MECHANISMS OF CALCIUM OSCILLATIONS IN MOUSE AND HUMAN EGGS

Dr Khalil Elgmati

MBChB, MSc

A Thesis Submitted in Fulfilment of the Requirements of Cardiff University for the Degree of a Doctor of Philosophy

Institute of Molecular and Experimental Medicine

School of Medicine

Cardiff University

2013

AUTHOR'S DECLARATION

I hereby declare that this Thesis has been composed by myself and is a record of work performed by myself under the title 'Mechanisms of Calcium Oscillations in Mouse and Human Eggs'. This work was carriedout under the supervision of Prof. Karl Swann. I conducted all research at Karl's laboratory in the School of Medicine in Cardiff University, Cardiff, UK.

.....Dr.Khalil Elgmati..... (candidate) Date …….28/02/2013…...…

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed ……..*Dr Khalil Elgmati* …… (candidate) Date ….....28/02/2013……

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD.

Signed ……..*Dr Khalil Elgmati* …… (candidate) Date …….28/02/2013…...…

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

Signed ……..*Dr Khalil Elgmati* …… (candidate) Date …….28/02/2013…...…

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed ……..*Dr Khalil Elgmati*…… (candidate) Date …….28/02/2013…...…

STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access previously approved by the Graduate Development Committee.**

Signed ……..*Dr Khalil Elgmati* ……..……. (candidate) Date …….28/02/2013…...…

ABSTRACT

Long lasting calcium (Ca^{2+}) oscillations are necessary and sufficient for mammalian egg activation and early embryological development. In mammals, phospholipase C zeta (PLCζ) has been identified as the likely endogenous trigger of Ca^{2+} oscillations at fertilization. Some cases of male factor infertility have been associated with the absence / reduced or presence a mutant form of PLCζ. In these cases sperm fails to activate eggs after intra-cytoplasmic sperm injection (ICSI). Artificial egg activation is the potential way to trigger Ca^{2+} oscillations and egg activation. Strontium (Sr^{2+}) is the main artificial agent for this purpose in rodent eggs. The work in this Thesis aims to examine the mechanism of PLCζ or Sr^{2+} ions to trigger Ca^{2+} oscillations in mammalian eggs. It was not clear how Sr^{2+} causes Ca^{2+} oscillations and why it is only effective in rodents but not human eggs or domestic animals. My studies show that Sr^{2+} is effective in causing Ca^{2+} oscillations in mouse eggs over a range of concentrations, but that its actions are influenced by the osmolarity of the medium. Low osmolarity enhances the ability of low concentrations of Sr^{2+} to cause Ca^{2+} oscillations. Further investigation revealed that Sr^{2+} influx is mainly through the reverse mode of the Na^{+}/Ca^{2+} exchange protein (NCX) which can be controlled by the membrane potential and Na^{+} gradient across the plasma membrane. Preliminary studies investigated the ability of a modified Sr^{2+} media that maximizes reverse mode NCX to trigger Ca^{2+} changes in human eggs.

In other studies, various PLCζ-luciferase cRNAs were injected into mouse and human eggs. PLCζ expression in mouse eggs was measured by imaging light due to luciferase

activity, and Ca^{2+}-oscillations were monitored with Ca^{2+} sensitive fluorescent dye. Aspects of the structure of PLCζ and the effects and the recent discovery of PLCζ sequence mutations were investigated. Preliminary studies were also carried out to test the ability of recombinant PLCζ protein to cause Ca^{2+} oscillations in mammalian eggs. It is hoped that these studies might open up new therapies for some male factor infertility couples that acconts 1-5% of failed ICSI.

ACKNOWLEDGEMENTS

Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people. I would like to first say a very big thank you to my PhD advisor, Professor Karl Swann, for supporting me during these past four years I spent undertaking my work in his lab at Cardiff University. Without his guidance and constant feedback this PhD would not have been achievable. Karl is a good teacher who encouraged and expected me to think more independently about my experiments and results. He is my primary resource for getting my science questions answered and was always on time in helping me stitch out this thesis. Many thanks also to Professor Tony Lai for his valuable input, kind advice and supervisory role as second supervisor.

This PhD study would not have been possible without the corporation collaborative work undertaken with Micheal Nomikos for generating enough data toward my PhD. My deep appreciation goes out to the local field research team members, especially my office and lab mates inparticular 'Dr. Yuangson Yu' who was always so helpful and provided me with his assistance throughout my research period in terms of scientific advice and knowledge and many insightful discussions and suggestions. Also and in advance I greatly appreciate the time of my PhD committee members for their acceptance to assess me in my Viva.

I gratefully acknowledge the funding received towards my post graduate study from my government 'Libya' for the trust given to me for continuous over 6 years of scholarship to get my MSc and the current PhD. I deeply thank my country Libya.

I would also like to say a heartfelt thank you to my Mum, Dad, Brothers and Sisters for their social sacrifice and providing unconditional love and always believing in me and pushing me to follow my dreams and for helping in whatever way they could during this challenging time. These past several years have not been an easy ride, both academically and personally.

Finally special thanks to my soul-mate, my wife Samira and my little daughter Saher for their kind support and patience as well as unlimited caring that I got from my beloved wife. Samira has been by my side throughout this PhD, even when I was irritable and depressed. She live every single minute of it, and without her I would not have made it this far. She is the best person out there for me during these past years and she is the one who appreciate my quirkiness and sense of humor. Heartfell love to my darling daughter for being such a good little girl that past eight years and making it possible for me to complete what I started. There are no words to convey how much I love you all.

I am also very grateful to who all helped me in during various stages of my PhD.

I dedicate this thesis to my family, my wife Samira and my daughter Saher for their constant support and unconditional love. I love you all dearly.

Dr Khalil Elgmati

PUBLICATIONS

Nomikos M, **Elgmati K**, Theodoridou M, Calver BL, Cumbes B, Nounesis G, Swann K and Lai FA. (2011a) Male infertility-linked point mutation disrupts the Ca^{2+} oscillation-inducing and $PIP(_2)$ hydrolysis activity of sperm PLCζ. *Biochem J.* 434.2; 211-7

Nomikos M, **Elgmati K**, Theodoridou M, Calver BL, Nounesis G, Swann K and Lai FA. (2011b) Phospholipase Cζ binding to $PtdIns(4,5)P_2$ requires the XY- linker region. *Journal of Cell Science* 124; 2582-90

Nomikos M, **Elgmati K,** Theodoridou M, Georgilis A, Gonzalez-Garcia R, Nounesis G, Swann K and Lai FA. (2011c) Novel regulation of PLCζ activity via its XY- linker. *Biochem J. Biochem. J.* 438; 427-432

Swann K, Windsor S, Campbell K, **Elgmati K,** Nomikos M, Zernicka-Goetz M, Amso N, Lai FA, Thomas A and Graham C (2012) PLCζ-induced Ca^{2+} oscillations cause coincident cytoplasmic movements in human oocytes. *Fertility Sterility* 97.3; 742-7

Nomikos M, Yu Y, **Elgmati K**, Theodoridou M, Campbell K, Vassilakopoulou V, Zikos C, Livaniou E, Amso N, Nounesis G, Swann K and Lai FA. (2013) Phospholipase Cζ rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril.* 99.1; 76-85

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS

cDNA	Complementary DNA
SERCAs	Sarco/Endoplasmic Reticulum Ca^{2+} ATPases
PMCAs	Plasma Membrane Ca^{2+} ATPases
GFP	Green flurescent protein
PLCζ	Phospholipase C zeta
OGBD	Oregon Green Bapta Dextran
IVF	*In Vitro* Fertilization
ICSI	Intra-Cytoplasmic Sperm Injection
NCX	Na^{+}/Ca^{2+} exchanger
TRPC	Transient Reciprocal Potential Cation Channel
Ca^{2+}	Calcium ion
Sr^{2+}	Strontium chloride (SrCl)
CG	Cortical granule
PIP_3	Phosphoinositide PI(3,4,5) trisphosphate
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PI 3	Phosphoinositide 3-kinases (class I)
ER	Endoplasmic reticulum
E_R	Reversal potential
MPF	Maturation promoting factor
MAPK	Mitogen activated protein kinase
NEBD	Nuclear Envelope Break Down
IP_3	Inositol (1,4,5) trisphosphate
IP_3Rs	Inositol-(14,5) trisphosphate receptors
cRNA	Complementary RNA
SR	Sarcoplasmic reticulum
SOCE	Store-operated calcium entry
DAG	Diacyl glycerol
PKC	Protein kinase C
PMGCs	Primordial germ cells
RyRs	Ryanodine receptors
NO	Nitric oxide

ABP	Androgen binding protein
FSH	Follicular stimulation hormone
LH	Lutinizing hormone
ZP	Zona pellucida
ROS	Reactive oxygen species
FRED	Fura-red
BAPTA	1,2-bis-(2-aminophenoxy)-*N,N,N′,N′*-tetraacetic acid
AM	Acetoxymethyl esters
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
kDa	Kilodalton
CICR	Calcium induced Calcium release
GTP	Guanosine tri-phosphate
SOAF	Sperm oocyte activating factor
PI-PLC	Phosphotidylinositol phospholipase C
PKC	Protein kinase C
PH	Pleckstrin homology
hPLCζ	Human phospholipase C zeta
SH2	Src homology 2
$PLC\zeta^{WT}$	Wild type phospholipase C zeta
GMAG	Genetic Manipulation Advisory Group
COSHH	Control of Substances Hazardous to Health
HFEA	Human Fertilization and Embryology Authority
JBIOS	Joint Biological Services
PMSG	Pregnant mare's serum gonadotropin
HCG	Human chorionic gonadotrophin
IP	Intra-peritoneal
M2	Gamete optimising medium from Sigma
HKSOM	Hepes buffered Potassium Simplex Optimised Media
SG	Sodium green
ICCD	Intensified cooled CCD camera
PLCζ-luc	Phospholipase C zeta tagged with luciferase
cAMP	Cyclic adenosine monophosphate

PCR	Polymerase chain reaction
PtdIns3*P*	Phosphatidylinositol 3-phosphate
a.a.	Amino acid
PtdIns5*P*	Phosphatidylinositol 5-phosphate
NB-DNJ	N-butyl-deoxynojirimycin
NB-DGJ	N-butyl-deoxygalactonojirimycin
c.p.s	Count per second
a.u	Arbitrary unit
GsMTx-4	Peptide toxin from Grammostola spatulata spider venom
TRPM	Transient receptor potential melastatin channels
rTRX	Reduced thioredoxin
DTT	Dithiothreitol
RhD	Rhod dextran
SAC	Stretch activated channels
PIV	Particle image velocimetry
Gd^{3+}	Gadolinium
FRET	Fluorescence resonance energy transfer
rtPCR	Reverse transcriptase polymerase chain reaction
PM	Plasma membrane

LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTION

1.1 Pre-embryonic development

A new life starts with the fusion of gametes of haploid chromosomes to create a diploid number of chromosomes and to triggers a series of molecular transitions within the egg to re-enter the cell cycle and begin embryonic development (Leguia and Wessel, 2007). The cell cycle stage at which the egg is held until it is fertilized is species specific, for example; G1 of the first mitosis in sea urchins and second meiotic metaphase in mammals (Stricker, 1999). Fertilization is the end-point of multiple molecular cascades that enable ejaculated spermatozoa to find and attach to the zona pellucida (ZP) (Tulsiani *et al.,* 1998) (Fig. 1.1)

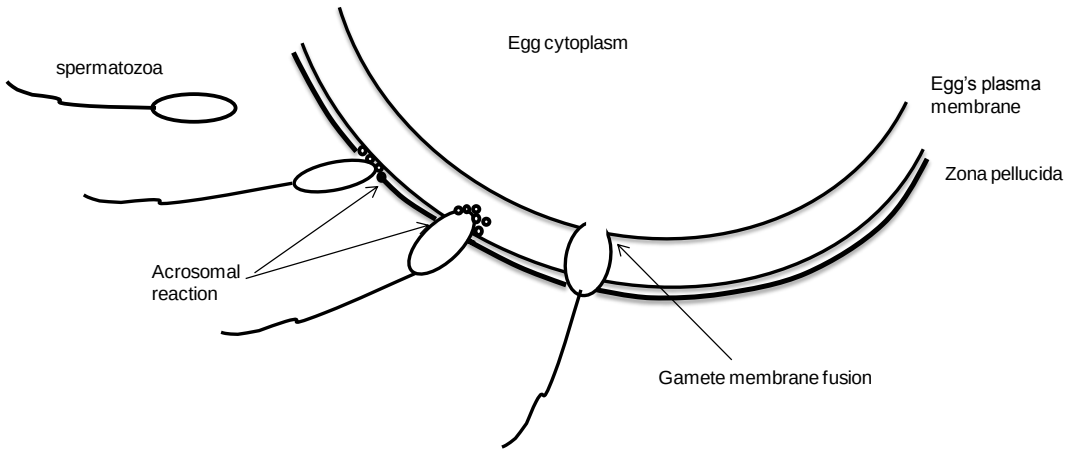


Figure 1.1 Descriptive diagram showing sperm-egg fusion in the fertilization process.

1.1.1 Oogenesis

In the human ovary, at 6-8 weeks gestation, rapid mitotic multiplication of primordial germ cells (PMGCs) gives rise to oogonia. In female embryos, during the pre-follicular stage between 7 and 9 gestational weeks, the oogonia (female germ cells) and

granulosa cells start arranging in cords and sheet patterns (Gondos, 1985). Subsequently, the oogonia undergo multiple mitotic divisions resulting in increases in their population. At the end of which and through these divisions at 11-12 weeks gestation, some oogonia stop and exit mitosis and reach the primary oocyte stage and enter the prophase of the first meiotic division (Stoop *et al.,* 2005), which takes place in the ovaries and continues into the second trimester (Rabinovici and Jaffe, 1990) see (Fig. 1. 2).

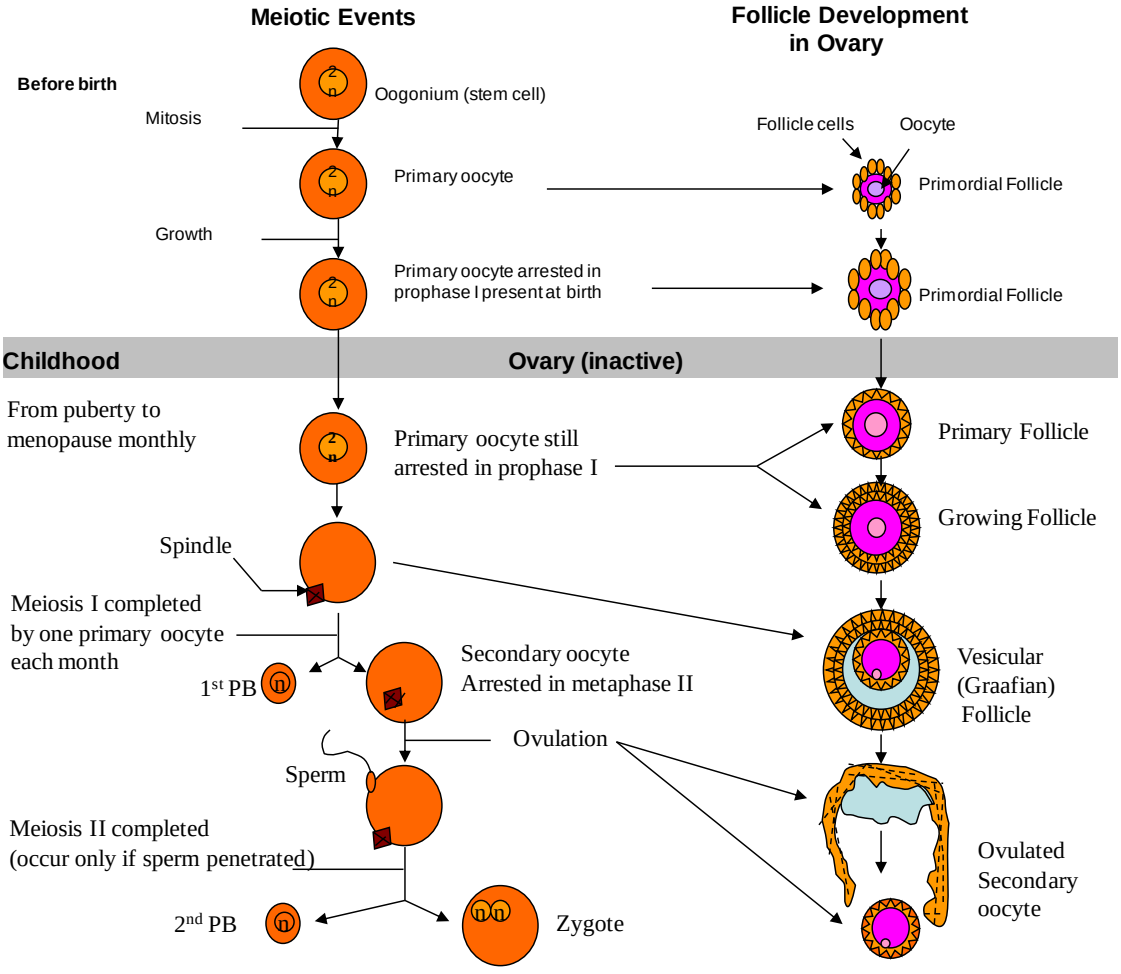


Figure 1.2 Schematic diagram illustrating oogenesis and fertilization processes.

The onset of folliculogenesis (the occurrence of primordial follicles) takes place between 16 and 21 weeks of gestation (Konishi *et al.*, 1986). Each primary oocyte (oogonia) is contained within a primordial follicle surrounded by layers of follicle cells (Fig. 1.2). Significant fluctuations in the population of germ cells are seen during follicular maturation. This is basically due to the continuous proliferation of premeiotic oogonia and the elimination process in the form of apoptosis, which occurs in both oogonia and germ cells at different stages of folliculogenesis (De Poll *et al.*, 1997). Physiologically, the destruction of singular or groups of cells appears to be a step of vital importance during embryonic and postnatal development in this count reduction (Raff *et al*., 1993). This takes place by the activation of a specific self-destruction programme, which involves many changes in form cellular activity such as cutting the DNA into oligonucleosomes by endonucleases (Peitsch *et al*., 1993). The primordial follicles undergo a stage of growth and during this time the primary oocytes are able to undergo a remarkable enlargement since no more mitosis occurs at this stage (Fig. 1.2). The ZP is formed while the egg is growing and passing through a stage called germinal vesicle (GV) stage which is characterised by a large nucleus (Fig. 1.3).

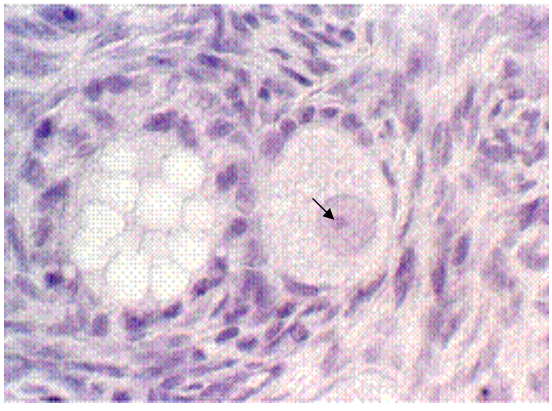


Figure 1.3 Egg in germinal vesicle stage. Prominent nucleolus in the nucleus (black arrow).

GV eggs are arrested at prophase I (PI arrest) of meiosis I. PI arrest is regulated by 3, 5 cyclic adenosine monophosphate (cAMP) levels within the oocyte (Conti *et al.,* 2002). The production of cAMP is either from the egg, or transferred into the egg through gap junctions from granulosa cells. The cAMP holds the oocytes at PI arrest by inactivating maturation promoting factor (MPF) see later (Pirino *et al.,* 2009).

During meiotic prophase in humans, the condensation of DNA occurs followed by the alignment of homologous chromosomes parallel to each other forming 23 bivalent pairs (each pair has twice the required DNA). Then each chromosome splits longitudinally except at the centromeres. Subsequently, cells are arrested in metaphase I (Fig. 1.4) before entering meiotic metaphase which lasts from birth until after puberty when ovulation start to occur.

At the onset of puberty in humans the total number of eggs falls to 300,000 and this reduction is mainly due to cell death through apoptosis (follicular atresia) (Billig *et al*., 1996). Roughly around 400 eggs are destined for ovulation. Resumption of meiosis occurs before ovulation, at the time of the LH surge (Pirino *et al.,* 2009) while the egg is still a primary egg. Chromosomes in the germinal vesicle nucleus become short and thick and the first stage of meiosis is completed to enter the Metaphase I (MI) stage. In this stage, the homologous chromosomes are separated into two equal sets of chromosomes, each containing twice the necessary chromatin ‘meiosis I end’ (Fig. 1.4) see next page.

To enter Metaphase II (MII), one of the chromosomes sets pinches off into a small cell in the perivitelline space (to make a polar body containing one chromosome set with

double amount of chromatin) producing the secondary egg (mature egg = MII) which is haploid but containing twice necessary chromatin (Fig. 1.5 left). MII eggs are identified by the presence of an extruded first polar body and have their diploid complement of chromosomes delicately arranged on the metaphase plate near the polar body (Peter *et al.,* 1997) (see the schematic diagram) (Fig. 1.5 right). MII arrested eggs subsequently undergo ovulation in expectation of fertilisation.

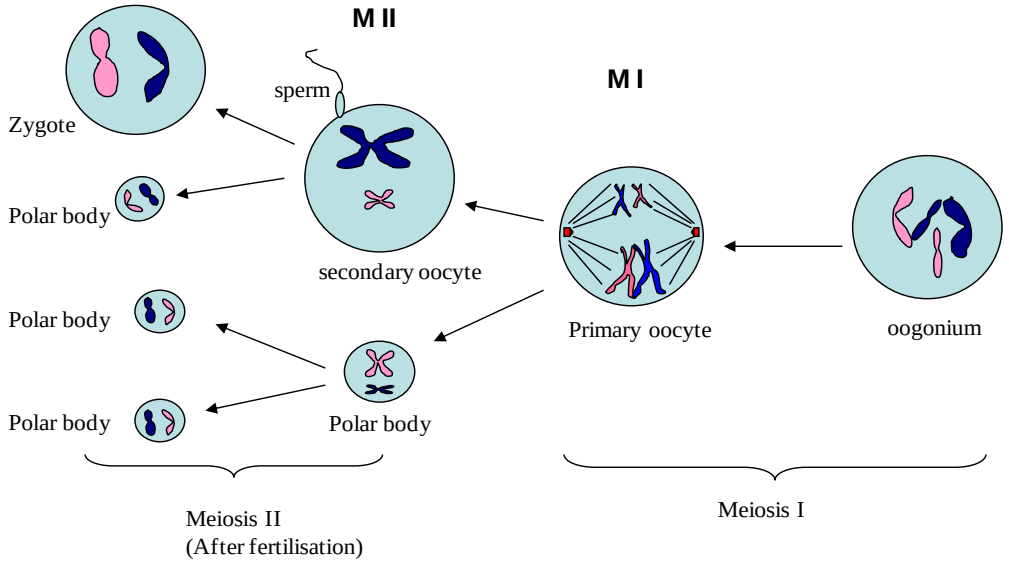


Figure 1.4 Schematic diagram illustrating meiotic processes and zygote formation. Note the metaphase I stage with metaphase plate.

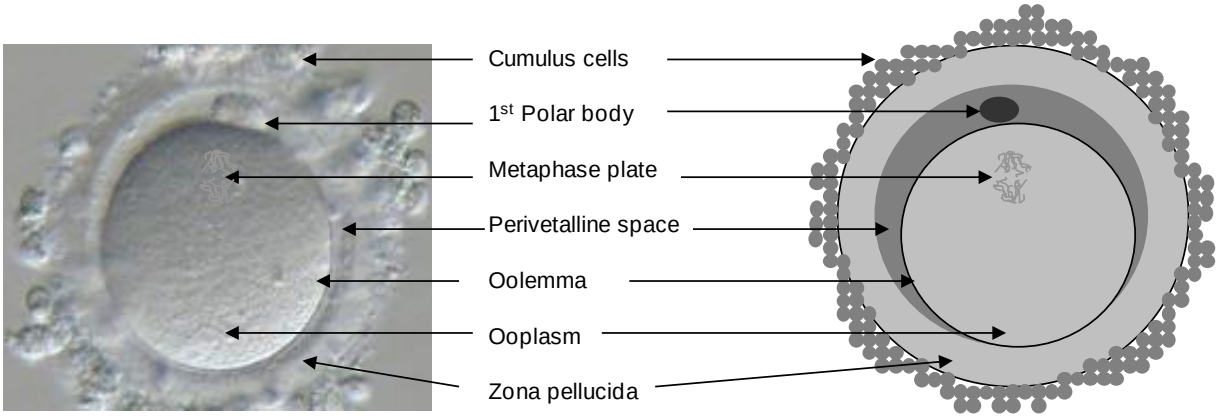


Figure 1.5 Mature secondary human egg with cumulus cells and a schematic illustration showing the structural components of the MII egg.

1.1.2 Spermatogenesis

Spermatogenesis is the process of sperm production from the embryonic precursors the primordial germ cells (PMGCs) (Donovan, 1998). Germ cells are the only cells that undergo meiosis. Spermatozoa have unique functional properties and morphology. In humans the PMGCs can be detected by the end of the 3rd week of gestation in the extra-embryonic mesoderm during early embryonic development. By 5 to 6 weeks they migrate by amoeboid movement to the genital ridge areas via hindgut and dorsal mesentery (Makabe *et al*., 1991). PMGCs multiply during the migration process, and show no sex determination by this time (Stoop *et al.,* 2005). A week later, the gonads and the sex establishment takes place. In male embryos, and under the effect of Sertoli cell precursors, the seminiferous tubules form (Falin, 1969). In the testis, PMGCs proliferate to gonocytes until third month of gestation when spermatogonia (the multi-potent stem cells of the germ line) are seen (Hiromitsu *et al*., 2007). Normal spermatogenesis is directed by genes on the Y chromosome, although many regulating proteins are derived from autosomal chromosomes, hormones, growth factors and vitamins act on the testis to regulate spermatogenesis (Skaletsky *et al*., 2003). The role of sex chromosomes in the premeiotic period has great importance for the sex differentiation processes; the ovarian egg of XY karyotype is differentiated whereas the testicular sperm of XX karyotype cannot differentiate (Isotani *et al*., 2005).

Spermatogenesis is the process of sperm differentiation in the testis. This systematic process is supported by Sertoli cells and by Leydig cells (interstitial cells of the testes). The functioning of these cell types is regulated by testosterone, which secreted by

leydig cells under control of LH stimulation (Hiromitsu *et al*., 2007). FSH (secretion from the pituitary gland) together with testosterone stimulates spermatogenesis by binding of FSH to Sertoli cells and producing several androgen binding proteins (ABP). These proteins bind to testosterone in the tubular lumen and stimulate spermatogenesis. Inhibin secreted from Sertoli cells suppresses FSH secretion; however, activin secreted from Sertoli, peritubular and Leydig cells opposes inhibin by inducing FSH production (Hiromitsu *et al*., 2007).

Spermatogenesis begins with the mitotic division of spermatogonia to produce diploid primary spermatocytes (46XY) (Stoop *et al.,* 2005). These cells undergo first meiotic divisions into two haploid cells (23X or 23Y) called secondary spermatocytes, consequently the second meiotic division occurs and spermatids are formed (containing 23 single half chromosomes).

In the postmeiotic period and during spermatogenesis the rounded spermatids convert to normal spermatozoa by significant morphological changes; the nucleus becomes compacted, the DNA condensed, the mitochondria gather and form an axoneme, the tail emerges by elongation of one of cellular centrioles and the acrosomal cap is created from the Golgi apparatus that surrounds the nucleus. This takes about 5–6 week to complete (Heller and Clermont, 1963). By the completion of the first sperm cell in the testis, the three different stages are arranged in the seminiferous tubules in a systematic pattern; the spermatogonia set in the tubular walls, the spermatids at the tubular lumens and the spermatocytes in the middle (Fig. 1.6) see next page.

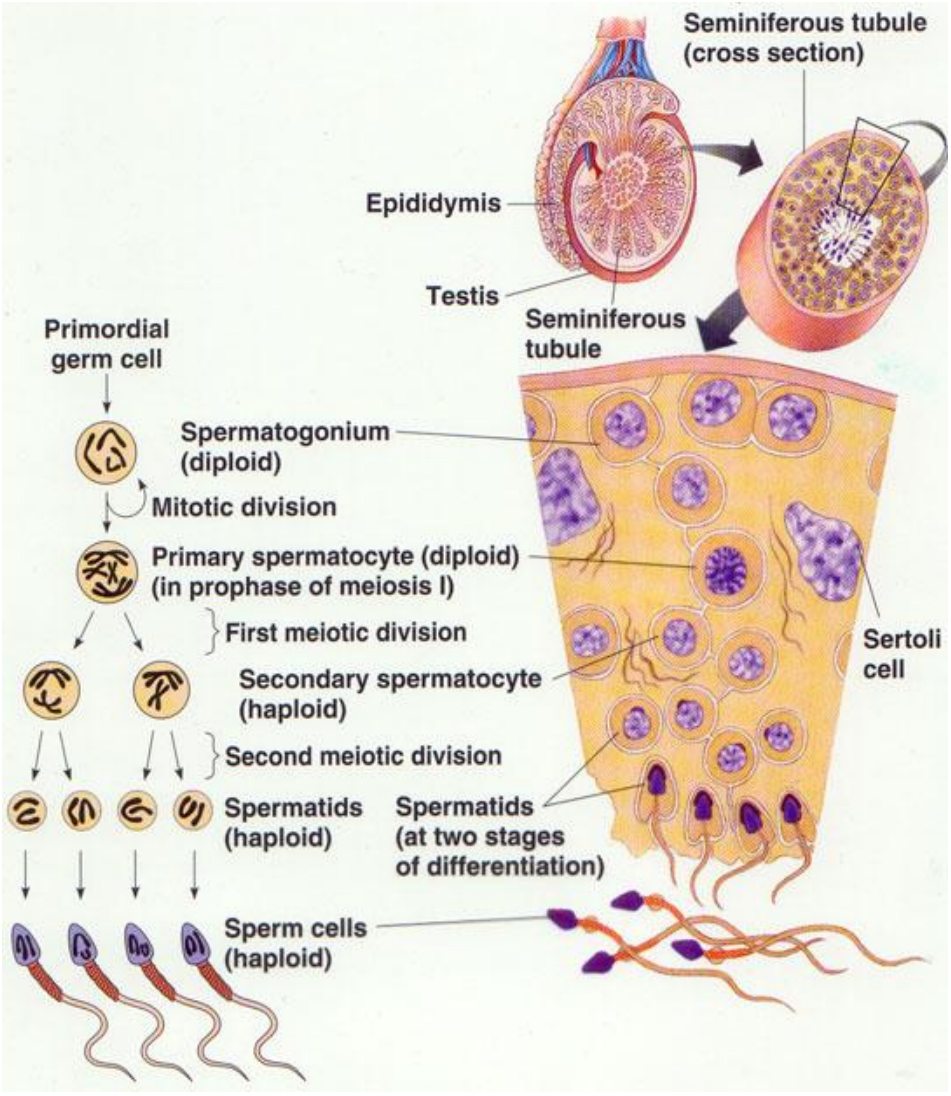


Figure 1.6 Diagrammatic illustration of spermatogenesis and germ cell arrangement in the tubule. (http://faculty.sunydutchess.edu/Scala/Bio102/PDF/Spermatogeneses.jpg)

During adulthood this process occurs in the seminiferous tubules which results in a large sperm counts and takes more than 2 months (Dym, 1994). Spermatogenesis itself is not sufficient to produce male gametes capable of fertilisation. Once sperm have completed their morphogenesis they detach from the seminiferous epithelium and are transported after passing through the rete testis to the cauda epididymis in testicular

fluid by peristaltic contractions. Sperm production needs approximately 65 - 74 days (Dym, 1994) in humans, 50 days of which are spent in the seminiferous tubule and the rest taken in travelling to the epididymis where sperm acquire motility and become capable of fertilization. In comparison to mouse only 34.5 days are sufficient for sperm formation (Reijo *et al.,* 1995). In human testicles 100 million sperm are made per day and each ejaculation releases 200 million sperm from the vas deferens through the epididymal caput (Reijo *et al.,* 1995).

Morphologically the sperm head is species specific; flattened rounded in man and domesticated animals such as the dog and hook shaped in mice and rodents to help each other to reach the ovulated egg. The dimensions of human sperm head is about 5 µm long, 2.5 µm wide and 1.5 µm thick (Austin and Short, 1982). In all mammals the sperm head contains a highly condensed DNA-protein mass called chromatin linked with protamines (Austin and Short, 1982) which are replaced by histone during fertilisation. The sperm tail is divided into a mid-piece and an end piece (Fig. 1.7). The mid-piece contains the mitochondrial helix and the end piece is mainly responsible for the sperm motility (Austin and Short, 1982).

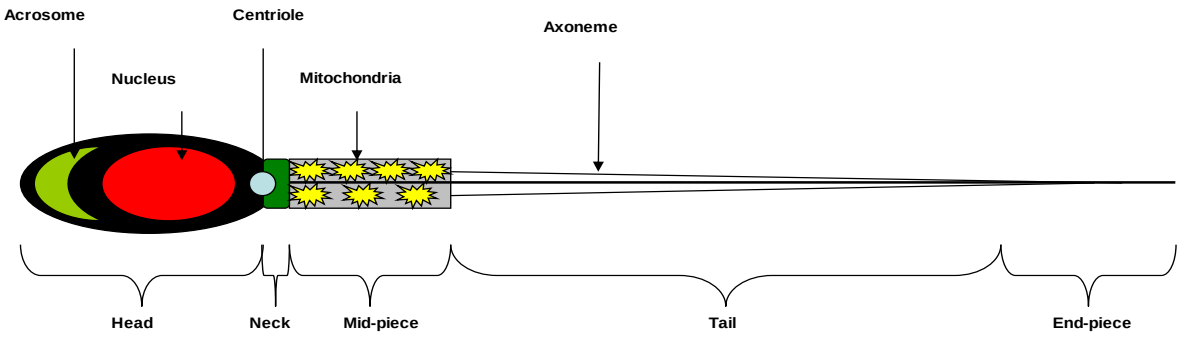


Figure 1.7 Schematic diagram of spermatozoa structural components.

1.1.3 Fertilization process

Fertilization is the process where the two different gametes (highly specialized cells) the egg and the sperm, unite to form a new individual (zygote) (Leguia and Wessel, 2007) that contains a genetic material derived from both parents.

Millions of sperm per ejaculate are released into the female reproductive tract. Only capacitated sperm are competent to initiate fertilisation, as they must penetrate both the cumulus masses and the ZP respectively. In humans, the metaphase II stage ovum (haploid) with its cumulus cells, is picked-up by the fimbrial end of the fallopian tube, and survives for about 18-24 hours waiting for sperm in the ampulla to initiate fertilization process. The activated sperm reach this area and the fertilization process starts. However, if fertilization does not occur, the ovum undergoes apoptosis and finally eliminated by the tube (Naokazu *et al.,* 2005).

As early as sperm penetrates the cumulus mass and the zona by means of chemical and mechanical cascades (acrosomal reaction see below), the remaining chromosome splits longitudinally and half the chromatin pinches off to form the second polar body (Naokazu *et al.,* 2005), and the second meiotic division of the ovum is completed (Fig. 1.4). Eventually, the nuclear contents of the sperm will enter the ooplasm, and then the haploid egg that contains correct amount of its chromatin will receive a similar amount of male chromatin from the penetrated sperm (haploid). Subsequently, the two haploid female and male pronuclei (PN) move toward the centre of the egg to form the zygote (Fig. 1.8) with the establishment of the diploid number of chromosomes. During this

migration event, DNA replication takes place and eventually the first mitotic division of newly formed organism is started (Abou-Haila and Tulsiani, 2000).

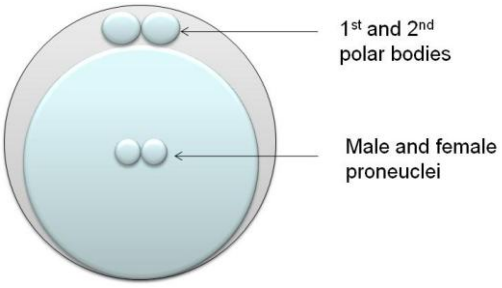


Figure 1.8 Schematic diagram of fertilized human egg.

The paternal DNA is required for normal embryonic development and must be remodelled at fertilisation since the DNA of the mature sperm is compacted and transcriptionally inactive (McLay and Clarke, 2003). The remodelling process involves replacing the major basic protein found in the nuclei of mature sperm cells (protamines) with histones. In mammals, fertilization involves several sequential steps. These include, capacitation of sperm in the female genital tract, binding of capacitated sperm to the ZP, sperm activation (acrosome reaction), ZP penetration and sperm-egg membrane fusion (Fig. 1.1) (Abou-Haila and Tulsiani, 2000; Naokazu *et al.,* 2005) subsequently egg activation events and re-entering the cell cycle.

1.1.3.1 Capacitation

Immediately after ejaculation mammalian spermatozoa are unable to fertilise the egg. Throughout testicular spermatogenesis, epididymal maturation, and capacitation in the female reproductive tract, sperm undergo a series of biochemical modifications. In the female reproductive tract, spermatozoa can survive for an average of 48 hours and they

have to travel from the vagina to the tubal ampullary portion to meet the ovulated ovum where only one sperm is needed to penetrate the ZP for normal fertilization. During this journey the sperms undergo a sequence of complex events known as capacitation process (Baldi *et al.,* 1996). This process increases the sperm activity and mobility. However, in IVF the collected sperm can undergo this capacitation by leaving them in a suitable media in the incubator that optimized with appropriate temperature 37°C and 5% CO_2 levels.

