shown to redistribute trans-golgi network resident proteins to the cytosol in a reversible fashion (Baron and Malhotra 2002). Propranolol prevents Golgi derived DAG production (Asp *et al.* 2009). Disrupting PA production has been shown to effect the $Ca^{2+}{}_i$ releasing ability of eggs in response to PLCζ. PA and DAG production are closely related interconnected pathways. It is possible that disrupting DAG/PA metabolism in any way could have a severe effect on the cell signalling events in the egg. For example propranolol also inhibited $Ca^{2+}{}_i$ oscillations stimulated by PLCδ1 which does not hydrolyse a vesicular source of $PI(4,5)P_2$ (Yu *et al.* 2012).

The Ca^{2+}releasing ability of eggs is cell cycle dependent. Mature MII arrested eggs are the only cells which are able to respond to PLCζ to produce Ca^{2+}_i oscillations like that seen at fertilisation (Carroll *et al.* 1994; Jones *et al.* 1995a; Wakai *et al.* 2012). It has previously been reported that eggs acquire this ability to respond to sperm during oocyte maturation (Carroll *et al.* 1994; Jones *et al.* 1995b; Wakai *et al.* 2012). The response of oocytes at different stages of maturation to PLCζ cRNA has previously been recorded (Wakai *et al.* 2012). However, oocytes sensitivity to PLCζ during oocyte maturation has not previously been quantified accurately. This is because immature oocytes express exogenous cRNA differently to mature eggs and it is not possible to quantify the expression of untagged cRNA easily. Therefore in order to quantify this response accurately the Ca^{2+}_i releasing response of oocytes at different stages of maturation was tested using luciferase tagged PLCζ cRNA. Adding a luciferase tag onto a sequence to create a fusion construct allows the amount of protein expressed to be measured by recording the bioluminescence produced by the luciferase enzyme. The greater the amount of luciferase the greater amount of bioluminescence emitted and as the luciferase is bound to PLCζ this quantification of bioluminescence emitted is also directly related to the amount of PLCζ protein in the egg (Swann *et al.* 2014) This allows comparisons to be made between oocytes expressing the same amount of PLCζ protein. Using this method it was found that all MII eggs oscillated with a frequency of approximately 4 spikes per hour. However, only 20 out of 26 MI oocytes oscillated in response to PLCζ expressed at very similar levels. These Ca^{2+}_ioscillations were slightly reduced in frequency compared to that seen in MII eggs and normally stopped within approximately 1 hour. However, remarkably only 3 out of 21 GV oocytes responded to PLCζ at these levels suggesting that there is a marked increase in Ca^{2+}_i releasing ability in oocytes after germinal vesicle breakdown. Furthermore this effect could not be rescued by over expressingPLCζ up to 30 times the physiological level as only 6 out of 25 oocytes oscillated. This is surprising as it has previously been suggested that egg acquire their ability to respond to PLCζ fairly gradually throughout maturation and that the responses to PLCζ in GV oocytes are reduced in frequency compared to MI oocytes and MII eggs (Wakai *et al.* 2012). This effect had previously been attributed to a reduction in the sensitivity of the IP3R in immature oocytes (Carroll *et al.* 1994; Wakai *et al.* 2012). In other words PLCζ produces IP_3in the same way however the IP_3R is less responsive to this and therefore releases less Ca^{2+}. The complete absence of Ca^{2+}_i oscillations recorded in most GV oocytes suggests that this is not the explanation for this difference in response. This is because if the IP_3R sensitivity was reduced in GV oocytes then you would expect that over

expressing PLCζ in order to produce a lot more IP_3would rescue the effect. However, this is not the case. Furthermore experiments using caged IP_3have shown that GV oocytes are able to respond even to low levels of IP_3. This further supports the notion that sensitivity changes in the IP_3R during oocyte maturation are not large enough to account for the dramatic increase in Ca^{2+}releasing ability of oocyte after germinal vesicle breakdown.

One reason for the proposed increase in sensitivity of the IP_3R during oocyte maturation is an increase in ER Ca^{2+} concentrations (Carroll *et al.* 1994; Jones *et al.* 1995b; Wakai *et al.* 2012) The level of ER luminal Ca^{2+} concentrations is believed to directly impact the sensitivity of the IP_3R (Missiaen *et al.* 1992). It has already been shown that ER Ca^{2+} concentration increase during oocyte maturation for example as oocytes mature they become more responsive to Ca^{2+}ionophore and thapsigargin (Jones *et al.* 1995b). The current experiments showed that there is a reduced amplitude of Ca^{2+}_i oscillations in response to PLCζ in both MI and GV oocytes. Furthermore the Ca^{2+}_i response stimulated by the un-caging of caged IP_3 was also reduced in amplitude in GV oocytes and MI oocytes compared to MII eggs. This supports the idea that ER Ca^{2+} concentrations increase during oocyte maturation however this is something which appears to happen more dramatically in the later stages of oocyte maturation. The biggest difference in the amplitude of Ca^{2+}release was recorded between MI oocytes and MII eggs, with GV oocytes only having slight reduced amplitude in comparison to MI oocytes. This suggests that the increase in ER Ca^{2+} concentrations occurs relatively late in maturation. Therefore an increase in ER Ca^{2+} cannot account for the dramatic increase in Ca^{2+}_i releasing ability occurring during transition to MI. One big difference between GV and MI oocytes is the change in cell cycle state. GV oocytes are arrested in interphase whereas MI and MII oocytes are in metaphase. This may be significant as it appears that an eggs ability to respond to PLCζ and sperm is cell cycle dependent (Jones *et al.* 1995a). For example zygotes in interphase do not respond to PLCζ however following nuclear envelope breakdown during the first mitotic cell cycle these Ca^{2+}_i oscillations return (Kono *et al.* 1996) This is because when the pronucleus forms PLCζ becomes sequestered here due to the presence of a nuclear targeting sequence in its structure (Larman *et al.* 2004). Fluorescently labelled PLCζ has been seen to sequester in the nucleus when the pronuclei form at egg activation (Yoda *et al.* 2004). It is also been suggested the same thing could happen in a GV oocytes were PLCζ accumulation in the GV and this could prevent Ca^{2+}_i oscillations (Yu *et al.* 2012). However, this does not appear to be the case with PLCζ- luciferase. The bioluminescence signal emitted by this construct can be seen uniformly throughout the cytoplasm of eggs and oocytes. However, if luciferase is

injected directly into the germinal vesicle of GV oocytes a bright signal can be clearly emitted from this region. Therefore, if PLCζ- luciferase is being sequestered in the nucleus it would be visible using these imaging methods. Furthermore at very high levels of PLCζ-luciferase expression the nucleus would become saturated and some PLCζ- luciferase would end up in the cytoplasm. Therefore it is very unlikely that nuclear localisation of PLCζ- luciferase is the reason it does not cause Ca^{2+}_i oscillations in GV oocytes. It is possible due to the length of the PLCζ- luciferase construct it is not able to enter the nucleus of GV oocytes easily whereas shorter length fluorescently tagged PLCζ constructs may be ableto get into the nuclear envelope. As a result fluorescently tagged PLCζ could accumulate in the GV (Yu *et al.* 2012). Other mammalian species such as humans do not contain a functional nuclear targeting sequence in PLCζ and therefore oscillations must be halted in these species by another mechanism (Cox *et al.* 2002). As intracellular

$PI(4,5)P_2$ is very likely to play a role in enabling PLCζ to release Ca^{2+}. It is possible that PLCζ is not able to release Ca^{2+}in GV oocytes because they lack of this pool of $PI(4,5)P_2$ (Yu *et al.* 2012). Immunostaining of $PI(4,5)P_2$ during oocyte maturation has shown that GV oocytes do not have a visible source of vesicular $PI(4,5)P_2$, instead all their $PI(4,5)P_2$ is located on the plasma membrane or contained within the germinal vesicle (Yu *et al.* 2012). As oocytes progress through MI some vesicular $PI(4,5)P_2$ becomes apparent however an abundance in $PI(4,5)P_2$ positive vesicles do not appear until the eggs are fully matured and arrested in MII (Yu *et al.* 2012). This result was reproduced with $PI(4,5)P_2$ immunostaining showing that once again $PI(4,5)P_2$ in GV oocytes localises to the germinal vesicle however little plasma membrane $PI(4,5)P_2$ staining was seen. This suggests that the vesicular source of $PI(4,5)P_2$ required for PLCζ activity is not existent in GV oocytes and therefore Ca^{2+} cannot be released effectively. However, these cells must be fixed and permeabilised in order to conduct immunostaining and this process can disrupt the distribution of membrane bound proteins (Sharma *et al.* 2008). As a result it proved difficult to obtain consistent results with all immunostaining experiment.

Because of this, the localisation of live cell markers for $PI(4,5)P_2$ were also investigated during oocyte maturation. FL-$PI(4,5)P_2$, DAN 20 and PBP10 were all injected into GV oocytes so the staining pattern of these probes could be compared to that of MII eggs. However, all these probes showed a vesicular source of $PI(4,5)P_2$ was present in GV oocytes that appeared similar to that seen in MII eggs. Furthermore no plasma membrane $PI(4,5)P_2$ was apparent in GV oocytes using these markers. Even if vesicular $PI(4,5)P_2$ is

present in GV oocytes it does not necessarily mean that this $PI(4,5)P_2$ is available to PLCζ. For example it is unclear where these $PI(4,5)P_2$ containing vesicles originate from in GV oocytes. Though there is strong support for the idea that the $PI(4,5)P_2$ containing vesicles in eggs originate from the Golgi this may not be the case in GV oocytes. There is evidence from immunostaining data that in GV oocytes the Golgi takes the form of cisternal stacks like that seen in somatic cells (Moreno *et al.* 2002; Payne and Schatten 2003). Staining with the lectin AC-lectin unlike immunostaining showed that the Golgi appears fragmented in GV and MI oocytes. Furthermore these vesicles appeared very similar to that seen in MII eggs. This indicates that the Golgi does not alter significantly in structure during oocyte maturation. However, the live cell Golgi marker GOLGI-ID® did show a difference in Golgi localisation between GV oocytes and MII eggs. In GV oocytes the staining pattern by this probe does not appear vesicular but instead appears to bind larger clustered structures throughout the cytoplasm. This suggests that the Golgi fragments during oocyte maturation and does not appear as vesicles prior to oocyte maturation. However, variability in results with different probes makes it difficult to draw conclusions on how the Golgi matures during oocyte maturation. As previously described however the structure of the ER does appear to alter during oocyte maturation with the ER becoming more reticular and clustered in MII eggs (FitzHarris *et al.* 2007). It is unclear exactly how the structural changes of organelles during oocyte maturation play a role in enabling PLCζ to release Ca^{2+} It is possible that structural changes are required to either make $PI(4,5)P_2$ accessible to PLCζ or ensure the correct localisation of targeting proteins for PLCζ. Alternatively germinal vesicle breakdown could result in the release of a targeting protein for PLCζ that may be required for PLCζ activity. This is something which needs to be explored in more detail.

It is apparent that rather than hydrolysing $PI(4,5)P_2$ at the plasma membrane like all other known PLC isoforms, PLCζ hydrolyses an alternative source of $PI(4,5)P_2$ (Yu *et al.* 2012). Many different probes for $PI(4,5)P_2$ have indicated that this $PI(4,5)P_2$ is located on small vesicles throughout the cytoplasm in mature eggs. It is unclear exactly what the origin of vesicles is, however, there is strong evidence that these could originate from the Golgi. The availability of this vesicular $PI(4,5)P_2$ may be a cell cycle dependent process which is a feature of MII arrested oocytes. Furthermore the inability of PLCζ to cause $Ca^{2+}{}_i$ oscillations in somatic cells or immature oocytes could be due to the inaccessibility of this vesicular $PI(4,5)P_2$.The functional effects of vesicular $PI(4,5)P_2$ availability are yet to be investigated in detail and exactly how PLCζ targets this specific source of $PI(4,5)P_2$ is yet to be fully understood.

6. ASSESSING POTENTIAL PROBES TO MONITOR IP_3 AND DAG DYNAMICS DURING PLCZ INDUCED $CA^{2+}{}_I$ OSCILLATIONS

Introduction

Secondary messengers are signalling molecules that play a key role in regulating the activity of other proteins and signalling pathways. During fertilisation, though $Ca^{2+}{}_i$ itself is a key signalling molecule, other messenger molecules are also produced that may play a role in egg activation. Two key secondary messengers are produced during PLCζ activity both of which are the cleavage products of $PI(4,5)P_2$. Firstly is the production of IP_3 from the inositol head component $PI(4,5)P_2$ which diffuses into the cytoplasm. IP_3 goes on to stimulate the IP_3 receptor and release Ca^{2+} from the endoplasmic reticulum giving the characteristic $Ca^{2+}{}_i$ oscillations seen at fertilisation (Miyazaki 1988). The second is the glyceride diacylglycerol (DAG) consisting of the glycerol backbone and fatty acid tails of $PI(4,5)P_2$ which remains in the plasma membrane (Ciapa and Whitaker 1986).

Though it is fully recognised that IP_3 is the means by which $Ca^{2+}$is released from stores during fertilisation the exact dynamics of IP_3 production during fertilisation remain unknown. This is significant as the pattern of IP_3 production could play a key role in determining the pattern of the $Ca^{2+}{}_i$ oscillations. Different models of $Ca^{2+}{}_i$oscillations have been proposed each of which makes different assumptions on the pattern of IP_3 production. One theory suggests that the rate of IP_3 synthesis is constant and therefore does not dynamically change during $Ca^{2+}{}_i$ oscillations (Iino 1990). In this model it is the intrinsic properties of the IP_3R that allow dramatic changes in Ca^{2+} release as the receptor is seen to have biphasic dependence on Ca^{2+} (Iino 1990). The opposing theory suggests that IP_3 is produced in an oscillatory manner which increases prior to each $Ca^{2+}{}_i$ transient (Meyer and Stryer 1988). In this model cytosolic $Ca^{2+}{}_i$ levels positively feedback onto PLC activity producing spikes in IP_3 which act as the pacemaker for $Ca^{2+}{}_i$ oscillations (Meyer and Stryer 1988).

In the past the only way to measure IP_3 production was biochemical assays using either isotope labelling or IP_3 radio-receptor assays (Balla *et al.* 1986; Bredt *et al.* 1989). Neither of these allows dynamic changes in the IP_3 of individual live cells to be measured, and they

generally require too much material to be used on mammalian eggs. As a result many genetic based probes have been developed to measure IP_3 dynamics in live cells. Most of these are based on the IP_3 binding domain of the IP_3R and adopt the fluorescence resonance energy transfer (FRET) technique. The benefit of FRET based genetically encoded probes is that FRET allows small changes over short distances to be measured relatively sensitively (Stryer 1978).

One of the first such probes was LIBRA which was successfully used to measure real-time IP_3 changes in acetylcholine stimulated SH-SY5Y cells (Tanimura *et al.* 2004). Similarly the IP_3R based IP_3 sensor 1 (IRIS), has been used to measure IP_3 changes in HeLa cells following stimulation of endogenously expressed histamine receptors or metabotropic glutamate receptor 5 (Matsu-ura *et al.* 2006).Other IP_3 probes have been developed in order to measure IP_3 dynamics in other cell types including the fluorescent IP_3 responsive element (FIRE) which has been used to measure IP_3 in COS cells, neonatal cardiac myocytes and cardiac ventricular myocytes (Remus *et al.* 2006). The FRETINO probe has been used to measure nuclear IP_3 dynamics alongside cytosolic IP_3 changes in neuronal dendrites (Sato *et al.* 2005).

IP_3 probes provide different evidence on how IP_3 levels change prior to Ca^{2+} release and these probes possibly behave in a cell specific manner. A GFP tagged probe based on the PH domain of PLCδ1 which translocates from the plasma membrane to the cytoplasm as IP_3 increases has shown that IP_3 oscillates with Ca^{2+}_i in Madin-Darby canine kidney epithelial cells (Hirose *et al.* 1999). However, this probe does not directly measure cytosolic IP_3 in the way that genetically encoded IP_3 binding domain based probes do. Direct measurements of IP_3 using the IRIS probeshowed no acceleration in the rate of IP_3 production with Ca^{2+}_ioscillations in HeLa cells (Matsu-ura *et al.* 2006). This result was verified in COS-7 cells using LIBRA (Tanimura *et al.* 2009). However, in HSY-EA1 cells the same probe found that there was repetitive spikes in IP_3 with Ca^{2+}_ioscillations (Tanimura *et al.* 2009). Therefore it remains unclear which model of IP_3 production is most common place during Ca^{2+}_i oscillations.

Some attempts have been made to measure IP_3 dynamics in mammalian eggs at fertilisation using these existing probes. The FRETINO probe has been used to look at IP_3 dynamics in mouse eggs during fertilisation and in response to PLCζ (Shirakawa *et al.* 2006). Only very small oscillations in IP_3 levels were detected alongside Ca^{2+}_i oscillations however these did get bigger with time (Shirakawa *et al.* 2006). It was also found that addition of

extracellular Ca^{2+} to eggs previously injected with PLCζ caused a large increase in Ca^{2+}_i and IP_3 which suggested a positive feedback loop between Ca^{2+}_i, IP_3 and PLCζ activity (Shirakawa *et al.* 2006). However, this probe was not able to measure any significant change in IP_3in response to individual Ca^{2+}_i oscillations during fertilization (Shirakawa *et al.* 2006).

The signalling role of DAG in egg activation is not yet clearly understood. Unlike IP_3, which has a direct known affect via Ca^{2+} release, DAG has multiple targets with many overlapping signalling pathways. Different species of DAG are produced in different cellular compartments via different mechanisms and therefore these species may play distinct signalling roles (Carrasco and Merida 2007). In somatic cells DAG production as a result of PLC activity via the hydrolysis of $PI(4,5)P_2$ generally occurs at the plasma membrane. As a result changes in DAG levels of PLC stimulated cells can be detected at the plasma membrane. This type of DAG is often referred to as "signalling" DAG and can be considered as distinct from the "metabolic" DAG produced on internal membranes via other metabolic pathways (Hodgkin *et al.* 1998). The role of signalling DAG is primarily considered to act as secondary messenger to activate PKC by recruiting it to the plasma membrane (Kraft *et al.* 1982).

Fairly simple indictors have been developed that allow signalling DAG to be measured dynamically in live cells. Most of these are based on the idea that DAG binds cysteine rich domains (CRD) for example the C1 domain of PKCs (Oancea *et al.* 1998). The tandem C1 domain of conventional PKCγ tagged with GFP has been used to detect the translocation of DAG in response to hormonal stimulation in Rat basophilic leukemia 2H3 cells (Oancea and Meyer 1998). However, as these probes only emit one wavelength of fluorescent light in order to measure dynamic changes in DAG the translocation of the probe has to be measured using high resolution microscopy. To try and get around this issue the DAG reporter (DAGR), a FRET based probe containing the C1 domain of PKCβ, has also been used to measure DAG levels in MDCK cells (Violin *et al.* 2003). DAGR translocates from the cytosol to the plasma membrane as DAG increases here (Violin *et al.* 2003). Interestingly there are differences in the affinity for DAG of C1 domains from different PKCs. The C1 domains of novel PKCs such as PKCδ actually have a higher affinity for DAG than conventional PKCs (Dries *et al.* 2007).

Probes based solely on the C1 domain are not able to discriminate between DAG produced in different cellular compartments as a result a series of membrane specific DAG probes

have been developed. DAGLAS probes are FRET based probes containing a CRD from PKCβ fixed between CFP and YFP molecules with α helix linkers (Sato *et al.* 2006). This core probe is then fused specific membrane targeting sequences to direct the probe to specific membranes. Once the probe is anchored to this membrane if it binds to DAG the probe undergoes a FLIP FLOP conformational change giving a change in FRET ratio (Sato *et al.* 2006). These probes have been successfully used to measure DAG dynamics in the different cellular compartments of MDCK cells (Sato *et al.* 2006).

More recently a series of new probes have been developed that are not based on FRET. Instead these biosensors are based on circularly permuted fluorescent proteins. When fluorescent proteins are circularly permuted the amino and carboxyl halves of the β barrel are switched over and linked with spacer (Baird *et al.* 1999). This allows the barrel to open and close more freely and leads to conformational changes. The fluorophore is still able to work effectively however due to an altered orientation the signal emitted can be enhanced several fold (Baird *et al.* 1999). By fusing with a protein sequence of interest these proteins can be used to develop probes to measure cellular dynamics (Baird *et al.* 1999). This technique has successfully been used to develop a Ca^{2+}_i sensor named Peri-cam, which contains a circularly permuted GFP fused to CaM and CaM binding peptide M13 (Nagai *et al.* 2001). This probe has been adapted and improved upon to create many more sophisticated Ca^{2+}_i sensors including GCaMP3, G-GECO and R-GECO (Akerboom *et al.* 2009; Zhao *et al.* 2011). One of these probes, G-GECO, has been adapted to produce a DAG sensor based on the same principle. In this probe the circularly permuted GFP from G-GECO was fused between C1 domain of PKCδ1 and its pseudo-substrate domain (Tewson *et al.* 2012). Both upwards and downwards versions of this probe were developed and both showed dynamic changes in fluorescence of at least 40% following carbachol treatment (Tewson *et al.* 2012). This change is greater than that previously recorded by FRET based probes.

It is not clear how DAG dynamics change during Ca^{2+}_i oscillations. Oscillations in DAG production along with Ca^{2+}_i oscillations have been detected following glutamate stimulation of astrocytes (Codazzi *et al.* 2001). GFP tagged C1 domain of both PKCγ and PKCδ showed oscillatory translocation between the cytosol and plasma membrane (Codazzi *et al.* 2001). Attempts have been made to measure changes in DAG levels in eggs during fertilisation. Oscillations in conventional PKC plasma membrane translocation, using GFP-tagged PKCγ and PKCα have been recorded in eggs during fertilisation (Halet 2004). This could be considered as an indirect measure of DAG production however, this PKC translocation

appears to be solely Ca^{2+} dependent mediated by the C2 domain and didn't indicate any changes in plasma membrane DAG (Halet 2004). Interestingly, PKC induced phosphorylation, as measured by the c-kinase activity reporter (CKAR), probe has been found to oscillate with Ca^{2+}_i oscillations in fertilising eggs. CKAR signal was seen to repetitively increase in both the plasma membrane and the cytoplasm. This is significant as it suggests DAG may be produced not just at the plasma membrane but also on internal membranes (Gonzalez-Garcia *et al.* 2013). Direct measurements of DAG using a GFP tagged tandem C1 domain of PKCδ ($C1_2$δ1-GFP) found no detectable change in DAG levels at the plasma membrane during fertilisation (Yu *et al.* 2008). However, if PLCζ is over-expressed beyond physiological levels, or $Ca^{2+}$is released via PLCδ1, changes in plasma membrane DAG are detected (Yu *et al.* 2008; Yu *et al.* 2012). This suggests that DAG is not being produced at the plasma membrane via $PI(4,5)P_2$ hydrolysis. Instead it is more likely that PLCζ is hydrolysing $PI(4,5)P_2$ on internal cellular membranes which are not being measured by this technique (Yu *et al.* 2008; Yu *et al.* 2012). DAG production in internal membranes of eggs during fertilisation is yet to be explored.

Though it is undisputed that IP_3 and DAG are produced via $PI(4,5)P_2$ hydrolysis, the dynamics of these signalling molecules have not yet been successfully measured in live eggs during fertilisation or PLCζ induced Ca^{2+}_i oscillations.

Aims

These series of experiments aimed to measure the live cell dynamics of DAG and IP_3 during PLCζ activity with the use of novel probes and some existing probes. These probes were used to look at the changes in these secondary messengers both during Ca^{2+}_i oscillations and in response to various other stimuli.

Results

Measuring DAG using C1 domain based inter-molecular FRET probe

C1b inter-FRET signal alters in response to phorbol esters

Some of the most basic DAG probes are based on a fluorescent tagged cysteine rich domain (CRD) such as a GFP tagged C1 domain of PKC (Oancea and Meyer 1998). For example the $C1_2$δ1-GFP probe was used to measure DAG changes in plasma membrane in eggs during fertilisation, however no change in DAG levels was detected using this probe (Yu *et al.* 2008). A new potential probe was developed (courtesy of MN) to try and measure whole cell changes in DAG levels in eggs at fertilisation. This construct was based on a

fluorescently tagged C1 domain.This principle has previously been used to measure $PI(4,5)P_2$ at the plasma membrane in somatic cells using an inter-molecular PH domain based FRET probe (van der Wal *et al.* 2001). Two constructs were produced with one C1 domain being tagged to either YFP (C1b-YFP) or CFP (C1b-GFP) collectively referred to as C1b inter-FRET (see fig 6.1B). When both of these constructs are expressed simultaneously and the YFP tag from one construct comes in close proximity with the CFP a change in inter-molecular FRET signal can be detected. A change in FRET indicates that the constructs are co-localising on a membrane due to DAG binding. To test the dynamic range of this probe, eggs were co-microinjected with 2µg/µl of C1b- YFP and C1b-CFP cRNA diluted in KCL hepes. Eggs were then left approximately 3 hours to allow protein levels of the constructs to accumulate. The injected eggs were then transferred onto a fluorescence imaging system and the FRET ratio was recorded. After around 15 minutes of recording 1 µM of phorbol myristate acetate (PMA) was added to the media surrounding the eggs. PMA acts as a DAG analogue therefore mimics the effects of DAG increase. There was a signal increase of around 6.6% in FRET ratio after the addition of PMA (see fig 6.2, Table 6.1). Though this shows that C1b inter-FRET can detect changes in DAG, this change was very low given that PMA at this concentration is such a strong stimulus which suggests that the dynamic range of the probe is relatively low.

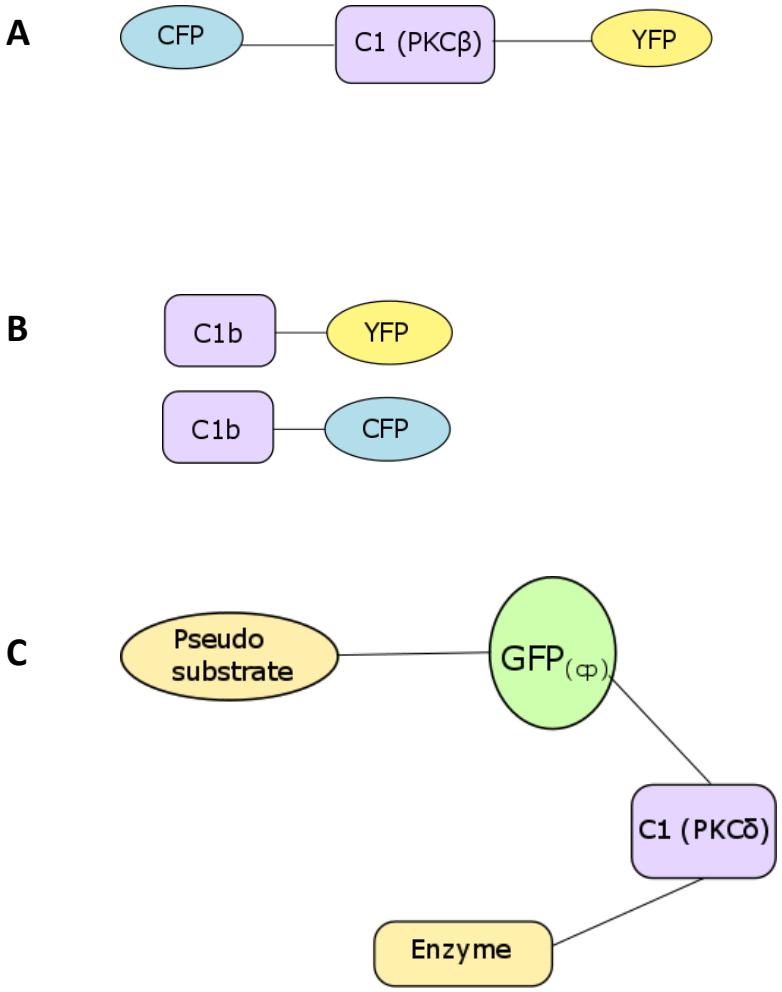


Figure 6.1 Schematic diagrams showing the structure of the genetically encoded constructs used to measure DAG

A) DAGR consisting of the C1 domain from PKCβ tagged with CFP and YFP fluorescent tags; **B)** C1 Inter-FRET probe consisting of two separate constructs that were co-injected both based on the C1b domain tagged with either YFP and CFP; **C)** Green 'Upwards' DAG containing the DAG binding C1 domain of PKCδ attached to a circularly permutated GFP.

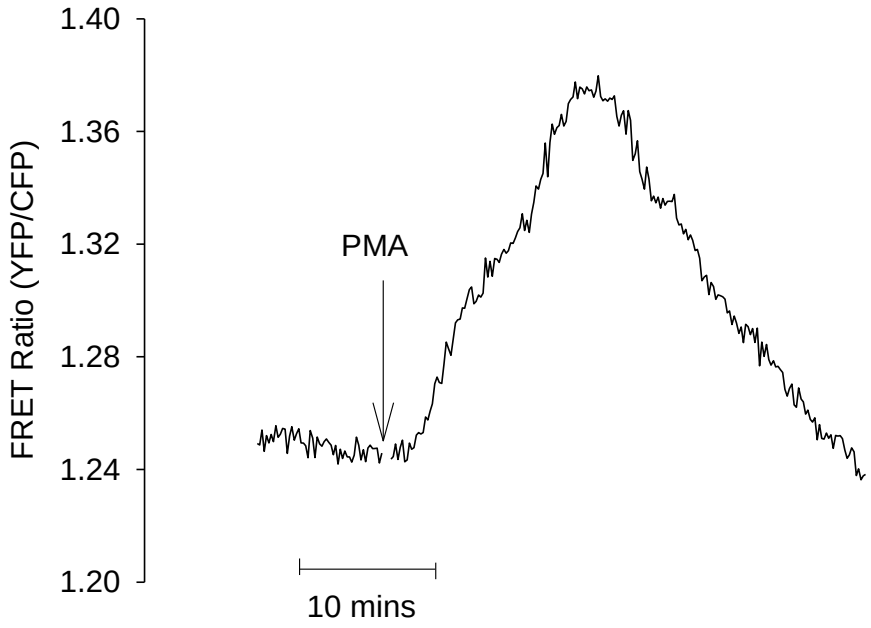


Figure 6.2 FRET changes of C1 Inter-FRET probe in response to PMA

An example fluorescence trace of an egg showing the change in FRET ratio of the C1 Inter-FRET probe in response to the addition of phorbol ester PMA (1µM) (n= 5), see Table 6.1 for analysis.

Probe	Stimuli	N	% Change	SD of % change
C1 inter-FRET	PMA	5	6.6	3.1
	mPLCζ	6	0	0.2
DAGR	PMA	9	28.5	8.7
	mPLCζ	5	1.39	0.9
Green 'Upwards' DAG	PDBµ	8	96.8	6.1
	mPLCζ	19	0.53	1.7

Table 6.1 Summary of the dynamic range of different DAG probes in response to different stimuli

Summary table showing the dynamic range of different DAG probes and the mean percentage change in signal in response to the phorbol esters either PMA (phorbol myristate acetate) (1µM), PDBµ (phorbol 12,13-dibutyrate)(2µM) or during mPLCζ cRNA (0.025µg/µl) induced Ca^{2+}_i oscillations. SD= standard deviation of that percentage change.

C1b inter-FRET signal does not alter in response to PLCζ activity

Next it was important to determine whether C1 inter-FRET could detect any changes in DAG levels in eggs at fertilisation. As Ca^{2+}_i oscillations at fertilisation are triggered by PLCζ, stimulation by PLCζ alone is a good way to parthenogenetically activate eggs and look at cellular dynamics during fertilisation. Therefore following microinjection of C1b inter-FRET as described previously and 4 hours of expression the eggs were injected with 0.025 µg/µl of mouse derived PLCζ (mPLCζ) mixed with the Ca^{2+}_i indictor Rhod dextran. These eggs were then placed immediately on the imaging system as well as recording the fluorescence of the FRET ratio the fluorescence from the fluorescence from the Ca^{2+}_i indictor was also recorded. This simultaneous recording allowed the FRET change during the Ca^{2+}_i oscillations to be recorded. To determine if changes in DAG could be detected with each Ca^{2+}_i transient the FRET ratio before the Ca^{2+}_i spike and after were noted for 3 Ca^{2+}_i oscillations from each egg. The difference between these 2 values was calculated and used to determine the percentage change. The percentage change for each Ca^{2+}_i oscillation was averaged across the egg and the group. C1 inter-FRET found 0% change in FRET signal with each Ca^{2+}_i oscillation (see fig 6.3, Table 6. 1) which suggests that C1 inter-FRET is not able to detect changes in DAG with Ca^{2+}_i oscillations. Alternatively, DAG levels may not change during Ca^{2+}_i oscillations.

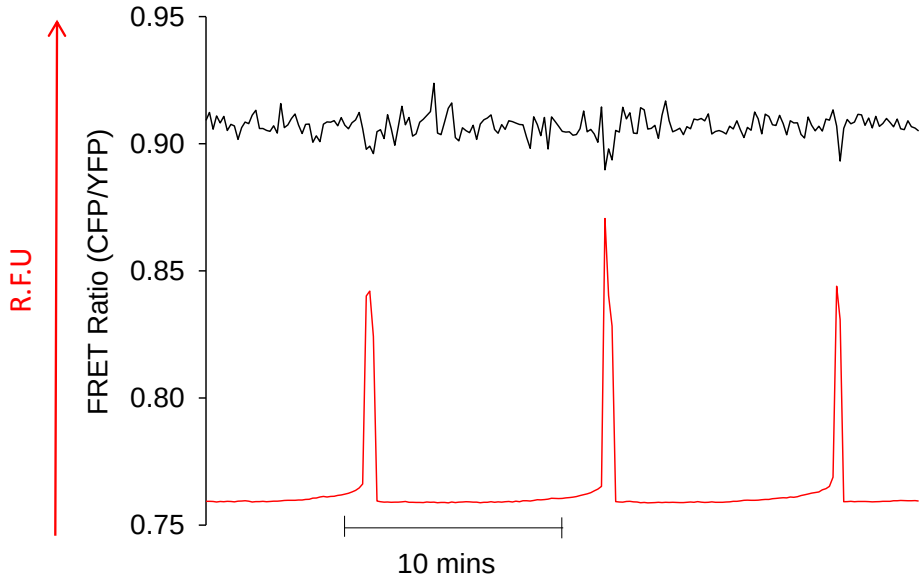


Figure 6.3 FRET and $Ca^{2+}{}_i$ changes of C1 Inter-FRET probe in response to PLCζ

A sample trace showing the change in FRET ratio of C1 Inter-FRET probe alongside mPLCζ induced $Ca^{2+}{}_i$ oscillations. $Ca^{2+}{}_i$ oscillations were initiated by the microinjection of 0.025 µg/µl RNA concentration and measured using the fluorescent $Ca^{2+}{}_i$indicator Rhod Dextran. R.F.U= relative fluorescence units, (n=6). See Table 6.1 for analysis.

Measuring DAG using DAGR probe

DAGR FRET ratio alters in response to phorbol esters

The DAG reporter probe (DAGR) is a FRET based probe that has been shown to measure whole cell changes of DAG in cell lines such as MDCK cells (Violin *et al.* 2003). However, this probe has never been used to try and measure changes in DAG levels in eggs during fertilisation. An experiment was conducted to determine whether DAGR could be successfully used to measure whole cell changes of DAG in eggs. In order to do this the genetic probe DAGR was microinjected into eggs in the form of cRNA (pipette concentration 2.5 μg/μl) and left to express for approximately 3 hours (see fig 6.1A). This gave time for levels of the DAGR protein to accumulate. The eggs were then placed on the fluorescence imaging system to determine the strength of the baseline fluorescence in the eggs prior to stimulation. In order to test the response of DAGR in egg, after half an hour 1 μM of PMA was added into the egg containing media. PMA acts as a DAG analogue therefore mimics the effects of DAG increase. DAGR responded to the stimulus by an average increase in FRET ratio (YFP/CFP) of 28.5% (see fig 6.4). This suggests that DAGR can be used to detect an increase in DAG in eggs.

DAGR FRET ratio does not alter in response to PLCζ activity

As DAGR was able to detect changes in DAG levels in eggs the next step was to see if DAGR was able to measure any alterations in DAG levels during Ca^{2+}_i oscillations seen at fertilisation. Once again the eggs were microinjected with 2.5 μg/μl DAGR cRNA and left to express for 4 hours. In order to look at how DAGR responded in line with Ca^{2+}_i oscillations at fertilisation eggs were then microinjected with 0.025 μg/μl mPLCζ RNA alongside the Ca^{2+}_i indicator Rhod Dextran. These eggs were then imaged so the Ca^{2+}_i dynamics and the FRET ratio could be recorded for several hours. During the first 2 hours of recording the DAGR ratio appeared to steadily increase over time before reaching a plateau. It is unclear how this increase in DAGR FRET ratio relates to the Ca^{2+}_i oscillations as in most of the eggs the FRET signal did not plateau until after the eggs had stopped oscillating (see fig 6.5A). In order to measure how the FRET signal changed with the Ca^{2+}_i oscillations, for each egg three Ca^{2+}_i oscillations were selected and the DAGR FRET signal before and after was noted. The FRET ratio percentage change for these 3 spikes was calculated and averaged across all the eggs. Only a very small percentage change of DAGR signal was seen with individual Ca^{2+}_i oscillations of 1.4 % (see fig 6.5B, Table 6.1). The same experiment was then repeated with human derived PLCζ (hPLCζ) as this has more potent activity in terms of releasing terms of

releasing Ca^{2+} (Cox et al 2002). hPLCζ was injected into eggs at a concentration of 0.02 µg/µl which caused strong Ca^{2+}_i oscillations. As with mPLCζ during the first 2 hours of recording there is a steady increase in the DAGR FRET ratio before reaching a plateau and then decreasing slightly (see fig 6.6A). There was no detectable change in DAGR FRET signal with each oscillation initiated with hPLCζ (see fig 6.6B). These experiments taken together suggest that PLCζ does not cause a detectable change in DAGR signal alongside Ca^{2+}_i oscillations.

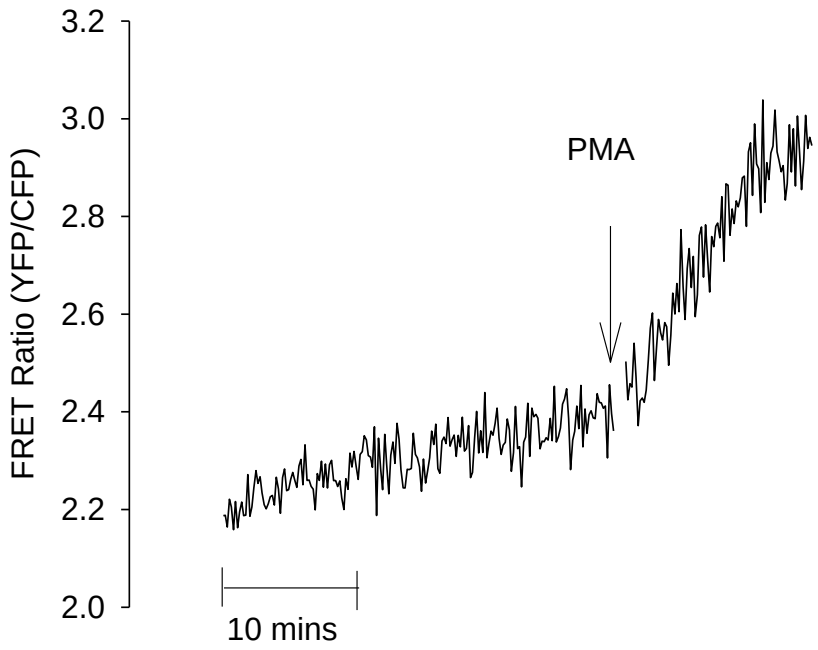


Figure 6.4 FRET changes of DAGR probe in response to PMA

A sample trace from an egg showing the FRET ratio change of the DAGR probe in response to 1 µM PMA (n=9) , see Table 6.1 for analysis

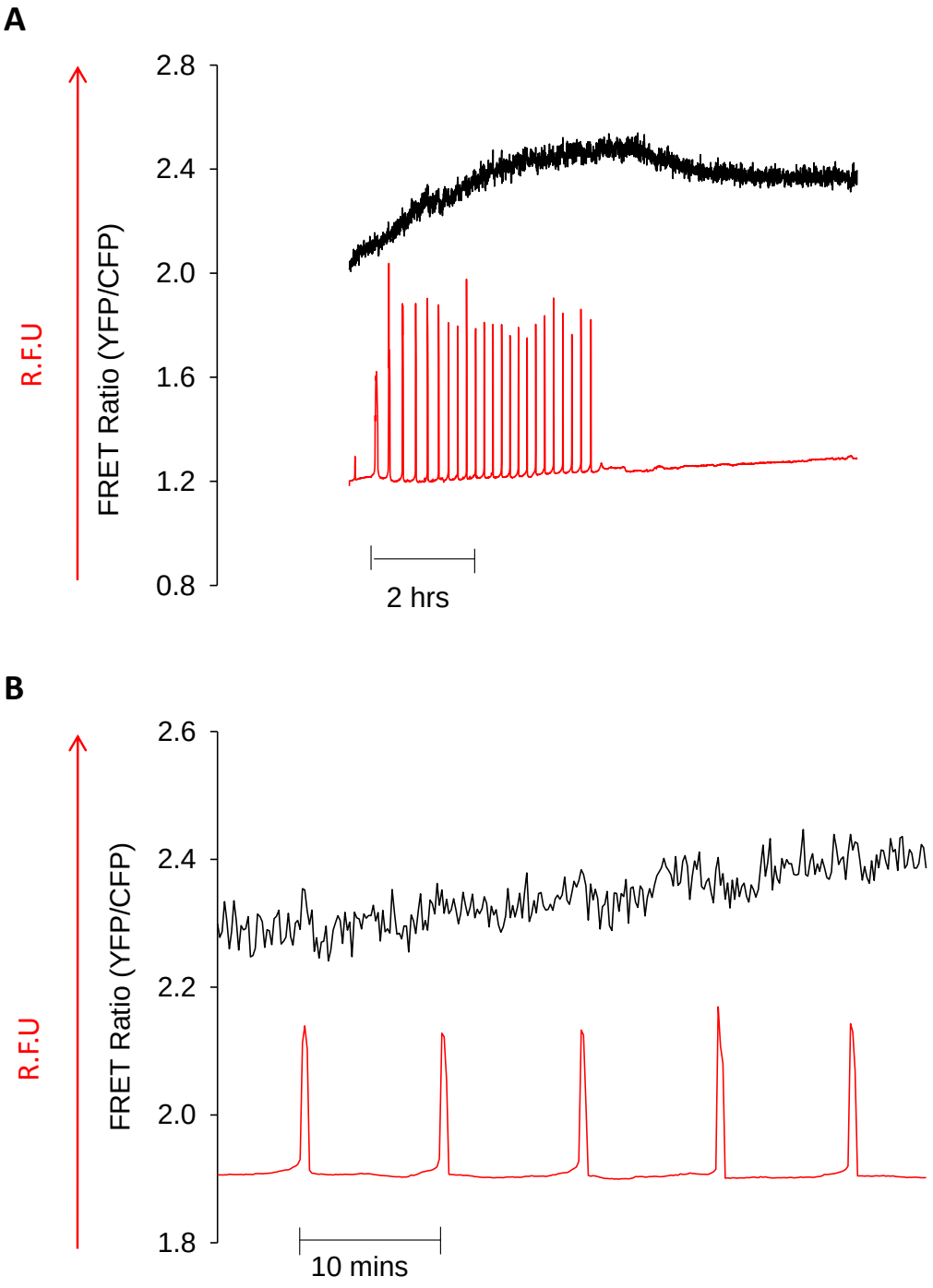


Figure 6.5 FRET and $Ca^{2+}{}_i$ changes of DAGR probe in response to mouse PLCζ

Example traces showing how the FRET ratio of the DAGR probe changes alongside $Ca^{2+}{}_i$ oscillations in an egg. $Ca^{2+}{}_i$ oscillations were initiated by 0.025 µg/µl mPLCζ RNA and measured using the $Ca^{2+}{}_i$ indictor Rhod Dextran. **A)** shows the change during the whole recording and **B)** shows a sample section of the same trace magnified R.F.U= relative fluorescence units (n=5), see Table 6.1 for analysis