1.1.3.2 Acrosomal reaction

The acrosome reaction is the process of normal fertilization, just before egg penetration, when the sperm is activated after capacitation (Fig. 1.1). It often involves the release of enzymes and exposure of surface antigens as a part of the cell ruptures (Naokazu *et al.,* 2005). It can be defined as a sequence of complex cellular and molecular incidents including changes of the sperm surface, oscillations in the intracellular Ca^{2+} concentration, cyclic nucleotides and protein de/phosphorylation reactions (Baldi, *et al.,* 1996). The acrosomal reaction in mammalian sperm is believed to be started *in vivo* by sperm binding to the ZP, the egg's extracellular matrix (Fukami *et al.,* 2010). Therefore ZP binding is restricted only to capacitated sperm undergoing the acrosome reaction (Tulsiani *et al.,* 1998). That means only sperm that have completed the acrosome reaction can penetrate the ZP and fuse with the egg plasma membrane (Wassarman *et al.,* 2001). Reactive oxygen species (ROS) have been suggested to have an important role in capacitation-related phosphorylation of several proteins in human (Aitken, 1989). Interestingly, Ca^{2+} has been reported to play a primary role in execution of the

acrosome reaction in sperm (Breitbart, 2002). However, recent studies have questioned the site at which the acrosome reaction occurs. A recent study used fluorescence microscopy was able to detect the onset of the acrosome reaction under experimental condition, and found out that most fertilizing spermatozoa underwent the acrosome reaction before reaching the ZP of cumulus-enclosed oocytes (Jin *et al.,* 2011).

1.1.3.3 Sperm-egg interaction and fusion

During fertilization, the interaction between egg's ZP and spermatozoa is a relatively species-specific incident (Swenson and Dunbar, 1982 and Abou-Haila and Tulsiani, 2000). Sperm-egg membrane fusion is thought to take place shortly after the sperm reaches the plasma membrane of the egg. The sperm fuses with microvilli on the egg surface. In other words, the capacitated sperm surface-receptors bind to their ligands on the ZP. Subsequently, the sperm plasma and the acrosomal outer membranes undergo multiple fenestrations, fusion and exocytosis of acrosomal contents. The surface over the equatorial part of the spermatozoon is prepared for the fusion step (Yanagimachi, 1994). It is a prerequisite step that enables the acrosome reacted sperm to penetrate the ZP and fertilize the egg. Hydrolytic enzymes with the hyperactivated flagellar motility of sperm tail play a major role in the ZP penetration and fertilization process (Tulsiani *et al.,* 2007). Once the sperm and egg membranes have fused, the sperm releases its content into the egg cytoplasm and in just a few minutes after fusion, the egg undergoes the first Ca^{2+} transient which begins the process of egg activation (Lawrence *et al.,* 1997).

1.1.4 Egg activation events

The fertilization and egg activation processes appears to be similar in many mammals. The similarity between human and mouse egg in some maturation aspects has made the mouse egg as an acceptable model to study the human egg maturation process. Since both exhibit meiotic arrest at metaphase II before ovulation and undergo ZP reaction to protect against polyspermy during fertilisation (Ducibella *et al.,* 1990). After sperm egg binding and fusion a sequential critical events took place in the egg towards the normal activation through specific biochemical pathways within the egg (Ben-Yosef and Shalgi, 1998). Amongst these events, characteristic increases in free cytosolic Ca^{2+} levels within the egg has been recognized by researchers to be the key stimulus and the universal feature for the egg-activation process (Runft *et al.,* 2002). These increases in Ca^{2+} level can lead to a series of events that are important steps towards embryo formation. These include; cortical granule (CG) exocytosis, and the resumption of the cell cycle by the completion of meiosis and expulsion of the second polar body. By the completion time of these events, both the formation of pronuclei and the synthesis of DNA will have started (Raz and Shalgi, 1998). These all help lead to the completion of the first cell cycle that initiates the early embryonic mitotic divisions (Yanagimachi, 1994; Ben-Yosef and Shalgi, 1998; Stricker 1999; Swann and Parrington, 1999).

1.1.4.1 Exocytosis

Following sperm penetration, one of the early characteristic and key events in the egg activation process takes place namely CG exocytosis (Raz and Shalgi, 1998). The

mammalian CGs are membrane-bound small specialized organelles within the cortex that contain enzymes such as proteinases and glycosidases. They are found in oocytes and eggs of all mammals (Leguia and Wessel, 2007), and in response to cytoplasmic Ca^{2+} transients undergo exocytosis into the perivitelline space (Ducibella *et al.,* 1990; Ducibella, 1991; Yanagimachi, 1994; Raz and Shalgi, 1998). The physiological function of exocytosis is mediated through a zona modification reaction which protects against polyspermic fertilization (Ducibella, 1991; Ben-Yosef and Shalgi, 1998). The maximal release of CGs takes place at the meitic metaphase II stage (Ducibella *et al.,* 1990).This kind of polyspermic protection has been reported in all mammalian species including human oocytes (Sathananthan and Trounson, 1985).

1.1.4.2 Role of Ca^{2+} in egg activation

It has been known that all forms of life during their resting state create a difference in Ca^{2+} concentration between the inside cytoplasmic free Ca^{2+} (50-100 nM) which is approximate of 10,000 fold lower than the outside extracellular Ca^{2+} concentration (1-2 mM). One consequence is that even the most primitive cells use Ca^{2+} as a messenger for sending signals from outside to inside the cell (Fukami *et al.,* 2010). Under fertilization event, the free cytoplasmic Ca^{2+} elevates to reach up to 1-10µM (Taylor *et al.,* 1993; Pepperell *et al.,* 1999). In general, a Ca^{2+} increase is important in cellular events such as in fertilization and cell division, and as well as in physiological events in cells such as in neurons, cardiac muscle, secretary cells (Stephanie *et al.,* 2008). Importantly, Ca^{2+} is a double-edged sword, because a suitable Ca^{2+} increase is good

signal transducer for various physiological functions (Fukami *et al.,* 2010), but high cytoplasmic free Ca^{2+} levels can also be toxic.

For decades, the physiological importance of Ca^{2+}-oscillations in egg activation has been an issue of importance. In all examined species the pivotal signal in fertilization is the rise in Ca^{2+}. As we know today that Ca^{2+} is a cell regulatory key ions and during fertilization its release is a prerequisite step for egg activation, releasing MII egg from the resting stage (meiotic arrest) (Miyazaki *et al.,* 2006) that all mammalian eggs are arrested at. The first clear role of Ca^{2+} was shown in 1974 when it as found that parthenogenic activation of star-fish, sea urchin and hamster eggs occurred with Ca^{2+} ionophore A23187 (Steinhardt *et al.,* 1974). Similar findings have been reported in fish (Ridgway *et al.,* 1977). Later on, different pathenogenetic activation protocols for eggs, such as ethanol (7%) and strontium (Sr^{2+}), were all shown to cause free cytoplasmic Ca^{2+} increases which eventually led to egg activation (Stricker, 1999; Saunders *et al.,* 2002; Miyazaki *et al.,* 2006). On the other hand, introduction of the Ca^{2+} chelating agent, EGTA, into the ooplasm was shown to have the opposite effect by stopping the egg activation at fertilization (Whitaker *et al.,* 1982 and Kline and Kline*,* 1992). All this extensive research lead to the conclusion, that Ca^{2+} release is necessary and sufficient for egg activation in mammals (Stricker, 1999; Miyazaki *et al.,* 2006). In fact, the first few Ca^{2+} spikes are essential for egg activation (Kline and Kline, 1992), whereas a long lasting Ca^{2+} rises are necessary for early embryo developments (Swann and Ozil, 1994).

1.1.4.3 Ca^{2+}-oscillations in mammals

An increase in intracellular Ca^{2+} is responsible for activating eggs of all species. The Ca^{2+} increases occur in two forms depending on species type, either as a single rise as seen in sea urchin, fish and frog eggs (Gilkey *et al.,* 1978; Jaffe, 1985 and Stricker, 1999), or as multiple transients as in ascidians and mammalian eggs (Swann and Ozil, 1994). These multiple transients are called Ca^{2+}-oscillations and in mammals take several hours to cease and they are characterized by larger amplitude and low frequent Ca^{2+} rises in compare to the ascidians (Swann and Yu, 2008). In mammals, the Ca^{2+} spikes' number, the amplitude and the frequency appear to be a species dependent and may have a direct impact on the developmental progression (Faure *et al.,* 1999), from events of egg activation to events associated with further embryogenesis (Runft *et al.,* 2002; Miyazaki and Ito, 2006). During normal mammalian fertilization repeated increases of the free Ca^{2+} level in egg cytoplasm from 1.0nM and reach up to 1.0 µM (Stricker, 1999; Runft *et al.,* 2002).

The frequency of oscillations at fertilization appears to vary within mammals, from 20 - 30 spikes per hour in hamster eggs (Miyazaki *et al.,* 1986), from 5 -30 spikes per hour in mouse eggs (Kline and Kline, 1992; Faure *et al.,* 1999), to about 6 spikes in rabbit (Fissore and Robl, 1993), 3 spikes in pig (Sun *et al.,* 1992), and as low as 2 spikes per hour in bovine eggs (Sun *et al.,* 1994; Faure *et al.,* 1999). There are several factors that may affect on the Ca^{2+} oscillations frequency. For example, in mouse eggs the extracellular Ca^{2+} concentration (Igusa and Miyazaki, 1983), the age of the eggs (Faure *et al.,* 1999), all appear to be responsible for the variability in the frequency of Ca^{2+}

oscillations. There is also variability in the first Ca^{2+} spike in mammalian eggs, which is generally of longer duration with small mini spikes on the top of the main increase. This tends to be followed by a series of shorter Ca^{2+} spikes without mini spikes (Fig. 1.9). Normally, the frequency and amplitude of Ca^{2+}-oscillations declines with time until they stop around the time of pronuclear formation.

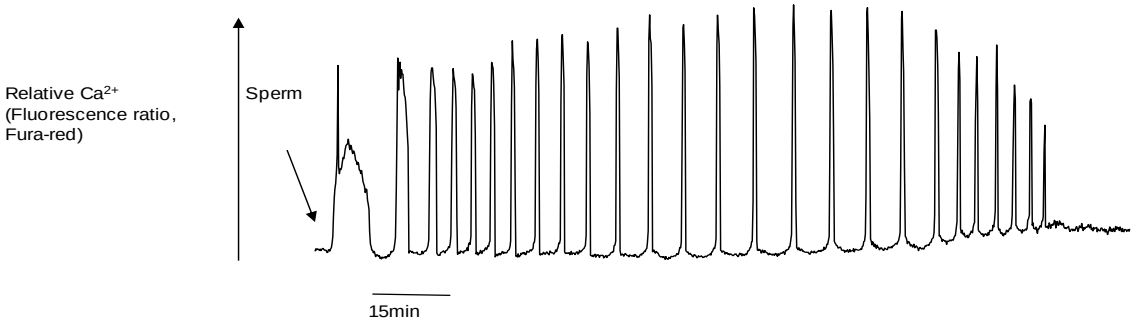


Figure 1.9 Ca^{2+}-oscillations initiated by sperm during IVF in a mouse eggs.

1.1.4.4 The importance of Ca^{2+}-oscillations during fertilization in mammals

In general, Ca^{2+} increase is important in cellular events such as in fertilization and cell division, and is involved as well as in many physiological events in cells (Stephanie *et al.,* 2008). It has been recognized that Ca^{2+} is the most known fundamental factor of egg activation (Loren and Lacham-Kaplan, 2006). It has been established that an increase in the intracellular free Ca^{2+} concentration is necessary for egg activation at fertilization in animals (Stricker *et al.,* 1999). These Ca^{2+} changes in mammals, occur as a prolonged series of oscillations after sperm-egg fusion which shown to be essential for egg activation and early embryonic development in mice (Kline and Kline 1992; Stricker *et al.,* 1999; Ducibella *et al.,* 2008). These oscillations are cell cycle dependent (Jones *et al.,* 1995). The eggs in pre-fertilization state are held in meiotic arrest by maturation

promoting factor (MPF). The triggered Ca^{2+} oscillations act by destroying MPF via cyclin B destruction and activate several protein kinases as part of downstream Ca^{2+} signalling. These include calmodulin-dependent protein kinase II, CaMKII, which seems to be critical to trigger meiotic resumption and early development in mouse eggs (Madgwick *et al.,* 2005; Ducibella *et al.,* 2006; Knott *et al.,* 2006; Ducibella *et al.,* 2008) by stimulation of ubiquitin-dependent degradation of cyclin B by the proteosome (Lorca *et al.,* 1993). These Ca^{2+}-oscillations have also been reported in human eggs during IVF (Taylor *et al.,* 1993) and ICSI (Tesarik *et al.,* 1994).

1.1.4.5 Ca^{2+}-waves

Each individual rise in the intracellular free Ca^{2+} in egg's cytoplasm in species such as frog and sea urchin moves from one pole to the other. They start from the point of sperm egg fusion and travels across the whole egg in a wave like pattern (Miyazaki *et al.,* 2006) (Fig. 1.10).

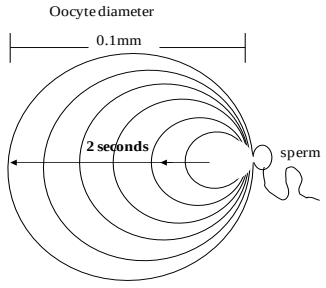


Figure 1.10 Schematic diagram representing the progression of Ca^{2+}-wave across the egg.

The first image of a Ca^{2+} wave was described by Gilkey and his colleagues in medaka fish eggs (Gilkey *et al.,* 1978), and it travels at a speed of 5-50 micrometer/seconds

(Whitaker *et al.,* 1993). It was shown that these waves are not affected by external Ca^{2+} confirming that Ca^{2+} rise comes from the intracellular source (Gilkey *et al.,* 1978).

However, in mammals the situation is more complex due to the occurrence of the recurrent elevations of free Ca^{2+} levels (Swann. *et al.,* 1994; Swann *et al.,* 1999; Miyazaki *et al.,* 2006). These transients are a result of releasing of Ca^{2+} from intracellular stores into all portions of ooplasm (Swann *et al.,* 1999). In mammals the average size of the eggs is around 0.1mm and Ca^{2+}-waves take around 2 seconds to cross the whole egg (Whitaker, 2006) (Fig 1.10). As mentioned previously the first Ca^{2+} wave start from sperm-egg fusion site, however the subsequent Ca^{2+}-waves initiate from different parts in the egg (vegetal hemisphere) which are rich in ER irrespective of sperm-egg fusion site (Stricker, 1999; Miyazaki *et al.,* 2006).

1.1.4.6 Cell cycle & Ca^{2+}-oscillations

At fertilization, the increased intracellular Ca^{2+} is needed only for short periods of time; since prolonged elevated Ca^{2+} levels, in most cases, results in cell death by activating DNA-fragmenting enzymes and proteases (Guerini, 1998). At mammalian fertilization the increases in Ca^{2+} levels lead to the completion of meiosis. In addition, it causes other egg activation events; e.g. CG exocytosis, which puts in place a mechanical block to polyspermy (Zucker and Steinhardt, 1978; Kline, 1988; Kline and Kline, 1992).

MPF is a protein complex that consists of a catalytic subunit cdk1 and a regulatory protein cyclin B1. MPF is responsible for transition from interphase into meiosis/mitosis (Nurse, 1990). In mature metaphase II eggs both cdk1, cyclinB and MAP kinase are required to stabilizing MPF activity (Palmer and Nebreda, 2000). Before fertilisation

MPF remains in highest level, however, Ca^{2+} transients at fertilization trigger cyclin B destruction via the activation of CaMKII that phosphorylates Emi2 and targets it for degradation (Doree and Hunt, 2002). This is followed by proteolysis of cyclin B by the proteasome and the MPF level then drops down to a minimal level as seen during interphase (Doree and Hunt, 2002; Jones, 2004). Subsequently, the fertilized egg completes meiosis by expulsion of the second polar body which is after 1.5 - 2 hrs after the start of fertilization. However, Ca^{2+}-oscillations continue beyond polar body formation to stop by the time of pronucleus formation around 2 hrs later, when the MAP kinase pathway is inactivated (Moos *et al.,* 1996).

The application of the spindle disrupting drug nocodazole leads to a block of cell cycle protein changes and to the inhibition of pronucleus formation, and this has shown to facilitate the continuation of Ca^{2+}-oscillations hours after the usual cessation time. This indicates a relationship between Ca^{2+} signals and the cell cycle. Most significantly it appears that Ca^{2+} transients stop when proneucleur formation occurs (Jones *et al.,* 1995). Additionally, Ca^{2+} released by different stimuli such as InsP3 and Sr^{2+}, all dramatically diminished after certain time (hours) in one-cell embryos which also signifies that the completion of meiosis II, as progress towards the first mitotic division is linked directly to the Ca^{2+} releasing ability (Carroll, 2001). Furthermore, Ca^{2+}-oscillations have been reported in different stages of cell cycle, as it they have been detected in fertilized mouse embryos 16 - 18 hrs after fertilization, which is around the time of nuclear envelope breakdown (NEBD) of the first mitotic division (Kono *et al.,* 1996).

1.1.5 Parthenogenetic activation

Parthenogenesis is the process by which an egg is activated without sperm or any contribution from the male genome (Swann and Ozil, 1994). There are three different procedures have been established to stimulate mammalian egg activation artificially, namely; electrical, mechanical, and chemical (Yamano *et al.,* 2000). The electrical stimulation depends on the Ca^{2+} influx through pore formation in the plasma membrane (Nasr-Esfahani *et al.,* 2010). It has been shown that long-lasting Ca^{2+} transients triggered by repetitive electric pulses not only enhance pronucleus formation but it can also facilitate later embryonic development, even in eggs who are resistant to parthenogenetic activation (Swann and Ozil, 1994; Ozil and Swann, 1995). The effectiveness of this procedure relays on medium ionic content, pore size and cell type. This method has been reported successfully on bovine and human oocytes (Zhang *et al.,* 1999; Yanagida *et al.,* 1999). However, it can also induce the reactive oxygen species (Nasr-Esfahani *et al.,* 2010).

The mechanical methods involve breaking the oocytes plasma membranes using a microneedle to facilitate the influx of Ca^{2+}, or involve by a direct microinjection of Ca^{2+} into the egg. The activation events have been observed on pig and human oocytes using this procedure (Mach'aty *et al.,* 1996; Dirican *et al.,* 2008). However neither the electrical nor mechanical methods are commonly used in clinical practice (Nasr-Esfahani *et al.,* 2010).

The third type and the most commonly used in clinical practice is the chemical method. This can involve different compounds such as 7% ethanol (Presice and Yang, 1994), Ca^{2+} ionophore A213187 (Kline and Kline, 1992; Perry *et al.,* 1999; Ahmady and Michael, 2007), cycloheximide (Moses and Kline, 1995), thimerosal (Fissore and Robl, 1993), and strontium "Sr^{2+}" (Kline and Kline, 1992; John *et al.,* 2003; Yanagida *et al.,* 2006; Ducibella and Fissore, 2008). However, the chemical method has been limited mainly to the experimental animal models and a few case reports on human eggs (Borges *et al.,* 2009; Nasr-Esfahani *et al.,* 2010). The chemical compounds act by Ca^{2+} releases from the intracellular stores, mainly the ER, this subsequently leads to the depletion of Ca^{2+} in ER which in turn promotes the extracellular Ca^{2+} influx (Nasr-Esfahani *et al.,* 2010). Interestingly, these agents could potentially be used to treat some cases of male factor infertility where the sperm is not able to activate the egg (Borges *et al.,* 2009; Nasr-Esfahani *et al.,* 2010). It has been found that couples with repeated failed ICSI could achieve pregnancy by artificial egg activation using Ca^{2+} ionophores (Perry *et al.,* 1999) and delivery of a healthy baby (Ahmady and Michael, 2007). Noteworthy, most chemical stimulators can only cause a single Ca^{2+} rise, such as with Ca^{2+} ionophores, and it is possible that such a single short Ca^{2+} rise fails to trigger the whole activation events (Sutovsky and Schatten, 1997). Similarly, 7% ethanol can only causes single large Ca^{2+} increase (Swann and Ozil, 1994). In contrast, others such as Sr^{2+} can triggers prolonged Ca^{2+}-oscillations that look similar to that seen in fertilization (Fig. 1.11 see next page). Sr^{2+} is known to be the most successful and efficient agent for causing mouse egg activation artificially (Kline and Kline, 1992; John

et al., 2003; Rogers *et al.,* 2004). Some reports show that parthenogenetic mouse embryos may implant but with severe growth retardation because the absence of the paternal chromosomes. This leads to a defect in the formation of extraembryonic tissue. Hence most embryos die before birth (Surani *et al.*, 1984; McGrath and Solter, 1984). From these findings, and other, it is clear that full embryogenesis needs one set of chromosomes from both parents that have undergone the appropriate imprinting.

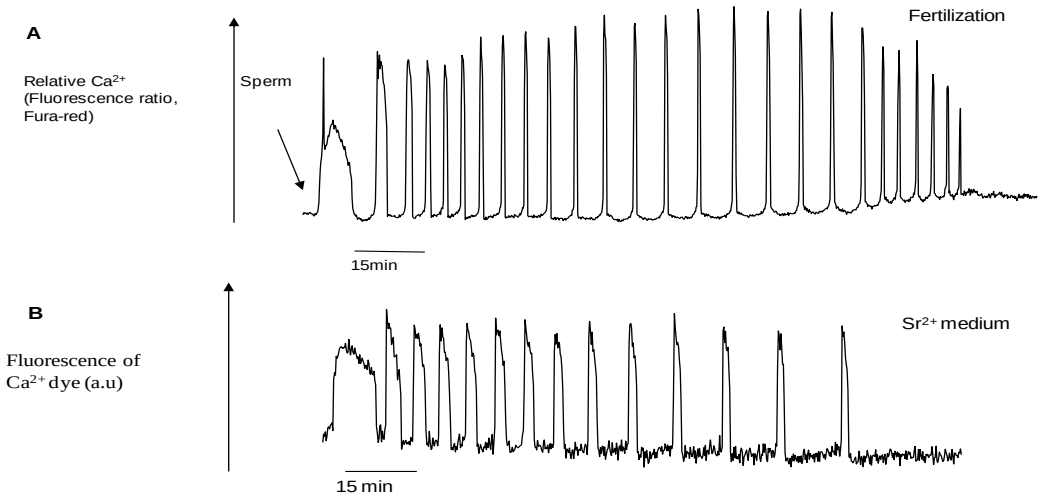


Figure 1.11 Ca^{2+}-oscillations patterns after IVF and Sr^{2+} activation.

A) Shows Ca^{2+}-oscillations initiated by sperm during IVF in a mouse egg. B) Shows the Ca^{2+}-oscillations in a mouse egg in response to 10mM Sr^{2+}.

1.1.5.1 Strontium (Sr^{2+})

Media containing Sr^{2+} is widely used in research labs as an egg activator in rodents. Normal mice offspring have been reported after ICSI (intra-cytoplasmic sperm injection) in combination with Sr^{2+} activation (Suganuma *et al.,* 2005). Treating mouse eggs with Sr^{2+}-triggers Ca^{2+}-oscillations that look similar to the normal pattern at fertilization (Kono *et al.,* 1996). It has been claimed that IP_3Rs is the mediator for Sr^{2+} action to trigger Ca^{2+} release and oscillations (Zhang *et al.,* 2005) (Fig. 1.12). In humans, several

published reports have claimed live births using a combination of ICSI and Sr^{2+} (10mM) exposure for 60 minutes, in cases of repeated ICSI failure (Yanagida *et al.,* 2006; Kyono *et al.,* 2008; Chen *et al.,* 2010). However, other studies have failed to show Ca^{2+} transients in human oocytes treated with 10 mM Sr^{2+} (Rogers *et al.,* 2004) and no other groups are using Sr^{2+} to activate human, pig or cow eggs.

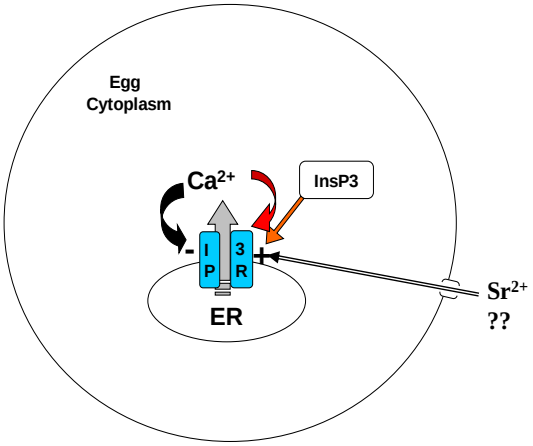


Figure 1.12 Schematic diagram showing the mechanism of how Sr^{2+} releases Ca^{2+} through IP_3R stimulation. Note, released Ca^{2+} has a stimulatory and inhibitory feedback effect on IP_3R.

IP_3Rs are thought to be present in all mammalian eggs. However, it is unclear why Sr^{2+} works reliably in rodent eggs, but not in eggs of other species including human. It is also unclear why the effective concentration of Sr^{2+} for activation of mouse eggs varies from 1.7mM to 20mM (Bos-Mikich *et al.,* 1995; Zhang *et al.,* 2005; Kishigami *et al.,* 2007). Based on the previous questions, part of my research project will be trying to answer these questions.

1.2 Human eggs

During fertilization in human, spermatozoa activate MII egg via a series of cellular events. These events started by series of rises in the free cytoplasmic Ca^{2+} in the first few minutes after gametes membranes fusion, originated from the sperm entry point (Lawrence *et al.,* 1997). Taylor and his colleagues (Taylor *et al.,* 1993) were able to show increases in cytoplasmic free Ca^{2+} in human eggs (Fig. 1.13). Aequorin was injected into human eggs as Ca^{2+} indicator; two groups of human eggs with and without zona were exposed overnight to capacitated sperm. Data analysis showed dramatic intracellular Ca^{2+} transients were recorded using a photomultiplier system. Interestingly, the amplitude, duration and frequency of these transients were similar in zona-intact and zona-free eggs. This was the first ever recorded Ca^{2+} oscillations in human eggs during IVF (Taylor *et al.,* 1993).

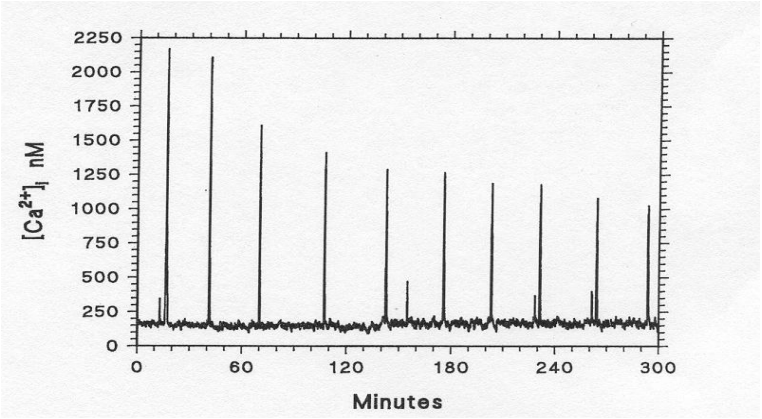


Figure 1.13 Representative figure of Ca^{2+}-oscillations in human eggs after IVF. (Taylor et al., 1993)

Ca^{2+} oscillations play a regulatory role on activation events as well as the developmental events (Ozil, 1990; Ducibella *et al.,* 2002). Ca^{2+}-oscillations have also been reported after ICSI on human oocyte using confocal microscope (Tesarik *et al.,*

1994). They found that after sperm injection a single non-oscillatory free intracellular Ca^{2+} rise recorded and that did not provoke the activation. This was also obtained after the injection of medium without spermatozoa. However after lag period of 4-12 hrs recording, the oscillatory pattern of Ca^{2+} changes was recorded and that provoke the human egg activation (Tesarik *et al.,* 1994). These oscillations were similar to the changes accompanying normal fertilization in mammalian. The interesting point here is the starting time point of Ca^{2+}-oscillations which delays from half to several hours after ICSI (Nagy *et al.,* 1994). However, these Ca^{2+} oscillations have been reported to start sooner if the sperm is damaged (Yanagida *et al.* 2001).

Similar to other mammals such as rodents, Ca^{2+}-oscillations at fertilizations of the human egg is induced by inositol trisphosphate ($IP_{3)}$, the catalytic product of phosphatidylinositol (4,5)-bisphosphate (PIP_2) by phospholipase C (PLC). These oscillations initiate a series of cellular events within the egg, that is along with exocytosis for monospermia (Raz and Shalgi, 1998), the egg re-enters cell cycle by resuming meiosis, sperm nucleus de-condensation, maternal RNA recruitment, formation of pronuclei and initiation of DNA synthesis before the cleavage step (Nasr-Esfahani *et al.,* 2010).

Regarding the parthenogenetic activation, both fresh and aged human eggs have also been reported to be activated parthenogenetically by various stimuli that increase intracellular Ca^{2+} level, such as calcium ionophores (A23187) (Winston *et al.* 1991; Borges *et al.,* 2009; Nasr-Esfahani *et al.,* 2010), and repeated electrical stimuli (Zhang *et al.,* 1999). However, despite the several published reports which claimed that human

oocyte can be activated artificially in patient with repeated failed IVF and/or ICSI (Zhang *et al.,* 1999; Yanagida *et al.,* 1999; Perry AC *et al.,* 1999; Rogers *et al.,* 2002; Yanagida *et al.,* 2006; Ahmady and Michael, 2007; Dirican *et al.,* 2008). There is as yet no clear evidence showing Ca^{2+}-oscillations triggered by artificial methods in human eggs. Notably, all these reported cases were reported in the combination with ICSI. This means that the activators of mammalian eggs have often not been shown to have the ability to induce any Ca^{2+} increase by themselves in human eggs. However, the use of combinations of these activators can be shown to improve the activation rates. Some of these combination protocols have shown better activation results, but with low success rates of subsequent preimplantation development in rabbits eggs (Nakagawa *et al.,* 2001). Attempts have been made to improve the parthenogenetic activation and the subsequent preimplantation developments in human eggs. However, injecting complementary RNA (cRNA) encoding for human phospholipase C zeta (hPLCζ) is the only parthenogenetic activator that can triggers Ca^{2+}-oscillations in human oocytes (Cox *et al.,* 2002) (for a full details on this see later in PLCζ sections). In addition, cRNA possess the ability to enhance embryo development up to the blastocyst stage (Rogers *et al*., 2004). However there are a few limitations in the way of the clinical PLCζ application (see general discussion chapter).

1.3 Ca^{2+}-pumps and Ca^{2+}-Channels

The viability of cells, including eggs requires a tight control and regulation of cytoplasmic free Ca^{2+} concentrations (Pepperell *et al.,* 1999). Intracellular Ca^{2+} levels

are maintained mainly by different type of ion transporters or channels. Ca^{2+} ions shuttle across endoplasmic reticulum (ER), mitochondrial and plasma membranes and regulated by channels, exchangers and pumps into and out of the cytosol (Cahalan, 2009). Normally in cells, a low level cytoplasmic Ca^{2+} is maintained by different mechanisms in organelle's and in plasma membranes. Ca^{2+} is removed from cytoplasm against its chemical gradient by ATP-driven systems into the SR/ER or extracellular space (Machaty *et al.,* 2002a). Among those in the plasma membrane are Na^{+}/Ca^{2+} exchanger, and the Ca^{2+}-ATPase (Pepperell *et al.,* 1999), both which play a keystone role in Ca^{2+} homeostasis process (Blaustein and Lederer, 1999). Interestingly, Ca^{2+} regulates wide range of cellular events such as cytoskeleton remodelling or release of vesicle contents, transcriptional changes and egg activation events (Machaty *et al.,* 2002a).

1.3.1 STIM and Orai

Ca^{2+} oscillations are mediated by the release of ER stores of Ca^{2+} and by external influx of Ca^{2+} (Igusa and Miyazaki, 1983) which involves a mechanism called store-operated calcium entry (SOCE), In this the depletion of intracellular stores of Ca^{2+} leads to Ca^{2+} influx (Berridge, 1995; Machaty *et al.,* 2002a). This is relevant to mouse eggs since a decrease in the extracellular Ca^{2+} inhibits oscillations, and completely blocking extracellular Ca^{2+} influx by Ca^{2+} channel blocker such as Lanthanum blocks oscillations (Machaty *et al.,* 2002a).

The sophisticated correlation between the ER and plasma membrane was established to show that the decrease in the Ca^{2+} content of ER, regardless of the cytosolic Ca^{2+} level, induces Ca^{2+} influx through the plasma membrane Ca^{2+} channels via SOCE feedback mechanism (Parekh and Putney, 2005). The exact molecular mechanism has been clarified by the identification of two proteins; STIM and Orai, both are a fundamental part of the calcium release activation channel (CRAC) activity. STIM1 is the Ca^{2+} sensor responsible for the molecular link between the ER Ca^{2+} store depletion, SOCE and the activation of CRAC channel in the plasma membrane i.e. stimulates SOCE but does not make the CRAC channel. Orai1 is the channel protein or the CRAC channel pore-forming subunit (Cahalan, 2009).

In mammals and some other species there are two homologues of STIM proteins; 1 and 2. STIM1 appears to be the primary sensor of Ca^{2+} in intracellular stores in mammalian cells (Roos *et al.,* 2005). It compromise of two proteins separated by single membrane, the N-terminus is predicted to be within the ER lumen and contains negative charges typical of an EF-hand with Ca^{2+}-binding domain The presence of an EF-hand motif in the ER lumen, suggests its role in sensing ER Ca^{2+}. When the ER store is full the Ca^{2+} binds to the EF-hand domain of STIM. However, once the ER Ca^{2+} is depleted the STIM releases the bound Ca^{2+} and triggers CRAC channel activation (Cahalan, 2009). Knocking down STIM1 has shown a complete loss of SOCE and CRAC activity following Ca^{2+}-store depletion.

The other component of the CRAC channel in mammals is Orai1. The co-expression of Orai1 and STIM1 dramatically improves CRAC and SOCE activity (Mercer *et al.,* 2006).

However, the co-expressed Orai1 and STIM1 gives electrophysiological features that do not match those originally described for CRAC since the CRAC activity declines upon substituting Ca^{2+} with other divalent cations such as Sr^{2+} or Ba^{2+}. This suggests that STMI and Orai mechanism for Ca^{2+} influx may not be the only mechanism and implies that additional molecules might be involved (Smyth *et al.,* 2006).

1.3.2 Transient receptor potential channel

There are a number of channels that can conduct Ca^{2+} into cells in response to store depletion; generally named as SOCEs. The transient receptor potential channel (TRP) is a gene product in *Drosophila* photoreceptors (Hardie and Minke, 1993) which includes seven related subfamilies: TRPC, TRPM, TRPV, TRPA, TRPP, TRPML, and TRPN (Estacion *et al.,* 2001; Hardie, 2007). Initially, the canonical (TRPC) was regarded as the best candidate for SOCE, because TRPC show a remarkable similarity to the voltage-gated Ca^{2+} channels, except it lacks the voltage sensor of the latter (Machaty *et al.,* 2002a). TRP was previously described only in non-mammalian species; however it has been expressed in mammals as well (Machaty *et al.,* 2002a&b). Hence, after the cloning of the *Drosophila* TRP gene it has been reported that the TRP homologues are expressed in mammals such as mouse where six related TRP genes identified (Zhu *et al.,* 1996), They are also found in rat (Funayama *et al.,* 1996) and human tissue such as ovary, testis, heart, and brain (Zhu *et al.,* 1995). Emptying internal stores either by IP_3 or thapsigargin has been shown to have a triggering effect on some TRPC channel proteins like TRPC1, TRPC4 and TRPC5 which all share a similar structure and function as store-operated channels (Strubing *et al.,* 2001; Beech,

2007; Hong-Tao Ma *et al.,* 2008). In contrast, TRPC3 which acts as a Ca^{2+}-activated non-selective cation channel, TRPC6, and TRPC7 appear to be activated primarily by DAG rather than store depletion (Hofmann *et al.,* 1999). In addition to Ca^{2+} permeability, the TRPC channels can also conduct a divalent cations such Sr^{2+} and Ba^{2+}. However, TRPC channel proteins appear dissimilar to CRAC electrophysiologicaly because CRAC channels have a lower conductance and are more specific than the TRP channels (Plant and Schaefer, 2003; Berridge, 1995).

Interestingly, some studies show there might be a relation between TRPC channels and STIM1 and Orai1, which facilitate Ca^{2+} influx by modifying channel properties (Yuan *et al.,* 2007). For example, STIM1 interacts with TRPC1, TRPC4, and TRPC5 and regulates their activities (Yuan *et al.,* 2007). Furthermore, STIM1 with both TRPC1 and Orai1 localised together and regulates SOCE. More surprisingly, the co-expression of Orai1 with TRPC3 and TRPC6 appears to change TRPC3 and TRPC6 function from receptor-activated, non-store dependent channels to a store-dependent manner (Liao *et al.,* 2007). The over-expression or knock down of TRPC, Orai1, and STIM1, studies have shown that TRPC5 supports a higher influx of Sr^{2+} than Ca^{2+} (Hong-Tao Ma *et al.,* 2008). In the contrary, a recent report has revealed no effect of either STIM1 or Orai1 on TRPC channels function (DeHaven *et al.,* 2009).

The expression of TRP homologue in mammalian eggs has been reported. It was shown that the cDNA TRP sequences from pig eggs are 92.0% and 96.2% homolgous to mouse and human TRP sequences respectively (Machaty *et al.,* 2002a).