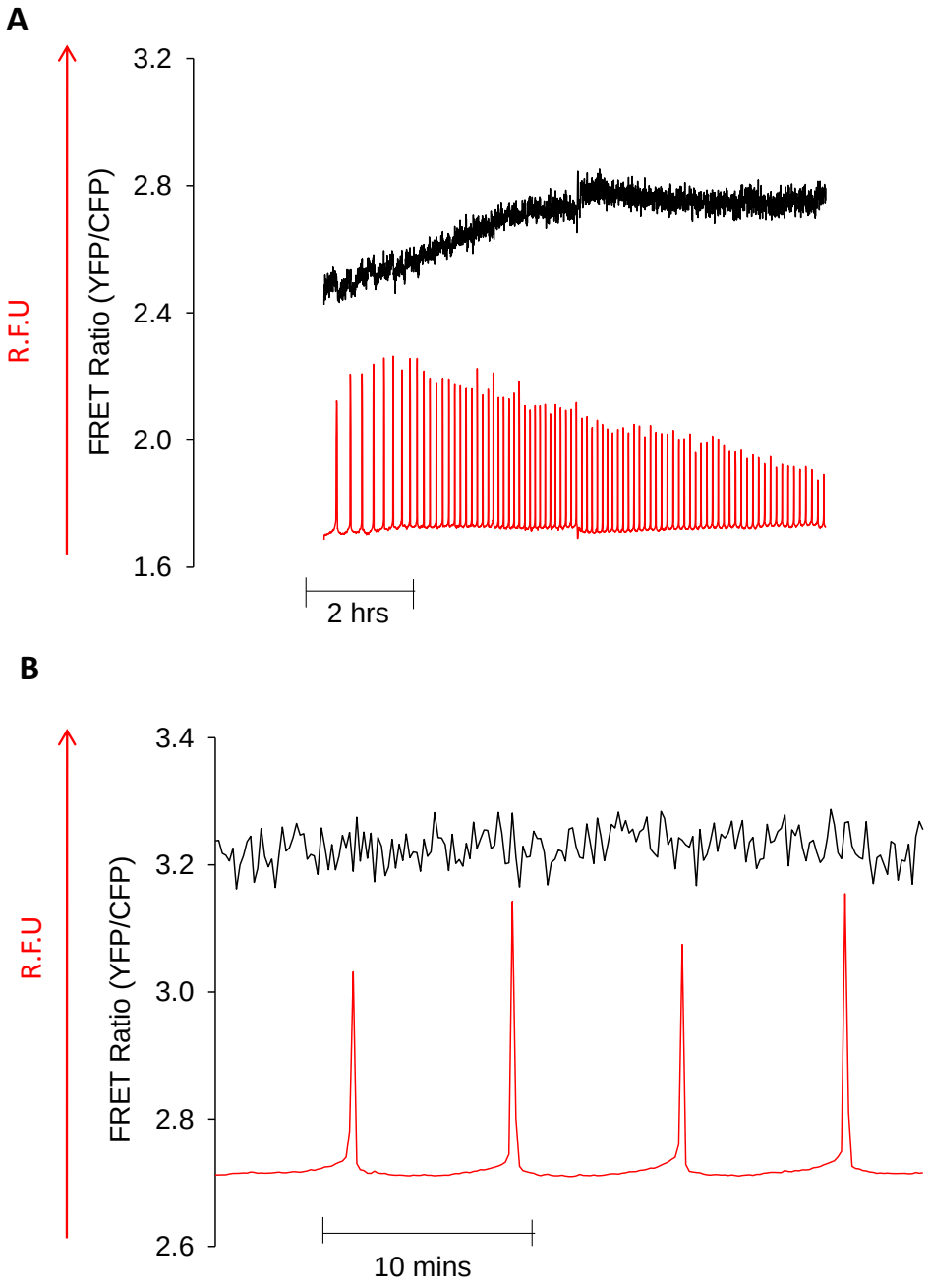


Figure 6.6 FRET and Ca^{2+}_i changes of DAGR probe in response to human PLCζ

Example traces showing how the FRET ratio of the DAGR probe changes alongside Ca^{2+}_i oscillations in an egg. Ca^{2+}_ioscillations were initiated by 0.02 µg/µl hPLCζ RNA and measured using the Ca^{2+}_i indicator Rhod Dextran. **A)**shows the change during the whole recording and **B)** shows a sample section of the same trace magnified, R.F.U= relative fluorescence units (n=7), see Table 6.1 for analysis

Measuring DAG using Green 'Upwards' DAG probe

The signal of Green 'Upwards' DAG alters in response to phorbol esters

In recent years a series of new probes have been developed to measure DAG, which rather than relying on FRET, contain only a single fluorescent protein which has been altered to produce a circularly permuted fluorescent protein. This adaptation produces a protein that is not only more fluorescent but that is also able to respond to conformational changes of the probe that occur on binding (Baird *et al.* 1999). This conformational binding results in a signal change which can be detected through fluorescence microscopy. Green 'Upwards' DAG is one such probe that has been successfully used to measure live cell changes in DAG levels in cell lines (Tewson *et al.* 2012). However, this probe has not been used to measure DAG changes in any eggs. To test this Green 'Upwards' DAG (Montana Molecular) was microinjected into eggs in the form of RNA (1 g/l) (see fig 6.1C). The probe was then left to express in the eggs for 3 hours before the eggs were transferred to the imaging system. Following imaging for approximately 30 minutes the phorbol ester, phorbol 12,13-dibutyrate (PDBμ) at a concentration of 2 uM was added to the media containing the eggs to mimic an increase in DAG. This caused an immediate large increase in the Green 'Upwards' DAG fluorescence signal of around 96.8 % (see fig 6.7, Table 6.1). This suggests that Green Upwards DAG is able to detect changes in DAG levels in eggs effectively.

Green 'Upwards' DAG does not detect changes in DAG during PLCζ induced $Ca^{2+}{}_i$ oscillations

Green 'Upwards' DAG has a relatively large dynamic range therefore it could be a good probe for detecting DAG changes during fertilisation. To investigate this eggs were injected with Green 'Upwards' DAG cRNA in the same way as previously (pipette concentration 2μg/μl) and left the express for approximately 3 hours. The eggs were then microinjected again with mPLCζ RNA (0.02 g/l) mixed with Rhod Dextran. The eggs were then transferred to the imaging system and the GFP signal from the Green 'Upwards' DAG was then measured simultaneously with the Rhod dextran signal. This enabled the change in Green 'Upwards' DAG signal to be measured with each $Ca^{2+}{}_i$ oscillation. The Green 'Upwards' DAG signal was then noted before and after 3 $Ca^{2+}{}_i$ oscillations for each eggs. These values were used to calculate the percentage change in signal for these $Ca^{2+}{}_i$ oscillations which was averaged over the egg and then the group of eggs. Very small changes in green fluorescent signal were detected of around 0.53 % with each $Ca^{2+}{}_i$ oscillation. However, this change is not considered a significant change in signal (see fig 6.8A). These small changes could be spill over from the red fluorescence channel; measuring $Ca^{2+}{}_i$ Furthermore there was a

constant drift up of Green 'Upwards' DAG signal during the course of recording which may indicate increased expression of the probe with time (see fig 6.8B).

To try and address the expression drift issue of the Green 'Upwards' DAG probe this experiment was repeated but the Green 'Upwards' DAG RNA was microinjected into immature GV oocytes. These oocytes were then allowed to *in vitro* mature (IVM) overnight until they reached MII phase. The eggs were then microinjected with 0.02 µg/µl mPLCζ RNA mixed with Rhod dextran to trigger Ca^{2+}_{i} oscillations in the eggs. The signals were then recorded and analysed as previously. Allowing the protein levels of Green 'Upwards' DAG to increase for the extended time period during IVM did reduce the problem associated with the drift in signal caused by increased expression with time (see fig 6.9B). However, in these eggs no detectable change in signal of Green 'Upwards' DAG was recorded with each Ca^{2+}_{i} oscillation as there was a very small decrease 0.17% (see fig 6.9A). Though there were some very small changes in the Green 'Upwards' fluorescence signal that corresponded with the Ca^{2+}_{i} oscillations it is difficult to determine whether these changes are significant.

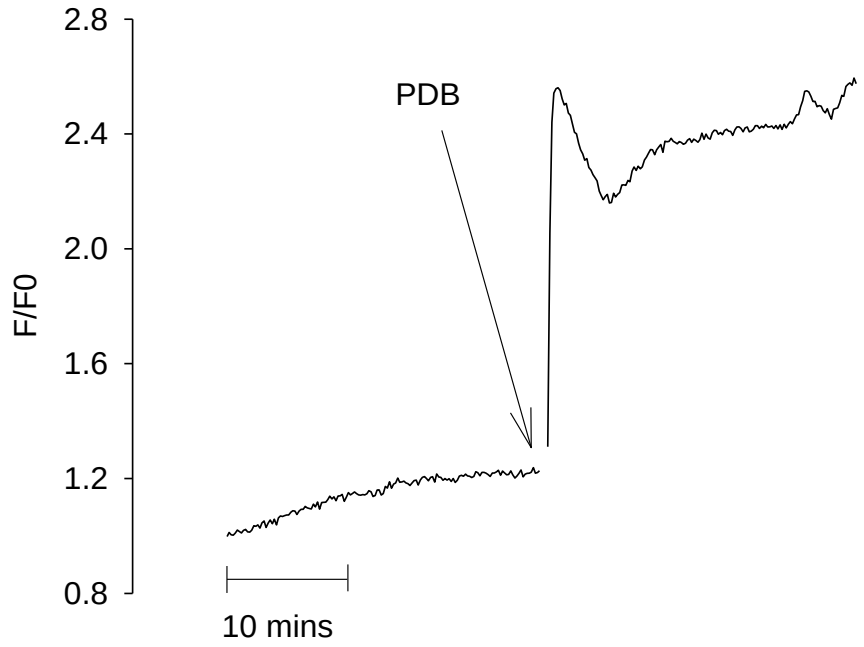


Figure 6.7 FRET changes of Green 'Upwards' DAG probe in response to PDBμ

A sample trace from an egg showing the change in fluorescence of Green "Upwards" DAG probe response to 2 μM PDBμ (n=8)

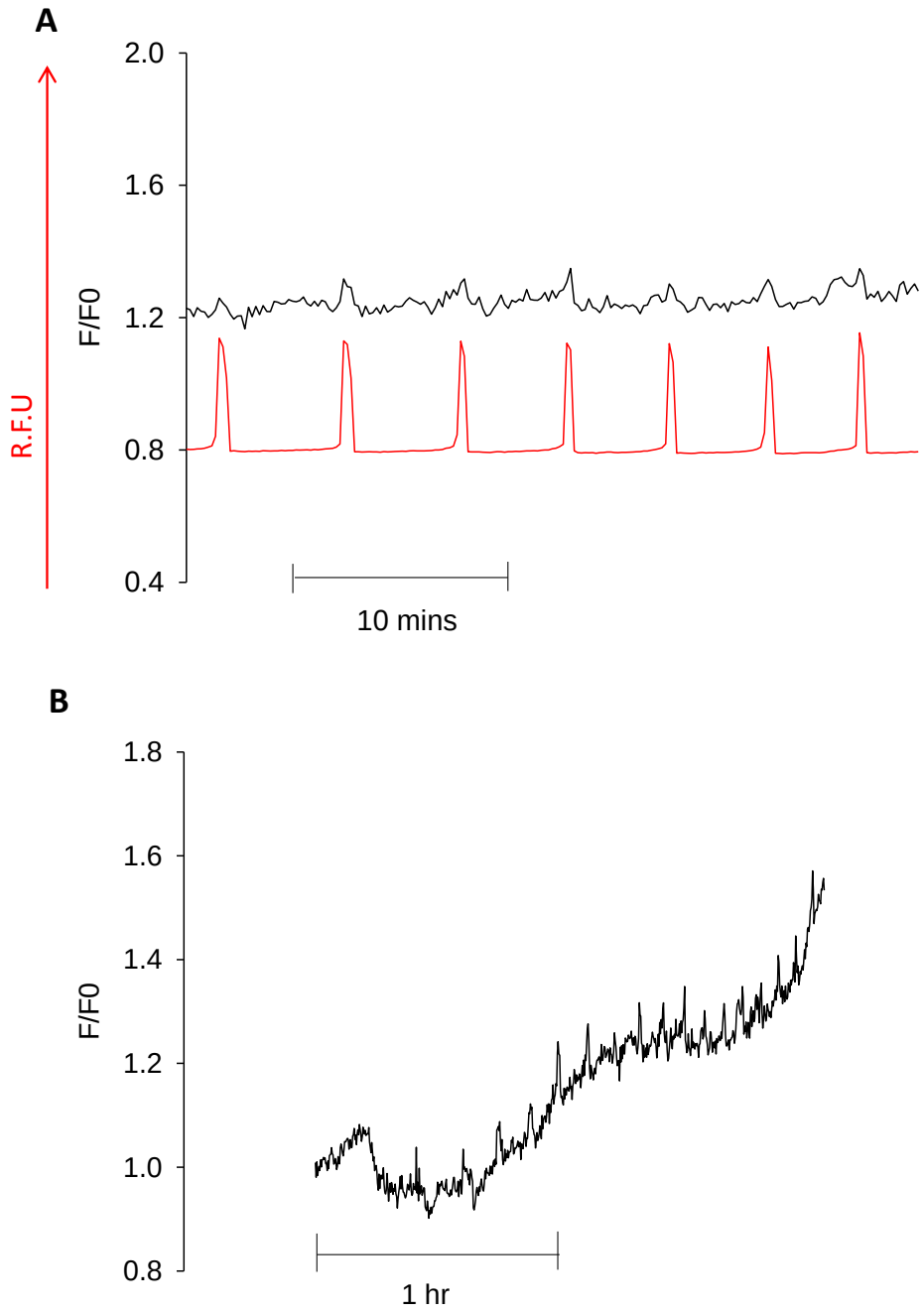


Figure 6.8 FRET and Ca^{2+}_i changes of Green 'Upwards' DAG probe in response to mouse PLCζ

Example traces from an egg expressing Green 'Upwards' DAG which show the changes in fluorescence signal of this probe alongside Ca^{2+}_i oscillations triggered by 0.02 µg/µl mPLCζ cRNA mixed with Rhod Dextran. **A)** shows the change in Green 'Upwards' DAG signal with Ca^{2+}_i oscillations and **B)** shows the change in Green 'Upwards' DAG signal alone over the course of the experiment , R.F.U= relative fluorescence units, F/F0= absolute fluorescence divided by mean baseline fluorescence (n=19)

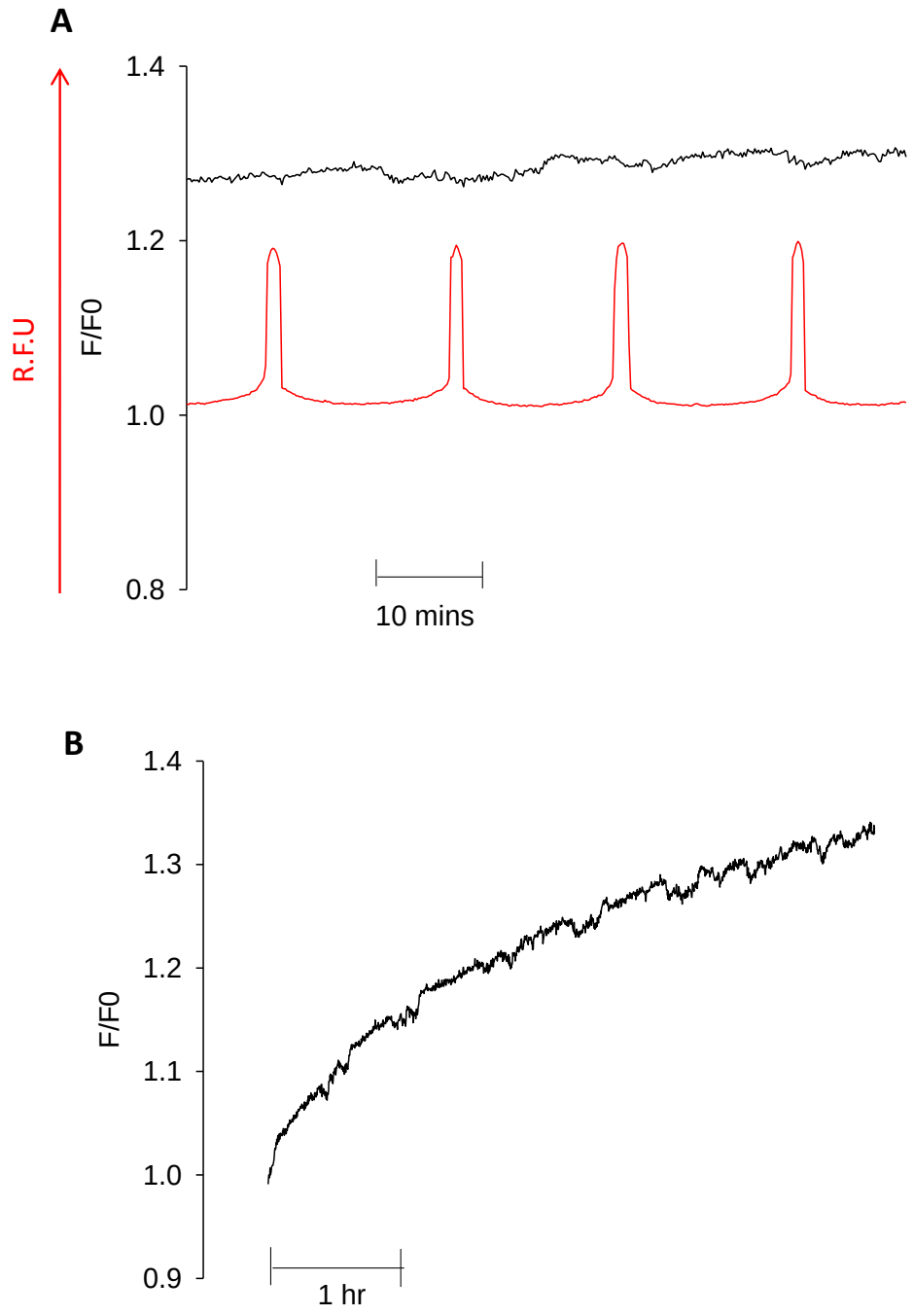


Figure 6.9 FRET and Ca^{2+}_i changes of in vitro matured Green 'Upwards' DAG probe in response to mouse PLCζ

Example traces from an egg expressing Green 'Upwards' DAG by *in vitro* maturation which show the changes in fluorescence signal of this probe alongside Ca^{2+}_i oscillations triggered by 0.02 µg/µl mPLCζ cRNA mixed with Rhod Dextran. **A)** shows the change in Green 'Upwards' DAG signal with Ca^{2+}_i oscillations and **B**) shows the change in Green 'Upwards' DAG signal alone over the course of the experiment . R.F.U= relative fluorescence units, F/F0= absolute fluorescence divided by mean baseline fluorescence (n=6)

Green 'Upwards' DAG doesn't detect changes in DAG during secondary oscillations induced by PLCζ overdose

If PLCζ is over-expressed past physiological levels in an egg its Ca^{2+}_i oscillations occur at a very high frequency and usually stop within approximately 2 hour. If the eggs continue to be imaged however, 'secondary' oscillations can occur (Yu et al 2008). It has previously been suggested that these secondary oscillations are different in nature to normal Ca^{2+}_i oscillations that occur during fertilisation. Secondary oscillations appear to be stimulated by conventional PKCs recruitment to the plasma membrane which become activated following an increase in Ca^{2+} influx (Yu *et al.* 2008). Due to the alternative mechanism of these secondary Ca^{2+}_i oscillations it was important to measure how DAG levels altered during these oscillations compared to regular Ca^{2+}_i oscillations induced by PLCζ. To do this the eggs were microinjected with Green Upwards DAG and PLCζ RNA as described previously however a much higher concentration of PLCζ RNA was used (0.8 μg/μl) to stimulate Ca^{2+}_i oscillations. As seen previously the Green 'Upwards' DAG signal was found to increase over the course of recording before reaching a plateau many hours later(see fig 6.10A). Interestingly, with the first Ca^{2+}_i oscillation alone a decrease in Green 'Upwards' DAG was seen of around 12 % however this was not seen with subsequent oscillations. This is possibly an arefact of green autofluorescence emitted from flavin adenine dinucleotide (FAD^{++}). Once secondary oscillations began no change in Green 'Upwards' DAG signal was recorded (see fig 6.10B). There was a slight decrease in overall Green 'Upwards' DAG signal during the onset of the secondary Ca^{2+}_i oscillations however this decrease was observed in all eggs during this time including those that did not have secondary Ca^{2+}_i oscillations (see fig 6.10B). This signal change is likely to be a result of a reduction in FAD^{++} autofluorescence.

Green 'Upwards' DAG detects little change in DAG when Ca^{2+}_i oscillations are inhibited by Propranolol and DAG kinase inhibitor

Ca^{2+}_i oscillations induced by PLCζ can be inhibited by two inhibitors as shown in Chapter 5).The first is propranolol which inhibits the enzyme LPP3 thereby inhibiting DAG production from phosphatidic acid (PA). The other is DAG kinase (DGK) inhibitor which inhibits the activity of the enzyme DGK which converts DAG to PA. In order to get a clearer idea of how these drugs inhibit PLCζ activity the Green 'Upwards' DAG probe was used to measure DAG dynamics in eggs during the action of these inhibitors. Eggs were microinjected with Green 'Upwards' DAG RNA and left to express as previously described. Eggs were then microinjected with mPLCζ RNA (0.025 μg/μl) and Rhod dextran to initiate

Ca^{2+}_i oscillations. Once the eggs had been oscillating for approximately 30 mins either DGK inhibitor (10 μM) or propranolol (300 μM) were added to the dish. In the case of DGK inhibitor the eggs were kept in media that was not covered in mineral oil instead a cap was placed on the dish to prevent evaporation. In all cases the drugs successfully inhibited the Ca^{2+}_i oscillations whilst keeping the baseline Ca^{2+}_i low (see fig 6.11). Interestingly though DGK inhibitor would be expected to have caused an increase in DAG actually this lead to a very small decrease in Green 'Upwards' DAG signal of 0.94% (fig 6.11A). In contrast propranolol, that should have caused a decrease in DAG production following addition, actually caused an immediate increase in Upwards' DAG signal of 8.5 % which quickly returned to basal levels (fig 6.11B). It is unclear what the explanation for this result is.

Green 'Upwards' DAG detects changes in DAG during PLCδ1 induced Ca^{2+}_i oscillations

PLCζ is unique amongst the PLC isoforms in being able to cause Ca^{2+}_i oscillations in eggs at the physiological levels found in the sperm in the absence of any further stimulation. PLCζ is most closely related to PLCδ1 and if PLCδ1 is expressed at very high levels in eggs it is able to cause some Ca^{2+} release in eggs. However, these oscillations do not look like Ca^{2+}_i oscillations elicited by PLCζ and appear to be biochemically quite different (Kouchi *et al.* 2004). For example, Ca^{2+}_i oscillations elicited by PLCδ1 generate DAG at the plasma membrane however no such DAG increase is found in PLCζ stimulated eggs (Yu *et al.* 2008). To investigate whether Green 'Upwards' DAG is able to measure changes in DAG levels in eggs stimulated by PLCδ1, eggs were micro-injected with Green "Upwards" DAG cRNA (2 μg/μl) and left to express as previously stated. After approximately 3 hours the eggs were injected with mPLCδ1 cRNA (2 μg/μl) mixed with Rhod dextran and then transferred onto the imaging system. Ca^{2+}_i oscillations were recorded however each oscillation was immediately followed by two to three smaller oscillations. Once the oscillations started a decrease in Green 'Upwards' DAG signal was recorded of approximately 14 % (see fig 6.12A). This signal then appeared to increase again slightly before decreasing again slightly when the next large Ca^{2+}_i oscillations occurred before increasing further (see fig 6.12A). Therefore once the initial decrease in signal occurred during the first Ca^{2+}_i transient the Green 'Upwards' DAG signal appeared to increase eventually up until the level recorded prior to the initiation of the Ca^{2+}_i oscillations (see fig 6.12A). Once again this decrease in green signal is likely to be result of a reduction in FAD^{++} autofluorescence.

Green 'Upwards' DAG does not detect changes in DAG during acetylcholine induced Ca^{2+}_i oscillations

Acetylcholine chloride (Ach) is able to trigger Ca^{2+}_ioscillations in eggs by stimulating the Gq coupled muscarinic acetylcholine receptors found on the plasma membrane (Felder *et al.* 1989). This causes the production of IP_3 which acts on the IP_3R to release Ca^{2+} (Gillo *et al.* 1987). As Ach leads to the production of IP_3 it is assumed that DAG must also be produced during this process. To test whether Green 'Upwards' DAG was able to detect any changes in DAG during Ca^{2+}_i oscillations initiated by acetylcholine eggs were microinjected with Green 'Upwards' DAG cRNA as previously described. Following expression for approximately 3 hours a second injection of Rhod Dextran was carried out before the eggs were immediately transferred to the imaging system. Once the eggs had been imaged for approximately 15 mins Ach (100 µM) was added to media surrounding the eggs. A very small decrease of 2.28% in Green 'Upwards' DAG signal was recorded during the first Ca^{2+}_i oscillation however no change was recorded with subsequent Ca^{2+}_i oscillations (see fig 6.13B). As a result Green 'Upwards' DAG does not appear to be able to detect any changes in DAG during Ach induced Ca^{2+}_i oscillations.

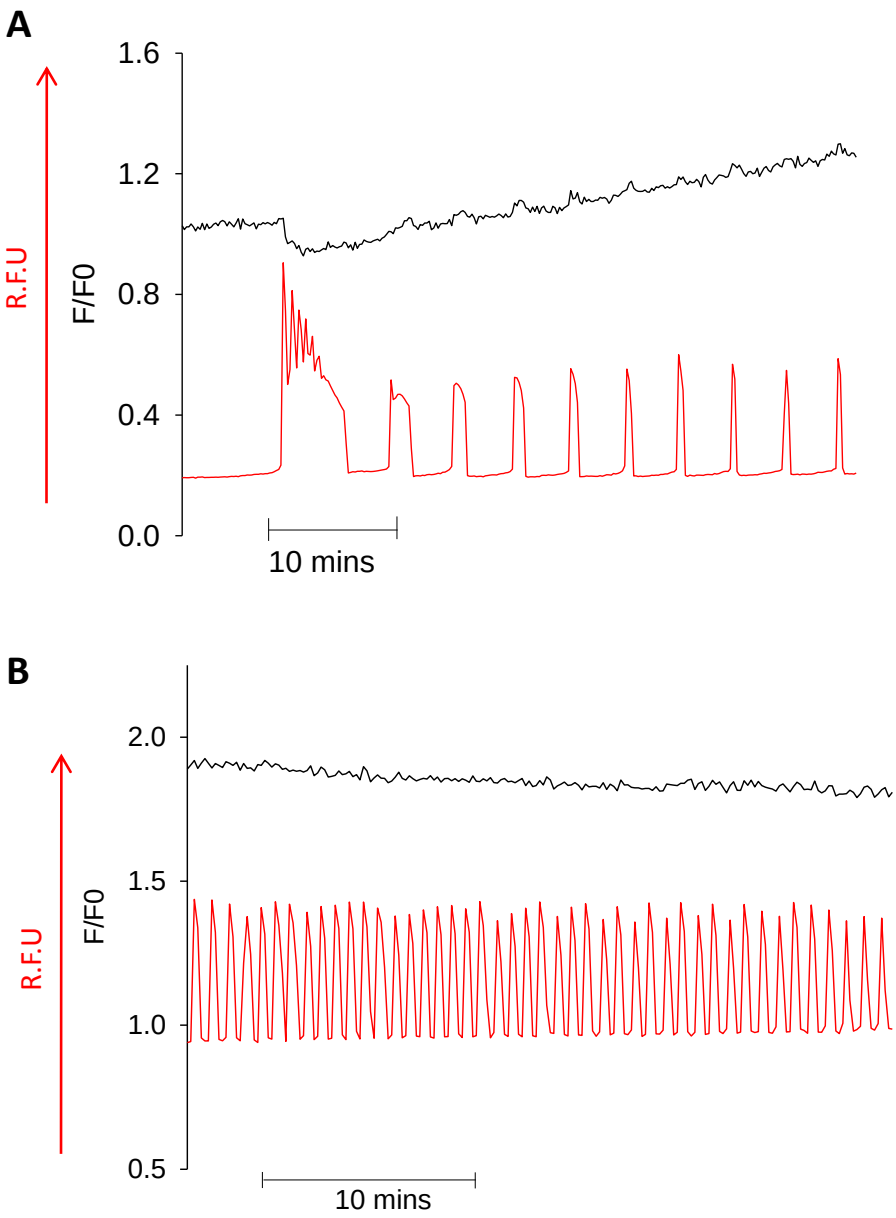


Figure 6.10 FRET and Ca^{2+}_i changes of in vitro matured Green 'Upwards' DAG probe in response to high dose mouse PLCζ

Traces showing how the Green 'Upwards' DAG signal changes in line with Ca^{2+}_i oscillations initiated by a high dose (0.8 µg/µl) of mPLCζ cRNA mixed with the Ca^{2+}_i indicator Rhod Dextran **A)** shows a magnified section of the beginning of this trace (primary Ca^{2+}_i oscillations) and **B)** shows a magnified section of the end of this trace (secondary Ca^{2+}_i oscillations) , R.F.U= relative fluorescence units, F/F0= absolute fluorescence divided by mean baseline fluorescence , (n=8)

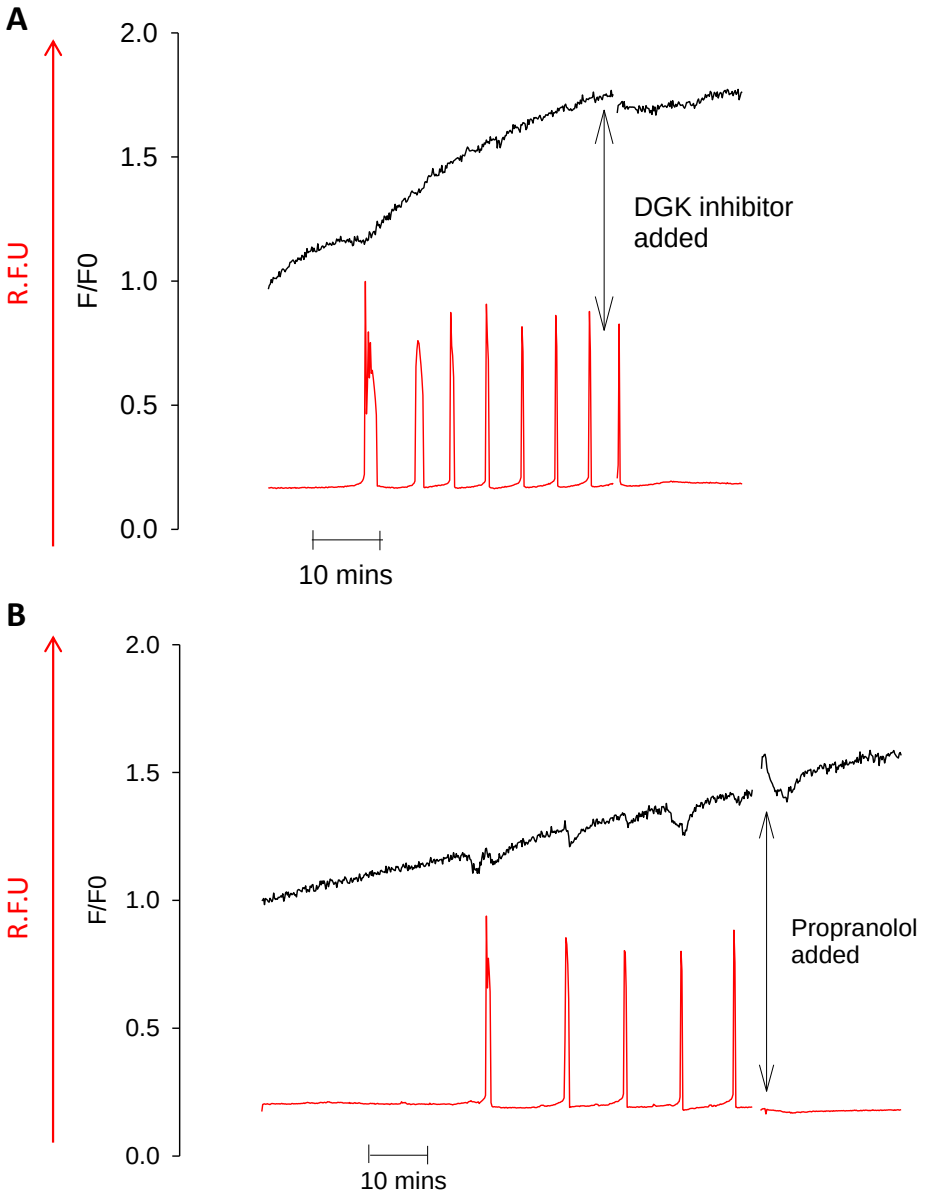


Figure 6.11 FRET and Ca^{2+}_i changes of Green 'Upwards' DAG probe in response to mouse PLCζ plus propranolol and DGK inhibitor

Traces showing how the Green 'Upwards' DAG signal changes in response to the induction of Ca^{2+}_i oscillations by mPLCζ cRNA (0.02 µg/µl) and following inhibition of Ca^{2+}_i oscillations by **A)** DAG Kinase inhibitor (100 µM) n= 5 or **B)** Propranolol (300µM) n= 9, R.F.U= relative fluorescence units

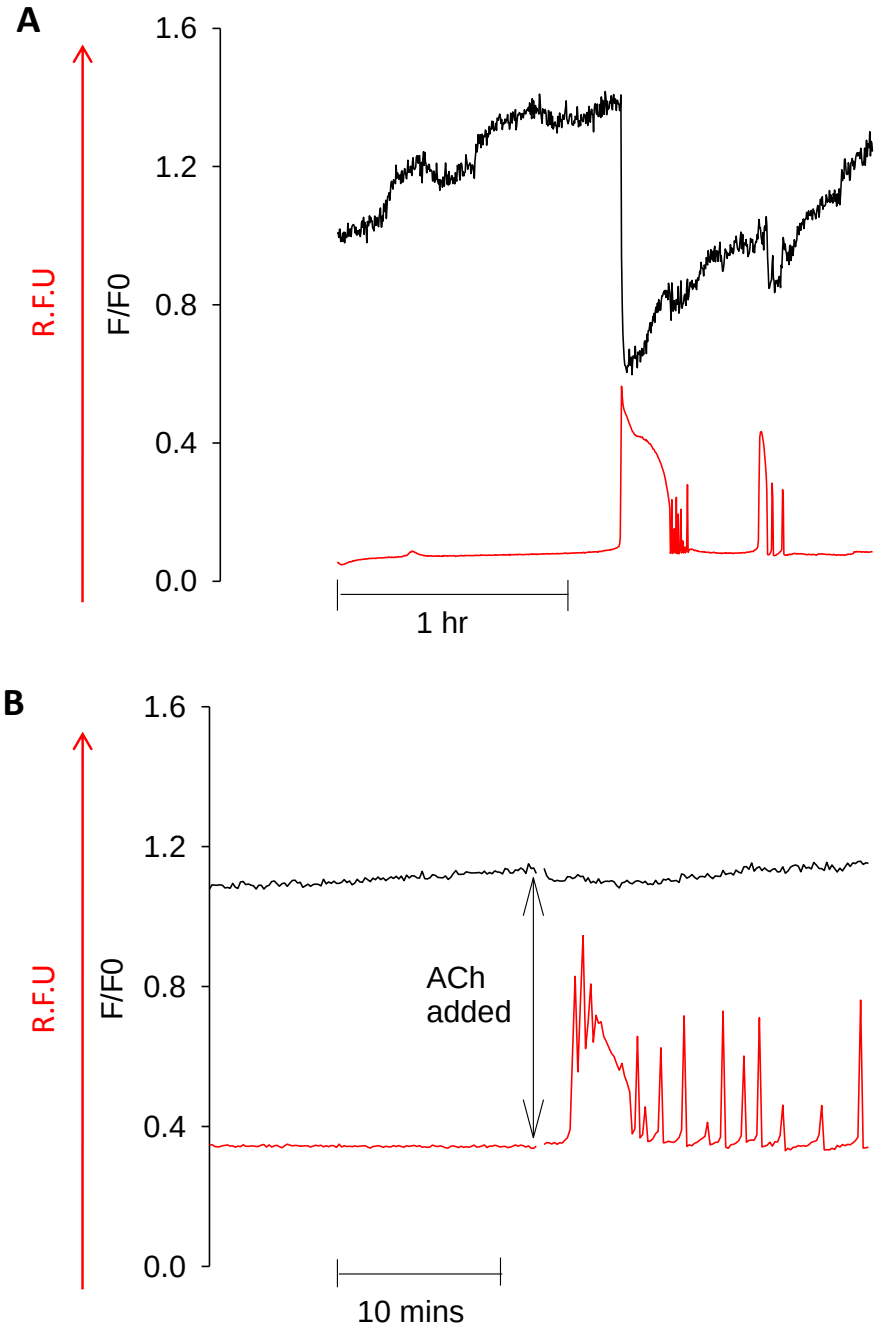


Figure 6.12 FRET and Ca^{2+}_i changes of Green 'Upwards' DAG probe in response PLCδ1 and Ach

Traces showing how the Green 'Upwards' DAG signal responds to other Ca^{2+}_i oscillations inducing agents **A)** PLCδ cRNA (2 µg/µl) (n= 5), **B)** Acetylcholine chloride (100µM) (n= 11). In both cases Ca^{2+}_i changes were measured using the indicator Rhod Dextran. R.F.U= relative fluorescence units, F/F0= absolute fluorescence divided by mean baseline fluorescence

Measuring IP_3 using a firefly split-luciferase IP_3 probe (F-SLIP)

In the last few years a new biosensor has been developed that is able measure IP_3 dynamics in live cell lines that is based on luminescence complementation (see fig 6.13) (Ataei *et al.* 2013). The probe is able to bind to IP_3 by the IP_3 binding core (IBC) which is tagged with the two halves of firefly luciferase at either terminus. Once IP_3 binds the IBC the probe undergoes a conformation change which brings the two halves of luciferase together (Ataei *et al.* 2013). When this happens the luciferase enzyme is active and able to produce light. The more IP_3 that is produced the higher the luminescence signal emitted. This probe has successfully been used to detect changes in IP_3 HEK293T showing a 11 fold increase when saturated (Ataei *et al.* 2013). This probe has never been used before to measure changes in IP_3 during $Ca^{2+}{}_i$oscillations at fertilisation. To investigate whether this probe could be used for this purpose, the firefly split-luciferase IP_3 probe (F-SLIP) cRNA was micro-injected into eggs (1 µg/µl) and left to express for approximately 3 hours. These eggs were then microinjected for a second time with the $Ca^{2+}{}_i$ indicator Oregon Green BAPTA Dextran (OGBD) mixed with mPLCζ cRNA (0.02 µg/µl). The eggs were then transferred to the imaging system and placed in media containing 1µM luciferin where fluorescence (for the OGBD) and luminescence (F-SLIP) were both measured by switching back and forth between fluorescence and luminescence imaging modes on a 10 second cycle. An overall increase in luminescence signal was recorded during the course of imaging that was still occurring 6 hours post-injection. Despite this increase the luminescence emitted by F-SLIP was very low even at the end of the experiment (maximum around 4 cps) (see fig 6.15). A small increase of 6.9 % was recorded in luminescence signal with each $Ca^{2+}{}_i$ oscillation (see fig 6.15) however there was a large degree of variability in this making it difficult to draw conclusions.

Figure 6.13 Structure of F-SLIP IP_3 probe

Schematic diagram showing the structure of the genetically encoded probe F-SLIP used to measure IP_3 dynamics. The probe consists of an IP_3 binding core (IBC) tagged with the N and C terminal halves of firefly luciferase joined with a 10 amino acid long linker.

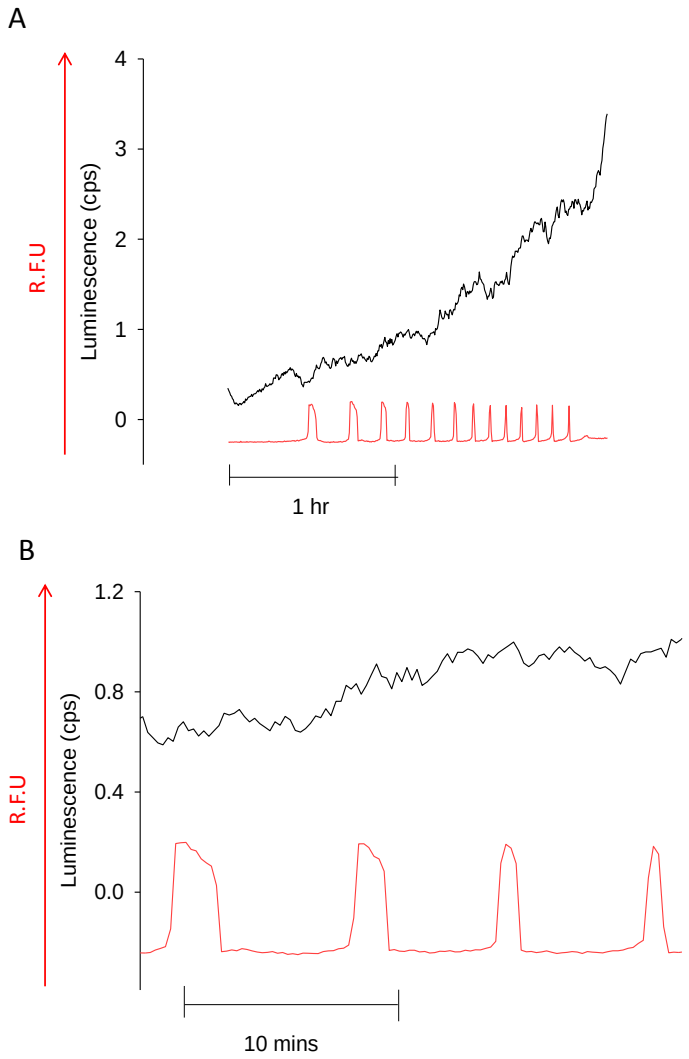


Figure 6.14 Luminescence and Ca^{2+}_i changes of F-SLIP probe in response mouse PLCζ

Sample traces showing changes in the luminescence signal from the F-SLIP probe alongside Ca^{2+}_i oscillations. Ca^{2+}_i oscillations were initiated by 0.02 µg/µl mPLCζ RNA and measured using the Ca^{2+}_i indicator Rhod Dextran. **A)** shows the change during the whole recording and **B)** shows a sample section of the same trace magnified, R.F.U= relative fluorescence units, cps= photon counts per second, (n=10)

Measuring IP_3 using a novel click beetle split-luciferase IP_3 probes (CB-SLIPs)

To try and increase the signal of F-SLIP the construct was redesigned to create a novel potential probe based in the same principles of F-SLIP. This probe contained the same IBC as F-SLIP however this core was tagged with 2 N termini of click beetle luciferase which is considered to produce a brighter signal than firefly luciferase (Miloud et al 2007). A series of click beetle split-luciferase IP_3 probes (CB-SLIPs) were produced each with a varying length of linker region to determine if any of these probes produced a higher signal than F-SLIP or measure a change in IP_3 in eggs during PLCζ induced $Ca^{2+}{}_i$oscillations. The constructs were designed and synthesised by MN (see fig 6.14).

CB-SLIP (2aa), CB-SLIP (4aa) and CB-SLIP (10aa) contained a 2, 4 and 10 amino acid long linker attaching the two click beetle luciferase fragments to the IBC respectively (see fig 6.14). The RNA of for each of these constructs was microinjected into eggs (1 μg/μl) and left to express for approximately 3 hours. After this time PLCζ cRNA (0.02 μg/μl) mixed with OGBD was microinjected into the eggs to cause $Ca^{2+}{}_i$ oscillations. The eggs were then placed in luciferin containing media on the imaging system whilst the luminescence and fluorescence of the eggs was measured for approximately 2 hours. During the course of imaging, for all constructs the luminescence increased steadily and eventually reached a very low maximum of about 10cps for CB-SLIP(2aa) (see fig 6.16), between 1-4 cps for CB-SLIP (4aa) (see fig 6.17) and a stronger signal of around 100 cps for CB-SLIP (10aa). In the case of CB-SLIP(2aa) was no clear change in luminescence during each $Ca^{2+}{}_i$ oscillation was recorded (see fig 6.16). CB-SLIP(4aa) showed an increase in 14.1 % however this effect was extremely variable and was not consistent (see fig 6.17). Again CB-SLIP (10aa only recorded a very small change (1.14 %) in luminescence signal with each $Ca^{2+}{}_i$ oscillation (see fig 6.18). This suggests that CB-SLIPs cannot accurately measure a change in IP_3 with $Ca^{2+}{}_i$ oscillations.