1.3.3 Ca^{2+}/Na^{+} exchange (NCX)

There are three genes have been identified in mammals that code for the Na^{+}/Ca^{2+} exchangers (NCX1, NCX2, and NCX3). However, the dominant in mammals is the NCX1 gene. All have similar sequential and functional properties: they have intracellular and extracellular surfaces with a relatively higher intracellular Ca^{2+} binding affinity (Machaty *et al.,* 2002a). The NCX is driven by electrochemical gradient of Na^{+} across the PM (Carroll, 2000). The NCX is an ion transport protein located in plasma membrane, and it acts by translocating Ca^{2+} ions across PM in an exchange process of 3 Na^{+} ions for one Ca^{2+} ion (Philipson and Nicoll, 1992; Carroll, 2000; Machaty *et al.,* 2002a). The continuation of this exchange process leads to the accumulation of the positive charges within the egg. This leads to the initiation of the efflux of Na^{+} by Na^{+}/K^{+} pump and activation of the reverse action of NCX (Fig. 1.14).

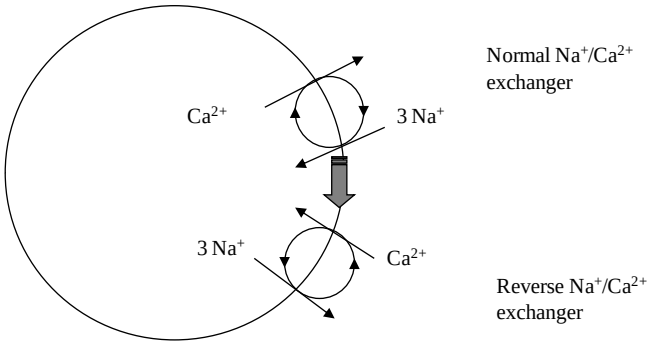


Figure 1.14 Schematic diagram show the direction of action of both normal and reverse modes of Na^{+}/Ca^{2+} exchanger. The big arrow indicates the switch from forward to the reverse mode.

In normal circumstances, the usual function of the NCX is to expel Ca^{2+} out and introduce Na^{+} in. However, in different conditions such as decreased extracellular Na^{+} or increased intracellular Na^{+}, a reverse mode of action occurs, i.e. the NCX brings

Ca^{2+} into the cell rather than extruding it from the cell (Blaustein & Lederer, 1999). This takes place when the exchanger reversal potential is similar to the resting membrane potential (Carroll, 2000). The reversal potential is the value of membrane potential at which the NCX is reverses its action to the reverse mode.

The NCX appears to expressed in the PM in a variety of animal cells including oocytes such as in hamster eggs in which the recovery of a Ca^{2+}-dependent hyperpolarization was delayed in Na^{+} free medium (Igusa and Miyazaki, 1983), and mouse eggs (Pepperell *et al.,* 1999) and in porcine eggs (Machaty *et al.,* 2002b).

In mouse and pig eggs functional evidence for NCX comes from studies which show Ca^{2+} extrusion is induced when Na^{+} was made rapidly available to the egg, after depletion, which means that NCX is activated. This concludes that NCX protein might contribute largely in Ca^{2+}-haemostasis during the fertilization process (Pepperell *et al.,* 1999; Machaty *et al.,* 2002a).

The NCX is driven by a Na^{+} gradient and based on previous study we can calculate the reversal potential of the exchanger precisely using this equation (Carroll, 2000)

(1) $E_R = (nE\mathrm{Na}^{+} - 2E\mathrm{Ca}^{2+}) /(n - 2)$

(2) $E\mathrm{Na}^{+} = 61 \log [\mathrm{Na}^{+}]_{out} /[\mathrm{Na}^{+}]_{in}$ & (3) $E\mathrm{Ca}^{2+} = (61/2) \log [\mathrm{Ca}^{2+}]_{out} /[\mathrm{Ca}^{2+}]_{in}$

E_R is the reversal potential, $n = 3$ (number of Na^{+} ions exchanged for each Ca^{2+} ion)

Both 2 and 3 were calculated assuming a temperature of 37°C

Replacing the known values for $[Na^{+}]_{in}$ = 8 mmol $l0^{-1}$, $[Na^{+}]_{out}$ =120 mmol $l0^{-1}$, $[Ca^{2+}]_{in}$ = 100 nmol $l0^{-1}$ and $[Ca^{2+}]_{out}$ = 1.7 mmol $l0^{-1}$ into equations (2) and (3) and then into (1), the E_R results will be – 40 mV.

Although, the (– 40mV) value of E_R is an estimated value, it is quite close to the oocytes resting membrane potential in mouse (Carroll, 2000) and in human eggs~ (-30 mV) (Feichtinger *et al.,* 1988). This suggests that the NCX could be close to working in either direction in mammalian eggs.

The $[Na^+]_{in}$ value is the critical value that E_R depends on, because in the literature it ranges from 4 to 12 mmol l^{-1}, which were taken from different observations in a variety of cell types (Sonn and Lee, 1988; Johnson *et al*., 1991). Similar to the TRPC channel, it has been observed in cardiac myocytes studies that NCX can transport Sr^{2+} out of and into the cytosol (Sheu and Blaustein, 1986; Niggli, 1989).

1.3.4 Ca^{2+}-channels on endoplasmic reticulum

There are several Ca^{2+} mediated channels in the endoplasmic reticulum (ER) membrane have been identified such as the inositol trisphosphate receptor (IP_3Rs) and ryanodine receptors in mammalian eggs (Miyazaki, 1991). The major Ca^{2+} release channel in all eggs studied to date is the IP_3R and its activity depends on the IP_3 production by PLC. IP_3R act as a receptor to bind IP_3 as well as a Ca^{2+} releasing channel. The binding of the IP_3 to the IP_3Rs is an early event in the fertilization process that triggers a conformational change in the IP_3Rs that allows Ca^{2+} to be released from the ER lumen and enter into the egg cytosol (Leguia and Wessel, 2007). The role of the IP_3 pathway in Ca^{2+} release has been examined by microinjecting IP_3 into the egg that showing the induction of Ca^{2+} release (Swann and Whitaker, 1986; Miyazaki, 1988; Wu

et al., 1997). On the other hand, injecting IP_3 antagonist or IP_3R antibody inhibits Ca^{2+} release at fertilization (Miyazaki *et al.,* 1992; Miyazaki *et al.,* 1993).

The other type of Ca^{2+} release channel that specifically triggered by ryanodine, hence named ryanodine receptors (RyRs) has also been identified in mammalian cells (Lai *et al.,* 1989). There are several other small molecules, such as nitric oxide (NO), cGMP and cyclic ADP ribose (cADPR) that have been shown to some extent to have the ability to release Ca^{2+} from the ER mediated by other Ca^{2+} releasing channels that are also located in the ER membrane. Protein kinase G (PKG) is proposed to phosphorylate and activate nitric oxide synthases (NOS) which converts the L-arginine amino acid into NO (Leguia and Wessel, 2007). The latter triggers the RyRs on ER membrane to release Ca^{2+} (Fig. 1.15). The NO pathway seems to be necessary and sufficient to triggers Ca^{2+} release in sea urchins egg (Kuo *et al.,* 2000) but not in vertebrates which include mammals (Hyslop *et al.,* 2001). Although, some reports show the existence of RyRs in mouse eggs (Swann, 1992). However, their role in Ca^{2+} oscillations triggered by fertilization is minimal. It could be because that the NO pathway is a late event in fertilization process (Leckie *et al.,* 2003).

1.3.5 Intracellular Ca^{2+}-homeostasis

In mammalian fertilisation, the Ca^{2+}-transients continue for 3 - 4 hours, and stop by the pronuclei stage (Marangos *et al.,* 2003; Dumollard *et al.,* 2004). The Ca^{2+} releases from the ER that cause cytosolic Ca^{2+} increases are either pumped back into the ER by sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs) or extruded out of the egg by

plasma membrane Ca^{2+} ATPases (PMCAs) (Carroll, 2001) (Figure 1.15). In mammalian eggs and embryos, the most prominent organelles are the mitochondria, ER and CG (Sathananthan and Trounson, 2000). The key regulator of intracellular Ca^{2+} homeostasis during fertilization, egg activation and early development is the ER. There are structural junctions between ER and mitochondria in mouse, hamster (Yang *et al.,* 1989) and human eggs (Motta *et al.,* 2000) which may suggest some functional association in Ca^{2+} signalling between them.

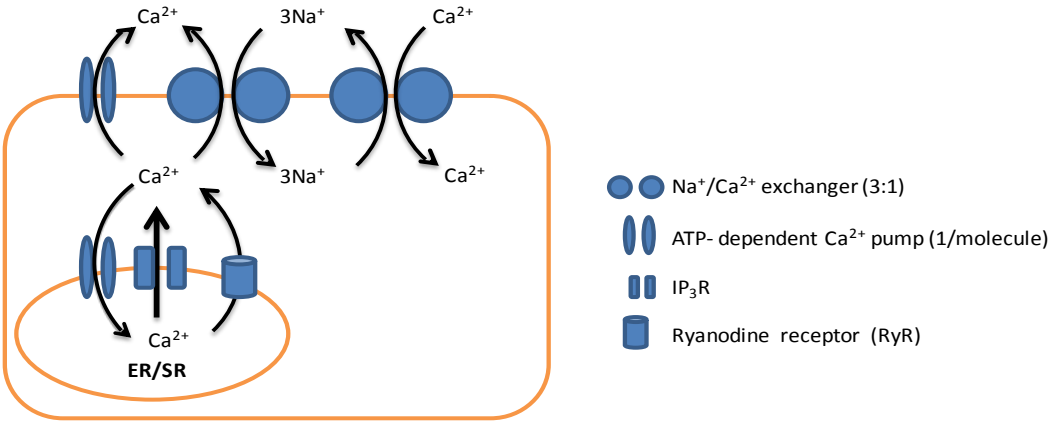


Figure 1.15 This is an illustrative diagram showing how cells/eggs precisely maintain its Ca^{2+}-homeostasis using different kinds of Ca^{2+}-channels/pumps.

Egg derived immature mitochondria are the precursors of mitochondria in all cell types in mammals (Carroll, 2000). Furthermore, any disruption of Ca^{2+} signalling due to dysfunctional mitochondria ends with apoptosis i.e. the mitochondria protects eggs from persistent rise in Ca^{2+} which lead to death (Liu *et al.,* 2001). Mammalian eggs show low metabolic rates and after fertilization as eggs develop the metabolic rate increases and mitochondria progress to maturity (Downs and Utecht, 1999) with changes in its

morphology to form cristae to cope with the increasing ATP requirement and oxygen consumption (Liu *et al.,* 2001).

1.3.6 IP_3 production

Cell membrane has a different forms of phospholipids such as phosphoinositide PI(3,4,5)P3 (PIP_3) and phosphatidylinositol (4,5)-bisphosphate (PIP_2). The former is the phosphorylation product of PIP_2 by the class I phosphoinositide 3-kinases (PI 3-kinases) (Whitman *et al.,* 1988). During the resting state in cells, the level of PIP_3 is undetectable. However, it produced immediately upon cell stimulation. In contrast PIP_2 level is relatively high in resting cells which counters 0.5 -1% from the total phospholipids. The importance of PIP_2 comes from the changes in the PIP_2 level by hydrolysis and synthesis in restricted areas (Fukami *et al.,* 2010).

There are two main types of Ca^{2+} release channels on the ER membrane. Ca^{2+} release is blocked by the inhibition of these channels IP_3Rs and RyRs (Galione *et al.,* 1993). In mammals, Ca^{2+}-oscillations are mediated by IP_3 induced calcium release (IICR) via the activation of IP_3 receptors on the ER (Miyazaki *et al.,* 1993; Carroll, 2001), which is known to be a major source for Ca^{2+} in mammalian eggs (Kline, 2000). The contribution from the extracellular Ca^{2+} during oscillations is probably to refill the IP_3-sensitive Ca^{2+} stores (ER) (Igusa and Miyazaki, 1983; Kline and Kline, 1992; Shirakawa and Miyazaki, 1995). Before the novel discovery of PLCζ it was unclear exactly how the sperm triggers IP_3 production in eggs in order to release Ca^{2+} (Saunders *et al.,* 2002; Yoda *et al.,* 2004). We now think that during fertilisation and just after sperm-egg fusion, the sperm

releases its content that contains PLC. The latter hydrolyse PIP_2 to generate IP_3 and diacyl glycerol (DAG). Raised levels of IP_3 stimulate IP_3Rs and Ca^{2+}-oscillations start. In fertilized mammalian oocytes, repetitive Ca^{2+} release from the ER causes Ca^{2+}-oscillations (Miyazaki *et al.,* 1992, Miyazaki *et al.,* 1993; Shiraishi *et al.,* 1995). The released Ca^{2+} enhances the PLC activity (Swann *et al.,* 1994), which in turn hydrolyses more PIP_2 to generate extra IP_3 within the egg which triggers IP_3R and release more Ca^{2+} and this cycle repeated for several hours (Whitaker *et al.,* 1993; Swann *et al.,* 1994; Fissore *et al.,* 1998; Swann *et al.,* 1999; Coward *et al.,* 2003).

IP_3Rs down-regulation can be achieved by injection adenophostin A or B, which are potent non-metabolizable fungal product (Shiraishi *et al.,* 1995) and agonists of IP_3Rs (Takahashi *et al.,* 1993) with higher binding affinity to IP_3Rs than IP_3. Such down-regulation has shown inhibition of Ca^{2+} release in mouse eggs at fertilization (Brind *et al.,* 2000; Jellerette *et al.,* 2000), including antibodies against IP_3Rs (Miyazaki *et al.,* 1992; Miyazaki *et al.,* 1993).

1.4 Overview on Intracellular Ca^{2+} changes detection

Today we know that Ca^{2+} signals is the key element in egg activation and embryo development (Stricker, 1999; Swann *et al.,* 1999). Ca^{2+} is a key factor for maintenance of long lasting Ca^{2+}-oscillations. Ca^{2+} is the main second messenger that controls the activation events (Miyazaki *et al.,* 2006). However, it is worth reflecting upon the historical back ground of Ca^{2+} measurement in relation to egg activation and development.

In the second decade of 20th century a US researcher team led by Jacques Loeb came up with the concept that, during fertilization, the sperm introduces a signal into egg and that this message may regarded as the key stimulus for egg activation and early embryo development (Sardet *et al.,* 2006). Additionally, this team was the first who has discovered the process of artificial activation of sea urchin eggs (parthenogenic activation) i.e. without sperm involvement, by modifying the chemical components of the culturing media (sea water) or by exposing eggs to ultraviolet rays (Loeb, 1914). Between 1930 and early 1950s it was believed that Ca^{2+} ions play an important role in cell regulation processes in living bodies (Epel *et al.,* 1998). Some tried to establish this idea experimentally, and it was Daniel Mazia who carried out most of his research on fertilization in sea urchins. He measured the differences in Ca^{2+} content between the extracts of fertilized and unfertilized eggs and found that that Ca^{2+} increases by 10 -100 fold in fertilized eggs (Mazia, 1937).

After the Second World War a group of scientists discovered a new chemiluminescent protein named aequorin (Shimomura *et al.,* 1963). Aequorin is the first photoprotein discovered that is Ca^{2+} sensitive and emits blue light (~ 480nm) in the presence of Ca^{2+} ions, even in the absence of oxygen. Later during the extraction process of the aequorin from glowing Jelly fish via the column chromatography, another green fluorescent protein was eluted before aequorin, which then purified and named green fluorescent protein (GFP) (Shimomura *et al.,* 1963).

This started a new era in the monitoring of Ca^{2+} changes within cells by relaying on its sensitivity to aequorin. Using aequorin as a Ca^{2+} indicator in 1967 it was experimentally

proven that Ca^{2+} is involved directly in the muscles contraction process. It was shown that the free Ca^{2+} levels rises in muscle cells just before the force increases and Ca^{2+} falls just before the force declines (Ridgway and Ashley, 1967; Ridgway and Gordon, 1984; Shimomura, 2006).

1.4.1 Fluorescent Ca^{2+}-dyes

Using aequorin requires specialist equipment, so in the early 1980s a newer generation of Ca^{2+} indicators (fluorescent dyes) were introduced such as quin-2, Fura-2 and indo-1. These were developed from the Ca^{2+} chelator BAPTA (fluorescent 1,2-bis-(2-aminophenoxy)-*N,N,N',N'*-tetraacetic acid) (Cobbold and Rink, 1987). Henceforth, Ca^{2+} oscillations began to be observed in all other species studied (Jaffe, 1985; Stricker, 1999) including plants (Dumas *et al.,* 2006).

With the introduction of the fluorescent dyes the intracellular free Ca^{2+} changes become relatively easy to measure, however these dyes were hydrophilic and did not cross the plasma membrane. To overcome this dilemma, a trick was used to turn them into lipophilic membrane permeable derivatives using acetoxymethyl (AM) esters. The complex is then cleaved by cytosolic esterases to release free dye in cytosol. However, this technique also faces a problem; for example, fura-2-AM accumulates in the intracellular organelles and can this give a false impression on Ca^{2+} levels (Cobbold and Rink, 1987). To get around this some of the Ca^{2+} indicators were conjugated to dextrans (long-chain polysaccharides of high molecular weight). These so called dextran-conjugated Ca^{2+} indicators appear particularly promising for Ca^{2+} measurements at

physiological temperatures for prolonged periods (Beierlein *et al.,* 2004). Currently, this class of indicators is widely used as the preferable Ca^{2+} dyes in large cells such as eggs. The ones I used in my Theses are oregon green bapta dextran (OGBD) (494-523nm) and Rhod Dextran (RhD) (550-580nm) and in only IVF experiments I used Fura-Red (488-660nm).

1.4.1.1 Measuring Ca^{2+} in eggs

It was impossible to monitor Ca^{2+}-oscillations in eggs until the availability of two good methods for Ca^{2+} measurement (calcium dyes and aequorin). Once scientists were able to measure cytoplasmic Ca^{2+} changes in eggs, they used aequorin to show that there is a large wave-like Ca^{2+} increase at fertilization in medaka fish eggs and (Ridgway *et al.,* 1977) and sea urchin eggs (Steinhardt *et al.,* 1977). Later aequorin was used show Ca^{2+}-oscillations in fertilizing mouse and hamster eggs (Miyazaki *et al.,* 1986). Later still fura2 and dextran derivatives were also used show Ca^{2+} increases at fertilization in sea urchin eggs, and Ca^{2+}-oscillations in fertilizing eggs of various mammalian species (Hafner *et al.,* 1988; Harrison *et al.,* 2002) respectively.

1.4.2 Bioluminescence (Chemiluminescence)

Bioluminescence is a natural process that occurs in living organisms in which they give off visible light from chemical energy. It is also a powerful *in vivo* imaging tool. The sensitivity ranges from 10nM to1mM with a very high efficient signal to noise detection level even with ~ 1-100 photons/sec. Bioluminescence has been used to monitor ATP (Dumollard *et al.,* 2004) and Ca^{2+} changes (Ridgway *et al.,* 1977) in cells and eggs.

1.4.2.1 The luciferase-luciferin system

Newton Harvey first discovered luciferin and an enzyme called luciferase from *Cypridina* (a small crustacean) (Harvey, 1926). He found that these two substances are produce strong bright blue luminescence (peak emission wavelength 482 nm) when mixed together in aqueous solution (Harvey, 1926) in an oxygen-dependent bioluminescent reaction (The luciferase-luciferin system). However, the luciferase is closely associated with green fluorescent protein (GFP). Therefore, in the presence of GFP a green light luminescence is released (peak emission wavelength 510 nm) (Shimomura, 1985). Luciferin has been extracted, purified, and crystallized from dried *Cypridina.* Similarly luciferase has also been extracted and purified (Shimomura, 2006).

The most common types of the living organisms that are bioluminescent are beetles e.g. click beetles and fireflies (Wilson and Hastings, 1998) that emit light flashes in a range of colours from green to yellow (550-580 nm) (Fig 1.16). Studies on North American firefly have shown that luciferase is located in organelles called peroxisomes in the photocytes that located on the ventral surface of the sixth and seventh abdominal segments (Wilson and Hastings, 1998).

There are several factors that augment luciferase-luciferin signals such as the pH , temperature (Seliger and Mc, 1960), ionic strength (Denburg and McElroy, 1970), protein concentration (Allue *et al.,* 1996) and chloride concentration (Ugarova *et al.,* 1981). On the other hand, luciferase-luciferin signals was also shown to be inhibited by high concentrations of oxyluciferin (Gandelman *et al.,* 1994). The optimal luciferin concentration in media has been suggested to be 100 μM for luminescent recordings

(Gandelman *et al.,* 1994). Firefly luciferase is a protein dimer of around 100 kDa. The luciferin-luciferase reaction takes place in 2 steps; first formation of adenosine monophosphate (AMP)-luciferin, followed by its oxidation to form oxyluciferin (excited state) (Fig. 1.16). Throughout my study I will use the luciferase developed from a North American firefly (*Photinus pyralis*) to measure the protein expression via a luciferin-luciferase reaction (Steghens *et al.,* 1998).

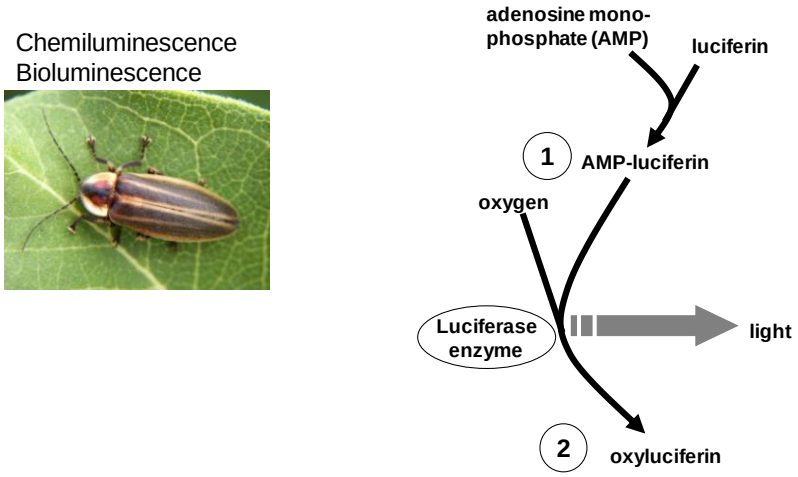


Figure 1.16 The luciferin-luciferase reaction. (Steghens *et al.,* 1998)

1.5 Signalling transduction mechanisms of Ca^{2+} release at fertilization in mammals

Along with the development of the ability for measuring cytosolic free Ca^{2+}, a few questions have come up concerning the source and the causative factor for this release at fertilization. Throughout the literature there have been three hypotheses concerning this issue. In the following paragraphs I will concentrate on these theories and discuss aspects that confirm or contradict each idea separately, and draw comparisons between different species where ever possible.

1.5.1 The Ca^{2+} influx theory

This theory is based on the principle of Calcium induced calcium release (CICR) whereby injecting a relatively small amount of Ca^{2+} into egg can trigger greater Ca^{2+} release. This theory describes a mechanism by which sperm plays a role as a conduit allowing an influx of extracellular Ca^{2+} into the egg through sperm membrane pores at sperm-egg fusion (Jaffe, 1991; Runft *et al.,* 2002), see (Fig. 1.17A). This idea is supported by the finding that taking fertilized oscillating eggs and incubating them in Ca^{2+} free media stops the oscillations. They can then be restarted by regular sustained Ca^{2+} injections (Igusa *et al.,* 1981). Further support comes from the finding that sperm egg fusion occurs before the Ca^{2+}-oscillations start (Lawrence *et al.,* 1997). According to Jaffe, in mammals and in other species, sperm pick up Ca^{2+} in the pre-fertilization period then introduced it after sperm egg fusion and this Ca^{2+} act as a trigger stimulus for internal Ca^{2+} store release (Jaffe, 1983). However, in mammals injection of Ca^{2+} only cause a single transient and never induces oscillations (Swann and Ozil, 1994). Furthermore, by injecting eggs with Ca^{2+} repeatedly it was shown that the oscillations pattern that looks different from normal fertilization (Igusa *et al.,* 1981; Swann *et al.,* 1994). In addition this idea was ruled out by the evidence that showed no localized increase of Ca^{2+} near the site of sperm-egg fusion in the mouse (Jones *et al.,* 1998; Runft *et al.,* 2002). Therefore, any Ca^{2+} increases after sperm-egg fusion do not seem to be a result of increased plasma membrane permeability to Ca^{2+}.

1.5.2 Sperm receptor model

This theory followes the principle of the hormone receptor system, in which a hormone binds to receptors on the plasma membrane, In this model, the sperm acts as a ligand that during fertilization firstly adheres to the plasma membrane via an egg membrane receptor (Foltz and Shilling, 1993; Schultz and Kopf, 1995). Following this model the sperm acts in the way of somatic cells that lead to the generation of IP_3. Since sperm-egg binding is mediated by surface proteins, the presence of some coupling protein such as guanosine tri-phosphate (GTP) binding proteins (G protein) in the egg cytoplasm, could be the link between the sperm-egg receptor structure and intracellular signalling, see (Fig. 1.17B).

Subsequently the receptors stimulate an egg specific phosphotidylinositol phospholipase C (PI-PLC) that, in turn, cleaves the lipid PIP_2 and generates IP_3 (Swann *et al.,* 1994; Rhee, 2001; Miyazaki *et al.,* 2006) and diacylglycerol (DAG) (Rhee, 2001) which activates protein kinase C (Miyazaki, 1991). There are two isoforms of PI-PLC have been postulated to play a role or involved in this process, one is PI-PLCγ which couples via tyrosine kinases (Swann *et al.,* 2002) and the other is PI-PLCβ which attaches to membrane receptors via G proteins.,

This model was supported by the finding that the injection of nonhydrolyzable GTP analogs to trigger G proteins has been shown to cause transient Ca^{2+} release in hamster eggs (Miyazaki *et al.,* 1993; Swann *et al.,* 2002). This idea also appears to have support from experiments in which a constant injection of IP_3 gives rise to Ca^{2+}-oscillations (Swann *et al.* 1990; Swann 1994; Miyazaki *et al.,* 2006). Inhibiting G

proteins by non-hydrolyzable GDP analogue also blocks Ca^{2+} release during normal fertilization in hamster eggs (Miyazaki, 1988; Swann *et al.,* 2002). However, subsequent injection of IP_3 showed no effect of GDP analogue on Ca^{2+} release (Miyazaki, 1988). Further, blocking IP_3 receptor using monoclonal antibody injection show an inhibitory effect on sperm induced Ca^{2+}-oscillations (Miyazaki *et al.,* 1992). This finding confirms that IP_3 receptor is certainly plays an important role in Ca^{2+} release during fertilization process in mammals (Swann *et al.,* 1994). In addition, Fissore and his colleagues have studied the importance on IP_3 in Ca^{2+} release using different kind of mammalian eggs. In rabbit and cow eggs they used heparin as a competitive inhibitor of IP_3 and have reported the inhibition of Ca^{2+}-oscillation at fertilization (Fissore *et al.,* 95; Leguia *et al.,* 2007).

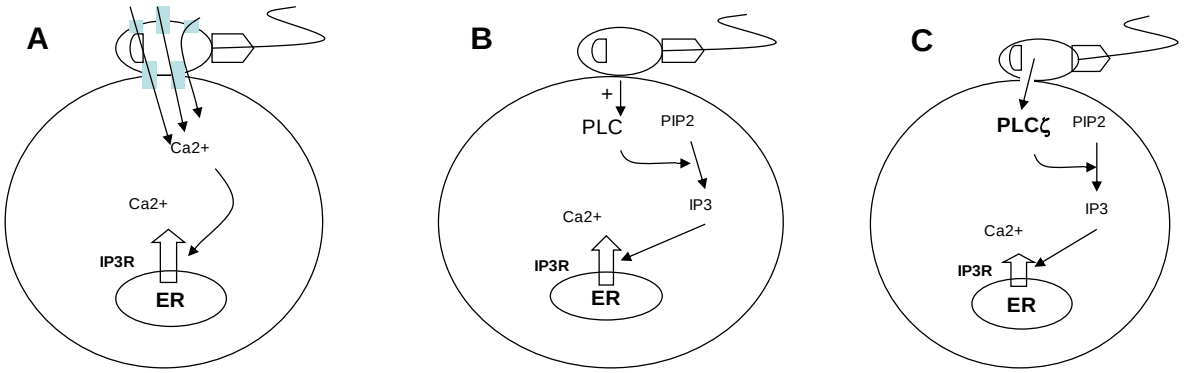
Although this idea of a G protein receptor seems plausible, it has been reported that there are some anomalies. For instance, Ca^{2+}-oscillations during normal fertilization of hamster eggs show variable frequencies, with no damping of oscillation amplitude over a period of more than 2hrs (Miyazaki, 1991). In contrast, the pattern after injecting GTP analogs, or IP_3, are always of high frequency and are always critically damped oscillations (Swann *et al.,* 1994; Swann *et al.,* 1999). In addition, the production of DAG together with IP_3 production as a result of phospholipase C activation by G protein leads to protein kinase C activation (Leguia *et al.,* 2007). And activating protein kinase C by phorbol ester was found to cause complete inhibition of Ca^{2+}-oscillations that mediated by G protein and vice versa with the inhibition of protein kinase C using sphingosine. In

contrast treating eggs with phorbol ester at fertilization show no effect on Ca^{2+}-oscillations.

The most convincing proof against the receptor theory is represented by the invention of intra-cytoplasmic sperm injection (ICSI), in which injecting a single sperm into the egg's cytoplasm lead to Ca^{2+}-oscillations and successful egg activation and live birth (Kimura and Yanagimachi, 1995; Stricker, 1999). From this point of view it can be claimed that Ca^{2+} release during normal fertilization in mammals is not consistent with sperm receptor models.

1.5.2.1 Sperm factor hypothesis

This is the most recent theory, and now seems most likely explanation for Ca^{2+} release in mammalian eggs. It proposes that following sperm-egg fusion the sperm introduces a Ca^{2+} releasing substance(s) (or soluble cytosolic factor) into the egg (Swann, 1990; Fissore *et al.,* 1998), see (Fig. 1.17C). In mammalian eggs, this idea has been studied considerably in the last two decades (Runft *et al.,* 2002). This model is consistent with data showing that sperm-egg plasma membranes and cytoplasm continuity is the prerequistic step for Ca^{2+} release in normal fertilisation (Miyazaki *et al.,* 2006; Lawrence *et al.* 1997). Furthermore, using dye transfer studies that enable to determine the time of sperm-egg fusion together with simultaneous Ca^{2+} monitoring in *in vitro* fertilization, show that sperm-egg fusion proceeds initial rise in Ca^{2+} by 1–5 min (Lawrence *et al.,* 1997; Jones *et al.,* 1998).



Ca^{2+} influx theory Sperm receptor model Sperm factor theory

Figure 1.17 Schematic illustrations of different theories of Ca^{2+} release.

This suggesting that sperm factor hypothesis describes a mechanism by which a sperm could introduce a factor into ooplasm that responsible for Ca^{2+} release and induce activation during fertilisation (Swann, 1990; Swann and Ozil, 1994).

The first clear evidence for this hypothesis came after the injection of sperm extracts from hamster or boar into hamster eggs was shown to induce Ca^{2+}-oscillations similar to the fertilization pattern (Swann, 1990). Since then, other research groups have confirmed this finding with other mammalian species (Swann, 1994; Homa and Swann, 1994; Sousa *et al.,* 1996; Palermo *et al.,* 1997; Sakurai *et al.,* 1999).

With the invention of intracytoplasmic sperm injection (ICSI) in early 1990s there was further strong evidence to the soluble factor hypothesis. It was shown that injection of one sperm into oocyte can cause Ca^{2+}-oscillations mimicking to those shown in normal fertilization (Stricker, 1999; Swann *et al.,* 1999). It was concluded that the soluble factor content of a single sperm is enough to maintain Ca^{2+}-oscillation for the period of the physiological range. Further evidence which firmly stands with this hypothesis has been

noticed by (Lawrence *et al.,* 1997) after carrying out IVF in mouse and they used confocal microscopy to show the egg and sperm cytoplasmic continuity with two independent fluorescence methods to monitor Ca^{2+} and found that the fusion precedes the onset of the first Ca^{2+} spike in mouse eggs. Moreover, they estimated the time elapsed after sperm-egg fusion to detect the first Ca^{2+} spike by several minutes (1-3) (Lawrence *et al.,* 1997; Miyazaki *et al.,* 2006). This information further suggested that sperm egg-fusion is necessary for normal fertilization and did not require the stimulation of egg plasma membrane receptors (Lawrence *et al.,* 1997; Leguia and Wessel, 2007).

In mammals, the sperm factor tends to be non-species specific (Swann, 1990; Wu *et al.,* 1997). Sperm extracts from different species including human have shown the ability to trigger Ca^{2+}-oscillations and egg activation in other species such as mouse eggs (Swann *et al.,* 1994; Fissore *et al.,* 1998; Swann *et al.,* 1999; Parrington *et al.,* 1999; Coward *et al.,* 2003). Pig sperm extracts have also been reported to trigger Ca^{2+} transients in mouse and cow eggs (Wu *et al.,* 1997). Fish sperm extracts are also able to trigger Ca^{2+}-oscillations in mouse eggs (Coward *et al.,* 2003). Further evidence includes the ability of sperm extracts from non-mammalian such as chicken and frogs can cause Ca^{2+}-oscillations in mouse and cow eggs (Dong *et al.,* 2000). It can also been shown that sperm extracts cause Ca^{2+}-oscillations in cells such as hepatocytes (Swann *et al.,* 2002). Furthermore, the injection of the sperm factor from flowering plants into mammalian eggs showed Ca^{2+}-oscillations (Li *et al.,* 2001). Sperm extracts, besides causing Ca^{2+} release, also cause full egg activation as indicated by cortical granule exocytosis, protrusion of the second polar body, pronuclear formation, and cell

cleavage (Meng and Wolf, 1997; Wu *et al.,* 1998; Sakurai *et al.,* 1999; Gordo *et al.,* 2000), and they trigger embryo development to up to the blastocyst stage (Fissore *et al.,* 1998). Importantly adding sperm extracts onto the surface of eggs has no effect what so ever (Swann, 1990). This further suggested that sperm factor needs to cross the membrane, and clearly this would not happens unless either one of these took place; gametes' membrane fusion or direct injection of sperm extracts.

1.5.2.2 Search for the sperm factor

In the past decade, progress has been made in identifying sperm factor related molecules. The current evidence suggests that Ca^{2+} release in mammalian eggs is by a mechanism in which an activating factor introduced from the sperm cytoplasm into the egg cytoplasm after sperm-egg fusion (Lawrence *et al.,* 1997). Several sperm proteins have been proposed as sperm factor molecules (Yoon *et al.,* 2008). Two major issues need to be resolved. First, the identification of a potential factor; and second, whether it is the physiological egg activator or not?

This factor was first shown to be protein in nature (Swann, 1990; Fissore *et al.,* 1998). This is because its activity is abolished after protease treatment or heating (Swann, 1990; Wu *et al.,* 1997; Swann *et al.,* 2002). According to Perry *et al.,* (1999) the sperm factor is a sperm oocyte activating factor (SOAF) and found to be located in spermatic perinuclear area. With detection of c-kit in mouse sperm, SOAF was proposed to be a truncated version of c-kit named tr-kit (Setti *et al.,* 1998). Additionally, mouse eggs undergo activation events when injected with extracts from cells expressing recombinant tr-kit. This activation ability of tr-kit has been reported to be blocked using

antibody against PLCγ and/or injection of SH3 domain of PLCγ, suggesting the role of PLCγ in activation process (Setti *et al.,* 1998). However, injecting these SH3 domain constructs into normally oscillating eggs show they have no effect on Ca^{2+}-oscillations. This finding was used to rule out the proposed identity of tr-kit as a physiological sperm factor. It is also noted that the injection of tr-kit has never been shown to cause any Ca^{2+}-oscillations.

It has been claimed that IP_3 is the sperm factor, or least a primary initiator of intracellular Ca^{2+} release at fertilization (Jaffe *et al.,* 2001). However, this is not consistent with findings in mammals where the sperm factor is clearly a protein of some form. However, injecting eggs with sperm extracts has been shown to cause a rise in the IP_3 levels, which suggests that sperm factor perform its action through IP_3 pathway (Wu *et al.,* 2001; Leguia and Wessel, 2007).

Within mammals, the sperm factor theory appears to be consistent across species. It has been reported that sperm extracts has phospholipase C (PLC) activity, suggesting that sperm factor is a form or subtype of PLC (Swann *et al.,* 1999). Hence, the differences between this theory and the sperm receptor model may be mainly regarding the source of PLC, and the way of sperm-egg interaction. This theory argues that PLC comes from directly from the sperm; meanwhile, sperm model claims that it is an egg source of PLC. The other difference is that in the former theory, is that the sperm attaches to the egg plasma membrane receptor to activates the egg's PLC, whereas in the sperm factor model the sperm plasma membrane fuses with the egg via a receptor and then delivers its PLC into the egg cytoplasm to trigger Ca^{2+} release.

The sperm factor hypothesis was hypothesized depending on three observations. First, the Ca^{2+}-oscillations in mouse start several minutes after sperm-egg fusion (Lawrence *et al.,* 1997). This suggests that fusion is a prelude to Ca^{2+} release. Second, with the novel discovery of ICSI, the normal Ca^{2+}-oscillations and egg activation are possible using ICSI techniques which bypass sperm-egg interactions at the plasma membrane (Kimura and Yanagimachi, 1995; Palermo *et al.,* 1996; Nakano *et al.,* 1997; Lawrence *et al.,* 1997). Finally, injecting the sperm extracts into eggs caused Ca^{2+}-oscillations and egg activation.

It has been suggested that the sperm extract activates eggs by the same pathway used at fertilization, since blocking of IP_3 receptor by antibody withholds Ca^{2+} release that was triggered by sperm extract in mouse eggs (Miyazaki *et al.,* 1992).