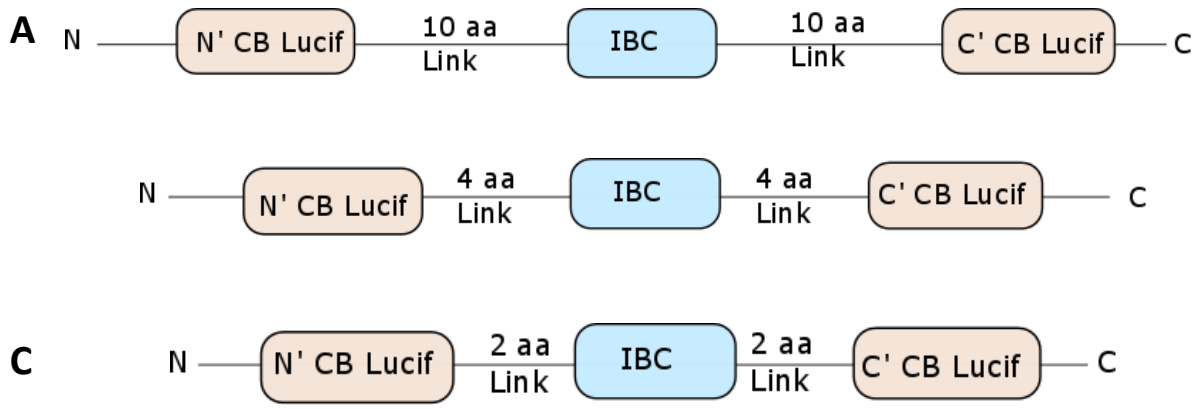


Figure 6.15 Schematic showing the structure of CB-SLIP IP_3constructs

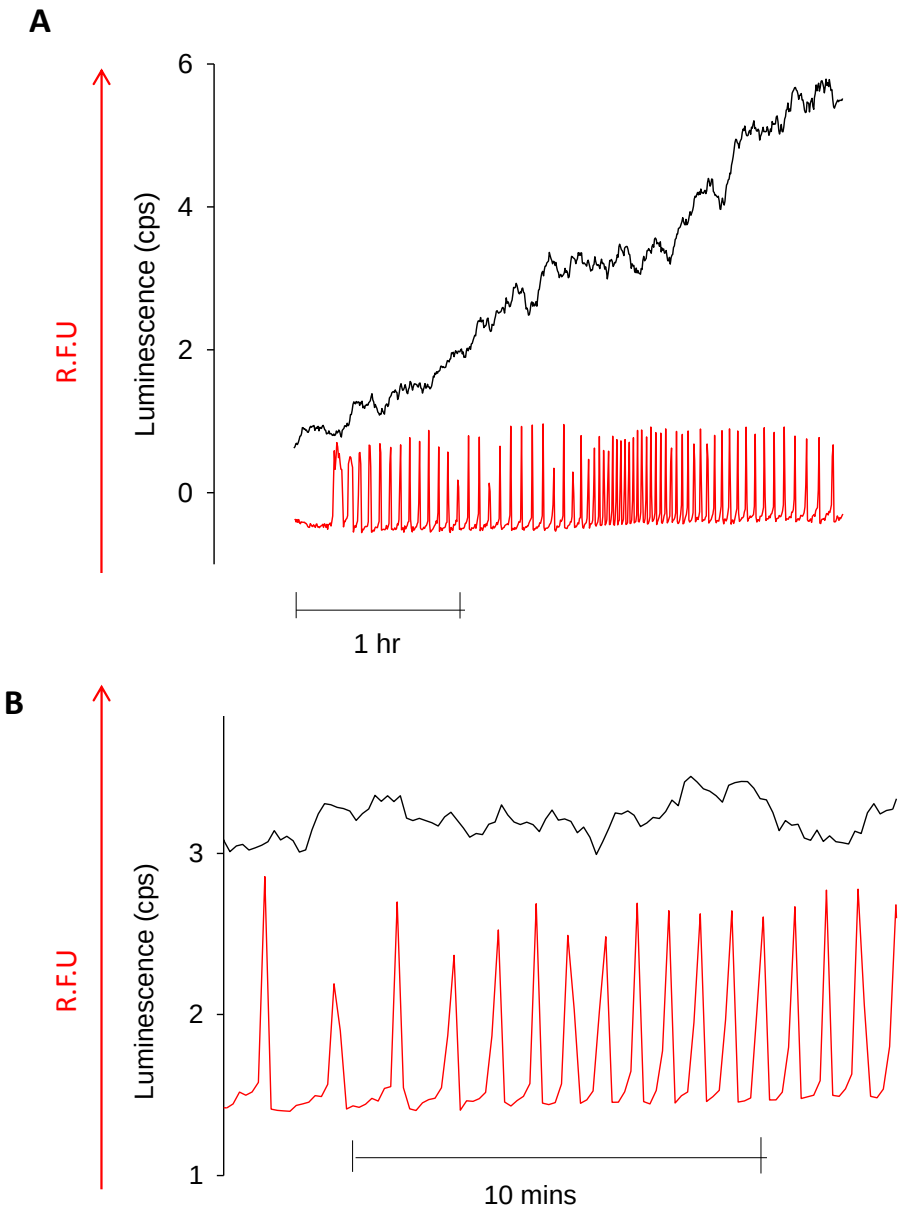


Figure 6.16 Luminescence and Ca^{2+}_i changes of CB-SLIP(2aa) probe in response mouse PLCζ

Sample traces showing changes in the luminescence signal from the CB-SLIP(2aa) probe alongside Ca^{2+}_i oscillations. Ca^{2+}_i oscillations were initiated by 0.02 µg/µl mPLCζ RNA and measured using the Ca^{2+}_i indicator Rhod Dextran. **A)** shows the change during the whole recording and **B)** shows a sample section of the same trace magnified , R.F.U= relative fluorescence units, , cps= photon counts per second (n=11)

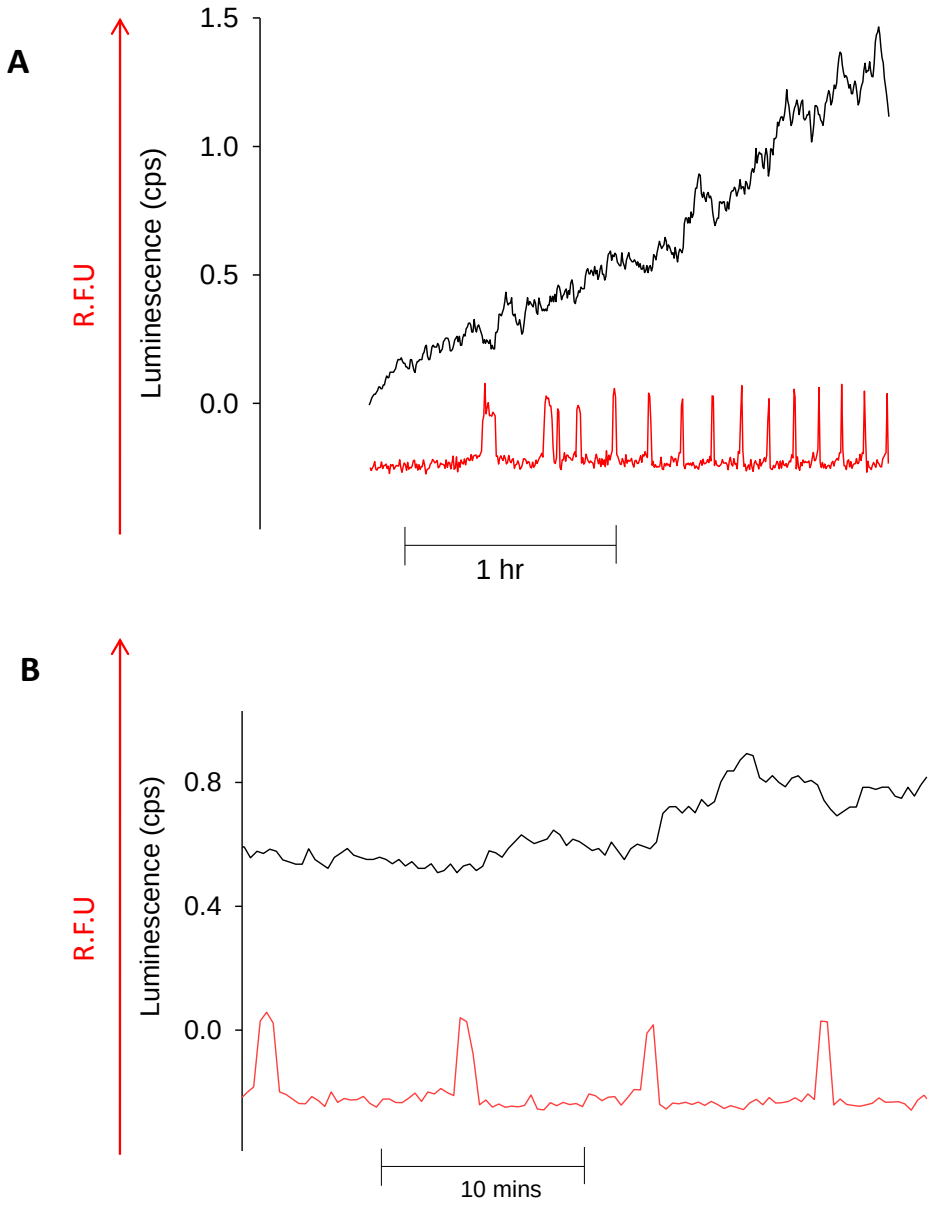


Figure 6.17 Luminescence and Ca^{2+}_i changes of CB-SLIP(4aa) probe in response mouse PLCζ

Sample traces showing changes in the luminescence signal from the CB-SLIP(4aa)probe alongside Ca^{2+}_i oscillations. Ca^{2+}_i oscillations were initiated by 0.02 µg/µl mPLCζ RNA and measured using the Ca^{2+}_i indicator Rhod Dextran. **A)** shows the change during the whole recording and **B)** shows a sample section of the same trace magnified R.F.U= relative fluorescence units, , cps= photon counts per second (n=10)

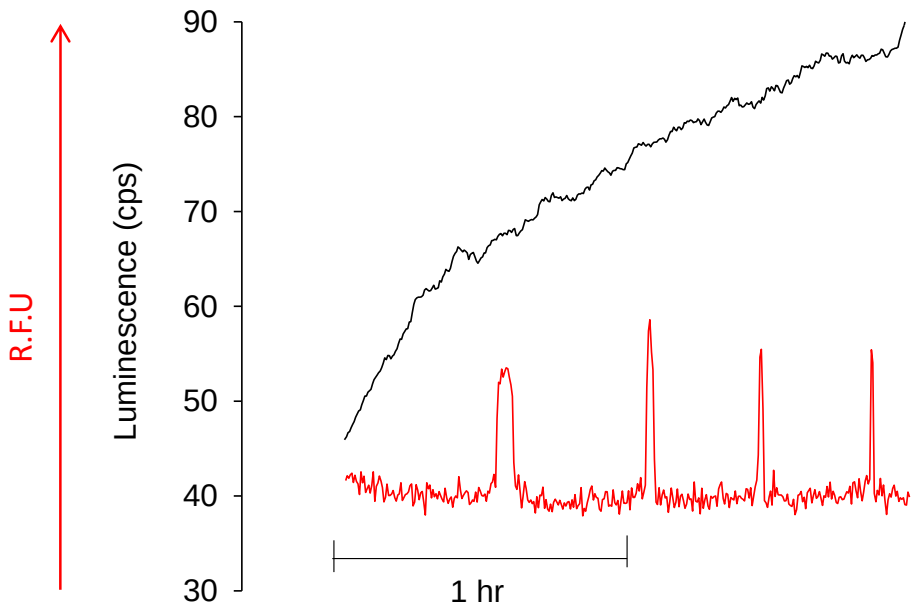


Figure 6.18 Luminescence and $Ca^{2+}{}_i$ changes of CB-SLIP(10aa) probe in response mouse PLCζ

Sample trace showing changes in the luminescence signal from the CB-SLIP(10aa) probe alongside $Ca^{2+}{}_i$ oscillations. $Ca^{2+}{}_i$ oscillations were initiated by 0.02 µg/µl mPLCζ RNA and measured using the $Ca^{2+}{}_i$ indicator Rhod Dextran. **A)** shows the change during the whole recording and **B)** shows a sample section of the same trace magnified, R.F.U= relative fluorescence units (n=10)

Discussion

As Ca^{2+}_i oscillations at fertilisation are a result of the hydrolysis of $PI(4,5)P_2$ by PLCζ activity it is undisputed that IP_3 and DAG are produced during this process however it is unclear exactly what the dynamics of these messengers are in live eggs. The lack of understanding the dynamics of these messengers leaves disputes around exactly what role they play dynamically as messengers and what impact this has on Ca^{2+}_i oscillation pattern or egg activation. Due to the often low cell number available of mammalian eggs it is unpractical to do some of the conventional biochemistry based experiments to measure the production of these messengers following fertilisation such as isotope labelling. Furthermore these methods do not allow accurate measures of messenger production to be made in live cells in response to different stimuli for example alongside a series of Ca^{2+}_i oscillations.

In order to measure messengers like IP_3 and DAG in real time genetically encoded probes need to used that can be expressed in cells. Many such probes have been developed to measure either IP_3 or DAG. These probes are usually tested by transfection into cell lines and measuring the signal of this probe in response un-physiological exogenous stimuli. Despite the vast array of probes available these are rarely used to measure physiological cell dynamics in physiological cell types and seem to produce cell specific effects in many cases. With this in mind these experiments aimed to find genetic based probes that had the ability to measure IP_3 and DAG dynamics in eggs in response to a physiological stimulus.

In many cell types DAG has been successfully measured using simple probes consisting of a DAG binding domain tagged to a fluorescent protein (Oancea and Meyer 1998; Oancea *et al.* 1998). These probes require the accurate measurement of probe translocation from the cytosol to a membrane (Oancea *et al.* 1998). In most cell types this is not an issue as the majority of signalling DAG is produced at the plasma membrane and translocation onto the plasma membrane is fairly easy to detect. However, in eggs at fertilisation there is a strong evidence to suggest signalling DAG is produced on intracellular membranes and not the plasma membrane (Yu *et al.* 2008; Yu *et al.* 2012). It is much harder to measure translocation dynamics of probes on and off intracellular membranes which requires specialist optical techniques and very high resolution imaging. Therefore in order to measure whole cell DAG levels in eggs it was necessary to use a probe that would produce changes in signal that could be detected on whole cell level. One technique that may be adopted is FRET (fluorescence resonance energy transfer). This requires two fluorophores

to come in close proximity such as coalescing on a membrane which produces a change in the ratio of one fluorophore signal to the other (Stryer 1978). This means FRET ratio changes can be detected in whole cells using simple epifluorescence imaging. Many different FRET based probes have been developed to measure all kinds of cell signalling dynamics. Indeed many FRET based DAG probes have been developed and used to measure whole cell DAG changes in cell lines (Violin *et al.* 2003; Sato *et al.* 2005).

For these experiments a very simple intermolecular FRET reporting system containing the C1b domain of PKC was used. Two constructs were created one tagged with YFP and the other with CFP and collectively termed C1b Inter-FRET. This probe only produced a small change in signal in response to the strong stimulus PMA. PMA is a phorbol ester which means it acts as a DAG analogue so should produce a near saturated response in signal of the probe, much greater than that seen by a physiological stimuli. This probe detected no change in probe signal during $Ca^{2+}{}_i$ oscillations initiated by PLCζ which would suggest that there is no detectable change in DAG levels in line with $Ca^{2+}{}_i$ oscillations. Similar results were recorded with the intra-molecular FRET based DAGR probe which has previously been used to measure DAG in MDCK cells in response to phorbol ester (Violin *et al.* 2003). When expressed in mouse eggs however, this probe only detected a small change in signal in response to PMA. Again, no dynamic change in DAGR signal was recorded during the PLCζ induced $Ca^{2+}{}_i$ oscillations stimulated either by human or mouse derived PLCζ cRNA. The fact both probes show no change in DAG levels during $Ca^{2+}{}_i$oscillations may mean that DAG levels do not significantly alter during PLCζ induced $Ca^{2+}{}_i$oscillations. However, due to the small change recorded in response to a strong stimulus it is just as likely that probes were not sensitive enough to detect any changes in DAG.

When recording FRET because 2 fluorophores are excited the sample is exposed to a large range of visible light which increases the chances of probe photo-bleaching. Furthermore the two fluorophores used may react differently to the high amount of light exposure. For example these may photobleach at different rates. If one flurophore photobleaches to a greater extent than the other this can disrupt the ratio of the 2 signals in the absence of stimuli. This effect could account for the upwards drift in the signal that was recorded during these experiments. However, it is very likely that this drift is due to differential expression of these two fluorescent probes. Nevertheless the drift makes it difficult to accurately calculate changes in signal in response to a stimulus. One of the issues with FRET based probes is the mechanism by with the signal change is created. FRET relies on the emission light from one fluorescent protein exciting a second fluorescent protein once

these two proteins come in close range. This can either occur between fluorescent proteins on two different molecules (inter-molecular FRET) or between two fluorescent proteins within the same molecule (intra-molecular FRET). Intra-molecular FRET, like that used by the DAGR probe, changes when the probe binds to a protein of interest and undergoes a conformational change which brings the two fluorescent proteins within range for FRET to occur. However, in addition to this intra-molecular FRET, undesired inter-molecular FRET between two fluorophores from different probe molecules can also occur if these co-localise. This produces a background signal that can disrupt the true intra-molecular FRET signal change. Furthermore FRET probes generally only produce a maximum signal change of around 20% which can be as low as 5% using physiological stimuli (Gonzalez Garcia 2013). In contrast to conventional fluorescent tagged probes and FRET based probes, circularly permuted fluorescent probes give off a much larger signal and allow for a greater dynamic change in signal (Baird *et al.* 1999). As a result these circularly permuted fluorescent proteins are being used more and more within biosensors. One example of such probe is the Green 'Upwards' DAG probe which has previously been shown to cause a 109% increase in signal in response to carbachol and PDBμ in M1 acetylcholine receptor expressing HEK 293 cells (Tewson *et al.* 2012). The current experiments found that a similar change of around 97% increase in signal in eggs expressing the probe in response to PDBμ alone. This would suggest that Green' Upwards' DAG is very responsive to changes in DAG levels in eggs however no significant changes in signal was recorded in response to PLCζ induced $Ca^{2+}{}_i$oscillations. However, in all traces even 5 hours post injection an upwards drift in Green 'Upwards' DAG signal was recorded. As this increase was seen in eggs irrespective of stimuli and a single wavelength indictor was used this is most likely due to protein expression drift. To try and avoid this situation GV oocytes were injected with Green 'Upwards' DAG and matured *in vitro* to express the probe and spontaneously mature to MII prior to PLCζ cRNA injection. This did help stabilise the Green 'Upwards' DAG signal when the eggs were imaged however no change in Green 'Upwards' DAG signal was recorded in response to PLCζ induced $Ca^{2+}{}_i$ oscillations in these eggs . This technique was not routinely used however as mouse eggs that mature via *in vitro* maturation are rarely the same quality of *in vivo* matured. For example many of the oocytes failed to mature fully following maturation. This could be due either to the high expression of exogenous RNA or the maturation conditions. For this reason *in vitro* maturation was not routinely used during these experiments.

The emission spectrum of Green 'Upwards' DAG overlaps with the FAD^{++} auto-fluorescence emitted by eggs during $Ca^{2+}{}_i$oscillations therefore there was a concern that the decrease in GFP signal from the FAD^{++} auto-fluorescence could confound the signal from the Green 'Upwards' DAG probe (Dumollard *et al.* 2004). Indeed this appeared to be the case in many of the green fluorescence signals emitted from the eggs and this effect was appeared to be variable between eggs. Therefore it would be advantageous to use an alternative circularly permuted fluorescent protein that had an emission signal that did not overlap with auto-fluorescence.

Physiological levels of PLCζ, equivalent to that found in the sperm, cause low frequency regular $Ca^{2+}{}_i$ oscillations that last for many hours in an egg (Saunders et al 2002). However, if much higher levels of PLCζ protein are expressed or introduced into eggs then a different pattern of $Ca^{2+}{}_i$ oscillations can occur. These oscillations are much higher frequency and usually stop within 2 hours (Yu et al 2008). After an hour or more these eggs can then start oscillating again producing very high frequency oscillations which looks very different in pattern to usual fertilisation induced $Ca^{2+}{}_i$oscillations (Yu et al 2008). It is believed that these $Ca^{2+}{}_i$ oscillations occur via an alternative mechanism to those that occur at fertilisation. There is evidence that secondary oscillations are initiated by DAG and PKC activation which increases Ca^{2+} influx (Yu et al 2008). Secondary oscillations can be mimicked by the addition of PMA to eggs that have ceased $Ca^{2+}{}_i$ oscillations as well as an increase in extracellular Ca^{2+} (Yu *et al.* 2008). Previously a GFP tagged tandem PKCδ C1 domain probe ($C1_2\delta$–GFP) has shown that DAG is produced at the plasma membrane during PLCζ induced secondary $Ca^{2+}{}_i$ oscillations (Yu *et al.* 2008). Furthermore secondary $Ca^{2+}{}_i$ oscillations can be prevented using the PKC inhibitor, cherlerythrine chloride (CHE) (Yu *et al.* 2008).

However, Green 'Upwards' DAG detected no change significant change in signal during primary or secondary $Ca^{2+}{}_i$ oscillations in eggs expressing high levels of PLCζ. Small decreases in signal were detected during primary oscillations however these looked like an arefact of FAD^{++} autofluorescence (Dumollard *et al.* 2004). A decrease in signal was recorded during the first $Ca^{2+}{}_i$ spike in most eggs. It is unclear why this would be the case for eggs expressing high levels of PLCζ and not the lower levels used in the other experiments. Furthermore, you would expect an increase in signal corresponding to an increase in DAG rather than a decrease. Unlike $C1_2\delta$–GFP which measures DAG by plasma membrane translocation it is not possible for Green 'Upwards' DAG to show where DAG is being produced. This means that Green 'Upwards' DAG is not able to discriminate between

measuring metabolic or signalling DAG. PLCζ induced DAG production should only take the form of signalling DAG but Green 'Upwards' DAG could also be measuring changes in metabolic DAG that disrupts the Green 'Upwards' DAG signal changes.

Propranolol and DAG kinase inhibitor are two drugs that are able to inhibit PLCζ induced Ca^{2+}_i oscillations and both have roles in disruption DAG metabolism however it is unclear what effect the drugs have on the DAG levels of Ca^{2+}_i oscillating eggs. The Green Upwards DAG probe only reported a small change in signal in response to DAG kinase inhibitor and propranolol but different types of effects were seen. Propranolol gave a small increase in Green 'Upwards' DAG signal, however, DGK inhibitor produced a small decrease in signal. This is contradictory to the known modes of action of these drugs. Propranolol inhibits the enzyme which converts phosphatidic acid (PA) into DAG whereas DGK inhibitor inhibits the enzyme that converts DAG to phosphatidic acid (Asp *et al.* 2009; Kaneko and Ishikawa 2016). As a result you would expect propranolol to decrease levels of DAG however this is likely to be an alternative source of DAG to that produced by PLCζ. Furthermore it remains unclear exactly how propranolol inhibit PLCζ induced Ca^{2+}_i oscillations. Rather than Ca^{2+}_i oscillations being inhibited as a result of DAG metabolic disruption propranolol may work by disrupting Golgi structure and membrane trafficking (Asp *et al.* 2009).

It is unclear if other stimuli that also release Ca^{2+} via IP_3 will result in a significant alteration in DAG levels. Interestingly Ca^{2+}_i oscillations initiated by PLCδ1 cRNA did cause an alteration in Green 'Upwards' DAG signal in the form of a significant decrease. It is unclear why PLCδ1 would lead to a decrease in DAG levels as it has previously been shown using the $C1_2\delta$–GFP probe that DAG levels at the plasma membrane increase during PLCδ1 induced Ca^{2+}_i oscillations (Yu *et al.* 2008). PLCδ1 is thought to hydrolyse plasma membrane $PI(4,5)P_2$ which would lead to DAG production in the plasma membrane. However, it is unclear whether $PI(4,5)P_2$ is hydrolysed in any other compartments of the cell in response to PLCδ1 and therefore whether DAG is made here also. It is worth noting that in order to trigger Ca^{2+}_ioscillations using PLCδ1 over 100 times more cRNA needed to the expressed. These are levels far past physiological and the decrease in signal may be a result of over-expression of the protein, which could also account for the decrease in signal recorded during the first Ca^{2+}_i spikes caused by the over-expression of PLCζ. Furthermore, the decrease in green fluorescent signal is likely to be an artefact of FAD^{++} autofluorescence (Dumollard *et al.* 2004)

Acetylcholine chloride (Ach) is another agent that is able to initiate Ca^{2+}_i oscillations in eggs via the production of IP_3 (Felder *et al.* 1989). These oscillations follow a different form to Ca^{2+}_i oscillations initiated by fertilisation or PLCζ. Green 'Upwards' DAG did not record any alterations in signal in these eggs stimulated by Ach either. This suggests that as with PLCζ induced Ca^{2+}_i oscillations, no alterations in DAG are seen with Ach induced Ca^{2+}_i oscillations either. However, it is unclear exactly how Ach causes Ca^{2+}_i oscillations in eggs and what determines the pattern of Ca^{2+}_i oscillations. It most likely that Ach stimulates IP_3 production by G proteins (Swann 1992).