1.5.2.3 Discovering of PLCζ as a sperm factor

The sperm factor theory has now been adopted by many groups over the last few years as a result of the identification of PLCζ, which was identified as a new isoform of PLC with a size range of (~70 kDa) (Saunders *et al.,* 2002).

PLCζ was first discovered by the analysis of a mouse expressed sequence tags (EST) database from mouse testis. A gene for a PLC-related sequence was found and an antibody made against a peptide antigen (from the putative PLC sequence C terminus). Using Western blots this band was only found in testicular tissue, which suggests that the ~70 kDa protein that enriched in sperm is the new PLC (Saunders *et al.,* 2002). This new PLC was termed PLCζ. After the identification of the novel sperm-specific PLCζ in

mouse, a genomic search for sperm- and testis specific PLCs has been carried out and has characterized a PLCζ in human (Cox et *al.,* 2004), and other species (Saunders *et al.,* 2007).

Interestingly, during the fluorescent based assays using sea urchin egg homogenates, the same (~70 kDa) protein was found to co-migrate with Ca^{2+} releasing activity. This was in contrast to the results from previous studies on PLCβ, γ and δ isoforms that found no co-migration with Ca^{2+} releasing activity (Wu *et al.,* 2001; Parrington *et al.,* 2002). Additionally, the ~70 kDa PLC protein seems unrelated to the more recently discovered PLCε which has a molecular mass ~250 kDa (Song *et al.,* 2001; Parrington *et al.,* 2002). Further supporting evidence for this novel discovery comes from three lines. Firstly, the removal of PLCζ from sperm extracts caused a total loss of Ca^{2+} releasing activity in eggs. Secondly, injecting eggs with cRNA encoding for PLCζ caused Ca^{2+}-oscillations undistinguishable from those seen in normal fertilization. Finally, PLCζ has been reported to possess the ability to support embryonic development (Saunders *et al* 2002; Rogers *et al*., 2004; Swann and Yu, 2008). Much more recently, a research study reported that human sperm that lacks PLCζ neither trigger Ca^{2+} release nor induce the first step of embryo development in mouse eggs (Yoon *et al.,* 2008).

1.6 Phospholipase C zeta (PLCζ)

It has been widely accepted nowadays, at least in mammals, that PLCζ is the main candidate for the endogenous agent or the physiological egg activator at fertilization

(Cox *et al*., 2002; Swann *et al*., 2006). It has been shown that cRNA encoding human PLCζ (hPLCζ) can causes a long lasting series of Ca^{2+}-oscillations in mouse (Cox *et al*., 2002) and 'failed to fertilize' human eggs (Fig. 1.18) (Rogers *et al*., 2004). Moreover, it can also trigger oocyte activation which leads to enhanced embryo development to the blastocyst stage (Rogers *et al*., 2004). But before I go in the details of PLCζ, I will give a general overview on the PLCs family and subfamilies.

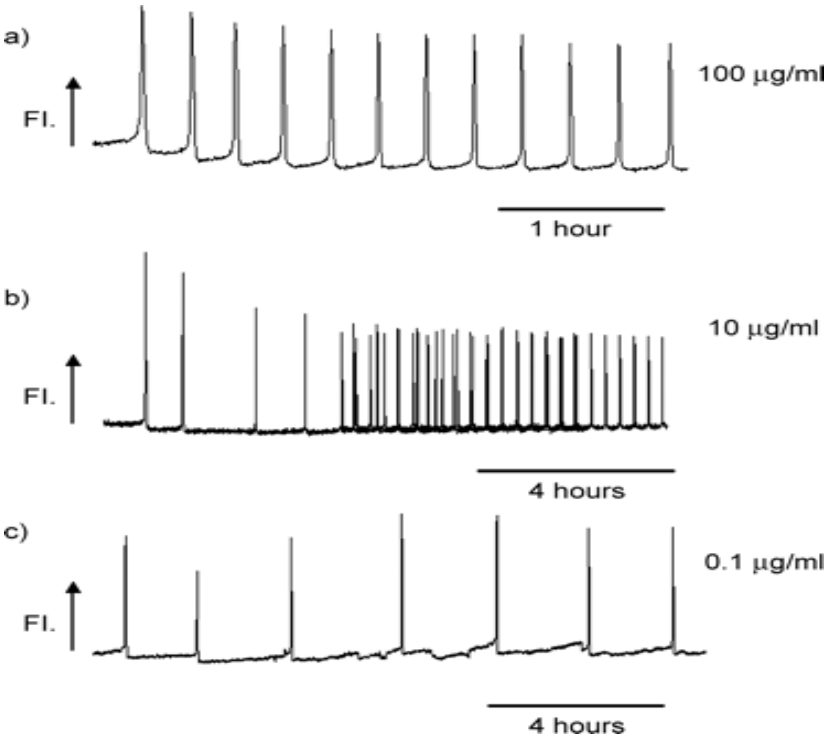


Figure 1.18 Ca^{2+}-oscillations in human oocytes triggered by different concentration of cRNA encoding PLCζ. (Rogers *et al.,* (2004) *Reproduction.*128 , 697-702).

1.6.1 Structure and function of PLCs in general.

At least thirteen PLC isoforms that have been discovered that are expressed in mammals. Based on their structure and regulatory activation mechanisms they have been classified into six different subfamilies, the δ (1, 3, 4), β (1, 2, 3, 4), γ (1, 2), η (1,

2), ε and more recently also ζ (Rhee, 2001; Hicks *et al.,* 2008). Since PLC is a soluble protein during the resting state it is usually distributed in the cytoplasm. However, upon cell activation it targets to the plasma membrane to hydrolyze PIP_2 which is a critical event for signal transduction (Fukami *et al.,* 2010). Each PLC isoform contains subtype-specific domains as well as conserved PLC domains (Leguia and Wessel, 2007; Fukami *et al.,* 2010), see (Fig. 1.19).

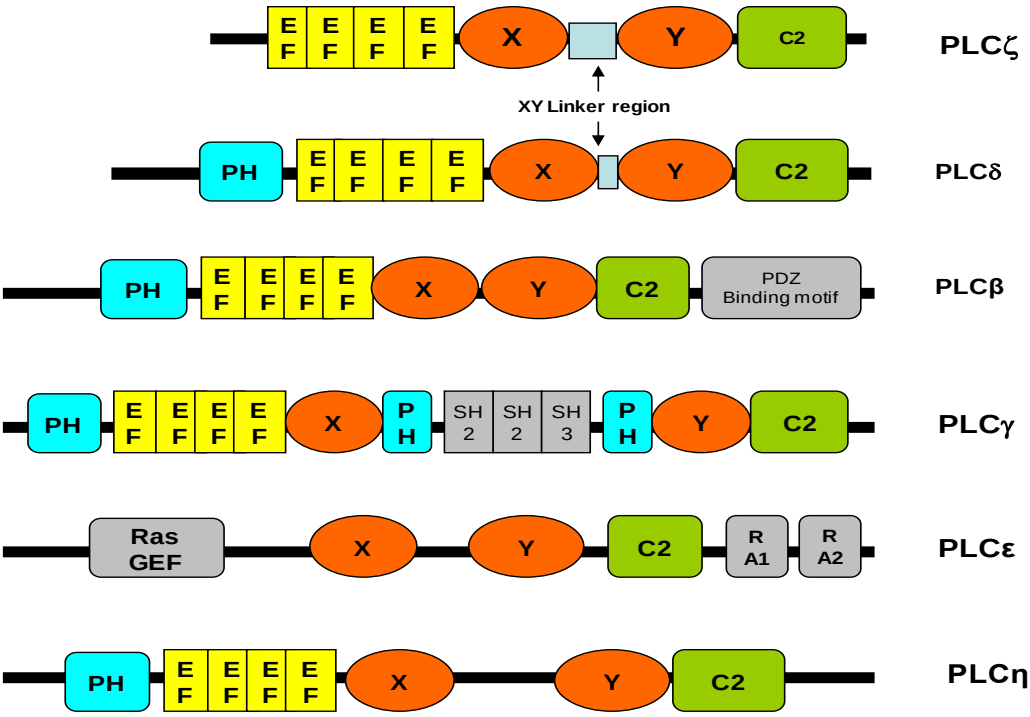


Figure 1.19 Schematic illustration of the domain structure of different PLCs. Catalytic and regulatory domains and their interacting molecules are shown. PH (pleckstrin homology domain), EF (EF-hand domain), X & Y (PLC catalytic domains), C2 (C2 domain), PDZ (PDZ-binding motif), SH (src homology domain), RasGEF (Ras-GTPase exchange factor-like domain) and RA (Ras-associating domains).

Although there is no high structural sequence homology between these isozymes, the amino acid sequence between similar domains of all isozymes is up to 50% homologous (Fukami *et al.,* 2010). All isozymes are composed of catalytic X and Y domains that come together to form the PIP_2 cleavage active site (X-Y domain), as well

as an EF-hand motif, and a C2 domain (constant region 2 of PKC) (Rhee, 2001). The C2 domain was first identified in protein kinase C. Most C2 domains bind Ca^{2+}. C2 domains have been also characterized as membrane-associating domains referring to their involvement in Ca^{2+}-dependent phospholipid interactions (Nalefski and Falke, 1996).

With the exception of PLCζ, all other PLCs proteins subfamilies have various combinations of regulatory domains including a pleckstrin homology (PH) domain and SH2 domains (Src homology 2) as in PLCγ (Rhee, 2001). PH domains in different PLCs are responsible for targeting to the membrane via polyphosphoinositides binding to PIP_2 or PIP_3 (Harlan *et al.,* 1994). SH2 domains bind to receptor protein tyrosine kinases and non-receptor tyrosine kinases like Src (Leguia and Wessel, 2007).

1.6.1.1 Regulation and activation of PLC isozymes

All PLCs are activated by specific stimulants and they generally have their own regulatory mechanisms (Rhee, 2001; Nomikos *et al.,* 2007). However, Ca^{2+} is needed in the activation of all PLC isoforms. Various regulatory mechanisms can be found within particular PLC subfamily. For example, Ras-related GTPases such as Rac activate PLCβ2, β3, and γ2, whereas PLCε is activated by Ras, Rap, and Rho (Hicks *et al.,* 2008). PLCβ subtypes are regulated differently by Gβγ and Gq, the components of the heterotrimeric G-proteins family (Offermanns *et al.,* 1997). Similarly, PLCη2 might be regulated also by Gβγ which is part of the G-protein coupled receptors since higher PLC activity has been reported with the co-expression of PLCη2 and Gβγ (Zhou *et al*. 2005). However, PLCη2 is very sensitive to Ca^{2+}; it is probably the only PLC that is equal to the

PLCζ Ca^{2+} sensitivity (Fukami *et al.,* 2010). PLCγ isozymes are activated by various receptor and non-receptor tyrosine kinases (Leguia and Wessel, 2007; Fukami *et al.,* 2010). In addition, The SH2 domains of PLCγ1 are necessary for the tyrosine auto-phosphorylation and activation (Fukami *et al.,* 2010). The detailed study on structural analysis of PLCε has revealed that the RA2 domain of PLCε binds to the Ras complex prior to the translocation and activation of PLCε on the membrane (Bunney *et al.,* 2006). Interestingly, both PLCδ and ζ are very sensitive to physiological Ca^{2+} levels, which might be part of their regulation and function. PLCδ in particular might be regulated by Ca^{2+} alone (Kim *et al.,* 1999). Its activation occurs in two steps, firstly PLCδ1 binds to the membrane via PH domain-PIP_2 interaction, followed by another interaction mediated by the C2 and catalytic domains of the PLCδ1 that expose the active site leading to PLCδ1 activation and eventually PIP_2 hydrolyzes (Lemmon *et al.,* 1997; Fukami *et al.,* 2010). In general PLCβ, γ and ε bind specifically to plasma membrane proteins in a protein-protein interaction, whereas PLCδ1 has PH domain that binds specificity with high affinity to the PIP_2 in a protein-lipid interaction (Nomikos *et al.,* 2007). On the other hand, a specific PLCζ activator is as yet unknown. The extreme sensitivity of PLCζ to Ca^{2+} even at the resting Ca^{2+} concentration might be sufficient for PLCζ activity in the absence of a stimulus (Saunders *et al.,* 2002).

1.6.1.2 PLC isozymes expression in mammalian tissues

The physiological function of many PLC isozymes is predicted by their tissue distributions. The expression of PLCβ isozymes is widely expressed in different mammalian tissue but mainly in the brain. On the other hand, PLCγ has been found

mainly in the brain and lungs (Suh *et al.,* 2008). While PLCδ isozymes are distributed more widely and abundantly in the brain, heart, lungs, skeletal muscle, and testes (Suh *et al.,* 2008), PLCε is highly expressed in the heart, lungs and kidneys (Song *et al.,* 2001). PLCη seems to be neuron-specific as it has been only observed in the brain tissue (Fukami *et al.,* 2010). Similarly, PLCζ seems to be sperm-specific as it has been found mainly in sperm, suggesting its specific role in sperm (Saunders *et al.,* 2002). However, it has been found with evidence of PLCζ in ovary of puffer fish (Coward *et al.,* 2011).

To understand the role of different PLCs in fertilization, several studies were carried out to identify these PLCs in the gametes and these studies have shown that in eggs both PLCβ and γ are expressed, while in sperm more isoforms have been identified such as PLCβ, γ, δ, and most recently ζ (Mehlmann *et al.,* 1998; Wang *et al.,* 1998; Wu *et al.,* 2001; Parrington *et al.,* 2002; Saunders *et al.,* 2002).

1.6.2 Structure and function of PLCζ

PLCζ is a very unstable protein *in vitro*, which is one reason why we inject cRNA encoding PLCζ to study its structure and function. PLCζ has the smallest size sequence among all other PLC isoforms that have been identified so far (Rhee, 2001) (Fig. 1.19). Mouse PLCζ composes of a sequence of 647 amino acids, with a molecular mass of 74 kDa that also includes the C-terminal peptide sequence (Saunders *et al.,* 2002). On the other hand, human PLCζ is even shorter with a sequence of 608 amino acids (Cox *et al.,* 2002). Interestingly, despite the fact that human PLCζ is the shortest PLCζ among

all other PLCζs of all species studied so far (Saunders *et al.,* 2007) it appears to be the most potent isoform in eggs. This enzyme protein is composing of four main domains (Fig. 1.20).

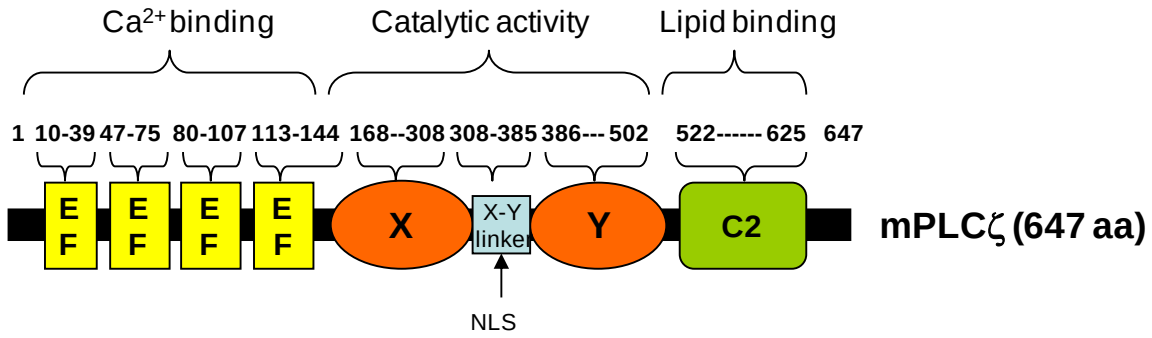


Figure 1.20 mPLCζ domains and amino acid compositions.

1.6.2.1 EF hands

There are four EF hand motifs at N terminus (Saunders *et al.,* 2002; Miyazaki and Ito, 2006). Many known proteins contain EF-hand motifs in their structure that are able to modify their conformation upon Ca^{2+} binding. Their structures are arranged in a helix-turn-helix motif. Structural analysis study of these motifs in PLCδ1 showed that they seem to be responsible for conformation changes rather than Ca^{2+} binding (Essen *et al.,* 1996; Saunders *et al.,* 2007). Previous report suggested that EF hand domains might act to target the membrane PIP_2 (Nomikos *et al.,* 2005). But a newer report suggested that PLCζ targets the intracelullar vesicular membrane (Yu *et al.,* 2012).

1.6.2.2 Catalytic domains

The catalytic X and Y domains of PLC is a barrel-like components consisting of α-helices and β-sheets in alternative patterns (Saunders *et al.,* 2007). The X and Y

catalytic domain are separated by a region named X-Y linker region. Significant sequence conservation is found in PLCζ -X and Y catalytic domains that make it distinct from other PLC isoforms (Saunders *et al.,* 2007).

1.6.2.3 X-Y linker region (XYl)

This linker region in PLCζ varies in length among species, where the longest is in the rhesus monkey, and the shortest in humans (Saunders *et al.,* 2007). The XYl region of PLCζ consists of 77aa residues extends from 308-385 of PLCζ sequence (Fig. 1.20). These regions are free of any predicted secondary structure. The XYl also shows the greatest variation between all PLC isoforms for a region connecting the catalytic domains. The most interesting sequence in this region is the nuclear localisation signal (NLS). The latter appears to be responsible for PLCζ localization into pronuclei during fertilization in mice (Swann *et al.,* 2006). To follow in this Theses I and my colleagues investigated the XYl region in further aspects toward PLCζ targeting and regulation.

1.6.2.4 C2 domain

C2 domains have been identified in a wide range of proteins including all isoforms of protein kinase C, phospholipase A and phospholipase C (Nomikos *et al.,* 2011b), and structurally they all are about 120 residues in length (Fig. 1.20), and are composed of a sandwich of two, four-stranded β-sheets. They are quite diverse in sequence which may explain the wide range of lipid specificity. However, these domains bind lipid in two ways, either Ca^{2+}-dependent or independent (Saunders *et al.,* 2007). The C2 domain in PLCζ seems to have a pivotal role in cell physiology since truncated PLCζ with a C2 domain deletion is unable to cause Ca^{2+}-oscillations in mouse eggs, despite preserving

its PLC activity *in vitro* (Nomikos *et al.,* 2005; Kouchi *et al.,* 2005). The screening of phosphoinositides for interaction with the PLCζ-C2 domain, through protein-lipid overlay assays, revealed that PLCζ-C2 can bind both phosphatidylinositol 3-phosphate (PtdIns3P) and phosphatidylinositol 5-phosphate (PtdIns5P) (Kouchi *et al*., 2005; Nomikos *et al.,* 2005), but not to phosphatidylserine (Saunders *et al.,* 2007) . However, there is no proof for the PLCζ C2 domain binding to membrane phospholipids through a Ca^{2+}-dependent manner (Kouchi *et al.,* 2005).

1.6.3 PLCζ Ca^{2+}-releasing activity versus other PLCs

PLCs sequence alignment analysis suggests that PLCζ has the closest sequence homology with PLCδ1 (47% similarity, 33% identity) (Saunders *et al.,* 2002). However, there are two distinct features noticed between the two isoforms. First is the existence of the N terminal PH domain in PLCδ1 that mediates its interactions with the plasma membrane, The second apparent difference is in the linker region between the conserved X and Y catalytic domains in both PLCs. In particular a cluster of basic residues is found in the PLCζ linker region proximal to the Y domain that PLCδ1 lacks (Saunders *et al.,* 2007). This explains why X-Y linker region in PLCδ1 is shorter than of its correspondent in PLCζ (Saunders *et al.,* 2002) (Fig. 1.21).

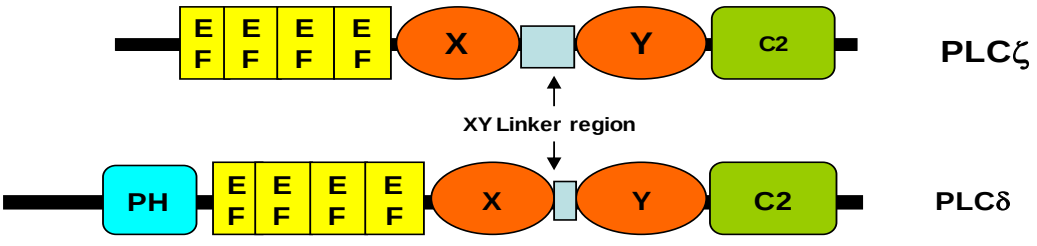


Figure 1.21 Difference between PLCζ and PLCδ1. Shorter XYl and the presence of PH domain in PLCδ1.

Based on the close homology between PLCζ and PLCδ1 through sequence comparisons between PLCζ and rat PLCδ1 catalytic domains, it is predcited that both enzymes share a similarly structured catalytic domain (Saunders *et al.,* 2007). Additionally, structural studies of the catalytic domain of PLCδ1 have been identified active-site residues that are essential for enzyme activity (Ellis *et al.,* 1998). The active-site residue analogous to one of these PLCδ1 residues, at Asp343, was mutated in mouse PLCζ to produce PLCζ^{D210R} (Saunders *et al.,* 2002) (Fig. 1. 22). Injecting PLCζ^{D210R} into mouse eggs showed a complete absence of Ca^{2+}-oscillations inducing activity.

In terms of Ca^{2+}-oscillations triggering ability, PLCζ has highest potency compared to any other PLC isoforms. PLCζ is the only PLC that able to be significantly activated even at a physiological Ca^{2+} levels (100 nM) (Saunders *et al.,* 2007). Injecting even tiny amounts of cRNA encoding PLCζ (0.02 mg/ml pipette concentration) can cause clear Ca^{2+}-oscillations in mouse eggs (Saunders *et al.,* 2002). In contrast, Ca^{2+}-oscillations triggering by PLCδ1 requires the injection of 20 - 50 times as much protein compare to PLCζ (Kouchi *et al.,* 2004). Another study showed that injecting PLCγ1 in amounts ~ 500 - 900 times higher than the PLC activity of single sperm content can cause Ca^{2+}-oscillations (Mehlmann *et al.,* 2001).

1.6.4 PLCζ Mutations

Given the fact that PLCζ is probably the endogenous egg activating agent, it is possible that some cases of male infertility could be related to some defects in PLCζ in sperm. A

recent study reported that some infertile patients had sperm lacking PLCζ, and that these sperm fail to activate eggs (Yoon *et al.,* 2008). Whilst there have been many reports on PLCζ structure and function, only a few studies have reported the effects of PLCζ mutations, whether they are made intentionally (e.g PLCζ^{D210R} (Saunders *et al.,* 2002) or discovered in a real patient (Heytens *et al.,* 2009). The latter case was an interesting study because it was based on one patient with repeated failed IVF/ICSI, and this patient was found to have non-globozoospermia. By conducting a study of the PLCζ gene in genomic DNA of this patient, a base change affecting the open reading frame of the DNA sequence was identified (Heytens *et al.,* 2009). This leads to a replacing of an adenine (A) by cytosine (C) at position 1193 in the DNA ORF sequence (Heytens *et al.,* 2009).Protein structure modelling predicted that this mutation is in the Y-catalytic domain (residues 348 - 465) of PLCζ at position 398 and involves substituting a proline for a histidine (PLCζ^{H398P}). This seems to be responsible for his infertility (Heytens *et al.,* 2009) (Fig. 1. 22).

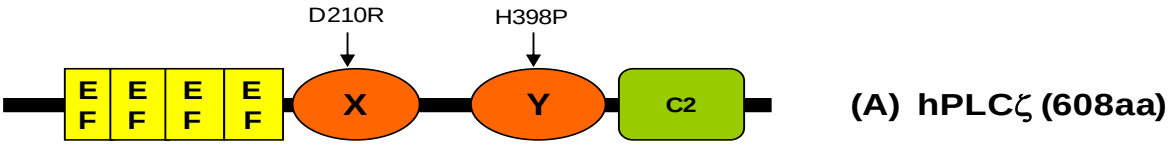


Figure 1.22 Positions of different mutations in hPLCζ.

In order to verify this experimentally, the researchers generated cRNA encoding hPLCζ in wild (hPLCζ^{WT}) and mutant (hPLCζ^{H398P}) forms and injected into two separate groups of mouse eggs for comparison. Interestingly, the wild type hPLCζ injected group exhibited Ca^{2+}-oscillations similar to those at fertilization, whereas, the mutant hPLCζ

injected group showed a marked decline in the hPLCζ activity, and in some eggs not a single Ca^{2+} response was recorded (Heytens *et al.,* 2009). These results clearly showed the negative effect of this mutation on hPLCζ activity. It is the first ever natural mutation that has been found in the hPLCζ sequence. This study also claimed that $PLC\zeta^{H398P}$ might have a dominant negative inhibitory effect on $PLC\zeta^{WT}$ (Heytens *et al.,* 2009). This was based on the finding that this mutation appears to be heterozygous. Since a spermatozoon is a haploid cell that only possesses one allele of a particular gene, it might be possibly that only half of the male germ cells are affected by this heterozygous mutation. However, there is an interchange of mRNA transcripts in developing spermatids that share cytoplasmic bridges; hence it is possible that this mutation from one allele might have a negative inhibitory effect on the other allele expressing the wild type PLCζ (Heytens *et al.,* 2009). However, recently a new PLCζ point mutation was discovered from the same patient that was maternaly inherited on a different allele and involved a substitution of a histidine by leucine at position 233 PLCζH233L (Kashir *et al.,* 2012). This avoids the need for a dominant negative theory assumed in previous study (Heytens *et al.,* 2009). More recently, a clinical study on fertile and non-fertile patients regarding PLCζ immunofluorescence showed a wide variability in PLCζ levels that were significantly higher in fertile group compared to non-fertile men. This result indicates that sperm from control men exhibited significant variance in total levels of PLCζ protein (Kashir *et al.,* 2013). Such variance may prevent the diagnostic application of quantitative PLCζ immunofluorescent analysis.

In the PLCζ result's chapter in this thesis, I examine this mutation as well as other mutations using analogous mutation in mouse PLCζ. In addition, I looked at some of the interesting structural and functional aspects of PLCζ in comparison with other somatic PLC isoforms such as PLCδ1.

1.6.5 PLCζ and potential infertility treatment

The discovery of PLCζ has given a promise of a new therapy for some cases of male factor infertility associated with failed activation of human eggs. Injecting cRNA encoding human PLCζ into aged human eggs that have failed to fertilize via IVF or ICSI has been shown to cause a prolonged series of Ca^{2+}-oscillations and parthenogenetic activation up to the blastocyst stage (Rogers *et al.,* 2002). In fact, it is the only available way to triggers Ca^{2+}-oscillations in human eggs artificially since other activators, such as Ca^{2+} ionophores, can triggers only a single Ca^{2+} rise. However, there are some obstacles that prevent the potential clinical application of PLCζ cRNA in IVF. Firstly, there is a possible ethical objection to the use of a genetic based substance in human eggs. Secondly, it is difficult to determine the exact amount of protein that is expressed after injection of hPLCζ cRNA in the human egg.

1.7 Aims

In this Thesis I have two main objectives. Both of these involve investigating in detail the mechanisms of Ca^{2+}-oscillations in mouse and human eggs. The first aim is to find a way to activate human eggs artificially by using Sr^{2+} to cause cytosolic free Ca^{2+}-

oscillations. To do this I carried out an extensive study of exactly how Sr^{2+} ions can trigger release from internal stores and causes Ca^{2+}-oscillations in mouse eggs. The results of these investigations were then applied to studies of human eggs. The second main objective is to further investigate how PLCζ causes Ca^{2+}-oscillations in mouse and human eggs. This is with a view to understanding the nature of cases of male factor infertility that involve PLCζ, as well as to providing practical steps to using PLCζ as a potential therapy by virtue of its ability to activate eggs. This second project involves the structural and functional analysis of PLCζ via investigating the effects of the identified hPLCζ mutations on Ca^{2+} releasing activity using mPLCζ. I also looked at how PLCζ targets the PIP_2 substrate, and how it is regulated compared with other somatic PLC isoforms. I further investigated the effect of some PLCζ domains swaps with PLCδ1 'the closest isoform', as well as between PLCζs from different species. Lastly, I started examining the use of recombinant human PLCζ protein in eggs.

Chapter 2

MATERIAL AND METHODS

2.1 Health and safety & Legal procedures

All chemical were handled and stored as per manufacturer's safety recommendations and PLCζ RNA samples were handled as per Genetic Manipulation Advisory Group (GMAG) guidelines. All experiments were carried out in accordance to the regulations of the Control of Substances Hazardous to Health (COSHH) and Cardiff university laboratory regulations. All animal (mice) were killed in Joint Biological Services (JBIOS) facility at Heath park, Cardiff University and in accordance to HM Home Office schedule one procedures and licence. Human oocytes work was started after obtaining a licence from Human Fertilization and Embryology Authority (HFEA), with licence number R0161. All collected human oocytes were treated in accordance with the HFEA Licence guidelines.

2.2 Materials

All chemicals and reagents were of the best grade available such as 'embryo tested' or 'cell culture tested'. They were obtained from Sigma (Sigma-Aldrich, Poole, Dorset, UK), unless stated otherwise. All Sr^{2+} experiments were carried out in Ca^{2+} free H-KSOM. However some constituents were varied depending upon the target experiment. All PLCζ experiments were carried out in normal H-KSOM. H-KSOM was prepared on daily bases in laminar air flow safety cabinet.

2.2.1 Super-ovulation and egg collection

Under this section there are two egg collection ways based on species types

2.2.1.1 Mouse eggs

MF1 female mice aged 6-8 weeks were injected for ovarian stimulation using 7.5 IU of pregnant mare's serum gonadotropin (PMSG; Intervet) via intra-peritoneal (IP) route, 48 hours later followed by IP injection of 7.5 IU of human chorionic gonadotrophin (HCG; Intervet) (Marangos *et al.,* 2003). About 15 hours after HCG injection mice were killed via cervical dislocation, and subsequently their oviducts were dissected and collected. Under the dissecting microscope (Fig. 2.1) the egg-cumulus complex were released from an oviduct ampulae at the site of the cumulus bulge by tearing with a 25-gauge needle (Summers *et al.,* 2000) into M2 medium containing hyaluronidase 0.3 mg/ml or 150 U/ml; where they were left for a few minutes to denude the cumulus cells from around the eggs (Lawrence *et al.,* 1997; Marangos *et al.,* 2003). The eggs then were collected and kept in 100µl droplets of M2 medium under embryo tested mineral oil at 37°C in a 5% CO_2 incubator (Binder CB150-Germany) (Summers *et al.,* 2000) awaiting injection or further treatment (Saunders *et al.,* 2002; Campbell and Swann, 2006).

2.2.1.2 Human eggs

Human eggs were collected from the IVF Wales clinic from patients whose eggs had failed to fertilise after IVF or ICSI. However, in addition some fresh eggs were also obtained from follicle reductions following over response to hormone treatment for intrauterine insemination. Couples who engaged in this project were all given a consent

form describing the project in detail, and only those who gave written consent for use of their all unfertilised eggs were involved in this research.

Using the dissecting microscope C-DSD230 (Fig. 2.1) fresh or IVF failed eggs were transferred into M2 medium (Sigma-Aldrich, Poole, Dorset, UK) containing hyaluronidase (0.3 mg/ml or 150 U/ml; embryo tested grade, Sigma) to denude the cumulus cells from around the eggs for 5-10 minutes. Then the eggs, including those from failed ICSI, were kept in a drop of M2 media under mineral oil at 37°C in a 5% CO_2 incubator ready for use in a specified project. Unless stated otherwise all the actual imaging experiments on human and mouse eggs in our laboratory were carried out in HKSOM media.

Figure 2.1 Nikon C-DSD230 dissecting microscope used to release and collect eggs.

2.3 Methods

Throughout my studies I carried out different experiments. In Sr^{2+} experiment the difference between experiments was mainly in the composition of H-KSOM and the experimental protocols. However, in PLCζ experiments the media protocols were essentially the same in all PLCζ experiment.

2.3.1 Microinjection

All injection procedures on mouse and human eggs were carried out in M2 media. MII eggs were manipulated for microinjection in a shallow drop of M2 covered by oil in the lid of the petri dish using manipulators fitted on an inverted microscope (TE2000-S ECLIPSE, Nikon UK Ltd) (Fig. 2.2). An electrical circuit is formed between a fine silver wire (placed in the injection drop and connected to the ground of an electrical amplifier) and the tip of the injection pipette once it touches the media (Swann *et al.,* 2009).

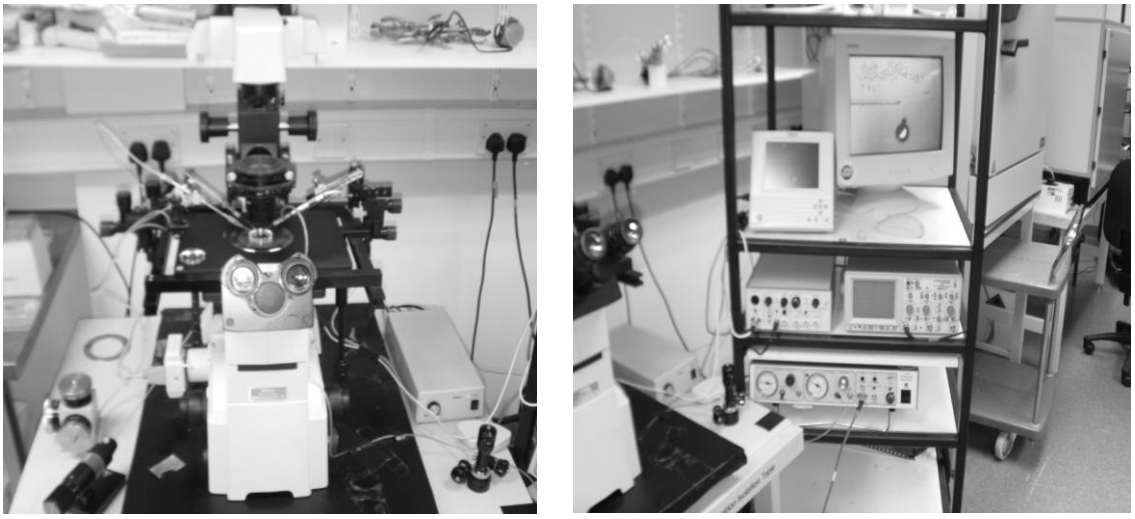


Figure 2.2 The injection system used routinely in our laboratory. A) The inverted microscope [Nikon ECLIPSE TE2000-S, Japan] fitted with pipette holders. B) The pressure injection system [Pneumatic PicoPump PV820 -WPI -USA], injector [intracellular Electrometer IE 251A] from WARNER Instrument Corporation, amplifier and the screens.

The holding pipette was pulled using a Narishige PN-30 puller (Intracel, UK) (Fig. 2.3A) and then prepared with an angle at the tip to suite the holding process using a Narishige MF-900 beveller (Fig. 2.3B) (Intracel, UK). Micropipettes containing an internal filament were pulled using a Sutter Instrument Corporation P-30 puller (Fig. 2.3C) and filled with 1-2 µl of the appropriate reagent.

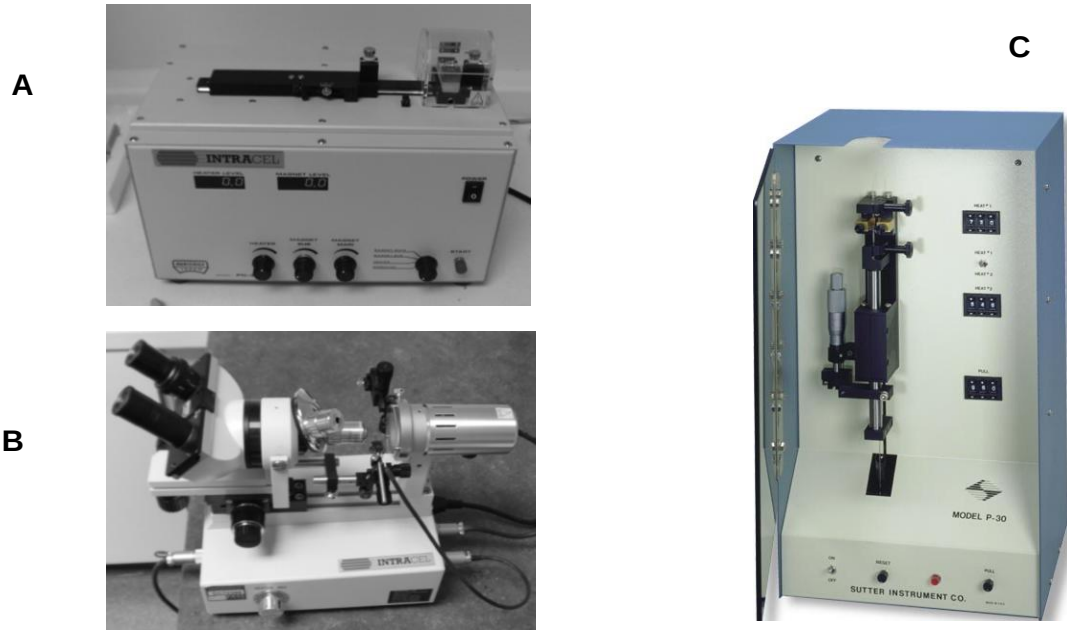


Figure 2.3 The different pullers we use in our laboratory. **A)** An image of the holding pipette (capillary) puller [Intracel PN-30 Narishige - Japan]. **B)** Photo of the holding pipette beveller [Intracel, Microforge, MF-900 –Narishige - Japan]. **C)** Photo of the injection pipette puller [SUTTER Instrument Corp P-30]

This micropipette was then held by special holder with a silver wire and this was placed onto an electrical preamplifier [Intracellular Electrometer IE-251A, Warner Instruments Corporation] which is clamped into the micromanipulator (Swann *et al.,* 2009). The MII arrested eggs were immobilized using suction with a holding pipette. Then the injection pipette pushed through the ZP and indented into plasma membrane. Insertion through the plasma membrane was achieved by electrical oscillations in the electric amplifier.