Data from all the current experiments suggests that there is no significant change in DAG during PLCζ induced Ca^{2+}_i oscillations. This supports previous evidence using the translocation of a $C1_2\delta$-GFP which recorded no change in plasma membrane DAG levels during PLCζ induced Ca^{2+}_i oscillations (Yu *et al.* 2008). However, it has previously been suggested that PLCζ hydrolyses a vesicular source of $PI(4,5)P_2$ and therefore would produce DAG on intracellular membranes and not that plasma membrane (Yu *et al.* 2012). Furthermore indirect DAG measurement of PKC phosphorylation using the CKAR probe showed that DAG levels did change during PLCζ induced Ca^{2+}_i oscillations in eggs and this change was predominantly in the cytoplasm (Gonzalez-Garcia *et al.* 2013). All the probes used during these experiments measure whole cell changes in DAG levels and were not specific to certain cellular membranes. DAGLAS is a FRET based probe that is able to measure DAG production at specific membranes by addition of a membrane targeting sequence (Sato *et al.* 2006). In order to specifically measure intracellular DAG production that may occur as a result of vesicular $PI(4,5)P_2$ hydrolysis, a similar probe could be developed that contains a targeting sequence for these vesicles. However, it is not yet fully established what the identity of these vesicles are and therefore would make it difficult to identify a suitable targeting sequence. Furthermore different species of DAG are present in the cell. Only 'signalling' DAG is thought to be produced as a result of PLCζ activity however many different species of metabolic DAG are also produced on intracellular organelles. For example YFP tagged DAG binding domain has shown that DAG is also produced in the Golgi (Gallegos *et al.* 2006). Nevertheless it is unclear what species of DAG this is, as a result producing a DAG probe which targets the Golgi for example would detect changes in all species of DAG present here not just signalling DAG. To escape this issue it would be necessary to discover a DAG binding domain which was able to bind specifically to one molecular species of DAG with a particular fatty acid composition. As of yet no such domain has been developed. Because no distinction was made during these experiments between

different species of DAG, all DAG production and metabolism was measured which may not have given an accurate measure of changes in signalling DAG dynamics. Alternatively the level of "metabolic" DAG may be so high that it is not possibly the distinguish changes in "signalling" DAG as the "metabolic" DAG signal would drown this out.

It is also possible that no change in DAG levels were seen because signalling DAG is being synthesised but quickly metabolised, that is the rate of DAG turnover may have increased. As a result no detectable changes in DAG levels could be seen with the probes. It is likely that a delicate balance between metabolism and synthesis is required to enable the eggs to oscillate effectively. For example high turnover for DAG may be necessary for a Ca^{2+}_i oscillating eggs to ensure that DAG levels do not get too high and lead to overstimulation of PKC (Yu *et al.* 2008). PKC activation can lead to Ca^{2+} influx which produced non-physiologically high frequency oscillations (Yu *et al.* 2008). Oscillations of this nature are harmful to a fertilising egg as too high frequency Ca^{2+}_i oscillations can lead to poor development in early embryos (Ozil *et al.* 2006). As a result it may be necessary to keep the DAG levels low to prevent Ca^{2+} influx and ensure low frequency Ca^{2+}_i oscillations. All probes used to try and measure DAG in these experiments were fluorescence based which often makes it difficult to measure small changes in signal as the background noise is relatively high. Furthermore single fluorophore probes such as Green 'Upwards' DAG are not ratiometic in their signal so are dependent on lamp intensity to determine signal strength. This makes it difficult to make comparisons between different experiments. This also makes it impossible to determine the true strength of the signal produced by probe. FRET on the other hand is dependent on the ratio between two fluorophores which may have different stabilities.

To try and avoid some of the issues that arise using FRET and fluorescence based probes more and more biosensors centred on bioluminescence (luminescence) are being developed. Luminescence tagged probes are effective at measuring small changes in signals as a very low level of background is produced. These probes also do not vary with lamp intensity and doesn't photo- bleach in the way fluorescent probes can. Luminescence based probes can even be used to look at protein –protein interactions for example the technique of bioluminescence resonance energy transfer (BRET) contains a donor luminescence sequence which emits light that excites the fluorescent acceptor protein(Xu *et al.* 2002). However, BRET is rarely used on individual cells due to the very low level of light emitted. Alternatively split bioluminescence (luminescence) complementation is another way to look at cellular interactions that is able to produce a greater overall signal

that BRET therefore is being used more and more to develop biosensors (Paulmurugan and Gambhir 2003). These probes have a very low background value so are more sensitive at measuring small changes in signal. One such probe is the IP_3biosensor which is tagged with the N and C terminal halves of firefly luciferase and has previously been used to measure IP_3 in cell lines (Ataei *et al.* 2013). This firefly split luciferase IP_3 probe (F-SLIP) has never been used in measure IP_3 changes in eggs before so was utilised to try and measure IP_3 dynamics in PLCζ induced Ca^{2+}_i oscillating eggs. When this probe was injected into eggs the luminescence signal was very low and no change was seen in the luminescence signal in line with Ca^{2+}_i oscillations. The very low baseline signal recorded could have made it difficult to detect any changes in the signal. This is because the square root of the mean number of photons counts is proportionate to the signal to noise ratio. Therefore if the mean signal is 10 counts per second then approximately 3 of these counts can be considered as stochastic noise. As a result for a probe producing a signal of around 10 counts per second a change in signal of more than 30 % would need to be produced in order to be detected a in one second window. In contrast a signal of around 1000 counts per second would only contain around 32 counts of stochastic noise and therefore a signal change of around 3% would be detectable. It is important to note however that in order to be sure an effect is genuinely significant this change would need to be much higher, at least 1 standard deviation more than the change that can be accounted for by stochastic noise.

In attempt to try and increase the luminescence signal a new set of probes were constructed that replaced the firefly luciferase for click beetle luciferase. Click beetle luciferase is thought to produce a brighter signal than firefly luciferase however this construct design had not previously been used to measure IP_3 changes in any cell types (Miloud *et al.* 2007). A series of constructs were developed based on this principle with varying amino acid linker lengths joining the luciferase to the IBC. Altering the linker length in luciferase complementation based probes can dramatically alter the sensitivity of the probe as this can optimise the efficiency of the two halves of luciferase joining during the conformational change.

Both CB-SLIP (2aa) and CB-SLIP (4aa) produced a very low luminescence signal in eggs following microinjection. No change was seen in CB SLIP (2aa) probe signal as a result of Ca^{2+}_ioscillations. A small change of 14.1 % was detected by CB-SLIP (4aa) however this result was extremely variable and due to the low signal count was not significant. With photons counts this low it would be necessary to have a signal change significantly higher than 50% before in one second before any meaningful change could be claimed. CB-SLIP

(10aa) however showed a relatively stronger signal in comparison to the other probes tested however this probe only reached a maximum signal of around 120 cps. This means that only a signal change of greater than 10% would be detectable with this probe. Only a very small change in signal was recorded with each Ca^{2+}_i oscillations of around 1% so this was not significant.

As no IP_3 probe expressed high enough to detect a change in IP_3 dynamics during Ca^{2+}_i oscillations it is unclear how IP_3 dynamics change during Ca^{2+}_i oscillations. There is an issue however with expressing probes such as this at very high levels. The probe senses IP_3 by binding to it via the IBC. If levels of IBC are very high there is a chance that IP_3 will be buffered and therefore not release Ca^{2+} effectively. This makes it difficult to measure IP_3 using such probes which have a high affinity for IP_3. Improved version of this domain has been developed that contains mutations which lower the binding affinity to IP_3 (Gulyas *et al.* 2015). Two versions of this probe were developed one based on FRET with Cerulean and Venus tags the other based on BRET where the Cerulean tag is replaced with *Renilla* luciferase (Gulyas *et al.* 2015). These probes were used on individual and cell sheets of HEK293T cells respectively to successfully measure IP_3 changes (Gulyas *et al.* 2015). It could therefore be possible in the future to develop a lower affinity IP_3 probe based on this IP_3binding domain that uses luminescence complementation. This could allow for greater expression of the probe without the concerns of buffering or at the expense of sensitivity.

Indirect measures of IP_3 via measurement of $PI(4,5)P_2$ hydrolysis have suggested that IP_3 oscillates with Ca^{2+}_i oscillations. Measuring IP_3 production using fluorescently tagged PH domain which binds to plasma membrane $PI(4,5)P_2$ and translocates to the cytosol on $PI(4,5)P_2$ cleavage suggests that IP_3 production and Ca^{2+}_i oscillations are phase locked in MDCK cells stimulated by ATP (Hirose *et al.* 1999). Similar effects have been recorded using FRET based probes in other cell types (Violin *et al.* 2003). However, this method is only able to measure IP_3 produced at the plasma membrane as a result of plasma membrane $PI(4,5)P_2$ hydrolysis. This does not appear to be the case during PLCζ induced Ca^{2+}_i oscillations as plasma membrane $PI(4,5)P_2$ is not hydrolysed in eggs at fertilisation (Yu *et al.* 2012). Interestingly when additional caged IP_3 is released in eggs already oscillating in response to PLCζ, a Ca^{2+}_i oscillation is triggered and the pattern of Ca^{2+}_i oscillations are reset in the process of phase resetting. During this process the oscillations do not change frequency. This suggests that oscillations in IP_3 set the pace for Ca^{2+}_i oscillations with a spike in IP_3 corresponding to a spike in Ca^{2+}_i (Swann and Yu 2008). This supports the idea that IP_3,

Ca^{2+}_i and PLCζ are part of a positive feedback loop that set the pace for Ca^{2+}_i oscillations (Hirose *et al.* 1999).

To conclude the data from these experiments did not find that DAG or IP_3 changed dynamically during PLCζ induced Ca^{2+}_i oscillations. However, this is most likely because the probes used were not sensitive enough to measure any changes that may occur during this process or a result of poor expression of the probes.

7. CONCLUSIONS

Summary of Findings

Though there is an overwhelming amount of evidence that PLCζ is the physiological sperm factor that releases Ca^{2+} in mammalian eggs at fertilisation the mechanisms by which it does so are still not fully understood. The subject of this thesis was to determine the structural characteristics of PLCζ and the cellular characteristics of the egg that enable Ca^{2+}_i oscillations at fertilisation. Positively charged residues in the EF hand domain and X-Y linker of PLCζ were found to play a crucial role in enabling PLCζ to release $Ca^{2+}$by ensuring sufficient binding of the enzyme to its substrate $PI(4,5)P_2$. An intracellular source of $PI(4,5)P_2$ was detected on small vesicles in the egg cytoplasm and these vesicles are likely to be the binding site of PLCζ. The ability for PLCζ to release Ca^{2+} is likely to be a cell cycle dependent feature that is acquired during oocyte maturation. The role of the potential alternative sperm factor PAWP was investigated however the data collected in this thesis indicated that PAWP is not able to release Ca^{2+}in eggs under physiological conditions. Finally attempts were made to measure DAG and IP_3 dynamics in eggs during PLCζ induced Ca^{2+}_i oscillations but none of the probes tested measured a significant change in either DAG or IP_3 during Ca^{2+}_i oscillations.

PLCζ as the Sperm Factor

It is now well established that egg activation in mammalian eggs is caused by a sperm derived factor that diffuses into at sperm egg fusion (Swann 1990a). In mouse eggs this sperm factor diffuses into the egg and causes Ca^{2+}_i oscillations within 1 to 2 minutes (Swann 1990a; Lawrence *et al.* 1997; Lee *et al.* 2001). The sperm factor appears to be universal in initiating egg activation in mammals. Injection of sperm extracts from a variety of mammalian species causes Ca^{2+}_i oscillations and egg activation in a range of (Swann 1990a; Homa and Swann 1994; Swann 1994). Mammalian sperm extracts can also cause egg activation in species from other animal groups such as marine worms and sea urchin eggs (Parrington *et al.* 1999; Witton *et al.* 1999; Stricker *et al.* 2000). In some mammalian species, such as hamster, the sperm factor is soluble however in other species for example mouse, this sperm factor is only extractable under reducing conditions (Swann 1994; Kimura *et al.* 1998). It would appear in both instances PLCζ is the sperm factor that causes Ca^{2+}_i oscillations (Saunders *et al.* 2002; Fujimoto *et al.* 2004). Indeed the ability for PLCζ to cause Ca^{2+}_i oscillations in mammalian eggs has been established in many species. The

results contained in this thesis consistently show that mouse or human derived PLCζ protein or cRNA are able cause Ca^{2+}_i oscillations reliably in mouse eggs even at very low levels of these proteins.

However, in order for PLCζ to be considered the physiological sperm factor that activates mammalian eggs at fertilisation it has to satisfy a number of characteristics. Firstly it must stimulate the production of IP_3 in order to release Ca^{2+} as it is well established that Ca^{2+}_i oscillations at fertilisation occur as a result of IP_3 induced Ca^{2+} release (IICR) (Miyazaki 1988). The Ca^{2+}_i oscillations initiated by sperm extract are also caused by IICR (Jones *et al.* 1998). Like all other PLC isoforms PLCζ is able to directly hydrolyse $PI(4,5)P_2$ to produce IP_3 (Lee *et al.* 2001; Saunders *et al.* 2002). Secondly PLCζ must be present in the correct part of the sperm. The perinuclear theca is the first part of the sperm to fuse with the egg and appears to contain active Ca^{2+}_i releasing ability (Kimura *et al.* 1998; Fujimoto *et al.* 2004; Kurokawa *et al.* 2005). Immunocytochemistry has shown that PLCζ is also present in this region of the sperm (Heytens *et al.* 2009; Kashir *et al.* 2012; Yoon *et al.* 2012; Escoffier *et al.* 2015b; Kashir *et al.* 2017). Finally the absence of PLCζ from the sperm must ablate its Ca^{2+}_i releasing ability. It has been difficult to develop a PLCζ null mouse model as these mutants do not complete spermatogenesis and therefore are infertile (Ito 2010). However, when the level of PLCζ in the sperm is reduced significantly using RNA interference the Ca^{2+}_i oscillations initiated by this sperm stop earlier than that initiated by control sperm (Knott *et al.* 2005). Furthermore depletion of PLCζ in sperm extracts with immuno-depletion using antibodies against PLCζ affects the Ca^{2+}_i releasing ability of these sperm extracts (Saunders *et al.* 2002). Sperm from mice lacking PLCζ is unable to cause Ca^{2+}_i oscillations initiated by ICSI (Parrington 2017, unpublished). Therefore, PLCζ is the only sperm derived protein which is able to cause Ca^{2+}_ioscillations in a physiological manner.

Despite this vast array of evidence supporting the role of PLCζ as the sperm factor another candidate has been proposed. Post-acrosomal sheath WW domain-binding protein (PAWP) appeared to fulfil many of the characteristics of a sperm factor candidate. For example PAWP is localised in the perinuclear theca of the sperm head specifically the post-acrosomal sheath of this region (Wu *et al.* 2007; Aarabi *et al.* 2014b). PAWP has also found to promote egg activation in a range of species following injection of recombinant protein (Wu *et al.* 2007). In mouse and *Xenopus* eggs this egg activation has been attributed to the stimulation of Ca^{2+} release (Wu *et al.* 2007; Aarabi *et al.* 2010). Most specifically human derived PAWP recombinant protein and cRNA was reported to cause Ca^{2+}_i oscillations in mouse eggs (Aarabi *et al.* 2014b). Furthermore, the Ca^{2+}releasing characteristics of sperm

could be abolished by interfering with PAWP activity by injecting a PPXY peptide into eggs that is supposed to interfere with PAWP binding in egg (Aarabi *et al.* 2014b).

However, the results shown in Chapter 3 indicate that the Ca^{2+}_i releasing abilities of PAWP could not be reproduced using a range of human and mouse derived PAWP constructs. Under no circumstances were Ca^{2+}_i oscillations initiated by PAWP even at very high levels of PAWP protein. Furthermore the Ca^{2+}_i oscillations initiated by both IVF and PLCζ were not affected by the PPXY peptide. There is also no clear pathway defined as to how PAWP is able to cause IP_3 production as PAWP does not possess any catalytic activity of its own (Nomikos *et al.* 2014a). It has been suggested that the PPXY motif in the proline rich region of PAWP binds to the WW1 domain of Yes-associated protein (YAP) in the egg (Wu *et al.* 2007). YAP possesses a SH3 domain that could then activate PLCγ via some unknown pathway which could then hydrolyse $PI(4,5)P_2$ to produce IP_3 (Aarabi *et al.* 2014b).

The role of PLCγ in activating mouse eggs has previously been speculated. Another proposed sperm factor candidate the tyrosine kinase tr-kit has also been found to activate mouse eggs and is believed to do so by activating PLCγ (Sette *et al.* 1997). Interestingly the injection of excess SH3 domains of PLCγ inhibits tr-Kit induced egg activation (Sette *et al.* 1998). However, no Ca^{2+}_i oscillations have been recorded in response to tr-kit.This suggests that in mouse eggs PLCγ activation through interactions with its SH2 or SH3 domains is unlikely. PLCζ on the other hand possesses inherent catalytic activity and is able to hydrolyse $PI(4,5)P_2$ in the egg directly without any further stimulus (Saunders *et al.* 2002). Furthermore the potency and reliability of PLCζ at eliciting Ca^{2+}_i oscillations makes it a good egg activator. Ca^{2+}release is so essential for correct egg activation in eggs it would make sense that the stimulus from this process would produce IP_3 in simple and reliable way. The data in this thesis PLCζ has found to consistently cause Ca^{2+}_i oscillations and activate eggs at physiological concentrations however this is not the case for PAWP. Finally gene knockout experiments have shown that PAWP null mice are perfectly fertile suggesting that PAWP plays no physiological role in activating eggs (Satouh *et al.* 2015). Unlike PLCζ PAWP appears to possess no Ca^{2+}_i releasing ability and does not possess all the characteristics necessary to be considered the mammalian sperm factor. Furthermore unlike PLCζ, PAWP is not present in the fraction of sperm that has Ca^{2+}_i releasing ability when injected into eggs (Fujimoto *et al.* 2004).

Homologues of the PLCζ gene have been also been found in non-mammalian species across the animal kingdom. For example PLCζ is expressed in fish such as teleosts, eels and pufferfish (Coward *et al.* 2003; Coward *et al.* 2011; Morini *et al.* 2015) amphibians such as

newts (Harada *et al.* 2007) and birds such as chickens and quails (Coward *et al.* 2005; Mizushima *et al.* 2008). However, it is not clear whether PLCζ plays a role in activating eggs physiologically in these species. For example, though the newt *Cynopspyrrhogaster*can release Ca^{2+}in response to mouse derived PLCζ RNA, fractionation of sperm extracts has shown that physiologically it is citrate synthase which triggers Ca^{2+}releases in these eggs (Harada *et al.* 2007). Though *Xenopus* PLCζ is able to cause Ca^{2+} release in mouse eggs it has been suggested that endogenous PLCγ is activated by Src in response to PA production (Bates *et al.* 2014). Despite the fact a PLCζ homologue is present in pufferfish it is expressed in the oocyte and not the sperm so it unclear what role this plays in egg activation (Coward *et al.* 2011). However, in at least some birds, for example quails, PLCζ is considered the sperm factor which activates the egg physiologically (Mizushima *et al.* 2008). It is important to note that birds are evolutionarily more closely related to mammals than amphibians and fish, as a result it is more likely that birds and mammals may share characteristics of egg activation. The evolutionary relationship between how eggs activate is not clear mainly because different eggs release Ca^{2+} via different mechanisms depending on the method of fertilisation and environmental niche that species occupies.

Mechanisms and models of Ca^{2+}_i signalling in eggs

The importance of Ca^{2+}_i signalling in the egg activation of animal species is undisputed (Stricker 1999). However, the mechanisms by which this Ca^{2+}_i increases varies dramatically between species. For example in some species such as *Drosophilia* fruit flies and *Sicyonia* shrimps Ca^{2+}_i induced egg activation occurs during ovulation not fertilisation (Lindsay *et al.* 1992; Heifetz *et al.* 2001). Of the eggs that activate following sperm-egg fusion some species rely on Ca^{2+} influx in order to increase Ca^{2+}_i, others rely on Ca^{2+}release from stores or a combination of both. For example limpets activate solely by Ca^{2+}_i influx which produces a uniform increase in Ca^{2+}_i (Deguchi 2007). In contrast other marine invertebrates like sea urchins release Ca^{2+} from stores (Whitaker and Steinhardt 1982). This is also true for *Xenopus*eggs (Busa and Nuccitelli 1985). Medaka fish and bivalves such as mussels use a combination of these mechanisms (Yamamoto 1954; Gilkey *et al.* 1978; Deguchi *et al.* 1996). Mammalian eggs increase Ca^{2+}_i solely through Ca^{2+}release in the form of Ca^{2+}_i oscillations (Igusa *et al.* 1983; Igusa and Miyazaki 1986). These oscillations cross the egg in the form of a wave that takes less than 1 minute (Deguchi *et al.* 2000). These Ca^{2+}_i waves are a product of IP_3 induced Ca^{2+} release and have a generative component that requires positive feedback onto PLCζ (Miyazaki 1988; Miyazaki *et al.* 1992; Swann and Yu 2008).

There are two different ideas for how these regenerative waves can be produced.

The first idea is reliant on the intrinsic biphasic nature of the IP_3 receptor (Iino 1990). Ca^{2+}_i acts directly on the IP_3R so when cytosolic Ca^{2+}_i levels are low increase in Ca^{2+}_i are able to act positively, in the presence of IP_3 open and the IP_3R (Bezprozvanny *et al.* 1991; De Young and Keizer 1992). However, when the Ca^{2+}_i is high the IP_3 receptor closes (Bezprozvanny *et al.* 1991).

This model often relies on changes in ER Ca^{2+}concentrations to act as the pacemaker of the Ca^{2+}_i oscillations and it is considered raising the ER Ca^{2+}concentrations increases the sensitivity of the IP_3R which results in the release in $Ca^{2+}$from these stores (Iino 1990). According to this model when ER Ca^{2+} levels are low eggs would not be-able to oscillate. However, this is not the case. If eggs experiencing PLCζ induced Ca^{2+}_i oscillations are exposed to thapsigargin, which depletes ER Ca^{2+} levels, these eggs continue to oscillate but with a reduced amplitude (Wakai *et al.* 2013). As this model is dependent solely on the properties of the IP_3 receptor it does not rely on fluctuations in cytosolic IP_3 instead assumes a constant production of IP_3. However, injecting the IP_3 analogue adenophostin, which mimics a constant level of IP_3 production does not cause Ca^{2+}_i oscillations that look like that at fertilisation (Swann and Yu 2008). Furthermore the biphasic properties of the IP_3R are only relevant at low levels of Ca^{2+}_i (Mak *et al.* 1998). Therefore it is unlikely that this model of IP_3 induced Ca^{2+}release is how the regenerative Ca^{2+}_i waves are produced in mammalian eggs at fertilisation.