Then eggs were injected using a PicoPump pressure injection system (WPI) that applies pressure pulse (~ 20 psi) for 100ms - 1 sec (Swann *et al.,* 2009) to inject 0.5-5% of the egg volume (depending on the injected reagent) (Halet *et al.,* 2002). For mouse eggs all injections were carried out within 1 hour from the egg collection time, whereas in human oocytes the injections process where carried out roughly after 24 or 36 hrs from the collection time from the donated women. However the injection procedures are similar in both cases.

2.3.1.1 The injection procedure for PLCζ experiments

The luciferase tagged cRNA (PLCζ-Luc) was diluted with an RNase-free injection buffer (0.5´ PBS; Ambion) (Larman *et al.,* 2004) and just before injection mixed with 1 mM (1:1) Oregan green BAPTA dextran (OGBD) (Molecular probes, www.probes.com) (Invitrogen Ltd, Paisley, UK) (Yu *et al.,* 2008). Then the micropipette containing an internal filament was filled with ~1 µl of the PLCζ-Luc, OGBD mixture. The injection was carried out as mention in previous page and the injection volume was estimated from the diameter of cytoplasmic displacement caused by the bolus injection (Larman *et al.,* 2004). This was consider appropraite because a bolus injection of 3-5% of egg volume with cRNA pipette concentration of 0.02 mg/ml appears to show similar Ca^{2+} oscillations triggering ability of a single sperm (Saunders *et al.,* 2002). In cases where more than one reagent needed to be injected and to reduce the potential harming effect of multiple injections, a co-injection protocol was carried out (Marangos *et al.,* 2003). Different experiments have different injected reagents, so for more details see the specific experimental procedures in results chapters.

2.3.1.2 The injection procedure for Sr^{2+} experiments

It is the same process as in PLCζ experiments except in that, eggs were only injected with Ca^{2+} indicator mainly OGBD (1 mM) since we only needed to monitor the intracellular Ca^{2+}. However in some Sr^{2+} experiments such as in case of intracellular Na^{+} monitoring, Na^{+} indicator sodium green (SG) was co-injected with Ca^{2+} dye Rhod dextran (RhD) in a 50:50 mixture.

2.3.1.3 The procedure for Sr^{2+} injection into mouse/human eggs

One of protocols we tried in order to stimulate mouse or human eggs was carried out by injecting Sr^{2+} directly into egg's cytoplasm. The injection procedures were similar to the standard procedure with slight differences. However, in this case the necessary parts of an injection system were fitted onto the Nikon inverted fluorescent imaging microscope (TE2000 -S, Nikon UK Ltd) (Fig. 2.4). An injection drop of 50-100µl of Ca^{2+} free HKSOM covered by mineral oil was placed in a suitable wide dish fitted with glass floor on the heating stage of the inverted microscope. An electrical circuit is formed between a fine silver wire (placed in the injection drop and connected to the ground for amplifier) and the tip of the injection pipette once it touches the media. Holding pipettes were pulled using a puller (Fig. 2.3A).

MII eggs were injected with fluorescent dye (OGBD) using the standard injection system (Fig. 2.2), then the pre-injected MII eggs were placed in the injection drop and immobilized by suction with the holding pipette. A very sharp micropipette containing an internal filament were pulled using a puller (Fig. 2.3C) and filled with few micro-litres of the $SrCl_2$ (100mM). This micropipette was then held by a holder provided with silver wire

and placed onto preamplifier which is clamped into the micromanipulator (Swann *et al.,* 2009).

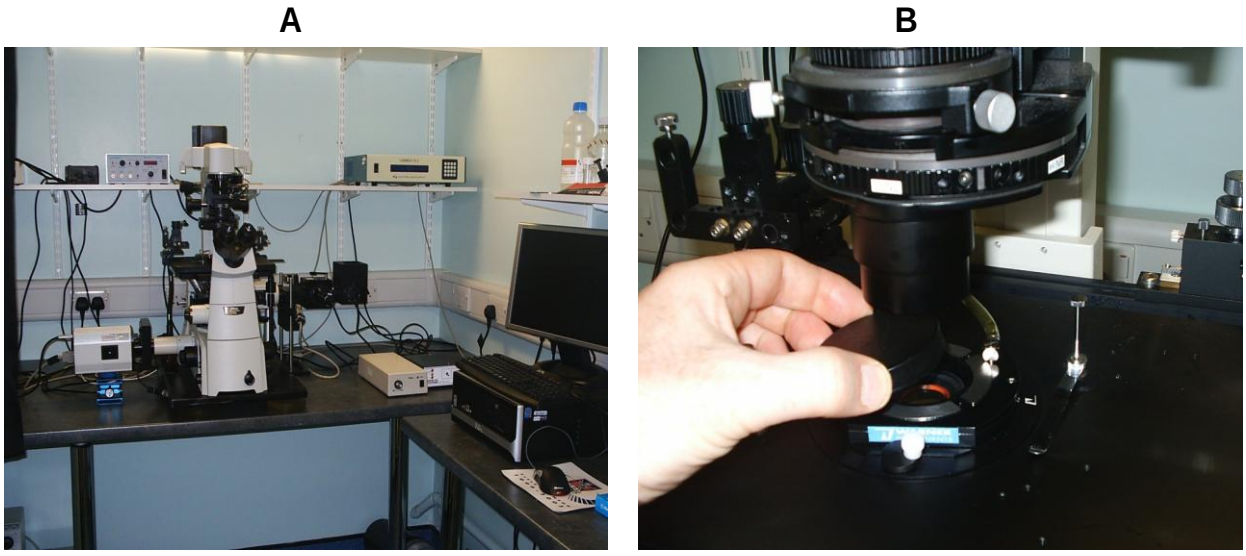


Figure 2.4 The fluorescence system in the laboratory. **A)** The complete system; the inverted microscope [Nikon ECLIPSE Ti – Japan], camera, light source, stage heater stabilizer [WARNER Instrument CL-100] and PC. This system is the one I used to inject Sr^{2+} into mouse and human eggs. **B)** Closer picture of the heating stage containing the dish with the cover lid in hand.

After holding the egg, the injection pipette pushed through the ZP and plasma membrane and the injection pipette was put into place in the egg for up to 30min before it was drawn back. During this time a series of positive current pulses were applied via the electrical amplifier to start injecting Sr^{2+} every second for 0.5 second continuously for 2000 pulses. This process causes continuous amounts of Sr^{2+} to enter the cytoplasm by ionotophoresis. Intracellular Ca^{2+} changes were monitored by changes in the fluorescence intensity throughout this time.

2.3.2 Media preparation

M2 media was aliquot into test tubes, ~10 ml each and kept in fridge. Each day one tube was taken out onto heated block for that day use. On a daily bases H-KSOM was prepared from the concentrated H-KSOM stocks. However, different types of this media were made depending on the intended experiment.

2.3.2.1 H-KSOM (Hepes-buffered Potassium Simplex Optimised Media).

HKSOM is the media I used in almost all of my experiments during my study. Hence I made large quantity of a concentrated H-KSOM stock. I added sterile water of up to 500ml to the following amounts (Table 2.1, see next page) of all the components to make concentrated stocks of H-KSOM (Lawitts *et al.,* 1993; Erbach *et al*., 1994). However, different preparations of the final HKSOM were optimised according to the intended experiment.

2.3.2.2 Sr^{2+} H-KSOM Preparation

In case of Ca^{2+} monitoring during Sr^{2+} stimulation experiments I prepared Ca^{2+} free H-KSOM in the same way of preparing normal H-KSOM but without adding 10µl of a 1.71 M of $CaCl_2$. I replaced that Ca^{2+} by adding Sr^{2+} to the media in different concentrations (0.5, 5.0 and 10.0mM) depending on the experiment protocol. However, based on pronuclear development, blastocyst formation and the cell number in parthenotes, it has been reported that the best optimum Sr^{2+} activation protocols in mouse eggs is 10mM with 2.5 hrs exposure in Ca^{2+} free media (Ma *et al.,* 2005).

In some experiments I modify the composition of H-KSOM to make H-KSOM with low Na^+ (~30mM) by opting out NaCl or else totally Na^+ free H-KSOM, details to follow. However, in order to keep the osmolarity within the normal range, I replaced Na^+ with choline or KCl. The osmolarity was monitored using calibrated freezing point osmometer (Gonotec Osmometers - Osmomat 030, GERMANY). Then the medium was kept on the heated block.

Chemical	**Working media (1X) pH 7.4**		**10X stock (stored at -20C, filter sterilised)**		**Sigma code**	**M W**
	mM	**g/l**	**g/500ml**	**ml/500ml**		
NaCl	95	5.55	27.75			58.44
KCl	2.5	0.185	0.925			74.56
KH_2PO_4	0.35	0.0476	0.238			228.2
Na Pyruvate	0.2	0.022	0.11			110.04
Streptomycin sulphate		0.05	0.25			
Penicillin G		0.063	0.315			
EDTA	0.01	0.0038	0.019			416.2
$MgSO_4$. $7H_2O$	0.2	0.0493	0.246			246.48
Na Lactate (liquid)	10.0			7.37		112.1
$NaHCO_3$	4.0	0.336	1.68			84.01
HEPES	20.0	4.76	23.8		H6147	238.31
Phenol red			0.055	5.0		354.38
L-glutamine	1.0	0.146	0.73			146.15

Table 2.1 Exact concentrations of the different components of the concentrated stock of H-KSOM.

2.3.2.2.1 Low sodium H-KSOM

Further in Sr^{2+} experiments and in some special circumstances, I prepared H-KSOM with low Na^+ content (~30mM) by not adding NaCl in the concentrated stock. The rest of

the constituents were the same as in Table 2.1. It is known that missing out NaCl from the media leads to hypoosmotic media (~ 200mOsm/kg).

In order to try and stimulate human eggs with Sr^{2+} a different protocol was used in which a special H-KOSM was prepared by modifying the H-KSOM to be totally Na^{+} free so as to enable the reversal of the Na^{+}/Ca^{2+} exchanger in Sr^{2+} media that could enhance Sr^{2+} influx (details in first result's chapter). The other benefit of this media is that the egg membrane potential should depolarize due to high K^{+} content and this could facilitate the exchanger causing Na^{+} efflux with Sr^{2+} influx. In order to maintain the osmolarity I replaced all Na^{+} salts with their K^{+} correspondents and finally made 10 times stock solutions of the modified H-KSOM by adding all the followings and sterile water of up to 500ml (Table 2.2).

Chemical	**Working media (1X) pH 7.4**	**Sigma code**	**10X stock (stored at -20C)**		**M.W**
	mM		**g/500ml**	**ml/500ml**	
KCl	2.5		0.932		74.56
KH_2PO_4	0.35		0.399		228.2
Pyruvic acid (liquid)	0.2		0.088	0.088/1.276= 0.07ml (70ul)	88.06
$MgSO_4$	0.2		0.12		120.37
Lactic acid (powder)	10.0		4.5		90.08
$KHCO_3$	4.0		2.0		100.12
HEPES K-buffered	20.0	H0527	27.64		276.4
Phenol red			0.055		354.38
L-glutamine	1.0		0.73		146.15

Table 2.2 Exact concentrations of the different components of zero Na^{+} modified H-KSOM.

2.3.2.3 H-KSOM for PLCζ experiments

On the day of an experiment I prepared H-KSOM from the concentrated stock by adding all the following: 1ml of H-KSOM (10X stock) (Table 2.1), 20µl of Na^+ Hydroxide 1.0N solution to buffer the media (pH 7.2 – 7.4), 10µl of a 1.71M of CaCl2, 10µl of a 0.2M glucose, and finally adding sterile water to achieve the required osmolarity. This was then mixed well and kept in the heated block for use during the whole day. On the day of use I took 1ml and mix it with 1µl of (100M) luciferin to get a final concentration of (100µM) luciferin (Dumollard *et al.,* 2004). I then placed a small drop under oil on the heated stage of the inverted microscope for recording.

2.3.3 Measurements of intracellular Ca^{2+} and luciferase expression

In all experiments H-KSOM was used as a medium during experimental recording. The injected oocytes were placed in a chamber with 500ml H-KSOM medium (Summers *et al.,* 2000) containing 100µM luciferin under mineral oil (Dumollard *et al*., 2004) on a temperature-controlled stage [Intracel] of an inverted microscope. On the same sets of eggs both luciferase expression (luminescence) and Ca^{2+} oscillations (OGBD fluorescence) were simultaneously measured using a Zeiss Axiovert S100 (Fig. 2.5) or Nikon TE2000 microscope (Fig. 2.6) equipped with a light source (halogen lamp) with emitted light directed towards a cooled intensified CCD camera (ICCD) with a bialkali-type or S20 photocathode-based intensifier cooled to 10°C or 0^oC respectively (Yu *et al.,* 2008 and Swann *et al.,* 2009). Ca^{2+} measurements were considered valid only if the

egg was also luminescent. The luminescence was measured as the light emission recorded in the absence of excitation light. Fluorescence signals were 10–100 times that for luminescence.

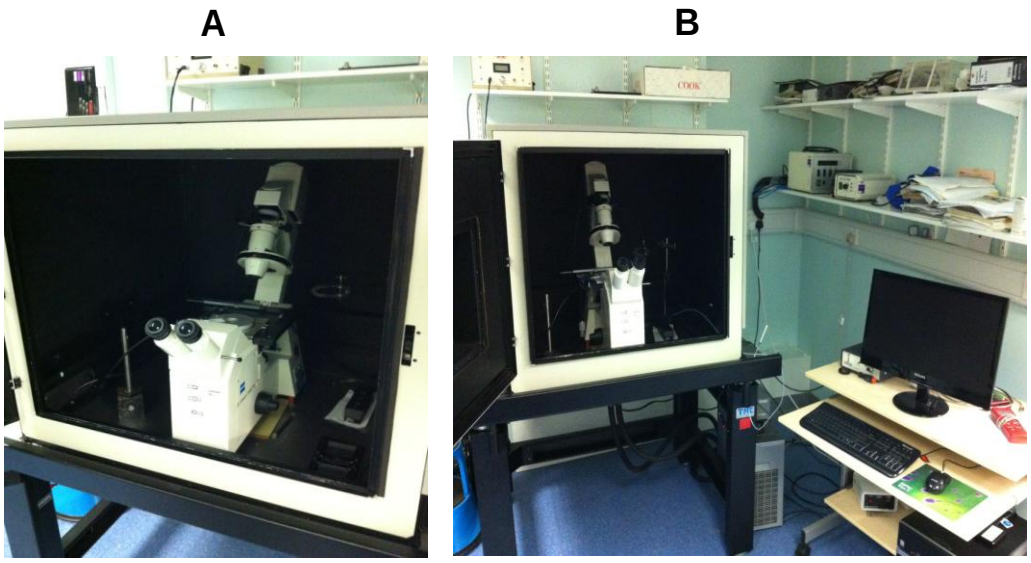


Figure 2.5 The Zeiss luminescence system in the laboratory. A) The inverted microscope [Zeiss - Germany] fitted within the dark box {Photek]. **B)** The complete system; the inverted microscope, ICCD camera, cooler system, light source, stage temperature controller [Intracel - Japan] and PC**.**

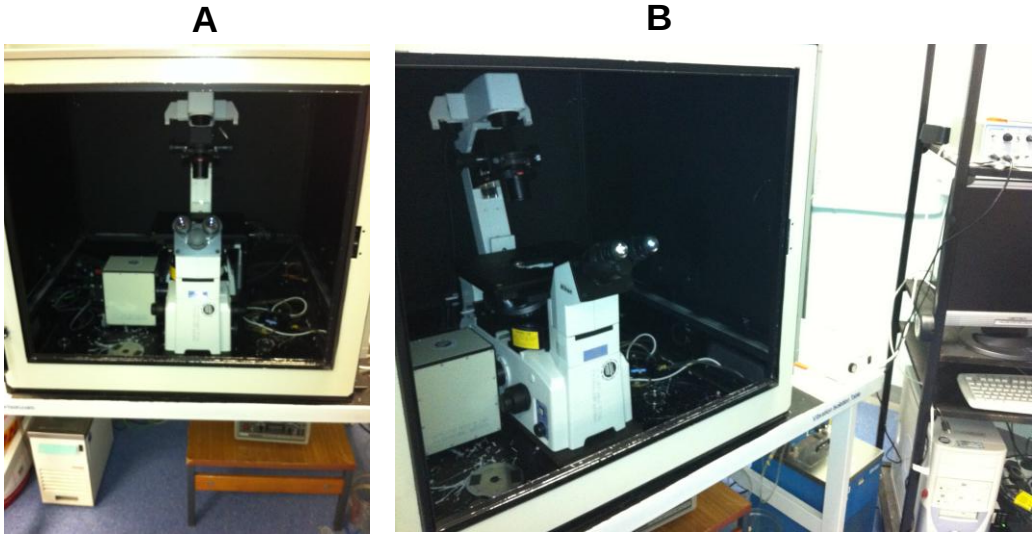


Figure 2.6 The Nikon luminescence system in the laboratory. A) Inverted microscope [Nikon ECLIPSE TE2000-S Japan] fitted within the dark box [Photek]. **B)** The complete system; the inverted microscope, ICCD camera, cooler system, light source, stage temperature controller [Intracel -UK] and PC.

Figure 2.7, is representative diagram of ICCD camera imaging process. Since the goal is to study the expression at low levels, a highly sensitive camera was needed. The combination of a high-gain amplifier with a good low-light image sensor is found with ICCD camera. The typical intensifier has three key components; a photocathode, a series of two-dimensional micro-channel plates and the phosphorescent output screen. The photocathode converts the received photon energy to electrons, which is then emitted from the photocathode and travels across an electric field through the two-dimensional micro-channel plate that has a high-voltage across its ends. A dramatic amplification of the electrons numbers can be obtained.

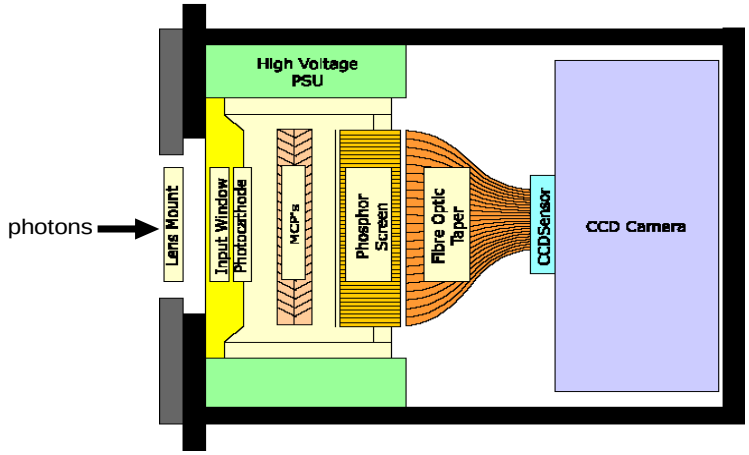


Figure 2.7 The Intensified CCD camera imaging process. A single photon hits the input window and is amplified to a million photons by the time it reaches the CCD sensor.

The amplified electrons impact with a phosphorescent output screen which is connected to a fibre optic faceplate to give an 'amplified image'. Since the ICCD cameras are highly sensitive to light, to obtain the image from eggs a completely dark environment is necessary. Hence, the microscope and ICCD camera were placed inside a custom-

made dark box which including all parts of the system. The dark box and associated software [Photek IFS-32] was supplied by Photek Ltd (www.photek.co.uk) (St Leonards on Sea, East Sussex, UK) (Campbell and Swann, 2006). The software controls the switching mechanism between fluorescence and luminescence recording (Fig. 2.8).

The fluorescence was displayed in arbitrary units of intensity and was setup to record in most experiments by exposing eggs to excitation light (450–490 nm) with 10% ICCD camera sensitivity for 10 seconds. The luminescence which represents the absolute number of measured photon counts per second (cps) was recorded also for 10 second by blocking the excitation light and switching the ICCD camera to 100% sensitivity (Yu *et al.,* 2008).

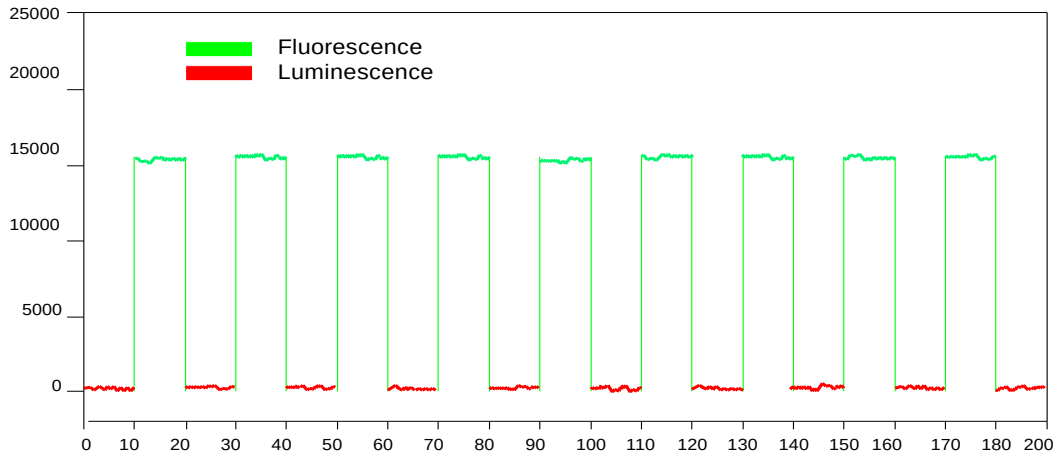


Figure 2.8 Switching between the fluorescence and the luminescence during PLCζ expression experiments.

To estimate the levels of luciferase protein that corresponds to a given luminescence level, a standard luminescence calibration curve was used. This standard was prepared by injecting a known amounts of recombinant luciferase protein (Sigma–Aldrich, Poole, Dorset, UK) into another group of mouse eggs and then the luminescence of these eggs

was measured under the same conditions as those injected with cRNA of mouse PLCζ-luc (Yu *et al.,* 2008). The standard luminescence calibration curve has yield this formula **(Protein [fg] = [luminescence "cps" x 0.169] + 0.288)** which I used for my estimation of the expressed proteins. In some of the Sr^{2+} experiments, the same system was used but only in fluorescence photon counting mode to continuously monitor the Ca^{2+} changes in eggs.

2.3.3.1 Fluorescence system and data analysis

The oocytes or zygotes were imaged continuously for 2hrs after microinjection using a Nikon ECLIPSE TiU epifluroescence microscope (Fig. 2.4) with a 20x 0.75 NA objective (Swann *et al.,* 2012). The dish was covered by a black plastic lid to minimise the stray light exposure. Fluorescence excitation from a halogen lamp was passed through a 490nm bandpass filter, reflected via a 505nm dichroic mirror, and collected with a 530nm bandpass filter.

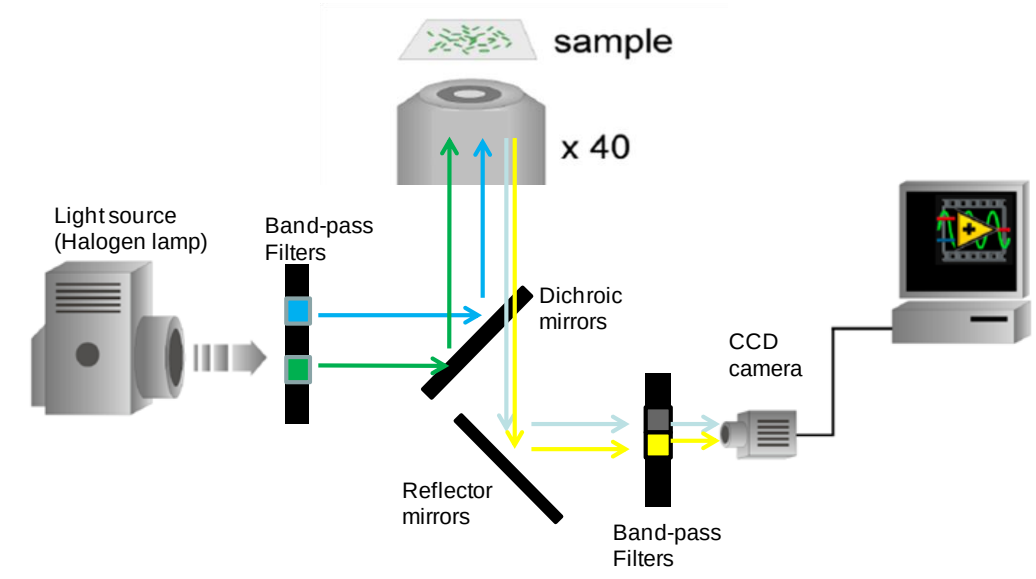


Figure 2.9 Pathway of the light through the fluorescence system.

A Coolsnap HQ2 CCD camera (Photometrics) was taking a series of images from the fluorescent eggs every 10 seconds with 100-200 msec exposures. Both image collection and initial analysis were carried out using InVivo software (Media Cybernetics) and images were stored as tiff stacks (Fig. 2.9).

The fluorescence images were analysed using a free software package called ImageJ (http://rsbweb.nih.gov/ij/). In brief, in order to measure the fluorescence intensity changes in each egg separately, a circle the same size as the egg was drawn around each image of an egg and in a background region near the eggs. Then the 'multi-measure' plugin was used to give a series of outputs for each egg as numerical values. These row data then were transferred into a SigmaPlot file where the background value was subtracted from each egg intensity value. The fluorescence intensity of the dye from the whole each egg was then plotted against time.

2.3.4 Complementary RNA (cRNA) synthesis

All PLCζ cRNA samples were prepared by Micheal Nomikos or Maria Theodoridou in Tony Lai's laboratory. Complementary RNA (cRNA) was synthesised from the open reading frame (ORF) of mouse PLCζ and c-myc tagged-PLCζ (Saunders *et al.,* 2002; Swann *et al.,* 2009). Each batch of cRNA produced was quantified by measuring its absorbance at 260nm in a 500µl quartz cuvette and calculated using this equation (RNA conc [ug/ml] = 40 x A260 x 500) (Nomikos et al., 2005; Swann *et al.,* 2009). Then the cRNA was checked for *in vitro* protein expression in a rabbit reticulocyte lysate system (Promega). A full detail of this has been described in previous publications (Saunders *et*

al., 2002; Nomikos *et al.,* 2005; Swann *et al.,* 2009). The cRNA encoding the 608-residue human PLCζ or 647-residue mouse PLCζ tagged via the C-terminus with firefly luciferase (PLCζ-Luc) was prepared as previously described (Nomikos *et al.,* 2005), diluted with an RNase-free injection buffer (0.5´ PBS; Ambion) (Larman *et al.,* 2004).

$PLC\zeta^{WT}$ and mutants were amplified by PCR from the corresponding pCR3 plasmid using Phusion polymerase (Finnzymes) to incorporate a 5′ EcoRI site and a 3′ SalI site. PCR products were cloned into pGEX-6P1 (GE Healthcare). The primers used for amplification of WT and mutant PLCζ were: 5′-ACATGAATTCATGGAAAGCCAACTTCATGA-3′ (forward) and 5′-TAACGTCGACTCACTCTCTGAAGTACCAAAC-3′ (reverse). Following linearization of WT and mutant PLCζs, cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and a poly (A) tailing kit (Ambion), as per the manufacturer's instructions.

Chapter 3

Sr^{2+} & Ca^{2+}-OSCILLATIONS IN MOUSE EGGS

3.1 Introduction

In mammals, sperm is the natural egg activator and works by triggering long-lasting oscillations in free ooplasmic Ca^{2+} (Kline and Kline, 1992; Miyazaki *et al*., 1993; Swann and Lai, 1997). However, mammalian eggs can also be activated via different artificial stimuli (Swann and Ozil, 1994). For many years Sr^{2+} has been known as the most successful and efficient activating agent, or substance, and it can parthenogenetically activate mouse, hamster and rat eggs' (Whittingham and Siracusa, 1978; Kline and Kline, 1992; Rogers *et al.,* 2004). It does this by inducing Ca^{2+} oscillations (Zhang *et al.,* 2005). Treating mouse eggs with Sr^{2+} triggers Ca^{2+} oscillations that look close to the normal pattern at fertilization (Kono *et al.,* 1996; Kyono *et al.,* 2008). Nowadays, Sr^{2+} is widely used in research labs as an egg activator in rodent eggs. Normal mice offspring have been reported after combinations of 10mM Sr^{2+} and ICSI of misshaped epididymal spermatozoa collected from alkylated imino sugar [N-butyl-deoxynojirimycin (NB-DNJ) and N-butyl-deoxygalactonojirimycin (NB-DGJ)] treated males (Suganuma *et al.,* 2005). These compounds are reversible male infertility-inducing agents. They work after short-term administration at the minimal dose and have been used as non-hormonal contraceptives (Suganuma *et al.,* 2005). There is no other report showing that Sr^{2+} causing Ca^{2+} oscillations in eggs from either human or domestic animals (Rogers *et al.,* 2004). However, some reports claim that Sr^{2+} can be used to activate bovine (Méo *et al.,* 2005), pig (Okada *et al.,* 2003) and human eggs with different means of Sr^{2+} applications. In the literature, different concentrations of Sr^{2+} have been reported as being required to activate mammalian eggs from 50mM (Méo *et al.,* 2005) down to

1.7mM (Bos-Mikich *et al.,* 1995). So it is unclear why Sr^{2+} only works in rodent eggs and what factors determine the concentration of Sr^{2+} used to activate eggs.

3.1.1 Critical levels of extracellular Sr^{2+} are required to trigger intracellular Ca^{2+} oscillations.

H-KSOM without Ca^{2+} is the media used in all Sr^{2+} experiments. I started by testing three different concentrations of Sr^{2+} (0.5mM, 5.0mM and 10mM) on mouse eggs in Ca^{2+} free H-KSOM. The detailed steps of eggs manipulation and the injection procedure were mentioned in the previous chapter. Mature MII mouse eggs were injected with the Ca^{2+} sensitive dye, OGBD. The injected eggs were then placed into a drop of media on the inverted microscope stage that contained the Sr^{2+} concentration for the intended experiment. The fluorescence recordings started as soon as possible (~1min).

After two hours of recording the experiment was stopped and the eggs were taken out from the Sr^{2+} media, and then the data were analysed. Not surprisingly my initial results were not very different from the previous studies. I found that at 0.5mM Sr^{2+} causes no oscillations in any eggs for as long as the 2 hrs recording period, 20/20 (100%). However, in 5mM Sr^{2+} and 10mM Sr^{2+}, Ca^{2+} oscillations were seen, but they were more frequent in 10mM Sr^{2+} (71.4%,10/14) and (84.6%, 22/26) respectively. The remaining eggs either died or not responded. These data suggests that Sr^{2+}-induced oscillations in mouse eggs in normal media requires a critical level of external Sr^{2+} of around 5-10 mM see (Fig. 3.1). The question that is still unsolved is why this critical Sr^{2+} levels is needed.

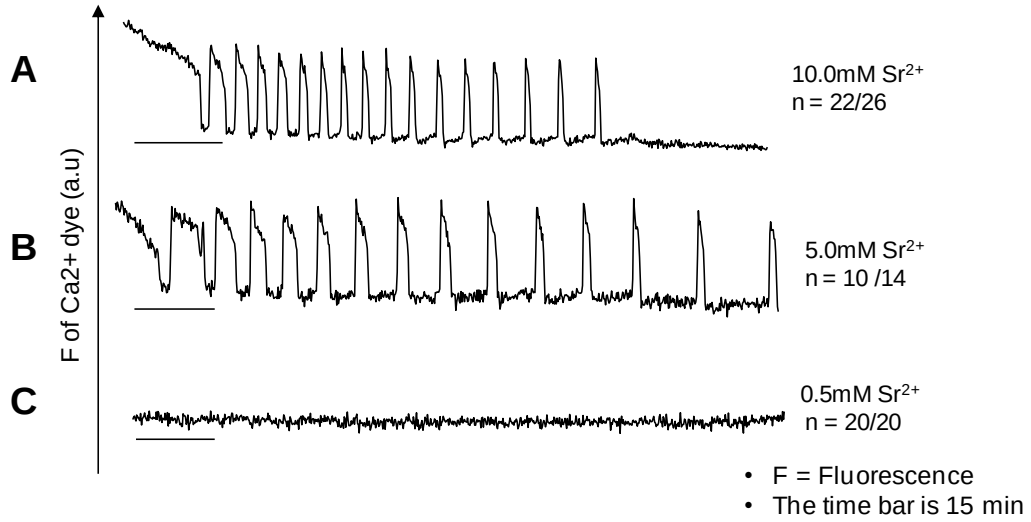


Figure 3.1 Sr^{2+} induced Ca^{2+}-oscillations in mouse eggs in Ca^{2+} free media (HKSOM) requires a critical level of external Sr^{2+}. A) Prompt frequent oscillations in 10mM Sr^{2+}, B) Prompt but less frequent in 5mM Sr^{2+} and C) no oscillations in 0.5mM Sr^{2+}.

3.1.2 How does extracellular Sr^{2+} trigger Ca^{2+} release?

Sr^{2+} is divalent cation similar to Ca^{2+}. It dissolves easily in the various culture media used for eggs. Treating mouse eggs with Sr^{2+} in optimized Ca^{2+} free media triggers Ca^{2+}-oscillations. Interestingly, it has been shown that Sr^{2+} acts via stimulating the IP_3R in cells (Marshall and Taylor., 1994) and this is consistent with findings in mouse eggs (Zhang *et al.,* 2005). So, in this case one can ask how Sr^{2+} could reach intracellular IP_3R and trigger Ca^{2+} release. One simple proposal is that Sr^{2+} influx is prerequisite step to release Ca^{2+} from the internal store (Fig. 3.2). Since eggs activate by adding Sr^{2+} in the culture media, it can be expected that in order to triggers Ca^{2+}-oscillations, Sr^{2+} needs to cross the plasma membrane. Another possible mechanism is that Sr^{2+} works via cell membrane receptor mechanism.

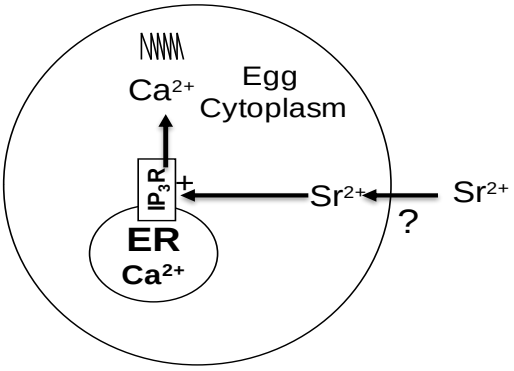


Figure 3.2 Suggested mechanism of Sr^{2+}-induced Ca^{2+}-oscillations in mammalian eggs. The question mark indicates the elusive potential mechanism of Sr^{2+} influx.

3.1.3 Objectives

Most of my work in this chapter is designed to establish that continuous Sr^{2+} influx is required for long lasting Ca^{2+}-oscillations in mouse eggs. In addition, I also aim to identify the possible pathway/s of Sr^{2+} entry into the mouse egg. In order trying to clarify this, I have applied different protocols. These included trying to bypass the membrane barrier by injecting Sr^{2+} directly into mouse eggs to rule out the potential membrane receptor theory. Other protocols include either blocking or facilitating the Sr^{2+} influx across the egg membrane. In addition, this chapter includes some other experiments related to nature of the Sr^{2+} triggered oscillations in mouse eggs. Finally, some of these protocols were also adopted for experiments on human eggs in a later results chapter.

3.2 Sr^{2+} injection into mouse eggs and Ca^{2+} release

Based on the idea that Sr^{2+} influx needs to occur across the egg membrane to trigger Ca^{2+}-transients, I have undertaken a more rigorous, challenging experiment. The idea was to inject Sr^{2+} directly into the cytoplasm bypassing the plasma membrane, and see

if this can trigger Ca^{2+}-oscillations. The injection of divalent cations including Sr^{2+} into mammalian eggs has been reported in hamster eggs (Georgiou *et al.,* 1987) and in pig eggs (Okada *et al.,* 2003). Georgiou and his colleagues studied the effect of Sr^{2+} upon the hamster egg's membrane potential and described a single transient hyperpolarization and fall in resistance similar to the effect of a single Ca^{2+} injection (Georgiou *et al.,* 1987). Okada and his colleagues were aiming to activate pig eggs, and they conclude that Sr^{2+} can activate pig eggs similar to Ca^{2+} via means of one Ca^{2+} transient (Okada *et al.,* 2003). This transient is different from the physiological pattern of Ca^{2+} changes during fertilizations.

My study was intended to activate mouse eggs with Sr^{2+}, not in the media, but through Sr^{2+} injection directly into the egg cytoplasm. The protocol has been described in the material and methods chapter. The challenging point in this protocol is the egg survival; some eggs lysed as a result of keeping the injection pipette inside the egg for long periods. In these experiments some injected eggs also died after their first response to Sr^{2+} injection. However, I did not include them in my data and I only counted those that survived after removal of the Sr^{2+} injection pipette.

Figure 3.3A results show that soon after starting the Sr^{2+} injection (2000 pulse every second for 0.5 sec) all injected eggs (15/15) had a rapid rise of in their Ca^{2+} signal, with a slow decline that resulted in a long duration first spike. This was then repeated with shorter spike durations between 5 and 8 times before oscillations stopped. It was of interest that some eggs could be re-injected with same concentration of Sr^{2+} using a new pipette and they all responded with a few extra Ca^{2+} spikes. This is the first

reported Ca^{2+}-oscillations in mouse eggs using Sr^{2+} injection. These results are consistent with the idea that the influx of Sr^{2+} is able to cause Ca^{2+}-oscillations and that Sr^{2+} works directly on the internal store rather than a plasma membrane receptor.