The other model by which the regenerative Ca^{2+}_i wave could be produced is by Ca^{2+}_i induced IP_3 formation (see fig 7.1). This model does not rely on the level of ER Ca^{2+}concentrations but instead is dependent on cytosolic Ca^{2+}_i concentrations (Meyer and Stryer 1988; Swann and Yu 2008). In this model IP_3 is produced in an oscillatory manner. During peaks in IP_3 levels the IP_3R are opened to release Ca^{2+} (Meyer and Stryer 1988). This produces an increase in Ca^{2+}_i which positively feeds back onto IP_3 production (Iino and Endo 1992). As a result if this model of regenerative Ca^{2+}_i increase is taking place in mammalian eggs at fertilisation you would expect there to be oscillations in IP_3 formation. Using a FRET based probe for IP_3 in mouse eggs has shown that there are small changes in IP_3 levels during Ca^{2+}_i oscillations. The results of Chapter 6 attempted to measure these dynamic using novel bioluminescent IP_3 probes. However, none of these probes appeared sensitive enough to measure changes in IP_3 during PLCζ induced Ca^{2+}_i oscillations.

It is significant that the rapid Ca^{2+}_I waves in mouse eggs that generate the upstroke of each Ca^{2+}_i oscillation occur with a 1 sec rise time (Dupont and Dumollard 2004). If Ca^{2+}_I induced

IP_3 formation is responsible for each wave then IP_3 must diffuse from its generation point to the IP_3R on the ER within less than 1 second (Dupont and Goldbeter 1994) . However, it has recently been shown that, intact cells, IP_3 can only diffuse approximately 3-5 µm in a 1 second time period (Dickinson *et al.* 2016). Therefore in order for Ca^{2+}_i oscillations to be initiated by Ca^{2+}_i induced IP_3 formation the production site of IP_3 needs to be less than 5 µm away from the ER containing the IP_3R. It is unlikely that IP_3 generated at the plasma membrane in mouse eggs (with a radius of 35um) could account for such a rapid Ca^{2+}_i rise time. It is therefore probably essential for an intracellular source of $PI(4,5)P_2$ to be in close proximity to the ER. Chapter 5 shows that a vesicular source of $PI(4,5)P_2$ distributed throughout the cytoplasm is present in mouse eggs and would be in close enough proximity to the ER in order to release Ca^{2+} by the mechanism described. Furthermore it appears PLCζ is able to hydrolyse this pool of $PI(4,5)P_2$. In fact there is evidence that this $PI(4,5)P_2$ is vital for PLCζ activity (Yu *et al.* 2012)

It has previously been suggested that Ca^{2+}_i dependent IP_3 formation is responsible for helping the propagation of the Ca^{2+}_i wave in sea urchin eggs (Whitaker and Irvine 1984). Seas urchin eggs also contain a source of $PI(4,5)P_2$ in their yolk platelets which could help facilitate the spread of the Ca^{2+}_i wave across the whole egg efficiently (Rice *et al.* 2000).Interestingly PLCζ from mammalian sperm extracts is able to hydrolyse this intracellular source of $PI(4,5)P_2$ in sea urchin homogenates (Rice *et al.* 2000). There is also evidence that a cytoplasmic organelle bound $PI(4,5)P_2$ in is present in *Xenopus* eggs (Snow *et al.* 1996). It is very likely that an intracellular $PI(4,5)P_2$ source is necessary for the regenerative Ca^{2+}_i increase in mammalian eggs (see fig 7.1). However, an intracellular source of $PI(4,5)P_2$ may also be necessary for the propagation of Ca^{2+}_i waves in all eggs that release Ca^{2+} by a wave.

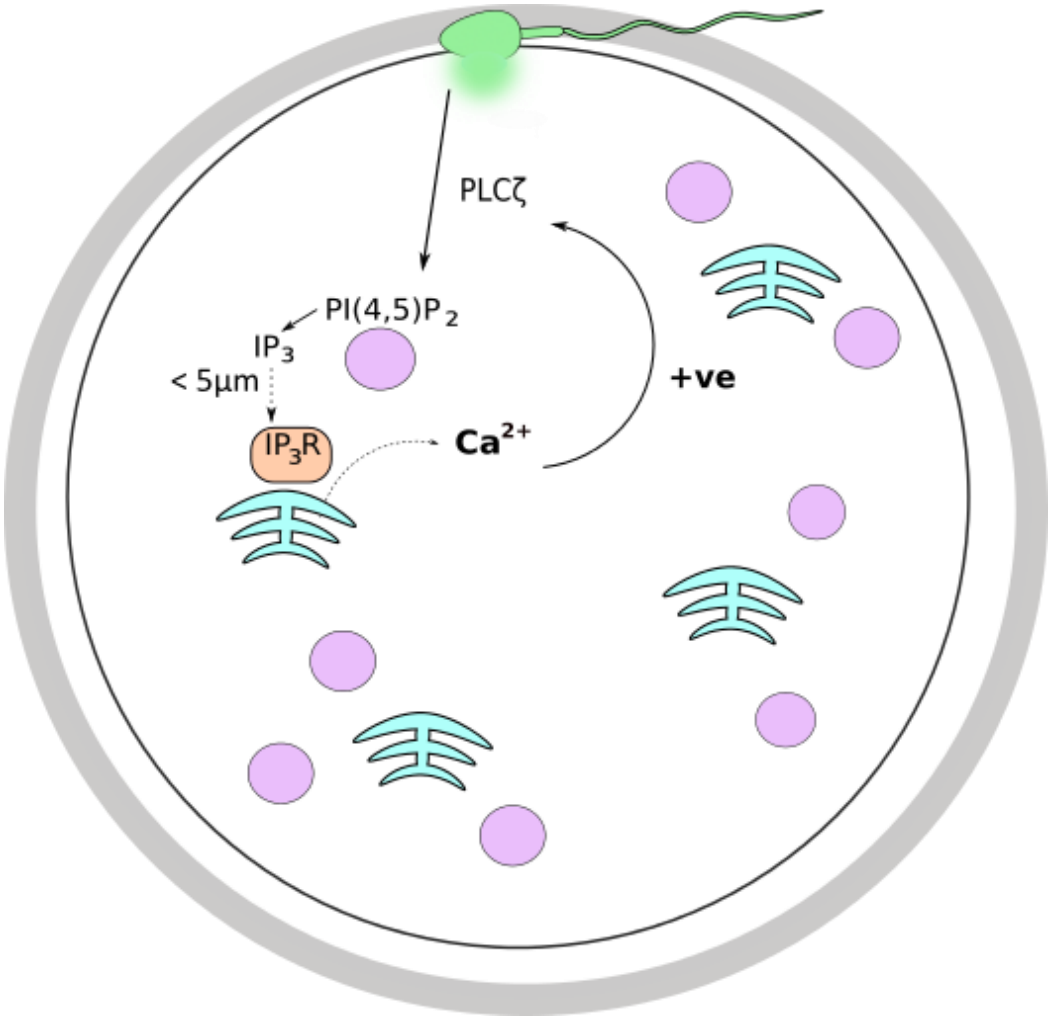


Figure 7.1 Diagram showing how PLCζ may release Ca^{2+} in mammalian eggs

Proposed model of PLCζ initiated Ca^{2+} release by Ca^{2+}_i induced IP_3 formation in mammalian eggs. In this model PLCζ diffuses into the egg and hydrolyses a vesicular source of $PI(4,5)P_2$ at low Ca^{2+}_i concentrations. This produces a surge in IP_3 this diffuses a distances of less than 5µm before binding to IP_3R on the ER (yellow) which releases Ca^{2+}. This Ca^{2+}_i increase positively feeds back onto PLCζ activity to produce more IP_3. When IP_3 levels get too high the IP_3R becomes desensitised and closes.

Cell cycle dependent organelle redistribution and Ca^{2+}release

Unlike other PLC isoforms PLCζ appears to be highly selective in which cell types it releases Ca^{2+}. For example it is only able to release $Ca^{2+}{}_i$ at physiological levels in eggs and will not release Ca^{2+}in CHO cells even at very high expression levels (Phillips *et al.* 2011). One of the main differences between mammalian eggs and somatic cells is their cell cycle state. Mammalian eggs are arrested in metaphase of the second round of meiosis. This is a highly unnatural state for cells as they usually remain in interphase for the majority of the time and progress through the other stages of the cell cycle, including metaphase, relatively quickly. During cell division cells undergo major cytoplasmic alterations and organelle redistribution. For example the network structure of the ER alters during mitosis and as the nuclear envelope breakdown proteins disperse from the NE into the ER (Ellenberg *et al.* 1997; Puhka *et al.* 2007). In contrast the Golgi apparatus fragments into very small vesicles known as the "Golgi haze" (Axelsson and Warren 2004). During this process many of the proteins that usually reside in the Golgi get absorbed into the ER (Axelsson and Warren 2004).

Unlike mitotic cell division which occurs very quickly meiotic cell division of oocytes occurs over a much more prolonged period of time. The cells also become arrested at various points in this cell cycle. For example GV oocytes are arrested in interphase of the first round of meiosis for many years. The oocytes then enter oocyte maturation which occurs over many hours before being arrested in the metaphase of meiosis II. There are significant disadvantages to being arrested in this phase of cell cycle for example there is an increased risk of chromosomal errors.

However, like mitotic cell divisions many cytoplasmic alterations occur during oocyte maturation which includes the redistribution of organelles. First of all the ER alters from forming a continuous network with the nuclear envelope that is seen in GV oocytes to forming clusters in the cortex of MII eggs (FitzHarris *et al.* 2007). These clusters are located close to the mitochondria (FitzHarris *et al.* 2007). The ER staining in Chapter 5 confirms this clustering of the ER during metaphase arrest in mouse eggs.

Like in mitotic cells the Golgi fragments during entry into meiosis. In GV oocytes the Golgi appears as small stacks called mini-Golgi but once the GV breaks down these stacks become fragmented into small vesicles (Moreno *et al.* 2002; Payne and Schatten 2003). The results in Chapter 5 support this notion as using both immunocytochemistry techniques on fixed cells and live Golgi markers show that the Golgi is vesicular in mature eggs. Interestingly these Golgi vesicles are more concentrated in the cortex like the ER and

mitochondria.

Oocyte maturation appears to be associated with an increase in Ca^{2+} releasing ability of oocytes specifically after germinal vesicle breakdown. It has previously been shown that the Ca^{2+}_i oscillations in immature mouse and hamster eggs are reduced in frequency, duration and amplitude compared to that in mature eggs in response to sperm, sperm extracts and PLCζ (Fujiwara *et al.* 1993; Jones *et al.* 1995b; Carroll *et al.* 1996; Wakai *et al.* 2012).The results in Chapter 5 verify this effect and show that mouse GV oocytes have more than a 30 fold reduction in sensitivity to PLCζ compared to MII eggs which is a far bigger reduction than ever reported previously.

This effect is not unique to mammalian eggs. Starfish eggs also acquire the ability to release Ca^{2+}during maturation(Chiba *et al.* 1990). This also appears to be true for *Xenopus* frog eggs. In immature *Xenopus*oocytes Ca^{2+} is released in response to IP_3in a series of oscillations. However, in mature eggs these Ca^{2+}_i puffs become concentrated to form a large single Ca^{2+}_i wave that takes several minutes to cross the egg (Machaca 2004). Ca^{2+}_i oscillations initiated by sperm in immature oocytes from the nemertean marine worm *Cerebratulus lacteus* are also reduced in amplitude and duration compared to that in mature eggs (Stricker 1996). Interestingly these oscillations initiated in immature oocytes do not have the same wave form as that seen in mature eggs (Stricker 1996). This is also something that has also been noted for immature mouse oocytes (Swann 1994).

Significantly in all the species noted above including mammals, mature eggs become arrested in during metaphase of either the first or second round of meiosis. This is in contrast to the GV oocytes which are usually arrested in prophase or interphase. This suggests that the ability for eggs to release Ca^{2+} could be a cell cycle dependent feature, particularly a feature of cells in metaphase. This is certainly true for ascidian eggs. Mature ascidian eggs are arrested in metaphase I and release Ca^{2+} in the form of oscillations when fertilised. However, as these eggs progress into the meiosis II these oscillations stop for approximately 5 minutes before resuming as the eggs enter metaphase II (McDougall and Levasseur 1998). This feature appears to be dependent on MPF levels as the Ca^{2+}_i oscillations persist whilst the levels of MPF and cease when the MPF levels fall (McDougall and Levasseur 1998).

Unlike most other species, mature sea urchin eggs complete the second round of meiosis before being fertilised and therefore are in interphase of the cell cycle when they are activated (Mazia 1974). It is unclear whether Ca^{2+} release alters during oocyte maturation in this species. The Ca^{2+} releasing channel/ryanodine receptor is concentrated to the cortex

in mature eggs however in immature oocytes this channel is absent in most cases or not localised (McPherson *et al.* 1992). The effect that this has physiologically on Ca^{2+} release is not fully understood as there appears to be both IP_3 sensitive and non-IP_3sensitive pathways for Ca^{2+} release in sea urchin eggs (Kuroda *et al.* 2001).

In mammalian eggs the increase in Ca^{2+} releasing ability during oocyte maturation has been attributed to an increase in IP_3 receptor sensitivity (Wakai *et al.* 2012). This appears to be the case in starfish, as immature oocytes require 100 times more IP_3than mature eggs to elicit the same Ca^{2+}_iresponse (Chiba *et al.* 1990). The increase in Ca^{2+}release in *Xenopus* eggs is also attributed to an increase in IP_3 sensitivity during oocyte maturation (Kume *et al.* 1997; Terasaki *et al.* 2001).

In mammalian eggs this increase in IP_3 receptor sensitivity is thought to be due to an increase in ER Ca^{2+} concentrations and phosphorylation status of the receptor (Wakai *et al.* 2012). Indeed the results in Chapter 5 show that the amount of Ca^{2+}_i in the stores does increase during oocyte maturation. However, this occurs in the later stages of maturation, between the first metaphase and the second metaphase not after GVBD when the greatest increase in Ca^{2+}_i releasing ability of oocytes is seen. Furthermore this relatively small increase in ER Ca^{2+} levels during maturation is not enough to attribute to the dramatic increase in PLCζ sensitivity during this time. Furthermore the sensitivity of IP_3R to luminal ER Ca^{2+}concentrations is a feature of the biphasic nature of the IP_3R and it is unlikely that this sets the pace for the Ca^{2+}_i oscillations (see previous section).

It is more likely that the cell cycle dependent nature of Ca^{2+}_i oscillations in eggs is due to the large structural changes in the cell that occur during this time. In many species for example, the structural reorganisation of the ER plays a significant role in enabling effective Ca^{2+}release. Reorganisation of the ER during oocyte maturation has been recorded in *Xenopus*frogs, starfish, hamster, nematode worms and mouse eggs (Jaffe and Terasaki 1994; Shiraishi *et al.* 1995; Stricker 1996; Terasaki *et al.* 2001; FitzHarris *et al.* 2003, 2007). In mouse eggs this ER re-organisation has been linked very closely with the increase in Ca^{2+}release (FitzHarris *et al.* 2003). However, this is unlikely to account for the stark difference in PLCζ sensitivity between phases of the cell cycle.

The fragmentation of the Golgi could however play a role in this. The results in Chapter 5 suggest that the vesicles containing intracellular $PI(4,5)P_2$ could originate from the Golgi apparatus. As the Golgi fragments during oocyte maturation it is possible that this plays a role in enabling effective Ca^{2+} release. For instance if $PI(4,5)P_2$ is contained in the Golgi vesicles, Golgi fragmentation may enable this $PI(4,5)P_2$is available for PLCζ binding.

Alternatively these Golgi vesicles may contain a targeting protein for PLCζ that only becomes accessible when the Golgi fragments. Chapter 5 shows that this vesicular Golgi is only present after GVBD however PI(4,5)P_2 markers suggest that PI(4,5)P_2 is localised in vesicular structures before that time. This may be because PI(4,5)P_2 synthesised in other compartments and is trafficked to the Golgi derived vesicles once it has fragmented.
It is also significant that the Golgi and PI(4,5)P_2 containing vesicles are distributed throughout the cytoplasm but concentrated in the cortex as this has two key implications. Firstly it allows concentration of PI(4,5)P_2 in close proximity to the ER which contains the IP_3R. This means that the IP_3 produced at these vesicles could have time to diffuse to the site of Ca^{2+} release in order to produce the fast Ca^{2+}_i rise time recorded. This means the Golgi, ER and mitochondria could all be within close proximity to each other and coupled to produce regular, paced Ca^{2+}_i oscillations. Secondly as this PI(4,5)P_2 is distributed throughout the whole egg cytoplasm a fast wave of Ca^{2+}_i release can occur from one side of the egg to the other.
One other key feature of cells in metaphase is the lack of an intact nuclear envelope. This membrane is usually continuous with the ER but when this envelope breaks down proteins within it get absorbed into the ER (Puhka *et al.* 2007). This means that cells that are in interphase and prophase have an intact nucleus. GV oocytes are arrested in prophase I and have a large intact nuclear envelope called the germinal vesicle however this envelope breaks down as these oocytes progress into metaphase in a process called germinal vesicle breakdown (GVBD). The results in Chapter 5 show that mouse eggs develop a dramatic increase in Ca^{2+}_i releasing ability after GVBD and one reason for this could be the localisation of PLCζ in GV oocytes compared to mature eggs. Mouse PLCζ contains a nuclear localisation sequence which targets PLCζ to the nucleus after pronuclear formation (Larman *et al.* 2004; Yoda *et al.* 2004). This sequestering of PLCζ in the nucleus ablates PLCζ induced Ca^{2+}_i oscillations.
There is also a suggestion that PLCζ could be localised to the nucleus in mouse GV oocytes and this may account for the dramatic reduction in PLCζ sensitivity seen in mouse GV oocytes (Yu *et al.* 2012). However, Chapter 5 shows that luciferase tagged PLCζ does not appear to localise to nucleus in GV oocytes possibly due to the size of the fusion protein. Though untagged PLCζ may localise to the nucleus in GV oocytes luciferase tagged PLCζ appears to be retained in the cytoplasm however it is still not able to produce Ca^{2+}_i oscillations. This suggests that other cytoplasmic differences are likely to account for the lack of Ca^{2+} release in these instances. In addition PLCζ doesn't localise to the nucleus in

oocytes and eggs of other mammalian species.

These cellular features are all a result of cells being arrested in a metaphase. It is possible that the reason most eggs, even those distantly related evolutionarily, are arrested in metaphase is because these cellular features are required for effective and sustainable IP_3 induced Ca^{2+} release through the whole egg at egg activation. For example the re-distribution of organelles during this time ensures that the production of IP_3, the IP_3R receptors and ATP generation site can all be in close proximity (see Fig 7.2). Furthermore this process may enable an intracellular source of $PI(4,5)P_2$ to become available and accessible. The need for arrest in metaphase may be particularly important in eggs that release Ca^{2+} in the form of oscillations. Particularly in order to set the pace and pattern of these Ca^{2+}_i oscillations, which could explain why the wave form of Ca^{2+} release is disrupted in immature mouse oocytes (Swann 1994). As a result it is possible that the structural reorganisation that occurs as eggs enter metaphase is vital for the model Ca^{2+}_i induced IP_3 formation that is likely to take place in those eggs that release Ca^{2+} as a wave.

Phosphoinositide turnover in eggs

IP_3 induced Ca^{2+} release requires the production of IP_3 from the phosphoinositide $PI(4,5)P_2$. This means that in order to release Ca^{2+} by IP_3 there must be an available source of $PI(4,5)P_2$ for hydrolysis. As a result it makes sense that there would be a high amount of phosphoinositide turnover in eggs. This turnover has been successfully measured in sea urchin eggs. The parent molecule for all phosphoinositides, PI, is essential for maintaining Ca^{2+}_i homeostasis in sea urchin eggs during fertilisation (Sillers and Forer 1985). Measurements have shown that there 600 fold increase in phosphoinositide turn over within the 1st minute of fertilisation (Ciapa *et al.* 1992). Furthermore there is a 5 fold increase in $PI(4,5)P_2$ production from PI in the first 10 minutes after fertilisation (Kamel *et al.* 1985). Another echinoderm, the starfish, experiences a biphasic increase in $PI(4,5)P_2$ levels in eggs at fertilisation. There is initial increase in $PI(4,5)P_2$ following sperm-egg fusion which then falls before at longer prolonged increase in $PI(4,5)P_2$ levels is produced (Chun *et al.* 2010).

An increase in PI turnover was also recorded in *Xenopus* frog eggs. Levels of both IP_3 and $PI(4,5)P_2$ were found to increase following fertilisation with a continuous rise in IP_3 during Ca^{2+} release and a two fold increase in $PI(4,5)P_2$ in the two minutes after the Ca^{2+}_i wave (Snow *et al.* 1996). Before fertilisation the levels of $PI(4,5)P_2$ in mature *Xenopus* eggs are higher than PI(4)P (Le Peuch *et al.* 1985). Interestingly PI turnover also seems to be

significant in protosome eggs such as surf clams. Unlike most eggs surf clam eggs are fertilised as GV oocytes with a GV intact. As a consequence GVBD occurs as an event of fertilisation. Nevertheless radio-labelling lipids in these cells has shown that PI and PI(4)P levels do not alter during fertilisation, however $PI(4,5)P_2$ levels appear to decrease (Bloom *et al.* 1988).

Attempts have been made to measure $PI(4,5)P_2$ levels at the plasma membrane in mouse eggs during fertilisation using fluorescent PH domain and no detectable loss of $PI(4,5)P_2$ was recorded (Halet *et al.* 2002). This technique is based on the premise that $PI(4,5)P_2$ is usually associated with plasma membrane which makes it easily accessible to PLCs (Watt *et al.* 2002). These probes have been used previously in somatic cells has been to show that $PI(4,5)P_2$ is hydrolysed at the plasma membrane to produce IP_3 that diffuses into the cytoplasm (Watt *et al.* 2002). However, this does not appear to be the case in mouse eggs (Yu *et al.* 2008; Yu *et al.* 2012). Furthermore depleting $PI(4,5)P_2$ in the plasma membrane has no effect on sperm or PLCζ induced $Ca^{2+}{}_i$ oscillations (Yu *et al.* 2012). As a result probes that measure plasma membrane PI turnover would not be appropriate to measure $PI(4,5)P_2$ hydrolysis and IP_3 production in mammalian eggs. In Chapter 6 attempts were made to measure whole cell PI turnover in mouse eggs in response to PLCζ by measuring DAG and IP_3 using probes. However, none of these probes appeared sensitive enoughtomeasure any significant changes in DAG or IP_3 during PLCζ induced $Ca^{2+}{}_i$oscillations. Oscillations in IP_3 production is a feature required for $Ca^{2+}{}_i$ induced IP_3 formation. However, it is possible that the turnover of phosphoinositides is so high that it is difficult to detect significant dynamic changes in $PI(4,5)P_2$, IP_3 and DAG.

As discussed previously spatial regulation of PI turnover such as intracellular IP_3 production may be important for wave front Ca^{2+}release and in setting the pace of $Ca^{2+}{}_i$ oscillations. This may also be a feature that is unique to mature eggs. As a result it is possible that phosphoinositide synthesis may be stimulated during oocyte maturation and there is evidence for this in some species. For example, limpet oocyte maturation is initiated by a change in intracellular pH which stimulates a 30 -50% increase in PI and PIP synthesis and a 50 fold turnover in phosphoinositides (Borg *et al.* 1992). However, these eggs do not increase $Ca^{2+}{}_i$ by a wave of Ca^{2+} release but by Ca^{2+} influx. There is also a significant increase in phosphoinositide turnover in *Xenopus*eggs during oocyte maturation indicated by an increased incorporation of myo(3H) inositol (Carrasco *et al.* 1990). There is also a suggestion that PI turnover in bovine eggs may play multiple roles in oocyte maturation (Homa *et al.* 1991). However, somatic cells and maturing oocytes still contain high levels of

plasma membrane $PI(4,5)P_2$. It may be the case therefore that this $PI(4,5)P_2$ is not localised sufficiently and a significant source of intracellular $PI(4,5)P_2$ must be present in eggs. Interestingly the results in Chapter 5 show that a vesicular source of $PI(4,5)P_2$ is present in immature oocytes however it is possible that this $PI(4,5)P_2$ is not localised correctly or in close enough proximity to PLCζ binding partners or the ER. The synthesis and sequestering of $PI(4,5)P_2$ in the correct membranes may be feature of oocyte maturation and could require membrane trafficking.

Chapter 5 also shows that disruptors of membrane trafficking and Golgi structure appear to inhibit $Ca^{2+}{}_i$ oscillations in eggs however it is still unclear what the mechanism of action of these drugs actually are. Furthermore it is unclear how much membrane trafficking is actually taking place in eggs. It may be possible for example that some anterograde and retrograde trafficking is occurring between the ER and the Golgi compartments shuttling proteins from one to other (see fig 7.2).

Interestingly unlike somatic cells, a probe based on the PH domain of OSBP shows that PI(4)P in eggs does not appear to be localised to the Golgi or at least the vesicular compartment of the Golgi that contains $PI(4,5)P_2$ (see Chapter 5). This is significant as PI(4)P is the precursor for $PI(4,5)P_2$ therefore it is possible that $PI(4,5)P_2$ is synthesised for example in the ER or another cellular compartment and is trafficked to the Golgi fragments where it can be hydrolysed by PLCζ (see fig 7.2) . This could offer an explanation for why disrupting membrane trafficking inhibits $Ca^{2+}{}_i$ oscillations induced by PLCζ (See fig 7.2).

It is possible that an upregulation of $PI(4,5)P_2$ synthesis during oocyte maturation acts to produce eggs that have the capacity to respond effectively to PLCζ. However, it is also likely that the maintenance of these oscillations is dependent on rapid re-synthesis of $PI(4,5)P_2$ and PI turnover during these $Ca^{2+}{}_i$oscillations (see fig 7.2). This could explain why it is difficult to record phosphoinositide turnover dynamically at fertilisation and why it is so difficult to deplete levels of $PI(4,5)P_2$ in eggs. This may also explain why no dominant negative effect has been recorded using PLCζ. Very high levels of catalytically dead PLCζ do not appear to effect wild type PLCζ activity. However, this may be because if there is a large amount of vesicular $PI(4,5)P_2$ in eggs and high rate of $PI(4,5)P_2$ synthesis it may prove very difficult to saturate or deplete this $PI(4,5)P_2$. It is also possible that as well as possessing a high level of the substrate $PI(4,5)P_2$ there is also an abundance of PLCζ binding partner present in target membranes.

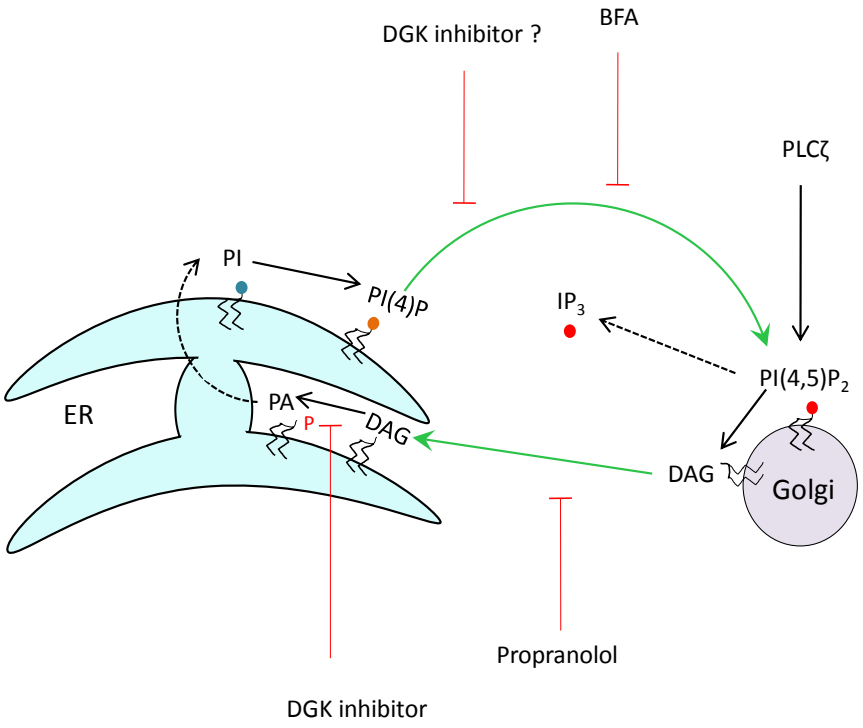


Figure 7.2 Proposed model of PI and DAG signalling between Golgi vesicles and the ER during Ca^{2+}_i oscillations in mammalian eggs

$PI(4,5)P_2$ synthesised from PI(4)P either at the ER or the Golgi. $PI(4,5)P_2$ generated at the ER may be transported to the Golgi by PI transfer proteins. Once in the Golgi $PI(4,5)P_2$ is available for hydrolysis by PLCζ that diffused into the egg at sperm-egg fusion. Hydrolysis of $PI(4,5)P_2$ produces IP_3 that diffuses into the cytoplasm and DAG which stays in the membrane. Interestingly DAG doesn't appear to stay the membranes of these vesicles. DAG the ER is converted into PA by the enzyme DGK. PA eventually gets recycled to PI which is the precursor for PI(4)P. The drug propranolol blocks formation of DAG by the activity of PAP. Propranolol also inhibits retrograde transport from the Golgi-ER. BFA inhibits anterograde transport from the ER-Golgi. The enzyme DGK might also be involved in anterograde transport by stimulating localised PA levels.

PLC mediated Ca^{2+}release in eggs

Many species that release Ca^{2+} from internal stores at fertilisation do so by IP_3 pathway therefore must require the activity of a PI- specific PLC. Most PLC isoforms are expressed in the target cell and respond to internal or external stimulation.

It appears that in most species, during egg activation, Ca^{2+}release is triggered by the activation of egg derived endogenously expressed PLCs. For example it has been suggested that PLCγ is involved in Ca^{2+} release in sea urchin eggs during egg activation (Carroll 1999). One mechanism by which PLCγ could be activated is through the stimulation of src family kinases (Abassi *et al.* 2000). However, PLCγ activity alone does not appear sufficient to account for all the Ca^{2+}release at fertilisation in sea urchins as inhibiting PLCγ activity reduces but does not ablate all Ca^{2+} release (Carroll 1999). Sea urchins eggs do express other PLC isoforms, for example PLCβ and PLCδ homologues have both been identified (Coward *et al.* 2004; Kulisz *et al.* 2005). However, it is not clear what role if any these PLC isoforms play in sea urchin egg activation. For example injection of PLCδ does not cause Ca^{2+}release in sea urchin eggs (Coward *et al.* 2007). Furthermore recombinant protein of PLCβ, PLCδ and PLCγ are not able to cause Ca^{2+} release in sea urchin egg homogenates (Jones *et al.* 2000).

Starfish egg activation also appears to be dependent on endogenous PLC activation. Inhibiting PLCγ activity using SH2 domains prevents egg activation at fertilisation (Carroll *et al.* 1997). As in sea urchin eggs PLCγ in starfish eggs appears to be activated by src family kinases (Giusti *et al.* 1999a; Giusti *et al.* 2000; Runft *et al.* 2004). This also appears to be the case in ascidian eggs (Runft and Jaffe 2000). PLCγ activation also appears to play a significant role in the egg activation of some amphibian species for example polyspermic newts (Ueno *et al.* 2014). There is some suggestion that Src family kinase activated PLCγ is also responsible for egg activation in *Xenopus* (Sato *et al.* 2000). The theory is that sperm activates phospholipase D which results in an increase in phosphatidic acid (Bates *et al.* 2014). PA then stimulates PLC γ by src family kinases (Bates *et al.* 2014). Membrane associated PLCs can be activated by an increase in PA (Jackowski and Rock 1989). Furthermore an increase in PA has previously been associated with an increase in $PI(4,5)P_2$ and PI hydrolysis(Jacob *et al.* 1993). However, SH2 domains do not appear to inhibit $Ca^{2+}{}_i$ oscillations stimulated at fertilisation (Runft *et al.* 1999). PLCγ activation also appears to play a role in polychaete annelid worm egg activation (Yin and Eckberg 2009).

This evidence would suggest that endogenously expressed PLCs, specifically PLCγ, appear to be involved in eliciting Ca^{2+} release during egg activation in many species. Despite the

expressing PLCγ PLCβ and PLCδ, this does not appear to be the case in mammalian eggs (Dupont *et al.* 1996). For example expression of excessive SH2 and SH3 domains, which act as an inhibitor for PLCγ, has no effect on Ca^{2+}_i oscillations initiated by sperm (Mehlmann *et al.* 1998). Furthermore inhibiting tyrosine kinase mediated PLCγ hinders sperm induced Ca^{2+}release however does not ablate Ca^{2+}_i oscillations (Dupont *et al.* 1996). Despite there being some suggestions that G protein activated PLCβ is involved in Ca^{2+}release in mouse eggs inhibiting PLCβ using Gq family G proteins does not inhibit Ca^{2+}_i oscillations (Moore *et al.* 1994; Williams *et al.* 1998). However, depleting PLCβ levels using iRNAs effect the amplitude of sperm induced Ca^{2+}_i oscillations however over-expression of PLCβ reduces the frequency and duration of these oscillations (Igarashi *et al.* 2007). Therefore it is possible that PLCβ does play some role in determining the pattern Ca^{2+}release in mammalian eggs however it is unclear what the significance of this effect is.

Unlike other animal groups Ca^{2+} release in mammals is initiated by an exogenous PLC. This may be significant for imposing the properties of Ca^{2+}release in eggs. For example there are some unique properties of PLCζ compared to other PLC isoforms and there are some unique features of Ca^{2+} release in mammalian eggs compared to that of other animal groups. For example PLCζ is unique amongst PLCs at causing Ca^{2+}release in mammalian eggs at physiological basal Ca^{2+}_i levels and it appears to be very potent at hydrolysing $PI(4,5)P_2$. There are several reasons for this, first is the increased Ca^{2+}_i sensitivity of PLCζ compared to other PLC isoforms (Nomikos *et al.* 2005). However, this factor is not sufficient to account for lack of other PLC activity. For example if this was the case then increasing basal level Ca^{2+}_i would stimulate the activity of other endogenous PLCs however there is no evidence for this. PLCδ and PLCγ are able to cause some Ca^{2+}release in mouse eggs if these are expressed at very high levels far past that physiological (Mehlmann *et al.* 2001; Yu *et al.* 2008).

Another feature that could ensure PLCζ activity is its localisation in the egg. Chapter 5 shows that PLCζ localises to small vesicles throughout the cytoplasm. This is different to other PLC isoforms which localise to the plasma membrane. They do this mainly through binding $PI(4,5)P_2$ here via a PH domain (Watt *et al.* 2002). However, as previously stated PLCζ does not appear to hydrolyse this source of $PI(4,5)P_2$ and does not possess a PH domain(Saunders *et al.* 2002; Yu *et al.* 2012). To compensate for this the results in Chapter 4 indicate that the XY linker and EF hand domain work in synergy to bind PLCζ to this vesicular source of $PI(4,5)P_2$. In other PLCs for example PLCδ1 the XY linker and EF hand do not have a role in $PI(4,5)P_2$ binding. For example positively charged residues in the EF hand

domain of PLCδ play a role in binding anionic phospholipids specifically phosphatidylserine (Cai *et al.* 2013). Binding of an intracellular source of PI(4,5)P_2 in this way could therefore be a unique feature of PLCζ activity. However, PI(4,5)P_2 binding by this region cannot discriminate between different pools of PI(4,5)P_2 so doesn't explain why PLCζ hydrolyses a vesicular source of PI(4,5)P_2 over plasma membrane PI(4,5)P_2. It also doesn't explain why PLCζ cannot hydrolyse the PI(4,5)P_2 in the plasma membrane of somatic CHO cells (Phillips *et al.* 2011). This suggests that membrane PI(4,5)P_2 is not available to PLCζ for hydrolysis.

PI(4,5)P_2 is a negatively charged protein that is exposed on membranes. Positively charged MARCKS protein at physiological levels has been found to bind to this PI(4,5)P_2 in order to shield it and in turn this inhibits PI(4,5)P_2 hydrolysis by PLCs (Glaser *et al.* 1996). PI(4,5)P_2 is released when MARCKS protein is phosphorylated by PKC (Glaser *et al.* 1996). Alternatively CaM binds to MARCKS with a strong affinity which rips it of PI(4,5)P_2 rapidly therefore freeing up PI(4,5)P_2 for hydrolysis (Arbuzova *et al.* 1997). As a result MARCKS protein acts as a reversible PI(4,5)P_2 buffer. Interestingly CaM binds to PLCζ via the CaM binding domain (Nomikos *et al.* 2007). PLCδ1 is inhibited by CaM which can be reversed by interaction with the small GTPase (Sidhu *et al.* 2005).

It is still unclear why PLCδ is able to hydrolyse plasma membrane PI(4,5)P_2 whereas PLCζ can't. This may be because the PH domain of PLCδ has a preference for PI(4,5)P_2 that is found in cholesterol rich membranes which may give it an increased affinity to plasma membrane PI(4,5)P_2(Rissanen *et al.* 2017). PLCζ does not contain a PH domain however other features of its structure could play a role in discriminating between PI(4,5)P_2 in different membranes. Alternatively PLCζ may require an additional targeting protein in order to be active that is not present in the plasma membrane. For example the C2 domain of PLCζ appears to play a role in enabling Ca^{2+}release by ensuring PLCζ membrane association (see Chapter 4). However, it is unclear how it does this. The only binding partners that the C2 domain of PLCζ has been found to bind with are PI(3)P and PI(5)P neither of which are distributed in a vesicular pattern (Kouchi *et al.* 2005), (Moon and Swann 2012 unpublished). It is possible there is an unknown binding partner for the C2 domain of PLCζ that is yet to be identified. This binding partner could play a role in reversing CAM inhibition in the same way Ral does for PLCδ (Sidhu *et al.* 2005). These structural and regulatory properties of PLCζ may explain why it is able to cause Ca^{2+}_i oscillations in mammalian eggs and also may determine the pattern of those oscillations. For example oscillations initiated by PLCδ1 in mouse eggs do not follow the same pattern as those initiated by PLCζ. This may be because PLCδ1 hydrolyses plasma membrane

$PI(4,5)P_2$ over a vesicular source of $PI(4,5)P_2$. As a result PLCδ may not stimulate Ca^{2+}release by Ca^{2+}_i induced IP_3 formation as this requires hydrolysis of an intracellular source of $PI(4,5)P_2$. This could explain, for example, why despite high expression levels PLCδ in eggs it only causes relatively low frequency oscillations (Coward *et al.* 2007; Yu *et al.* 2008). It could also explain why PLCδ induced Ca^{2+} release in mouse eggs does not lead to down regulation of the IP_3 receptors in the way PLCζ does.

It has previously been suggested that based on the 3D crystal structure of PLCδ1 (see fig 7.3) that it may bind to membrane derived $PI(4,5)P_2$ by a" tether and fix" and fix model (Essen *et al.* 1996). In this model the PH domain of PLCδ1 tethers it to the $PI(4,5)P_2$ initially and then the other domains act help fix it in place so $PI(4,5)P_2$ is able to come in contact with the active site and be hydrolysed. A similar model may be relevant for PLCζ. For example the C2 domain may act to tether PLCζ to the membrane by an unknown target without which it may not localise at all. The X-Y linker and EF hands domain then bind $PI(4,5)P_2$ by electrostatic interactions which fix PLCζ in place and allow $PI(4,5)P_2$ to be accessible to the active site (see fig 7.4).In this model specific membrane targeting by the C2 domain could explain how PLCζ discriminates between vesicular $PI(4,5)P_2$ and plasmam membrane bound $PI(4,5)P_2$.

In most animal groups that release Ca^{2+}in response to the activation of egg derived PLCs this increase occurs in the form of a single Ca^{2+}_i increase that crosses the egg as a wave front. Mammalian eggs are unique in that they release Ca^{2+}as multiple waves that are paced to produce low frequency oscillations. Furthermore these Ca^{2+}_i waves cross the egg quickly (Deguchi *et al.* 2000). This is very different to the single Ca^{2+}_i wave that takes several minutes to cross the egg in sea urchin eggs). In contrast however marine nemertean worms release Ca^{2+} in the form of Ca^{2+}_i oscillations in response to mammalian sperm extracts as well as at fertilisation (Stricker 1996; Stricker *et al.* 2000). Human sperm extracts have also been found to cause Ca^{2+}_i oscillations in ascidian eggs similar to those elicited by sperm (Wilding *et al.* 1997). Interestingly these Ca^{2+}_i oscillations could not be mimicked solely by IP_3injection (Wilding *et al.* 1997). This suggests that the initiationof Ca^{2+}_i oscillations in eggs are dependent on the cellular properties of the egg itself. For example the initiation of Ca^{2+}_i oscillations may be dependent on the rapid re-synthesis of $PI(4,5)P_2$. Furthermore the low frequency, high amplitude Ca^{2+}_i oscillations seen in mammalian eggs at fertilisation may also be dependent on the unique features of PLCζ induced Ca^{2+} release. This includes PLCζ hydrolysis of intracellular $PI(4,5)P_2$ resulting in Ca^{2+}_i induced IP_3 formation and the positive feedback on PLCζ activity.

The Ca^{2+}_i oscillations in mammalian eggs initiated by PLCζ at fertilisation appear to stimulated by a novel IP_3 signalling pathway that is different to that of other PLC isoforms. This pathway is very likely to depend on the hydrolysis of a vesicular, intracellular source of $PI(4,5)P_2$. The ability for PLCζ to hydrolyse this $PI(4,5)P_2$ appears to be a result of structural properties of PLCζ itself and the cell biology of a mature egg. Hydrolysis of this vesicular source of $PI(4,5)P_2$ by PLCζ makes it more than possible that Ca^{2+}_i induced IP_3formation is responsible for creating the regenerative Ca^{2+}_i waves seen in eggs at fertilisation.

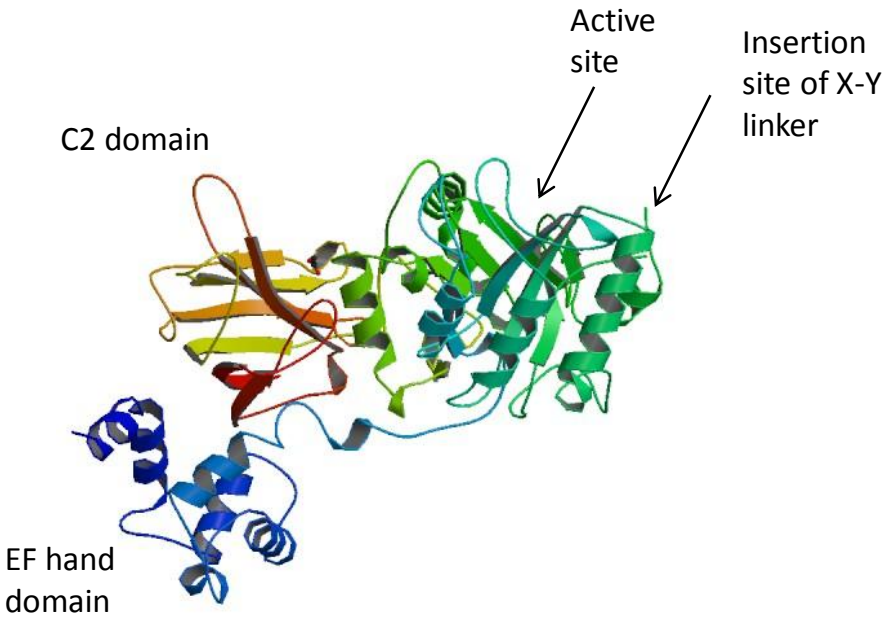


Figure 7.3 3D structure of PLCδ1

3D structure of rat derived PLCδ1 without a PH domain as determined by x-ray crystallography (PDB ID: 2ISD, Essen et al 1996)

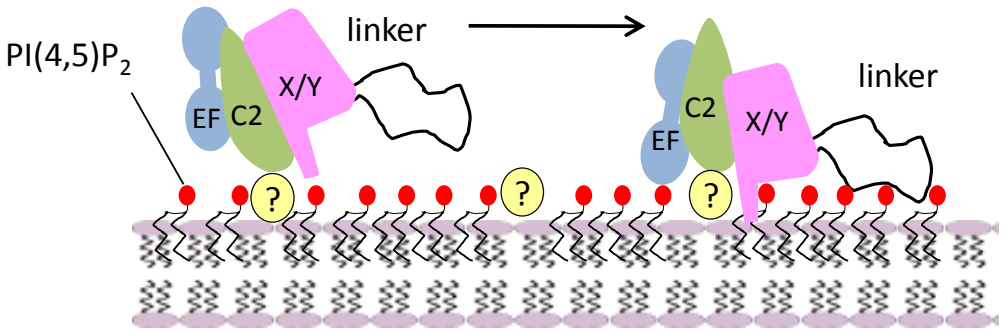


Figure 7.4 Schematic showing how PLCζ may bind to PI(4,5)P$_2$by a "tether and fix" model

The C2 domain may bind to some unknown membrane bound protein (?) which targets and tethers PLCζ to the membrane, the EF hand and X-Y linker then bind to PI(4,5)P_2, fixing PLCζ in place and putting the X/Y catalytic domain in close enough contact to hydrolyse PI(4,5)P_2 in the active site.

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