3.2.1 KCl injection into mouse eggs and Ca^{2+} release

To confirm that Ca^{2+}-oscillations shown in the above Sr^{2+} injection experiments were triggered by Sr^{2+} and not membrane potential changes driven by the current injection (Georgiou *et al.,* 1987), I used the same technique for Sr^{2+} injection but replaced 100mM Sr^{2+} with 100mM KCl. My results showed that all KCl injected eggs [5 eggs] gave no Ca^{2+} response. However, those eggs that were subsequently re-injected with Sr^{2+} 100mM (2/5) start to oscillate as soon as current pulses were started, see (Fig. 3.3B). These results confirm that these oscillations were Sr^{2+} triggered Ca^{2+}-oscillations and not caused changes in membrane potential or by current injection.

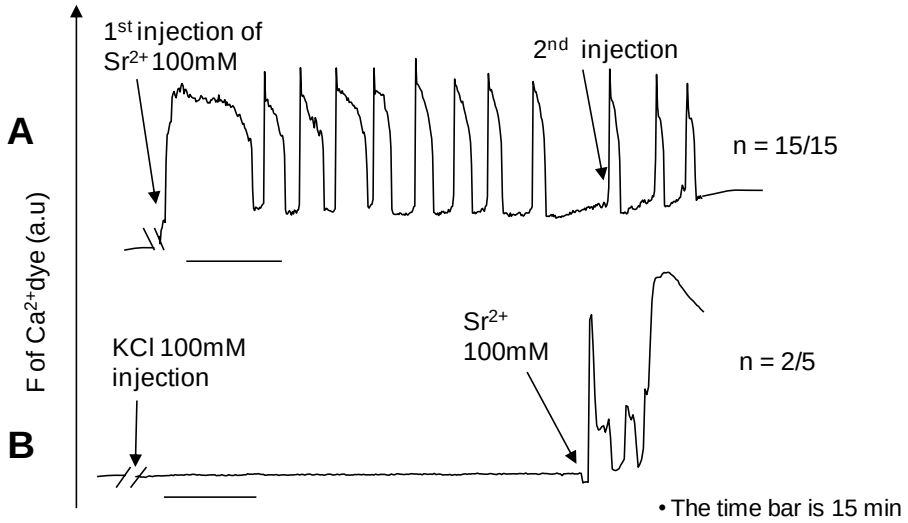


Figure 3.3 Ca^{2+}-oscillations in mouse eggs triggered by direct Sr^{2+} injection. A) Ca^{2+}-oscillations in eggs start straight away after the onset of the injection and stopped after a couple of spikes. It also shows that oscillation can be recover by re-injection Sr^{2+}. B) Shows that mouse eggs do not respond to KCl injection, however, oscillations start after subsequent Sr^{2+} injection.

3.3 Sr^{2+}-triggered oscillations; are they Ca^{2+} or Sr^{2+} transients?

It can be argued that since Sr^{2+} is similar to Ca^{2+} it could substitute for Ca^{2+} to refill the internal store during Sr^{2+} triggered oscillations in eggs. If this is the case then the oscillations seen in mouse eggs treated with Sr^{2+}, particularly those followed the first few spikes, could be Sr^{2+} transients rather than Ca^{2+} transients. From a survey of the literature, there is no apparent dye that can easily distinguish Ca^{2+} from Sr^{2+}. The only suggested way to clarify this is to use a Ca^{2+} ionophore that has much lower Sr^{2+} affinity compared to Ca^{2+}. The only suitable ionophore is the ionomycin with an affinity ratio of Sr^{2+} to Ca^{2+} of 1: 48 (Liu and Hermann, 1978).

3.3.1 Sr^{2+}-triggered fluorescence and ionomycin

Ionomycin is a monocarboxylic polyether antibiotic that has shown, using ion competition experiments, to have a high affinity binding to Ca^{2+} with insignificant affinity for Sr^{2+} (Liu and Hermann, 1978). It has been used as an ionophore to complex with Ca^{2+} and and carry it across lipophilic membranes (Liu and Hermann., 1978). However, some reports show that ionomycin works by a store-regulated Ca^{2+}-influx mechanism and not through plasma membrane cation translocation (Morgan and Jacob, 1994). The reason for examining the effect of ionomycin on Sr^{2+} experiment is that some researchers have suggests that in Ca^{2+} free Sr^{2+} media the Sr^{2+} could replaces Ca^{2+} in the internal store and result in Sr^{2+}-oscillations replacing Ca^{2+}-oscillations. In order to investigate whether these oscillations are Ca^{2+} transients I have carried out a series of

experiments using ionomycin to test the if the Ca^{2+} store is loaded with Ca^{2+} or not after a series of Ca^{2+}-oscillations has been induced by Sr^{2+}.

The first set of experiments were started by triggering Ca^{2+} oscillations with Sr^{2+} until oscillations stopped after about 2 hrs. I then added 5µM ionomycin. The fluorescence signal then went up for a while before restoration to the previous fluorescence intensity. After a detailed analysis of each egg signal's, the results showed a prompt rise in Ca^{2+} signal in almost all exposed eggs (90.9%, 10/11) which then recovered to the base line in few minutes time (Fig. 3.4A). This shows that the previous oscillations and this spike is Ca^{2+} spike. It also suggests Sr^{2+} does not substitute Ca^{2+} in the internal store which still has Ca^{2+} in it even after Ca^{2+}-oscillations triggered by Sr^{2+} have already stopped.

The second set of experiments was carried out by adding 5µM ionomycin to mouse eggs before exposing them to Sr^{2+}. The results showed that there was a big jump in Ca^{2+} signal in all eggs (which is normal response) before their Ca^{2+} levels recovered to lower cytoplasmic level. When 5mM Sr^{2+} was subsequently, added the majority of eggs start to respond to Sr^{2+} with the 1^{st} Ca^{2+} rise but they failed to restore Ca^{2+} back to the base line levels and eventually all eggs died (8/8) (Fig. 3.4B).

These two set of experiments were repeated twice and always gave similar results. These results could be interpreted as follows. In Fig. 3.4A, even though the Ca^{2+}-oscillations stopped, the internal stores (ER) are still filled with Ca^{2+} because if they contained Sr^{2+} ionomycin would not be effective in causing an increase in fluorescence of OGBD.

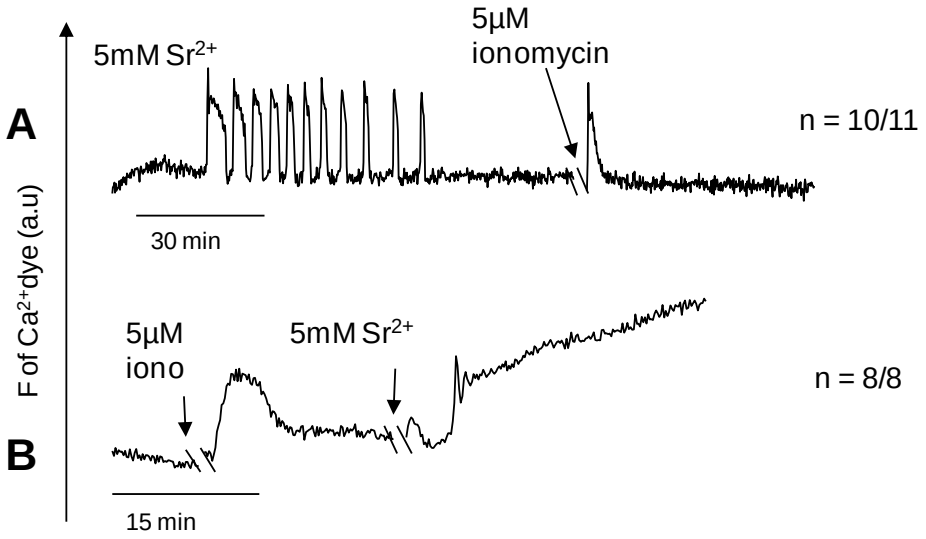


Figure 3.4 The effect of ionomycin on Ca^{2+}-oscillations in mouse eggs triggered by 5.0mM Sr^{2+}. A) 5µM ionomycin added after Sr^{2+} 5.0mM in Ca^{2+} free eggs show a clear transient with similar amplitude to preceding oscillations. B) 5µM ionomycin was added to eggs before Sr^{2+} 5.0mM in Ca^{2+} free media.

In Fig. 3.4B, the emptying and permeabilization of Ca^{2+} stores by ionomycin shows that there are no fluorescence oscillations when there is no Ca^{2+}. This then suggests that Sr^{2+} cannot replace the Ca^{2+} in the ER to enable oscillations. These data support the idea that the oscillations triggered by Sr^{2+} in mouse eggs are truly Ca^{2+}-oscillations, and not oscillations of Sr^{2+} in and out of the ER.

3.4 The relationship between Sr^{2+}-triggered Ca^{2+}-oscillations and medium osmolarity

It is a normal reaction or phenomenon that eggs will swell up or shrink if placed in low or high osmolarity media respectively. A previous study suggested that reduced osmolarity can potentiate Sr^{2+} induced oscillations (Bos-Mikich et al., 1995). It's known that the normal osmolarity of the experimental or culturing media is in the range from 280 to 300mOsm/kg, which is also the normal osmolarity in mammalian serum. To investigate

the effect of osmolarity upon Sr^{2+} induced oscillations, I have deliberately changed the osmolarity of the recording media by adding or subtracting NaCl from the media, whilst making measurements of the osmolarity values using a calibrated freezing point osmometer.

To be able to study appropriately the effect of osmolarity on Sr^{2+}-induced Ca^{2+}-oscillations, I prepared three new concentrated (10x) stocks of H-KSOM. Firstly, H-KSOM with normal Na^{+}, secondly, H-KSOM with a low Na^{+} concentration (~30mM) and finally, H-KSOM with no Na^{+} in which all Na^{+} salts were replaced with K^{+} salts to get zero Na^{+} and high K^{+} H-KOSM, see material and methods chapter (Table 2.1 & 2.2). Different H-KSOM recording media were then prepared with different osmolarities from 200 up to 400mOsm/kg. Here I used either KCl or choline to adjust the osmolarity values. i.e I add KCl or choline based on the target osmolarity level.

My initial results were consistent with the potential facilitating effect of lowering media osmolarity on Sr^{2+} stimulation that has been reported (Bos-Mikich *et al.,* 1995). Not only that but they also show that Ca^{2+}-oscillations can be triggers even with very low extracellular Sr^{2+} concentrations (0.5mM), by lowering media osmolarity.

For these studies Ca^{2+} was monitored in eggs in response to different concentrations of Sr^{2+}, at a variety of osmolarities. Figure 3.5, shows that 0.5mM Sr^{2+} cannot trigger Ca^{2+}-oscillations in mouse eggs placed in 280mOsm/kg HKSOM since there was no response in almost all eggs (16/17) 94.1% (Fig. 3.5A next page). However, 0.5mM Sr^{2+} media does trigger oscillations in the majority of eggs (20/25) 80% with delayed onset when the osmolarity was taken down to 250 mOsm/kg (Fig. 3.5B next page), and a

further decrease down to 200mOsm/kg lead to a prompt series of oscillations in the majority of eggs (19/21) 90.5% (Fig. 3.5C). The threshold Sr^{2+} concentration to trigger Ca^{2+}-oscillations seems to be between 0.5mM and 1.0mM Sr^{2+}. Since, in similar experiments in normal osmolarity (≥ 300mOsm/l) shows that Sr^{2+} cannot cause Ca^{2+}-oscillations at 1mM concentration (15/15) (Fig. 3.6A next page), however by decreasing the osmolarity to 280 mOsm/kg a clear Ca^{2+}-oscillations response was seen in the majority of treated eggs (32/38) 84% (Fig. 3.6B next page).

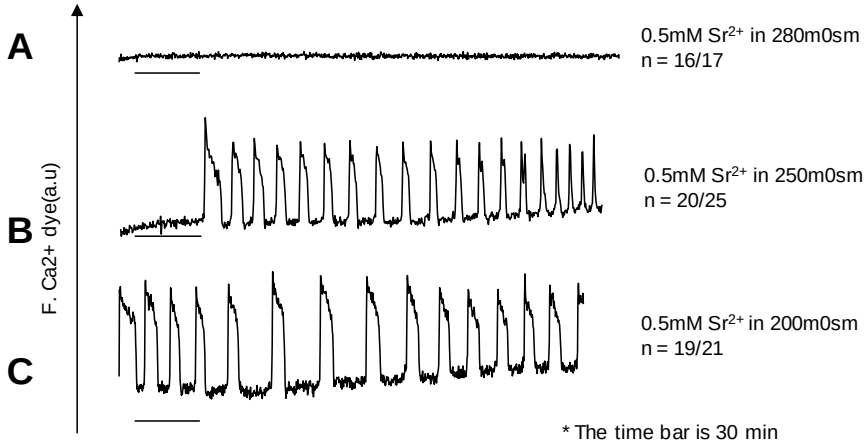


Figure 3.5 Mouse eggs response to 0.5mM Sr^{2+} in different osmolarity values. A) In normal osmolarity media (280mOsm/kg) eggs showed no response, B) in 250 mOsm/kg eggs showed a delayed onset with clear Ca^{2+}-oscillations and C) in 200mOsm/kg eggs showed prompt onset Ca^{2+}-oscillations.

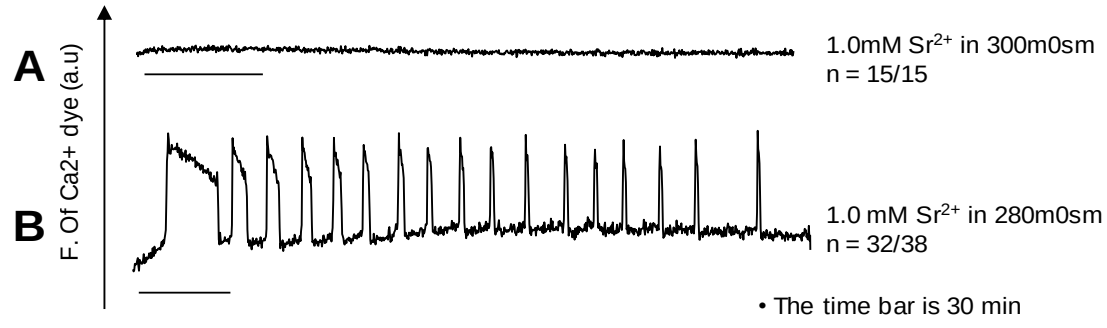


Figure 3.6 Mouse eggs response to 1.0mM Sr^{2+} in different osmolarity media. A) In 300 mOsm/kg eggs showed no response, whereas in B) in 280 mOsm/kg 1mM Sr^{2+} is able to trigger clear Ca^{2+}-oscillations in the majority of eggs.

On the other hand, increasing the osmolarity of media required increasing Sr^{2+} concentrations to triggers Ca^{2+}-oscillations in mouse eggs. Figure 3.7, shows that 5.0mM Sr^{2+} is able to cause Ca^{2+}-oscillations in most mouse eggs kept in HKSOM with osmolarity of 325mOsm/kg (85.7%, 12/14) (Fig. 3.7A), and in some eggs in 350mOsm/kg (5/16) 31.25% (Fig. 3.7B), but it did not causes oscillations in eggs placed in the range of 380mOsm/kg (9/9) (Fig. 3.7C).

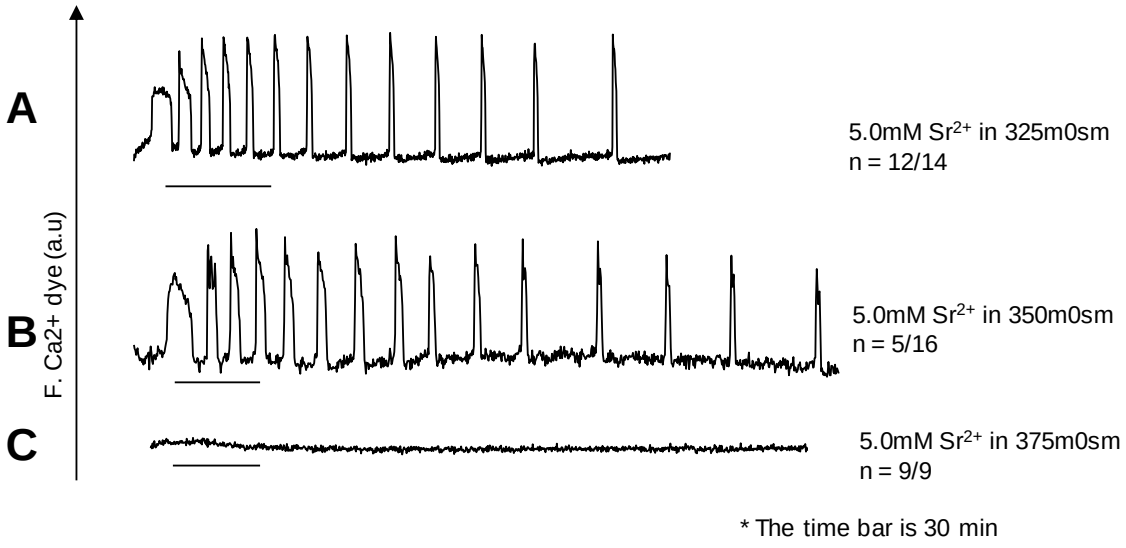


Figure 3.7 Mouse eggs response to 5.0mM Sr^{2+} in different osmolrity media. A) In 375mOsm/kg eggs showed no response, B) in 350mOsm/kg some eggs showed clear Ca^{2+}-oscillations but less frequent compared to C) those placed in 325mOsm/kg

In the same way 10mM Sr^{2+} was unable to triggers Ca^{2+}-oscillations in a very high osmolarity media ~ 400mOsm/kg (17/19) (Fig. 3.8A), but it does in the lower osmolarity media of 375mOsm/kg (11/21) and 350mOsm/kg (8/11) (52.38% and 72.72%) respectively (Fig. 3.8B&C).

To summarize the above results of the effect of osmolarity on Sr^{2+} triggering Ca^{2+} release, Fig. 3.9 (see page 120) shows a line graph that illustrates the relation between

osmolarity and the concentrations of Sr^{2+} required to trigger Ca^{2+}-oscillations in mouse eggs. The bars and points are the standard deviation of the mean respectively.

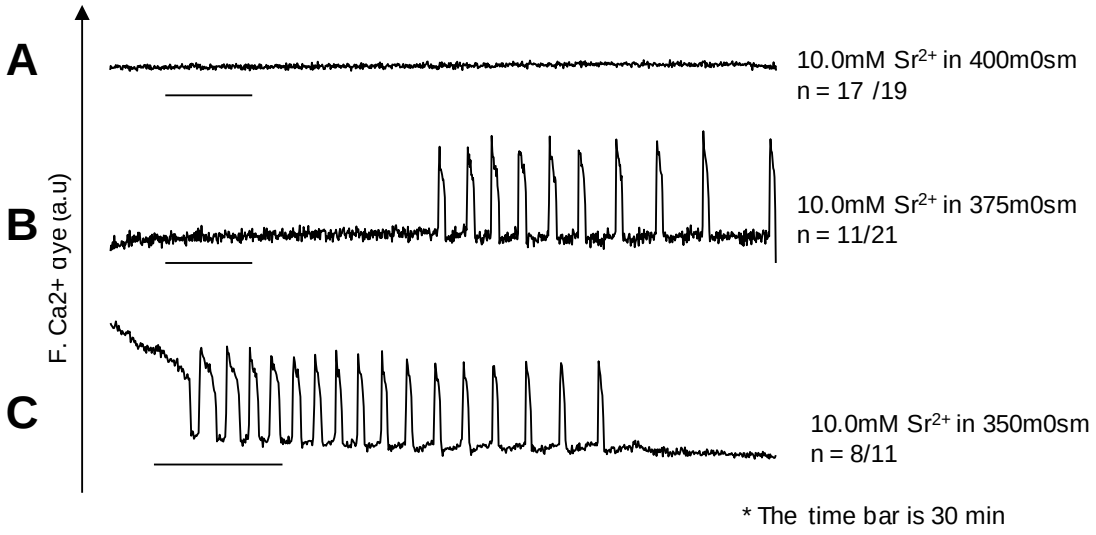


Figure 3.8 Mouse eggs response to 10.0mM Sr^{2+} in different osmolarity values. A) In 400mOsm/kg showed no response. B) in 375 mOsm/kg there is a late response and C) in 350mOsm/kg there is a prompt response with clear Ca^{2+}-oscillations

The spike number in 2hrs (frequency) is used as a standard measure of response in these Sr^{2+} concentration experiments. The simple interpretation of this graph is that the Sr^{2+} concentration that is able to trigger Ca^{2+}-oscillations in mouse eggs is directly related to the osmolarity of the medium used. For example, the media osmolarity threshold of Sr^{2+} triggering Ca^{2+} release at 1mM is around 280mOsm/kg. The addition or subtraction of NaCl to change the media osmolarity to generate these results suggests that some of the effect of media osmolarity on Sr^{2+} influx is related to the Na^{+} concentration. In this case Na^{+} ions could be an important factor in the effectiveness of Sr^{2+} as mouse egg artificial activator. This suggests that it is also worth investigating the

replacement of Na^+ with other ions that are capable to stabilizing the media osmolarity, such as choline or K^+.

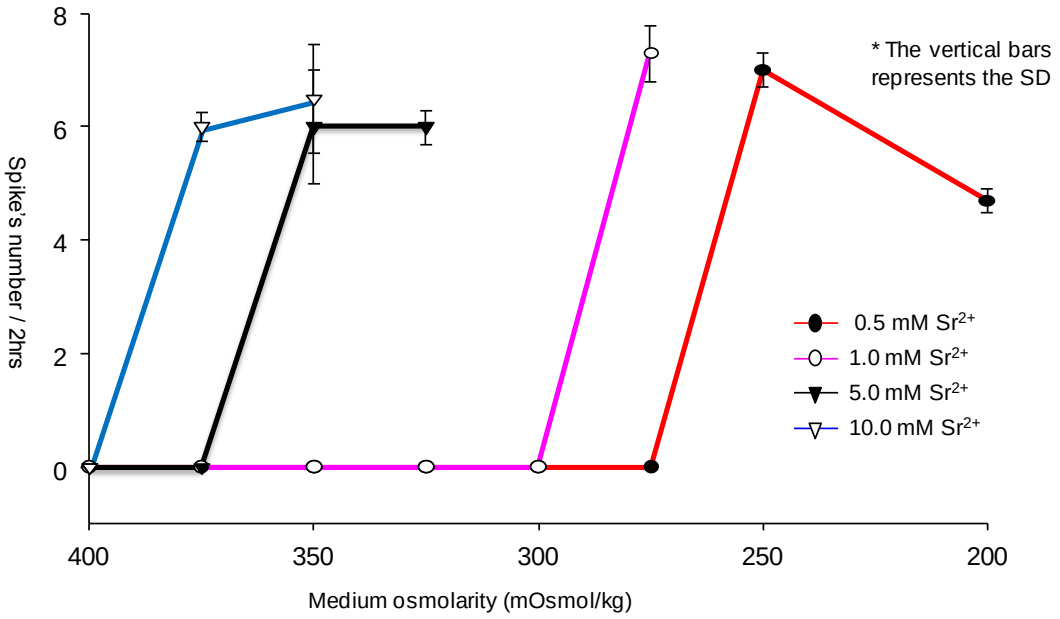


Figure 3.9 The relationship between Sr^{2+} concentrations and medium osmolarities on the Ca^{2+}-oscillations frequency triggered by Sr^{2+} in mouse eggs.

3.4.1 The effect of choline in low Na^+ media on Sr^{2+}-triggered Ca^{2+}-oscillations

To confirm the effect of Na^+ on Sr^{2+} influx, it was required to make changes to the Na^+ concentration whilst keeping osmolarity levels constant. An available substitute for Na^+ is a choline. The choline ion is unlike the Na^+ ion in that it does not cross the cell membrane via channels, and it also works as cell membrane protector (Stachecki *et al.,* 1998). This idea was investigated with a simple experiment, by reducing Na^+ concentration from 130mM to ~30mM. Choline was added to the media to replace the Na^+ to maintain osmolarity. As before eggs were placed in this media and after 2 hrs of

recording Ca^{2+} levels the data were collected and analyzed. The interesting result of this manipulation was that 0.5mM Sr^{2+} (in normal osmolarity media) was found to trigger Ca^{2+}-oscillations in mouse eggs in which the majority of Na^{+} is substituted with chloine (11/16) (Fig. 3.10), the remaining eggs either died or not responded. It is worth recalling here that in Sr^{2+} media with normal osmolarity, Sr^{2+} 0.5mM is completely unable to triggers any Ca^{2+}-oscillations. This experiment was repeated twice with similar results.

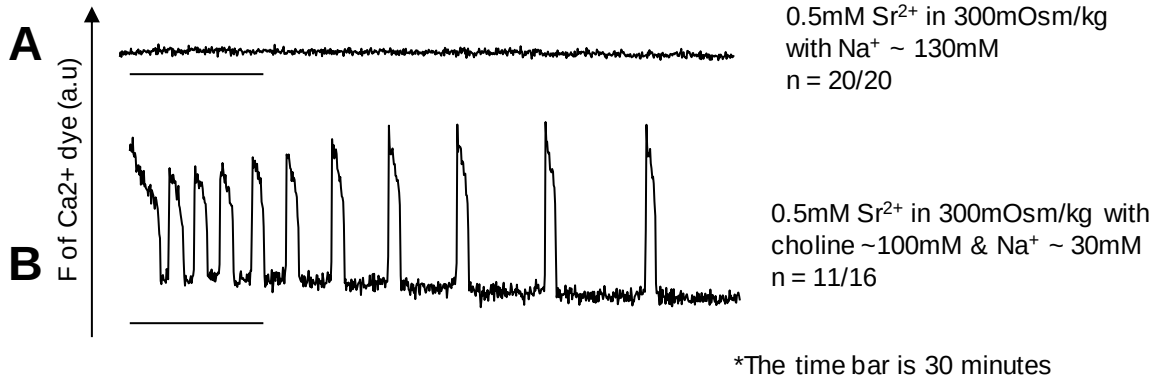


Figure 3.10 The effect of choline (osmolarity stabilized) on the ability of Sr^{2+} to induce Ca^{2+}-oscillations. A) Sr^{2+} 0.5mM cannot trigger Ca^{2+} increases in eggs in Ca^{2+} free HKSOM media (with normal Na^{+} and osmolarity values). In B) by substituting Na^{+} with choline 0.5mM Sr^{2+} triggering Ca^{2+}-oscillations in most eggs.

3.4.2 The effect of high K^{+} in low Na^{+} media on Sr^{2+}-triggered Ca^{2+}-oscillations

Similar to choline experiments I also used KCl to adjust and normalize the Sr^{2+} media osmolarity with a low Na^{+} (~30mM) content. The same protocol was used as in the above experiments. The initial experiments (Fig. 3.11A) showed no Ca^{2+}-oscillations in normal osmolarity with normal Na^{+} contents and 0.5mM Sr^{2+} media (11/12) 91.66%. However, (Fig. 3.11B) illustrates that 0.5mM Sr^{2+} triggered Ca^{2+} oscillations in the majority of eggs in normal osmolarity maintained with K^{+} (>100mM) and ~ 30mM Na^{+}

(35/38) 92.1%. These data support the idea that a reduction in Na^+ concentration alone significantly promotes the action of Sr^{2+} ions in mouse eggs.

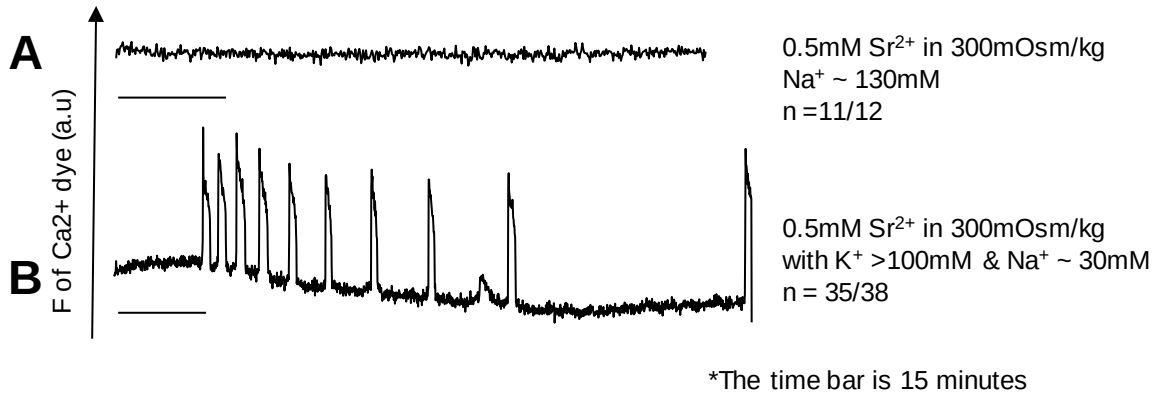


Figure 3.11 The effect of KCl as an osmolarity stabiliser on 0.5mM Sr^{2+}. A) Sr^{2+} (0.5mM) cannot elicit Ca^{2+}-oscillations in eggs in normal Na^+ normal osmolarity media. B) a representative trace of Ca^{2+}-oscillations in eggs triggered by 0.5mM Sr^{2+} in low Na^+ (~30mM) and normal osmolarity media containing high K^+ (>100mM).

3.4.3 Na^+ (0mM) media facilitates Sr^{2+}-triggered Ca^{2+}-oscillations in mouse eggs

The facilitating effect of Sr^{2+} triggered Ca^{2+}-oscillations in low Na^+ media experiments with either choline or high K^+ could be due to the promotion of Sr^{2+} influx via the activation of the reverse mode of the NCX. This was tested by the preparation of a completely Na^+ free (0mM) H-KSOM media that should maximize the exit of Na^+ and promote Sr^{2+} influx. Here I just used KCl in zero Na^+ media since it was impossible to use choline to replace all the Na^+ salts. In this media all Na^+ salts were replaced by K^+ salts for two reasons. Firstly it was important to normalise the osmolarity. Secondly it was helpful to depolarise the membrane potential of eggs and it is known that high K^+ can cause membrane potential depolarisation. With this media we can promote the

NCX in a chemical way, by promoting Na^+ exit, and in an electrical way, by reducing the membrane gradient that drives the normal mode of NCX operation.

Figure 3.12A&B shows that 0.5mM Sr^{2+} media can trigger Ca^{2+}-oscillations in 310mOsm/kg media if the buffer is totally Na^+ free and contains high K^+ (16/19) 84.21%. Although the majority of eggs responded after a varying time, a few eggs (3/19) did not respond. Similar results were obtained by repeating the experiments twice. These data suggests that Na^+ is the key element for Sr^{2+} action on mouse eggs and this could be because zero Na^+ media can activate the reverse NCX and facilitates Sr^{2+} influx in Ca^{2+} free media.

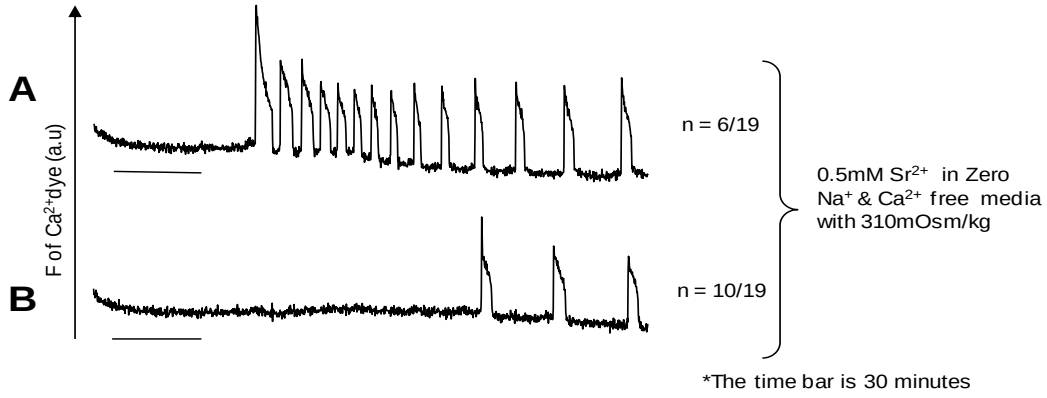


Figure 3.12 The effect of zero Na^+ media on the ability of Sr^{2+} to trigger Ca^{2+}-oscillations. Both A&B traces are from the same experiment which shows that 0.5mM Sr^{2+} can trigger Ca^{2+}-oscillations even in high osmolarity media (310m0sm), that if it is Na^+ free and in which Na^+ is replaced with K^+. A) Represents example of eggs that started to oscillate early and with high frequency. B) Exemplify the eggs that oscillated late and with a lower frequency.

3.4.4 The relationship between Sr^{2+}-triggered Ca^{2+}-oscillations and mouse egg swelling

In the above experiments it was clearly noticed that after two hours of incubation many mouse eggs in Sr^{2+} media became swollen. Additionally, if they were kept for long

periods of time in Ca^{2+} free Sr^{2+} media, most if not all eggs become swollen see (Fig. 3.13, before and after). I decided to study the possible connection between this phenomenon and Ca^{2+} oscillations in mouse eggs and establish if there is a causal relationship.

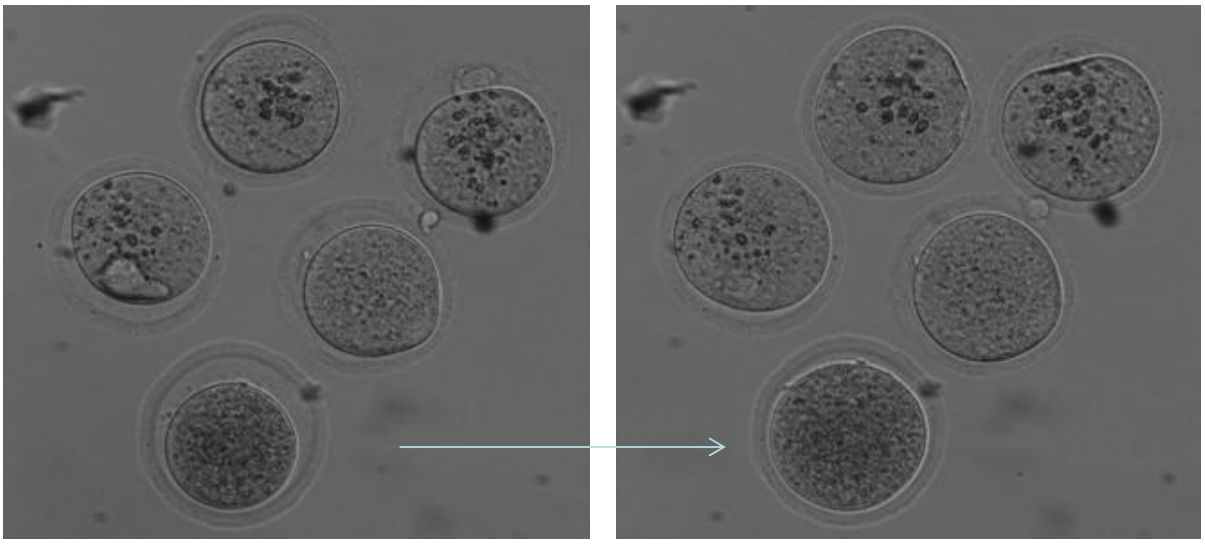


Figure 3.13 Images are of mouse eggs in normal osmolarity H-KSOM (not Sr^{2+} media) and then after lowering the osmolarity. Eggs swell up in low osmolarity H-KSOM media. Similar phenomenon where also notice in Sr^{2+} 0.5mM experiments and typically seen if eggs kept in for a long time (>2hrs).

The simplest way to study the relationship between Ca^{2+} and swelling was to co-inject eggs with two fluorescent dyes (one Ca^{2+} sensitive and the other non-Ca^{2+} sensitive). I could then use one to measure Ca^{2+} and the other to provide a clear outline of the egg in order to measure its cross sectional area. The two dyes need to have different emission spectra i.e. the non-Ca^{2+} binding dye spectrum should not overlap with the Ca^{2+} dye emission spectrum. The most suitable non-Ca^{2+} binding dye to use alongside OGBD is Alexa594. Mouse eggs were co-injected with OGBD and Alexa594 together (1:1) and transferred into a drop of Ca^{2+} free H-KSOM with a borderline osmolarity

~270mOsm/kg containing 0.5mM Sr^{2+}. I used this osmolarity to have a delayed effect of Sr^{2+} in trigger Ca^{2+}-oscillations and yet facilitate egg swelling. Any changes in the egg size should not change the fluorescence intensity of Alexa594 dextran, but egg swelling should result in an increase in the size of the fluorescent cross surface area of the target egg. By monitoring the outline of eggs against time we can plot a line graph using image-J soffware and shows the volume changes in each egg separately.

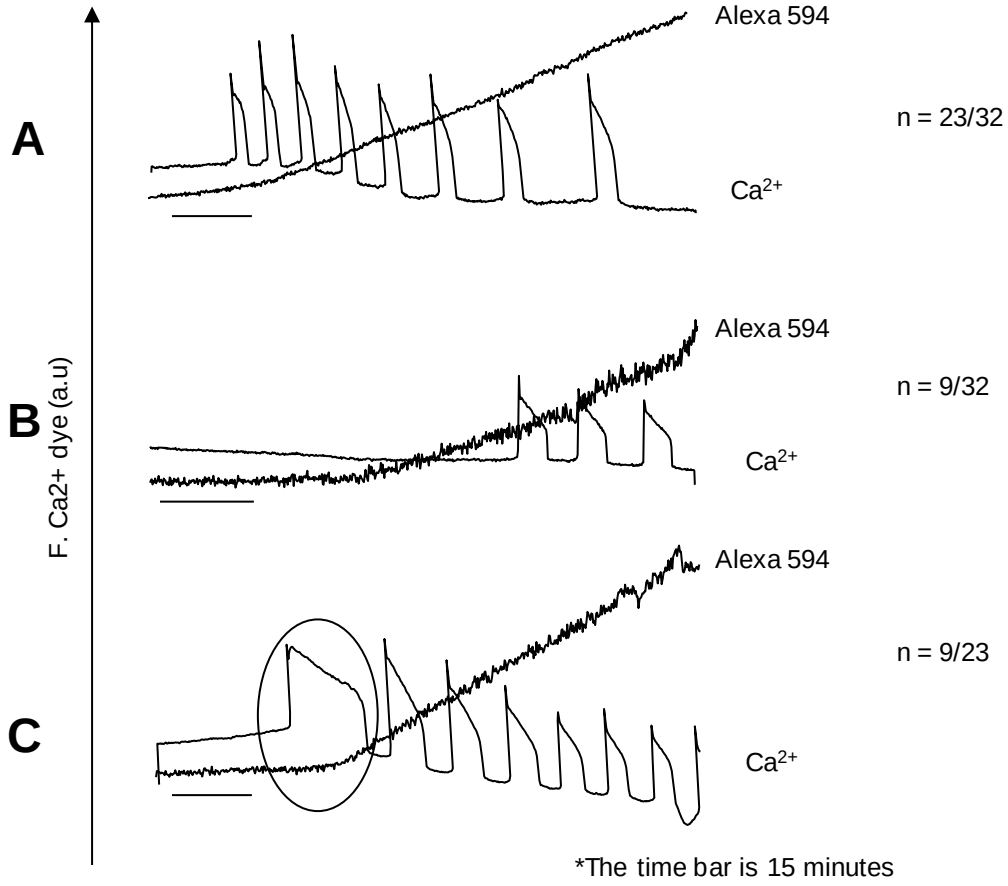


Figure 3.14 The relation between 0.5mM Sr^{2+} triggered Ca^{2+}-oscillations and egg swelling in ~270mOsm/kg media. In A) the Alexa594 dextran signal (thick line) increased gradually just after the time of Ca^{2+}-oscillations onset. B) Shows that some eggs start to swell up before oscillations onset. C) Represents part of (A) eggs and shows that in 39% of eggs swelling starts just after the onset of the first Ca^{2+}-oscillations.

After 2 hrs of recording the data were collected and analysed as usual. The results shows that most eggs started their first Ca^{2+} spike before the swelling started (71.87%, 23/32) (see Fig. 3.14A). However some eggs started to swell up just before they started to oscillate (9/32) 28.12% (see Fig. 3.14B). However in some eggs the swelling started towards the end of the first Ca^{2+} increase' (Fig. 3.14C). The other interesting point of note is that the longer the 1^{st} Ca^{2+} spike the prompt in the rise of the curve for the Alexa 594 fluorescence (9/32) 39.1% see within the circle in (Fig. 3.14C).

These finding suggests that although the majority of eggs undergo swelling during Sr^{2+} induced Ca^{2+}-oscillations, and whilst egg swelling may facilitate ongoing Sr^{2+} influx in mouse eggs, there is no indication that oscillations are triggered by an increase in cell volume.

3.5 The nature of Strontium influx pathways

Sr^{2+} is a divalent cation similar to Ca^{2+} and it can potentially cross cell membranes using different types of Ca^{2+} channels. However, the specificity and affinity of these channels to Sr^{2+} are often different from Ca^{2+}. Based on previous reports the mouse egg membrane may contain a variety of ion channels and exchangers, however, the full spectrum of Ca^{2+} influx channel types in the plasma membrane of the mouse egg is unknown. The confirmed Ca^{2+} channels or exchangers in mouse eggs include the stretch activated channels and the NCX (Pepperell *et al.,* 1999; Carroll, 2000; Machaty *et al.,* 2002). TRP channel expression has also been described in mammalian eggs

(Zhu *et al.,* 1996). Before returning to a more detailed to study of the NCX, I decided to investigate the role of stretch activated channels in Sr^{2+} influx.

3.5.1 Stretch activated channels and Sr^{2+} influx

The stretch activated channels are known to be present in cell membranes in many cell types and they can be osmotically activated (Kondoh *et al.,* 2003). As mentioned previously our protocol in Sr^{2+} experiments was to keep mouse eggs in Sr^{2+} media for 2 hrs, and I had noticed that at the end of this time most eggs increased in size, even in the normal osmolarity media. This occurs particularly when I used borderline osmolarity with particular Sr^{2+} levels, for example a combination of 260mOsm/kg and 0.5mM Sr^{2+}, where some eggs oscillated and other did not. To look into this further I analysed these experiments by correlating the each eggs appearance with their Ca^{2+} responses. Interestingly, the results show that those eggs who swollen that the ones who showed Ca^{2+}-oscillations and vice versa i.e. those who didn't oscillate they didn't swollen either. I then decided to carry out a key experiment which was to suddenly change the osmolarity of the Sr^{2+} media whilst recording Ca^{2+} levels. I did this by transferring OGBD injected mouse eggs into a 300µl drop of Sr^{2+} (0.5mM) media (HKSOM) with a normal osmolarity (300mOsm/kg). Then the experiment was started for and after 1 hr I added 150µl of the same media but with low Na^{+} (30mM) to decrease the osmolarity to ~220mOsm/kg. After over 1 hr of further recording the experiment was stopped.

Figure 3.15, shows that all eggs failed to oscillate for the first ~30 min, but after deliberately reducing the osmolarity the majority of eggs began to oscillate (38/45), and

at the same time all become larger in size (swollen). This result suggests that a lower osmolarity media potentiates Sr^{2+} influx that elicits Ca^{2+} release. The data also suggests that the stimulation of Ca^{2+}-oscillations requires an appropriate combination of Sr^{2+} and media osmolarity (Fig. 3.9).

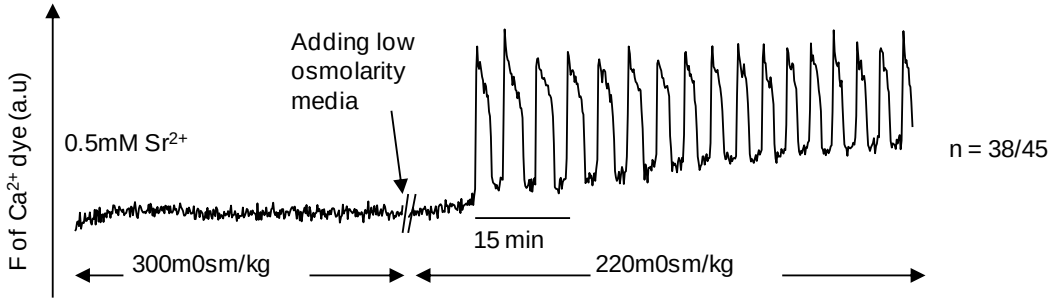


Figure 3.15 The effect of osmolarity on Ca^{2+}-oscillations driven by Sr^{2+} influx augmentation. The switch to a lower osmolarity media triggered Ca^{2+}-oscillations with low extracellular Sr^{2+} concentrations (0.5mM).

The possible explanation of this type of effect could be that, by decreasing the media osmolarity the eggs size increases and this then stretches the egg membranes. This stretch could l then open ion channels which could facilitate Sr^{2+} influx across the plasma membrane and triggers Ca^{2+} release. This idea prompted further investigation of the channels involved in influx in eggs.

3.5.2 The effects of non-selective Ca^{2+} channel blocker on Sr^{2+}-triggered Ca^{2+}-oscillations

There are several substances are known to be a non selective Ca^{2+} channel blockers such as gadolinium (Ga^{3+}) (Biagi and Enyeart,1990) and lanthanum (La^{3+}) (Machaty *et al.,* 2002). These can be used to investigate the role of TRPC in Sr^{2+} influx. The most

common inhibitor used is the Ga^{3+} ion, which is a positively charged and also a known blocker of stretch activated Ca^{2+} channels (Yang and Sachs, 1989). Its effects were investigated upon Ca^{2+} responses of mouse eggs kept in Sr^{2+} media. To start I used final concentrations of Ga^{3+} of 50µM and La^{3+} of 1mM to look at the effects on Ca^{2+} oscillations. These concentrations were chosen based on available literature on other cells. The protocol I used was to transfer OGBD injected mouse eggs into Sr^{2+} media on the recording stage. Then ~30 min after Ca^{2+}-oscillations had started I added either Ga^{3+} or La^{3+} and then kept the recording going for another hour. Unfortunately these initial experiments were not successful. This was because of the formation of crystals or either Ga^{3+} or La^{3+} in the Sr^{2+} media (Ca^{2+} free HKSOM). Ga^{3+} and La^{3+} are ions with three positive charges. This makes it easy for them to precipitate with the negatively charged ions, such as phosphate, that are present in HKSOM. So, to overcome this obstacle we used a modified media that is phosphates and sulphates free (Okada *et al.,* 2003). The Ca^{2+} free version of this buffer is NaCl 137mM, KCl 5.5mM, $MgCL_2$ 1.2mM, and HEPES NaOH 7.5mM, (Kondoh *et al.,* 2003) with 0.2 mM pyurvate as the main nutrient and the osmolarity was adjusted to 297mOsm/kg. Before repeating the previous protocols with the new buffer, I confirmed that both Ga^{3+} and La^{3+} can dissolve in this modified media. By carrying out the same protocol with the new buffer, the results showed that there are clear effects of both ions upon Sr^{2+}-induced Ca^{2+}-oscillations in mouse eggs. In detail, after a few Ca^{2+} oscillations, the addition of Gd^{3+} (50µM) or La^{3+} (1mM) clearly stopped the oscillations, generally after one or more Ca^{2+} spikes either with Gd^{3+} (24/30) 80% of eggs) or with La^{3+} (14/19 73.7% of eggs) (Fig. 3.16A&B).

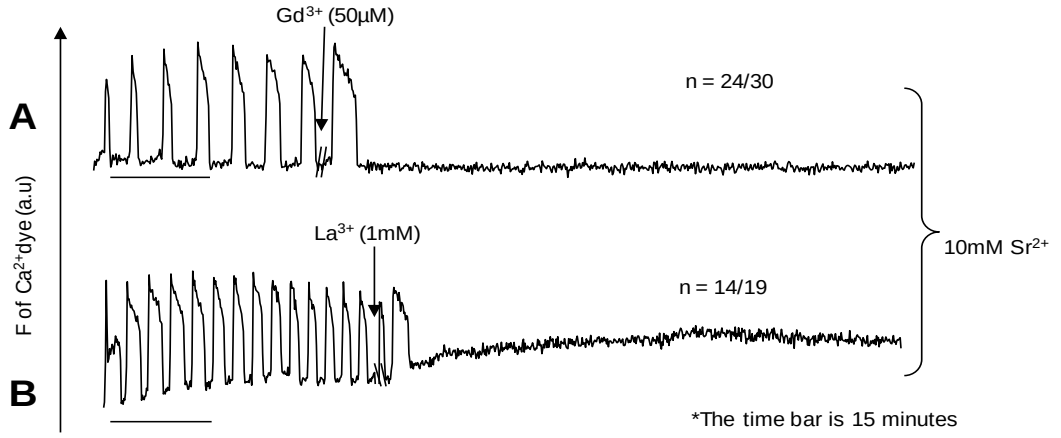


Figure 3.16 The effect of non-selective TRPC blockers on Ca^{2+}-oscillations caused by 10mM Sr^{2+} in mouse egg places in Ca^{2+} and phosphate free buffer. A) After oscillations were established, adding 50uM Gd^{3+} stopped oscillations after one or more Ca^{2+} spikes. B) Similar results were obtained by adding 1mM La^{3+} after oscillations Ca^{2+} had been established.

However, it was noted that for the remaining eggs (6/30) and (5/19) there was a some response to Gd^{3+} and La^{3+}, but this consisted of an increase in Ca^{2+} level that never recovered and eventually lead to egg death. These results suggest a pivotal role of a continuous Sr^{2+} influx for the continuation of Ca^{2+}-oscillations. Although these Ca^{2+} channel blockers are a non specific, these results do suggest that some Ca^{2+} influx channels are present in mouse eggs and that they are required to support Sr^{2+} influx and Ca^{2+}-oscillations.

3.5.3 The effect of selective TRP Ca^{2+} channel blockers upon Sr^{2+}-induced Ca^{2+}-oscillations

To be more precise about which type of Ca^{2+} channel is involved in Sr^{2+} influx I tried to block different kinds of channels selectively using known selective Ca^{2+} channel blockers. The presence or the selectivity of these channels towards Sr^{2+} influx was

judged by the effect of the particular blocker on Sr^{2+}-induced Ca^{2+}-oscillations in treated mouse eggs.

3.5.3.1 The responses of Sr^{2+}-induced Ca^{2+}-oscillations in mouse eggs to GsMTx-4 application

GsMTx-4 is a peptide toxin from *Grammostola spatulata* spider venom that blocks stretch activated ion channels (Suchyna *et al.,* 2000). TRPC1 it is widely expressed in different mammalian tissues, this makes it as one of best example of a stretch activated ion channels (Maroto *et al.,* 2005), making it as a potential candidate for inhibition by GsMTx-4. Some reports show that GsMTx4 blocks TRPC1 as well as TRPC5 (Gomis *et al.,* 2008). TRPC5 has been shown to allow Sr^{2+} influx into mast cells in conjunction with STIM1and Ori1 (Ma *et al.,* 2008). TRPC6 is also a GsMTx-4 sensitive stretch activated ion channel (Alessandri-Haber *et al.,* 2009).

To investigate GsMTx4 I followed the previous protocol using OGBD injected eggs and placing them into Sr^{2+} media at a concentration of 5.0mM. After oscillations had started I added *5*µM GsMTx4 (final concentration) which was mixed well in the dish before continuing recording. However, the result of adding this peptide was that there was no inhibitory effect of GsMTx-4 on Sr^{2+}-induced Ca^{2+}-oscillations. The majority of eggs (77.7%, 7/9) continued oscillating after adding the peptide (see Fig. 3.17). This experiment was repeated with 20µM of GsMTx-4 and the outcomes were the same. Then I modified the protocol by adding 20µM of GsMTx-4M from the beginning to the Sr^{2+} media before even transferring the eggs. The results showed that all eggs in this experiment displayed a continued increase in Ca^{2+} signal and eventually all died. In

general these data provide not support for the idea that TRPC play a role in Sr^{2+} influx in mouse eggs.

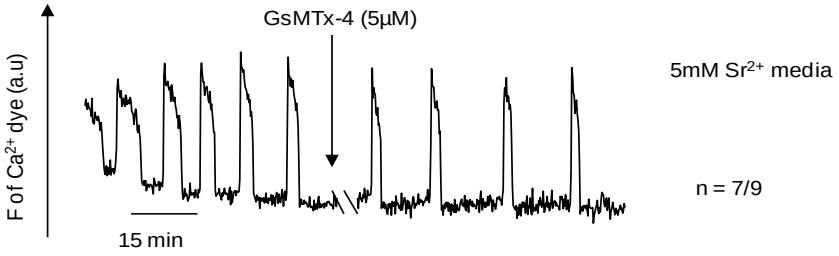


Figure 3.17 Representative trace showing the response of Ca^{2+}-oscillations induced by 5mM Sr^{2+} in mouse egg to which 5uM of GsMTx-4 peptide was added.

3.5.3.2 The effect of other specific Ca^{2+} channel blockers on Sr^{2+}-triggered Ca^{2+}-oscillations

Carvacrol is a novel inhibitor of mammalian TRPM7 (Melastatin) channels (Parnas *et al.,* 2009). TRPM7 is a divalent cation-selective ion channel that is permeable to essential ions such as Ca^{2+} and Mg^{2+}, physiologic metals such as Zn^{2+} and Mn^{2+}, as well as non-physiological ones such as Ni^{2+}, Cd^{2+}, Ba^{2+}, and Sr^{2+} (Penner and Fleig, 2007). Furthermore, TRPM7 has been shown to be expressed in mouse eggs (Evsikov *et al.,* 2006).

I investigated the possibility of stopping Sr^{2+}-triggered Ca^{2+}-oscillations by blocking TRPM7 in mouse eggs using carvacrol. The experiment protocol was the same as previous experiments, and after Ca^{2+} oscillations triggered I added carvacrol to the dish with a final concentration of 500µM. The results were inconclusive since some eggs showed declined in the oscillations frequencies whilst some did not, (5/19) 26.3% and (14/19) 73.68% respectively. Similar results were obtained twice, however, the eggs' responses were variable each time, i.e. (10/23) 43.47% responded and (13/23) 56.52%

did not respond. It could be that Sr^{2+} influx occurs through various membranous pores and TRPM7 is just one of many influx pathways.

I also tried to block store-operated Ca^{2+} entry (SOCE) channel using their specific inhibitor SKF96365 This drug has been found to block voltage-gated Ca^{2+} entry, TRP channels and K^{+} channels (Singh *et al.,* 2010). I used this drug in a concentration range from 10 to 20µM. Unfortunately, the drug kept precipitating in any media including M2 media, HKSOM, and Ca^{2+} free HKSOM and even in the modified phosphate and Ca^{2+} free buffer that was used with Ga^{3+} experiments.

3.5.4 Attempts at TRPC channel activation.

It was difficult to know the exact channel/s that Sr^{2+} used to influx across the membrane of mouse eggs. Since using inhibitors end with limited success, an alternative approach is to try to stimulate specific channels. TRPC5 and TRPC1 have been shown to respond positively to application of reduced thioredoxin (rTRX) or dithiothreitol (DTT) (Xu *et al.,* 2008). TRPC5, TRPC4 and TRPC1 proteins contain a pairs of cysteine residues near glutamic acid 543. These cysteine residues may be covalently linked by a disulphide bridge that can be broken by reduction by a reducing agent that then enables enhanced channel activity such as described for TRPC5 (Xu *et al.,* 2008). I tried to induce Sr^{2+} influx by activating some of the TRPC with these agents in situations where Sr^{2+} is otherwise unable to trigger Ca^{2+} oscillations, such as in normal osmolarity Ca^{2+} free media with 0.5mM Sr^{2+}.

3.5.4.1 The effect of DTT on Sr^{2+}-stimulated Ca^{2+}-oscillations

To investigate the possible role of TRPC5 on Sr^{2+} influx in mouse eggs, eggs were injected with OGBD as a Ca^{2+} indicator and transferred to Ca^{2+} free media with an osmolarity of 250mOsm/kg and containing 0.5mM Sr^{2+}. Then after over 1.5 hour from the experiment starting time, DTT was added to the dish at a final concentration of 1mM. The data showed that after establishing Sr^{2+}-induced Ca^{2+}-oscillations the addition of 1mM DTT clearly accelerates the frequency of oscillations (11/13) 84.6% (Fig. 3.18A). The mean spikes number in pre and post DTT is 2.6/1hr (s.e = 0.24) and 6.3/1hr (s.e = 0.65) respectively, with an overall increase by 2.4 folds. However, some responding eggs (4/11) 36.36% showed a persistent Ca^{2+} signal increase that eventually lead to cell death (Fig. 3.18B).

This experiment was then repeated with Sr^{2+} 1mM, and so I added DTT (1mM) after 1 hour from starting time, and then continued recording for another hour. The data show that 87.5% (14/16) of treated eggs responded to DTT by accelerating the frequency of their Ca^{2+} oscillations from (5.4/1hr) to (9.5/1hr) before and after DTT respectively (Fig. 3.18C). The overall increase was 1.7 times. Similarly, (6/14) 42.85% of eggs showed a sustained Ca^{2+} increase that lead to cell death.

Overall, these data with DTT show a clear augmentation effect on Sr^{2+}-induced Ca^{2+}-oscillations rises. These results also suggest that DTT potentially possess some toxic effect on mouse eggs if the later is kept in for a longer time. This suggests that the TRPC5 could be involved in Sr^{2+} influx, but they cannot be used to conclude or exclude the presence of TRPC5 in the mouse egg plasma membrane.

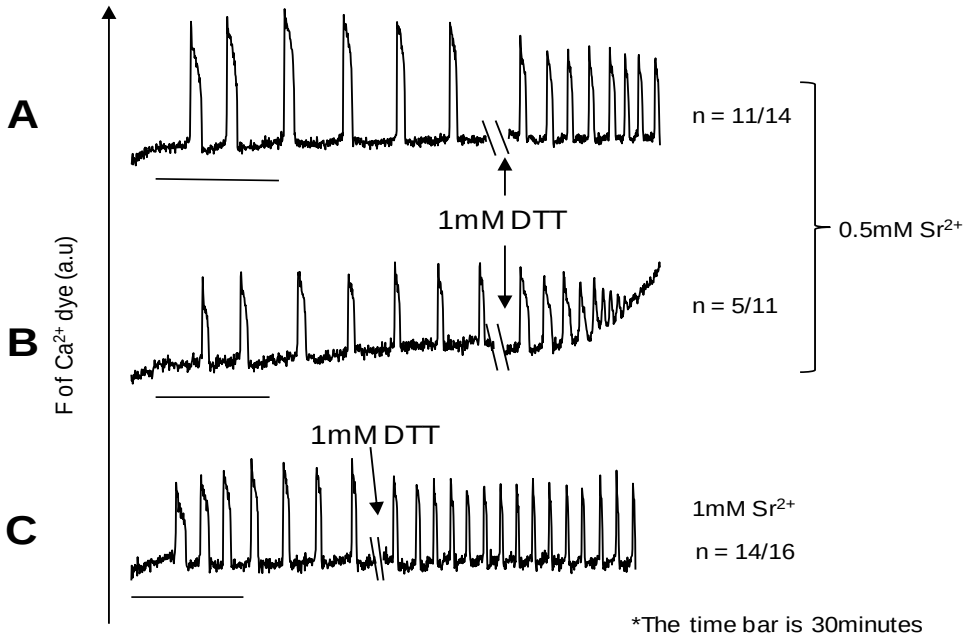


Figure 3.18 Representative data showing the responses of mouse eggs to DDT whilst undergoing Sr^{2+} induced Ca^{2+}-oscillations. A) Shows representative mouse egg responding to 1mM DTT in 0.5mM Sr^{2+} by increased frequency. B) Shows and example of an egg the (A) that died after responding to DTT. C) Shows an example of mouse egg responding to DTT (1mM) in 1mM Sr^{2+} which showed and increased frequency of Ca^{2+}-oscillations.

3.5.4.2 Thioredoxin (TRX) and Sr^{2+} influx

Thioredoxin is an important intracellular redox protein with established biological roles in physiological processes and it is also a secreted protein. It is reduced by the NADPH-dependent flavoprotein thioredoxin reductase which produces the reduced (r) form of thioredoxin (TRX). The rTRX has a capability to cleave disulphide bridges and hence work like DTT as a TRPC5 activator. It is regarded as a novel endogenous extracellular regulator of TRPC5 (Xu *et al.,* 2008).

Bearing in mind the possible toxic effect of the DTT, I used rTRX at a concentration of 1µg/ml. The protocol was similar to DTT experiments in which OGBD injected mouse eggs were transferred into the recording drop containing Sr^{2+} (0.5mM in Ca^{2+} free

media) with an osmolarity of 275mOsm/kg. After the experiment had recorded for ~30min I then added 1µg/ml (final concentration) of rTRX and then recorded for another 30min. The results showed that all eggs survived after adding rTRX, and a clear effect of rTRX on Sr^{2+} influx was noticed. After recording for ~1hr, over half of the tested eggs (13/25, 52%) started oscillating after rTRX addition. However, 20% (5/25) of eggs that were already oscillating even before adding rTRX, continued to oscillate with no clear changes in the frequency. Only 28% (7/25) do not respond after adding rTRX (Fig. 3.19A, B and C).

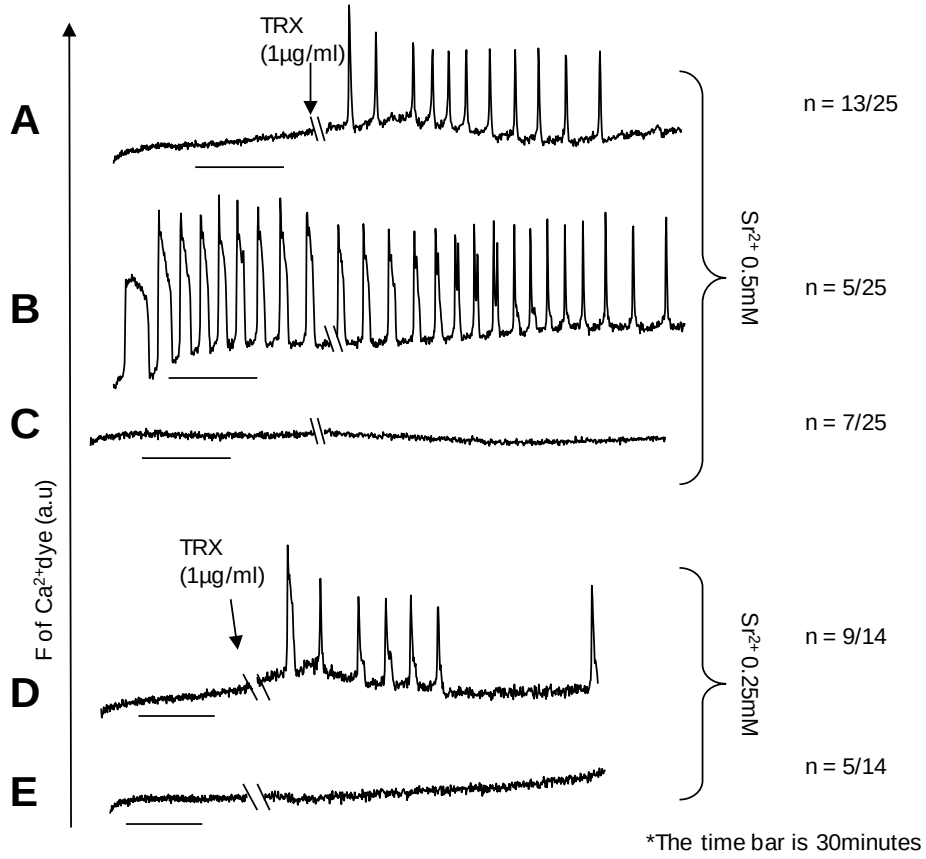


Figure 3.19 The effect of rTRX on Sr^{2+} influx and Ca^{2+}-oscillations. A, B and C shows the combination effects of Sr^{2+} (0.5mM) and rTRX (1µg/ml) on Ca^{2+}-oscillations. More than 50% of eggs responded by triggering oscillations and less than 30% did not respond. D and E were similar protocols to previous experiments but with (0.25mM) Sr^{2+} to prevent eggs from undergoing prior oscillations. Similar findings were seen. Only 35% of eggs do not respond to rTRX in such lower Sr^{2+} concentration.

To stop eggs oscillating before adding rTRX, I repeated this experiment with much lower Sr^{2+} concentration (0.25 mM) whilst keeping the other conditions the same. The results were promising in that the majority of eggs (9/14, 64.3%) started oscillating after adding 1µg/ml, and only 35.7% (5/14) did not respond (Figure 3.19D and E) respectively.

However, the time of response was variable between eggs. This variability could be related to intrinsic factors of eggs. These results suggest that TRPC5 could be present in mouse eggs and Sr^{2+} could conduct through TRPC5. However, it does not mean that TRPC5 is the only channel that allows Sr^{2+} to influx through into mouse eggs. To summarize the blocking and facilitating or promoting Sr^{2+} influx results, it can be proposed that Sr^{2+} may flow through TRPC5 but that this is not likely to be the only channel. Different kinds of Ca^{2+} channels might be involved.

3.5.5 The role of the NCX in Sr^{2+}-induced Ca^{2+}-oscillations

Previous work I presents suggested that the stimulation of Ca^{2+} oscillations by low Na^{+} media, containing choline 'Fig.3.10' could be due to the reversal of the NCX. As mentioned earlier in the first chapter, NCX is a plasma membrane transporter that moves Ca^{2+} in or out of the cell, depending on membrane potential and trans-membrane ion gradients. In mouse eggs the intracellular Na^{+} ion concentration plays a key role in determining the direction of NCX as well (Carroll, 2000). In other words, a minimal increase in the intracellular Na^{+} concentration could reverse the NCX. Similarly, the membrane potential can reverse the NCX, and this is likely to happen when the

exchanger reversal potential is similar to the resting membrane potential as suggested for mouse eggs (Carroll, 2000).

The hypothesis illustrated in (Fig. 3.20A&B) and suggests that in the various Sr^{2+} experiments, Sr^{2+} can substitute for Ca^{2+} and be taken into the cell on the reverse mode of the NCX. This would allow Sr^{2+} to enter the egg in exchange with intracellular Na^{+}. Based on this idea I studied the effect of applying a specific blocker of the reverse mode of the NCX on Ca^{2+} triggered by Sr^{2+} influx.

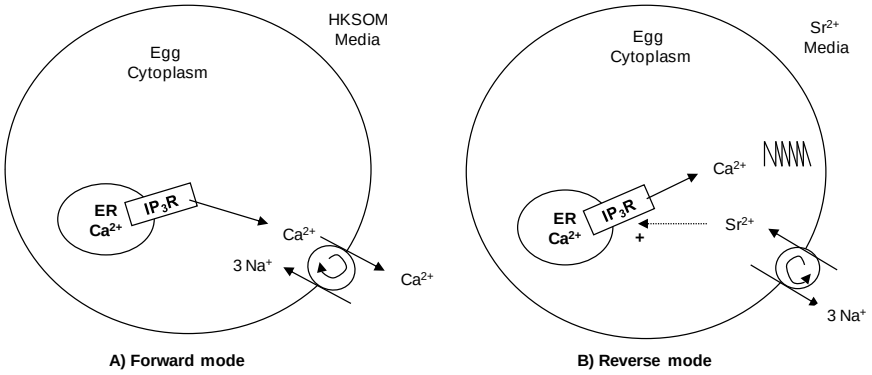


Figure 3.20 Schematic diagram of the hypothesis for Sr^{2+} entry. A) Normal (forward) mode of NCX. B) Reverse mode NCX by which Sr^{2+} could enter the egg and trigger Ca^{2+} release.

3.5.5.1 The effects of KB-R7943 on Sr^{2+}-induced Ca^{2+}-oscillations in mouse eggs

KB-R7943 is a selective blocker of a reverse mode of NCX (Barrientos *et al.,* 2009). The effect of KB-R7943, on Sr^{2+} triggered Ca^{2+} release in mouse eggs was investigated using different protocols and timings of applying of KB-R7943. I used this blocker after and before oscillations had started, and also washed it out after blocking Ca^{2+} oscillations. The results showed a clear effect of KB-R7943. Figure 3.21A, shows the effects of KB-R7943 (20µM) on Ca^{2+}-oscillations triggered by Sr^{2+} (0.5mM). It clearly

shows the inhibition of Ca^{2+}-oscillations in that after addition there was just one extra spike in 16 out of 20 eggs (80%). This protocol was repeated with Sr^{2+} concentrations of 5.0mM and 10.0 mM, and both gave similar results. Figure 3.21B, also shows the effect of KB-R7943 (20µM) upon eggs from the start of the incubation in Sr^{2+} media. The results show that KB-R7943 blocked (0.5mM) Sr^{2+}-induced Ca^{2+}-oscillations in all used eggs (15/15). Similar results were obtained with various Sr^{2+} concentrations (5.0 and 10.0mM). Finally, Fig. 3.21C shows that oscillations could be recovered by washing eggs with KB-R7943-free Sr^{2+} media to remove the drug. Furthermore, the recovered oscillations can be re-blocked by the re-addition of KB-R7943 (20µM). This experiment was repeated twice and gave similar results.

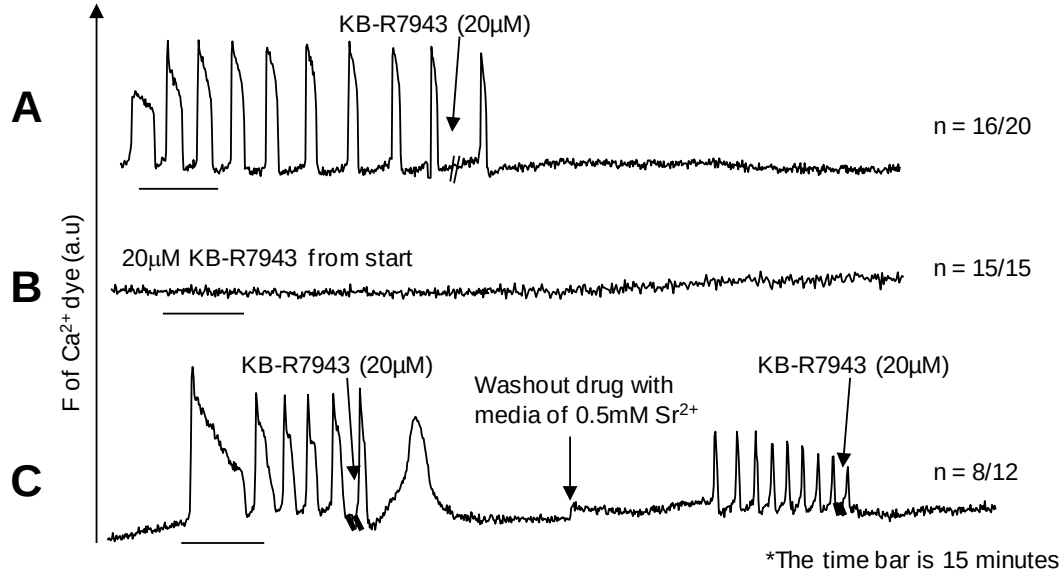


Figure 3.21 The effect of KB-R7943 on Ca^{2+}-oscillations triggered by 0.5mM Sr^{2+}. All experiments were started in 0.5mM Sr^{2+} media. A) adding KB-R7943 ~ 1hr after oscillations, B) Adding KB-R7943 in Sr^{2+} media from the beginning, C) Is in 3 stages, the 1st stage is similar to A, in the 2nd stage KB-R7943 was washed out which recovers the oscillations, and finally in the 3rd stage the re-addition of KB-R7943 re-blocked Ca^{2+}-oscillations.

In my study of KB-R7943's effect on Ca^{2+}-oscillations triggered by Sr^{2+}, I found that the optimal concentration of this blocker was 20µM for mouse eggs. Concentrations of 15µM or less did not blocks oscillations, whereas 25µM or more, not only blocked the oscillations but lead to the death of the some of the eggs. These data suggest that reverse mode of the NCX plays a key role in mediating Sr^{2+} influx that leads to Ca^{2+}-oscillations in mouse eggs.

3.5.5.2 The effects of SN-6 on Sr^{2+}-induced Ca^{2+}-oscillations in mouse eggs

The drug SN6 is also a NCX inhibitor which is structurally related, and has similar properties to, KB-R7943. It also has been shown to block the reverse mode of NCX (Barrientos *et al.,* 2009). I tested SN-6 (20µM) on Sr^{2+}-triggered Ca^{2+}-oscillations in mouse eggs using the same protocol as used in KB-R7943 experiment. I did one experiment and the results showed that SN-6 can also stop Ca^{2+}-oscillations. This result suggests again that Sr^{2+} enters egg cytoplasom via the reverse NCX, since blocking Sr^{2+} influx stops Ca^{2+}-oscillations. Although, most exposed eggs survived after this experiment, I stopped using SN-6 for further experiments because at the end of the experiment I noticed that SN-6 began to precipitate in the media.

3.5.6 Does intracellular Na^{+} oscillate during Sr^{2+}-triggered Ca^{2+}-oscillations?

Given the above data a further investigation into the involvement of Na^{+} and the reverse mode of the NCX and its exchange with Sr^{2+} was required. If there is significant movement of Na^{+} out of the cell via the NCX then it is possible that Ca^{2+}-oscillations

triggered by Sr^{2+} could be accompanied by intracellular Na^{+} changes. In order to monitor intracellular Na^{+} concentrations, sodium green (SG) a specific fluorescent sensitive Na^{+} indicator (Despa *et al.,* 2000) was used to measure intracellular Na^{+} together with a different emission spectrum intracellular Ca^{2+} indicator 'Rhod dextran' (RhD). However, monitoring intracellular Na^{+} is not an easy target, since we know that the normal intracellular value ranges within a narrow window.

In this set of experiments I mixed both dyes together (1:1) with final concentration of 1mM, and then injected them into mouse eggs as part of the routine protocol. The eggs were then transferred into a recording dish containing 10mM Sr^{2+}. The SG signal was measured at a wavelength 530nm and RhD at 600nm. After recording for 2 hours continuously, the experimental data were collected and analysed. Our obtained results showed clear Ca^{2+}-oscillations for RhD and shallow fluctuations in SG signals were seen in almost all tested eggs (15/16) 93.75%. However, these Na^{+} changes were clearly following the baseline signals of RhD in each egg (Fig. 3.22A).

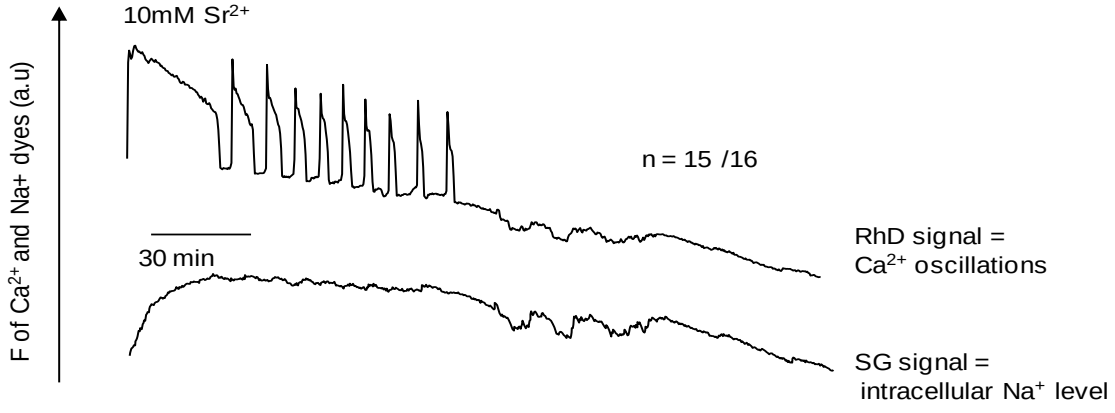


Figure 3.22 The affect of 10mM Sr^{2+} on mouse eggs injected with Na^{+} indicator (SG) and Ca^{2+} dye (RhD) (1:1). Shown figure is a representative mouse egg displaying clear Ca^{2+}-oscillations (RhD) with shallow oscillations in the SG signal.

This protocol has been repeated twice and gave similar results. These results suggest that Na^+ fluctuations accompany Sr^{2+} influx via NCX in mouse eggs. However, it is not clear how significant these intracellular Na^+ fluctuations are during Sr^{2+}-induced Ca^{2+}-oscillations.

3.5.7 The effect of Na^+ ionophore (monensin) on Sr^{2+}-triggered Ca^{2+}-oscillations

Monensin is a monovalent ion-selective ionophore that facilitates the translocation of Na^+ ions across the cell membrane. Based on this I hypothesized that monensin would facilitate Na^+ influx, increase cytosolic Na^+, and this should promote the reverse mode of the NCX. In an exchange of Na^+ with Sr^{2+} this could facilitate Sr^{2+} influx and triggers Ca^{2+}-oscillations in mouse eggs.

To investigate this hypothesis I carried out series of experiments. Ca^{2+} dye injected mouse eggs were placed in Sr^{2+} optimized H-KSOM media with osmolarity (~270mOsm/kg) containing 0.5mM Sr^{2+} with normal extracellular Na^+. The experiment started and was kept running for ~ 1 hr before I added monensin in the media at a final concentration of 10μM. After another hour of recording, the experiment was stopped and the results were analysed. The results showed that some eggs (8/17) 47% start oscillating even before monensin (Fig. 3.23A). The rest (53%) of the eggs (9/17) then started oscillating just after monensis addition (Fig. 3.23B). Regarding the former eggs that oscillated before the addition of monensin could be because of the osmolarity level (270mOsm/kg). The interesting point in this experiment is that compared with pre-

monensin addition, the oscillating eggs showed an increase in the oscillation frequency after monensin by (75.5%). All eggs were then incubated in HKSOM over night in the incubator (5% CO_2, 37C°) and on the next day 12 eggs (70.85%) had progressed to the two cell stage, 4 eggs (23.53%) stayed in MII stage and 1died. Similar results were obtained after the experiment was repeated once.

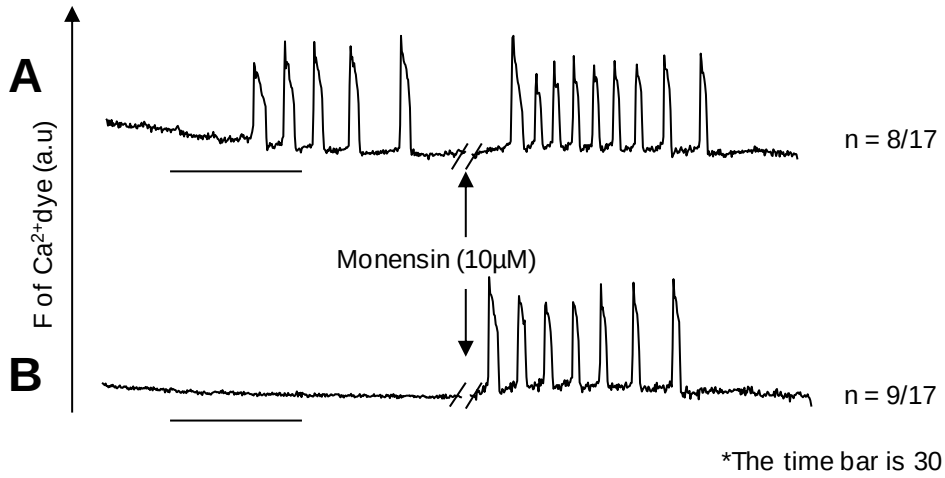


Figure 3.23 The effect of Monensin (10µM) on Sr^{2+} (0.5mM) induced Ca^{2+}-oscillations in low Na^{+} HKSOM Sr^{2+} media. A) Shows an egg that has an increase in oscillation frequency after monensin addition. B) Show Ca^{2+} oscillations triggered after monensin addition in an egg that has not responded to Sr^{2+} alone.

The second set of experiments was performed with higher osmolarity (300mOsm/kg) to prevent eggs from oscillating before monensin addition. A similar protocol was followed and the results showed that (9/14) 64.28% of eggs started oscillating after monensin addition, and that (5/14) 35.71% of eggs did not respond even after monensin addition. These results could be explained by the reversal of the NCX due to an increase in intracellular Na^{+}. This data is consistent with the idea that the NCX is a significant mechanism by which Sr^{2+} can cross the oolemma in mouse eggs.

3.6 Discussion

It has previously been suggested that Sr^{2+} is the most effective parthenogenetic mouse egg activator (Whittingham and Siracusa, 1978; Kline and Kline 1992; Swann and Ozil, 1994; Rogers *et al.,* 2004). I carried out most of the above work based on the theory that Sr^{2+} influx is required to initiate Ca^{2+}-oscillations. I also suggest that a continuous Sr^{2+} influx is necessary for the continuation of the long lasting Ca^{2+}-oscillations in the established Sr^{2+} activation protocols in mammalian eggs. I have also provided some ideas on the mechanism of Sr^{2+} influx in mouse eggs.

3.6.1 Sr^{2+} Influx can cause Ca^{2+}-oscillations

The most challenging experiment I did was the direct intra-cytoplasmic injection of Sr^{2+} (100mM pipette concentration). Prompt Ca^{2+}-oscillations were seen in all survived injected eggs, however, the continuous leaking of Sr^{2+} is required for the continuation of Ca^{2+}-oscillations to cause full activation, since the 2000 pulses were only able to elicited 5 to 9 spikes in injected eggs. However, a couple of more spikes were triggered by re-injecting Sr^{2+}. The restarting Sr^{2+} injection from the same pipette was not always successful, but this is not the case with a new injection pipette. These results clearly suggest that once a sufficient amount of Sr^{2+} is in the cytoplasm it can trigger Ca^{2+}-oscillations. It implies that the factors that affect the ability of Sr^{2+} to trigger oscillations could be related to factors effecting Sr^{2+} influx.

3.6.2 Ca^{2+}-Oscillations are affected by medium osmolarity and Na^{+} contents

It was noticed that keeping mouse eggs in Sr^{2+} optimized media for longer periods of time leads to egg swelling. This was especially true for those eggs that underwent Ca^{2+}-oscillations. It is know that the egg plasma membrane contains different kind of Ca^{2+} channels including stretch activated channels (SAC). I investigated this by deliberately altering the osmolarity of the media via either addition or subtraction of Na^{+} contents (i.e. changing media Na^{+} concentration). The data showed a clear correlation between Sr^{2+} triggered Ca^{2+}-oscillations and the osmolarity levels. Specifically, in a low osmolarity media, even a low Sr^{2+} (0.5mM) concentration can easily trigger Ca^{2+}-oscillations. The possible explanation for this result is that the mechanical effects of egg swelling could stretch these channels resulting in them opening their pores. This in turn could allow Sr^{2+} influx.

3.6.3 The involvement of SAC and TRPC

A clear impact of Sr^{2+}'s action on Ca^{2+}-oscillations in mouse eggs was noticed after the exposure of eggs to plasma membrane Ca^{2+} channel blockers. Blocking Ca^{2+} channels with nonspecific blocker Gd^{3+}, which is known to block different categories of Ca^{2+} channels, was shown to be effective at completely stopping of Sr^{2+} triggered Ca^{2+} oscillations. This suggests that Gd^{3+} is blocking of Sr^{2+} influx. However, various more specific blockers were unable to block Sr^{2+} influx and stop Ca^{2+} transients. One explanation of this is that Sr^{2+} might cross the plasma membrane by different channels.

On the other hand, demonstrating that Sr^{2+}-induced Ca^{2+}-oscillations can be enhanced by facilitating Sr^{2+} influx was successful in some experiments with agents like DTT and rTRX. The activation of TRPC5 channel by DTT or rTRX shows either an increase in frequency in pre-oscillating eggs, or else the triggering of Ca^{2+}-oscillations in non-oscillating eggs in normal osmolarity (290-300mOsm/kg) media. Hence, any means to enhance Sr^{2+} influx may be useful as a way of promoting Ca^{2+}-oscillations.

3.6.4 The oscillations are Ca^{2+}, not Sr^{2+} fluorescent signals

Since there is no specific indicator for Sr^{2+} that is selective over Ca^{2+}, I tried to distinguish between Ca^{2+} and Sr^{2+} using Ca^{2+} ionophore (ionomycin), which has Ca^{2+} affinity of 48 times higher compared to Sr^{2+} (Liu and Hermann., 1978). I tried different protocols using ionomycin with Sr^{2+} on mouse eggs. The addition of ionomycin to eggs after Sr^{2+} triggered oscillations already stopped resulted in a large Ca^{2+} dye fluorescence spike before the signal. This suggests Ca^{2+} is still present within the Ca^{2+} store. This then implies that the Ca^{2+} store has not been replaced by Sr^{2+}.

3.6.5 The key influx mechanism of Sr^{2+} is the NCX reverse mode

Finally, Sr^{2+} triggered Ca^{2+}-oscillations were also seen after the activation of the reverse mode of NCX in 0.5mM Sr^{2+} with normal osmolarity media. This was demonstrated in an experiment with either a low, or zero Na^{+} media. The oscillations in these experiments were completely and reversibly abolished by using a specific reverse mode inhibitor of NCX (20µM of KB-R7943) (Fig. 3.21).

In addition, by applying the Na^+ ionophore (monensin) to eggs, Sr^{2+}-triggered Ca^{2+}-oscillations that were increased in frequency, or else caused new transients in non-oscillating ones (Fig. 3.23). This suggest that Sr^{2+} substitutes for Ca^{2+} in the reverse mode of NCX, which could mean that Sr^{2+} influx can occur via SAC and TRPC channels, as well as the reverse mode of the NCX. However, the reverse NCX seems to be that a major route for Sr^{2+} influx, since Ca^{2+} oscillations were completely abolished after addition of 20µM of KB-R7943. The effects of this drug represent the only successful inhibition of oscillations with a specific drug.

3.6.6 The role of intracellular Na^+ in Sr^{2+}-induced Ca^{2+}-oscillations

Some extra experiments were done based on the role of Na^+ in Sr^{2+} triggered Ca^{2+}-oscillations. These were suggested by the effects of osmolarity and the by the reverse NCX experiments. The idea was to see if intracellular Na^+ would oscillate during Sr^{2+}-induced Ca^{2+}-oscillations. To investigate this possibility a specific Na^+ indicator was required. SG was the best Na^+ indicator I could use since it has fluorescence with a different wavelength from the Ca^{2+} dye RhD. The interpretation of these results suggest that intracellular Na^+ fluctuates within a narrow range, since the SG signal shows shallow fluctuations, and these seems to follow the Ca^{2+} base line levels (see Fig. 3.22). The other study which was carried out to try and confirm the role of intracellular Na^+ in Sr^{2+}-induced Ca^{2+}-oscillations was to raise cytosolic Na^+ levels artificially. The principle of this study was based on using the Na^+ ionophore, Monensin. Adding monensin (10µM) into in low Na^+ H-KSOM Sr^{2+} (0.5mM) media shows a clear augmentation effect

upon Sr^{2+}-induced Ca^{2+}-oscillation activity. This effect was noticed by the way monensin either triggered Ca^{2+}-oscillations or increased the frequency of the current oscillating eggs (Fig. 3.23).

To sum up, putting all these experimental results together, I can conclude that in order for Sr^{2+} to trigger Ca^{2+} releases it has to cross the plasma membrane to reach the IP_3R. The influx mechanism is mediated through different Ca^{2+} membrane channels and exchangers. Amongst all of these the NCX, in the reverse mode is the major contributor in mouse eggs.

Chapter 4

PLCζ AND Ca^{2+}-OSCILLATIONS

4.1 Overview of Phospholipase Cs

As mention in the main introductory section that there are 13 PLCs isozymes, grouped into 6 isoforms. All PLCs are cytosolic enzymes that require membrane association to access their phospholipid substrate. PLCζ has the smallest molecular mass and most primary domain organization among mammalian PLC isozymes. PLCζ consists of a tandem pair of EF hand domains at the N-terminus, followed by catalytic X and Y domains connected by specific amino acid sequence and called XY linker region, and a C-terminal C2 domain. All of which are common to the other PLC isozymes (Saunders *et al*., 2002; Swann *et al.,* 2006; Suh *et al.,* 2008).

Although, PLCζ possess a similar domain organization to PLCδ1, there are two notable exceptions in that PLCζ lacks an N-terminal pleckstrin homology (PH) domain and has a longer XY linker region compared to PLCδ1. The PH domain of PLCδ1 has been shown to plays a major role in membrane binding to PLCδ1 whenever PIP_2 is available (Pawelczyk and Lowenstein, 1993; Lomasney *et al.,* 1996). However, PLCβ2 binds strongly but non-specifically to lipid membranes via interaction with a G protein (Singh and Murray, 2003), whereas PLCγ binds to membranes specifically with high-affinity to PIP_3 (Bae *et al.,* 1998; Falasca *et al.,* 1998). PH domains are a significant mediator of membrane binding. They are well-defined structural modules comprising of over 120 amino acid residues and have been identified in >100 different proteins (Rebecchi and Pentyala, 2000).

The somatic PLCβ, δ and γXYl region has been shown to mediate auto-inhibition of PIP_2 hydrolytic activity specifically. It is proposed that the electrostatic repulsion of the

negatively charged residues of the XYl in these PLCs is stopped from making close contact with the negatively charged PIP_2 substrate (Hicks *et al.,* 2008). The presence of additional regulatory domains SH2, SH3 (Src homology) in the PLCγ XY linker region regulates PLCγ via tyrosine phosphorylation (Ozdener *et al.,* 2002) and the identification of PLCγ inhibition via the SH2 domains has led to a proposed general mechanism of PLC auto-inhibition mediated by the XYl region (Gresset *et al.,* 2010).

Several studies are carried out in this chapter on PLCζ, all were performed as a collaboration with Tony Lai's group. All molecular constructs for the PLCζ work including the RNA preparation in this thesis were carried out by Michael Nomikos and Maria Theodoridou. All created PLC constructs including wild type, were tagged at the C-terminus with luciferase to enable real-time monitoring of the relative protein expression by luminescence quantification (Swann *et al.,* 2009). It is worth mentioning here that each study in this chapter was done with a newly prepared batch of cRNA for PLCζ. As a consequence there are some differences in the wild type luminescence /egg and Ca^{2+} spike number.

A key role and involvement of PLCζ in fertilization has been further supported by two recent clinical reports that have linked either a reduced protein (PLCζ) level (Yoon *et al.,* 2008) or mutated forms of PLCζ with cases of human male infertility (Heytens *et al.,* 2009).

4.2 Male infertility-linked point mutation that disrupts the calcium oscillation-inducing activity and PIP_2 hydrolysis activity of sperm PLCζ

A recent report has identified one infertility case following failed ICSI treatment that was associated with a point mutation in the PLCζ catalytic Y domain. The replacement of histidine with a proline residue at a position 398 (H398P) correlated with the absence of Ca^{2+} oscillation-inducing activity of human PLCζ as tested in mouse eggs (Heytens *et al.,* 2009). His^{398} is conserved in PLCζ from various mammalian species as well as in PLCδ1 (Nomikos *et al.,* 2011a).

The resultant constructs were tested *in vivo* by injecting the cRNA encoding for constructs into mouse eggs along with Ca^{2+} sensitive dye (OGBD). I then analysed their effects on Ca^{2+} oscillation-inducing activity and correlated this with the levels of protein expression. It is important to get a proper assessment of the Ca^{2+} oscillation-inducing activity of each of these PLCζ constructs, including the wild type and mutants, and to verify that these constructs were faithfully expressed as functional proteins in cRNA-microinjected mouse eggs. Consequently, PLCζ-luciferase fusion constructs were prepared to enable accurate quantification of the relative protein expression in the same eggs were Ca^{2+} was measured. Our primary interest was to look at the Histidine to proline substitution at positon 435. However, for comparative analysis, we also introduced a new mutation on the same site by replacing His^{435} with a neutral non helix-destabilizing residue, alanine, to produce $PLCζ^{H435A}$. Furthermore, in these studies a negative control experiment was carried out, and for this purpose we used an additional

charge-reversal mutant that produces an inactive PLCζ enzyme 'PLCζ^{D210R}' that has been described previously (Saunders *et al.,* 2002). Additionally, we also examined the effect of the histidine to proline type of mutation on the activity of PLCδ1 by replacing its equivalent conserved His^{542} with proline to yield PLCδ1^{H542P}. Finally, we investigated potential dominant-negative inhibitory effects of PLCζ^{H435P} on the Ca^{2+} oscillation-inducing activity of mouse PLCζ^{WT} cRNA and mouse sperm PLCζ during *in vitro* fertilization.

In this study cRNA was prepared for PLCζ^{WT}, PLCζ^{H435P}, PLCζ^{H435A}, PLCζ^{D210R} and PLCδ1^{H542P}. To avoid using PLCζ^{WT} at concentrations that might cause the overstimulation of Ca^{2+}-oscillations, PLCζ^{WT} was diluted with RNAase free KCl and microinjected at a level that could match the Ca^{2+}-oscillations frequency that is typically induced by a single sperm. The final concentration of PLCζ^{WT} was co-injected into mouse eggs with OGBD in a 50:50% mix.

4.2.1 Methodology

In order to study the effects of the above mutation in molecular terms, Nomikos and his colleagues introduced the infertility-linked human PLCζ^{H398P} mutation into its equivalent position in mouse PLCζ. This is at His435 and gives rise to a mutation referred to as PLCζ^{H435P} (Figure 4.1). The full details of how this mutation was made are given in (Nomikos *et al.,* 2011a). In brief, cloning of PLCζ, PLCδ1, and mutant constructs were amplified by PCR from the original cDNA clone (GenBank™ accession number AF435950) using Phusion polymerase (Finnzymes). The PCR products were cloned into pCR3-PLC vector after the amplification of the luciferase open reading frame.

Mouse PLCζ-luciferase in pCR3 was then subjected to site-directed mutagenesis (QuikChange II; Stratagene) to generate the PLCζ^{H435P}, PLCζ^{H435A} and PLCζ^{D210R} mutants. PLCζ^{WT} and mutants were amplified by PCR from the corresponding pCR3 plasmid and were cloned into pGEX-6P1 (GE Healthcare). Similarly, rat PLCδ1 in pGEX-5X2 was subjected to site-directed mutagenesis to generate PLCδ1^{H542P}. Following linearization of wild type and mutant PLCζs, The cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and a poly(A) tailing kit (Ambion), as per the manufacturer's instructions (Nomikos *et al.,* 2005 and Nomikos e*t al.,* 2011a).

The resultant constructs were then handed over to me for *in vivo* testing, I co-inject them individually into unfertilized mouse eggs with Ca^{2+} dye (OGBD) as usual protocol see material and method (chapter 2).

4.2.2 Objectives

In the present study, my objective was to investigate the newly reported human PLCζ mutation quantitatively using mouse PLCζ. In addition, my aim was to investigate the potential negative effects of the H435P mutation on the PLCζ^{WT} activity by determine whether an equivalent or excess level of PLCζ^{H435P} protein expressed in mouse eggs can interfere with the Ca^{2+} oscillation-inducing activity of either PLCζ^{WT} or sperm (IVF). Finally, as a comparative study, we looked at the possible effects on PLCζ Ca^{2+}-triggering activity of inserting a neutral amino acid [alanine] in the same His435 residue position.

4.2.3 Results

4.2.3.1 H-to-P mutation abolishes Ca^{2+} triggering activity of PLCζ

Figure 4.1 shows that all $PLC\zeta^{WT}$-injected mouse eggs (17/17) displayed prominent Ca^{2+} oscillations, with the first Ca^{2+} spike occurring at a luminescence average of (0.35 c.p.s) around 30 min post injection time (also see Table 4.1) This level of luminescence is estimated to be equivalent to a protein expression level of around 35 fg/egg (see materials and methods for how we estimate this). This figure is similar to what previously reported for myc tagged PLCζ (Saunders *et al.,* 2002). In contrast, microinjecting cRNA encoding either $PLC\zeta^{H435P}$ or $PLC\zeta^{D210R}$ failed to cause any Ca^{2+}-oscillations in all injected eggs (17/17) and (22/22) respectively (Fig. 4.1 & Table 4.1). The lack of response for these two constructs remains the case even after relatively high levels of protein expression such (10.7 c.p.s and 8.6 c.p.s/egg). This is in comparison to only (7.33 c.p.s/egg) for the wild type PLCζ (Table 4.1). However, microinjecting the $PLC\zeta^{H435A}$ mutant caused Ca^{2+}-oscillations in all injected eggs (22/22) (Fig. 4.1), exhibiting a closer potency to $PLC\zeta^{WT}$. The main exception was that the first Ca^{2+} spike was detected after a protein expression level of (0.73 c.p.s./egg) and the oscillations starting time was just less than an hour after the time of injection (Table 4.1). These results show that the mouse $PLC\zeta^{H435P}$ mutation is completely inactive in mouse eggs, whereas the $PLC\zeta^{H435A}$ mutation retains most of its Ca^{2+} oscillation-inducing activity. These experiments were repeated twice and similar results were obtained.

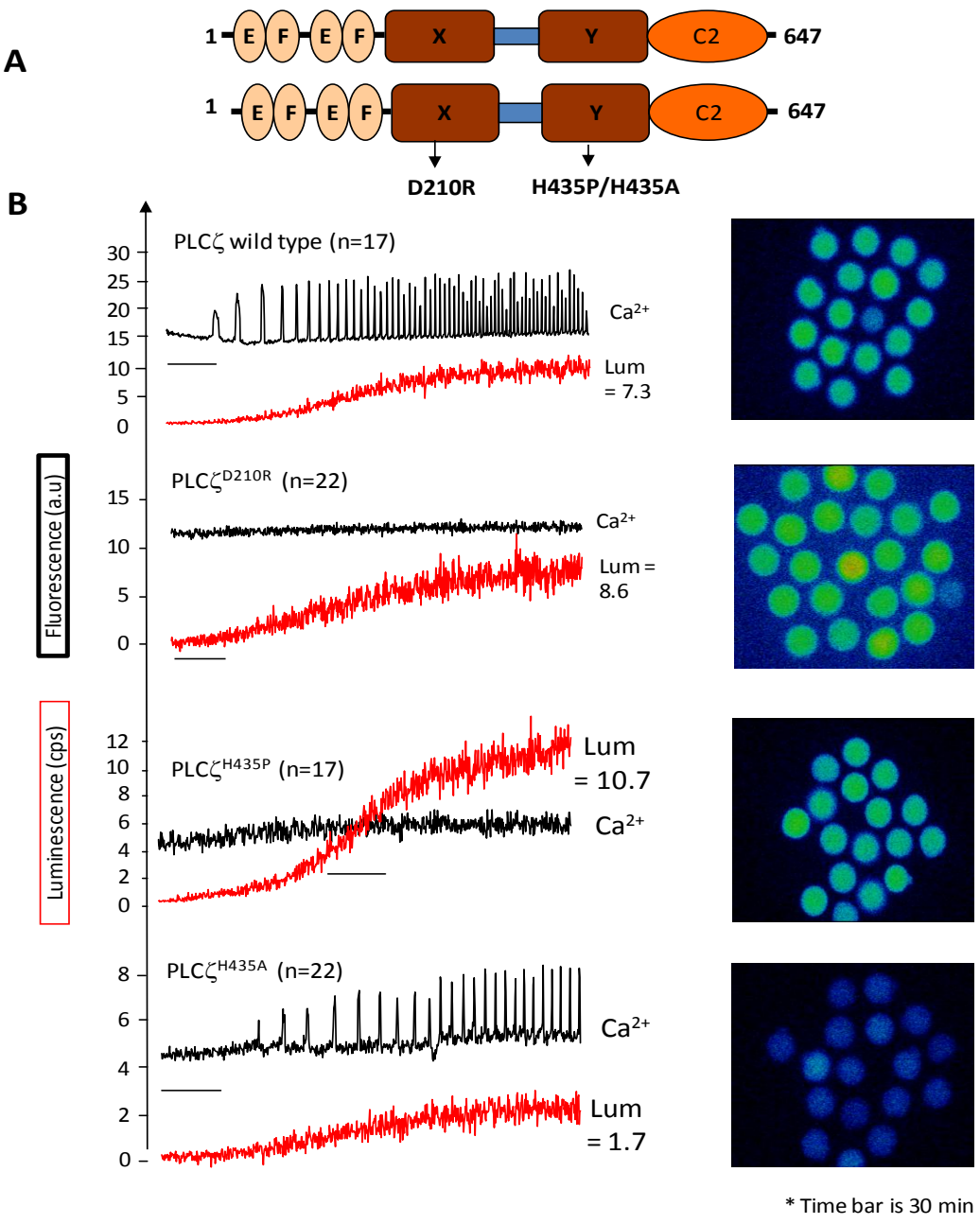


Figure 4.1 Ca^{2+} oscillation-inducing activity of PLCζ-luciferase and mutants in unfertilized mouse eggs. (A) Schematic representations of mouse PLCζ domain structure of wild type and mutant H435P ,H435A and D210R (control mutation) and identifying their locations within the catalytic Y and X domains. (B) The left-hand panels show representative fluorescence (a.u.; arbitrary units) and luminescence (c.p.s.) recordings reporting the Ca^{2+} concentration changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum) respectively in a mouse egg following microinjection of the indicated PLCζ-luciferase cRNA (encoding either $PLC\zeta^{WT}$, $PLC\zeta^{D210R}$, $PLC\zeta^{H435P}$ or $PLC\zeta^{H435A}$). Right-hand panels show integrated images of luciferase luminescence from eggs microinjected with the corresponding PLCζ-luciferase cRNA. The peak luminescence (Lum) recorded is shown in c.p.s.

Luciferase-tagged PLCζ	no of Ca^{2+} spikes in 1st 2 hrs	Lum peak (c.p.s.)	Protein peak expression (fg)	Time of 1st spike (min)	Lum at 1st spike (c.p.s.)	Protein expression = 1st spike (fg)	Total no of eggs
PLCζ wt	19.0 ± 0.14	7.33 ± 0.38	152.6	~30	0.35 ± 0.037	34.8	17
PLCζ ^{H435P}	-	10.7 ± 0.62	229.9	-	–	-	17
PLCζ^{H435A}	9.8 ± 0.65	1.74 ± 0.14	58.2	~55	0.73 ± 0.038	41.1	22
PLCζ^{D210R}	-	8.6 ± 0.55	174.1	-	–	-	22

Table 4.1 Properties of PLCζ-luciferase and mutants expressed in mouse eggs. Ca^{2+} oscillation-inducing activity (Ca^{2+} spike number in 2 h; time to first Ca^{2+} spike) and luciferase luminescence levels (peak luminescence; luminescence at first spike) are summarized for mouse eggs microinjected with each PLC-luciferase construct (see Fig. 4.1).

4.2.3.2 H435P has no potential dominant negative effects on the PLCζ^{WT} and sperm Ca^{2+}-oscillatory activity

To investigate whether PLCζ^{H435P} can alter the Ca^{2+} oscillation-inducing activity of PLCζ^{WT}, we co-microinjected into mouse eggs an equal mixture of cRNA encoding luciferase-tagged PLCζ^{H435P} and PLCζ^{WT} (Fig. 4.2, top panel). The co-injected cRNA produced Ca^{2+}-oscillations with comparable properties with those observed with PLCζ^{WT} alone (Fig. 4.1, top panel and Table 4.1), showing a time to the first peak of ~30 min (Table 4.2). This suggests that the expression of PLCζ^{H435P} at similar levels to PLCζ^{WT} does not interfere with Ca^{2+}-oscillations. To determine whether an excess of PLCζ^{H435P} was required to block PLCζ^{WT}-induced Ca^{2+}-oscillations, we performed sequential cRNA microinjections. PLCζ^{H435P} was injected into eggs first, followed after a period of 1 or 2 hrs by a second injection of PLCζ^{WT} (Fig. 4.2, middle and lower panels). I performed this protocol in order to get prior expression of PLCζ^{H435P} for 1 or 2 hrs before there could be

any expression of the PLCζ^{WT}. Carrying out this protocol did not interfere with the induction activity of PLCζ^{WT} cRNA which triggered normal Ca^{2+}-oscillations that started in average of ~30 min after injection. Table 4, shows that, in these double cRNA injection experiments, the cumulative luminescence for both PLCζ^{H435P} and PLCζ^{WT} at the time of first spike (7.06 and 18.68 c.p.s./egg) for 1 and 2 h respectively) was much higher than for control PLCζ^{WT} alone (0.35 c.p.s.; Table 4.1). This suggests that an excess of PLCζ^{H435P} protein does not interfere with PLCζ^{WT}-induced Ca^{2+}-oscillations. The estimated PLCζ^{H435P} protein level of 344 fg in the egg after 2.5 h (2hrs before +30minutes after recording) (Fig. 4.2 and Table 4.2), when the first spike is observed ~30 min after injection of PLCζ^{WT}, is well above the (~35 fg/egg) estimated to be required for Ca^{2+}-oscillations with PLCζ^{WT} alone (see Fig. 4.1 and Table 4.1). This result therefore suggests that the PLCζ^{H435P} protein even when expressed at a 10-fold excess remains unable to block Ca^{2+} oscillation-inducing activity of PLCζ^{WT}.

Luciferase-tagged PLCζ	no of Ca^{2+} spikes in 1st 2 hrs	Lum. peak (c.p.s.)	Protein peak expr-ession (fg)	Time of 1st spike (min)	Lum at 1st spike (c.p.s.)	Protein expression = 1st spike (fg)	Total no of eggs
PLCζ^{H435P} & PLCζ^{WT} mix	21 ± 1.7	3.4 ± 0.14	86.26	~30	0.68 ± 0.045	40.4	19
PLCζ^{H435P} after 1hr PLCζ^{WT}	17.2 ± 1.0	15.8 ± 0.47	295.8	~30	7.06 ± 0.72	148.2	21
PLCζ^{H435P} after 2hrs PLCζ^{WT}	15.5 ± 1.09	28.1 ± 1.6	453	~30	18.68 ± 1.2	344.6	13

Table 4.2 Properties of PLCζ^{WT} and PLCζ^{H435P} co-expressed in mouse eggs. Ca^{2+} oscillation-inducing activity (Ca^{2+} spike number in 2 h; time to first Ca^{2+} spike) and luciferase luminescence levels (peak luminescence; luminescence at first spike) are summarized for mouse eggs microinjected with each PLC-luciferase construct (see Figure 4.2). Values are means±S.E.M.

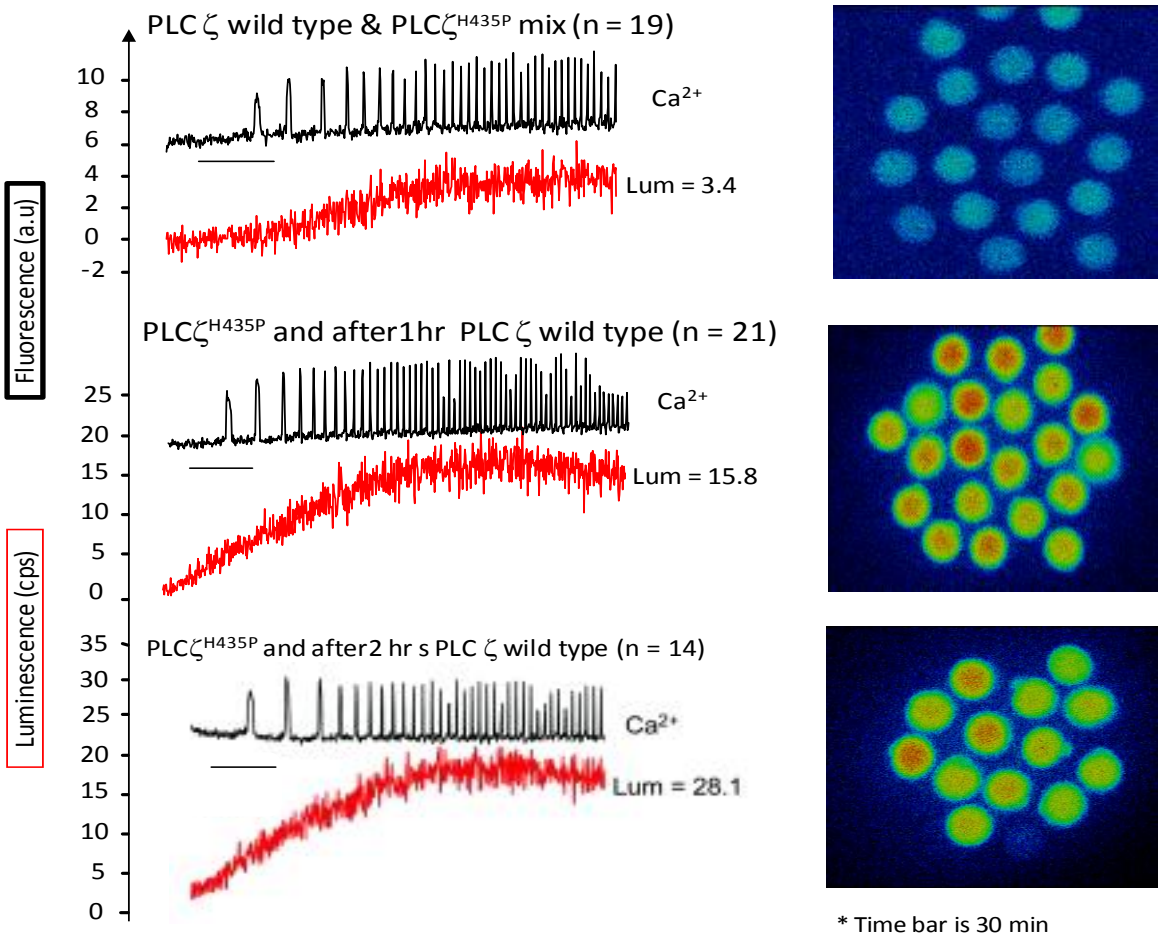


Figure 4.2 Co-expression of PLCζ^{H435P} and PLCζ^{WT} in mouse eggs

Left-hand panels show representative fluorescence and luminescence recordings reporting Ca^{2+} concentration changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum) respectively in a mouse egg. The egg was co-microinjected with equal amounts of PLCζ-luciferase cRNA encoding PLCζ^{WT} and PLCζ^{H435P} (top panel), or was initially microinjected with cRNA for PLCζ^{H435P} followed, after a period of 1 h (middle panel) or 2 h (bottom panel), by the microinjection of cRNA for PLCζ^{WT}. Right-hand panels show the integrated image of luciferase luminescence from eggs microinjected with PLCζ^{H435P} and PLCζ^{WT} cRNA. The peak luminescence (Lum) recorded is shown in c.p.s.

To examine whether normal sperm-induced Ca^{2+}-oscillations are affected by the presence of PLCζ^{H435P}, IVF experiments with mouse sperm were also performed. As with the above experiments the cRNA (PLCζ^{H435P}) was injected together with OGBD two hour prior to adding sperm into the dish in order to get prior expression of PLCζ^{H435P}. In this experiment we used a fluorescence imaging system (TE2000 -S, Nikon UK Ltd).

The injected eggs were then transferred into the dish containing control mouse eggs (only injected with OGBD) fitted in the fluorescent system. The two groups of eggs were securely separated and adhered onto the bottom of the dish floor after zona removal with Acid tyrodes solution. This was then followed by sperm addition. After two hours of recording, successful IVF was obvious by the presence of Ca^{2+}-oscillations.

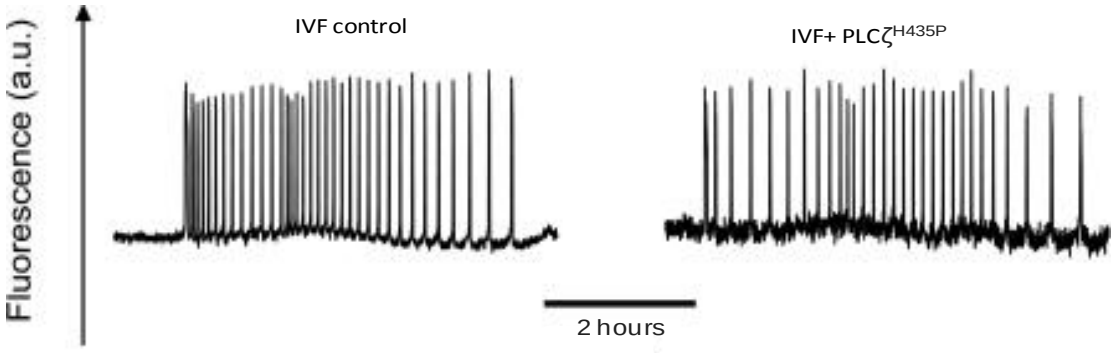


Figure 4.3 Effect of PLCζ^{H435P} on sperm-induced Ca^{2+} oscillations. Mouse eggs were either untreated (IVF control) or injected with PLCζ^{H435P} cRNA (IVF+PLCζ^{H435P}) 3hrs prior to the start of recording. PLCζ^{H435P} expression produced luminescence of 41.7±1.8 c.p.s. (value is mean±S.E.M., n=11). Fluorescence recordings [arbitrary units (a.u.)] reporting Ca^{2+} concentration changes were monitored after the addition of capacitated mouse sperm. Following IVF, both control and PLCζ^{H435P} cRNA-injected eggs exhibited robust Ca^{2+}-oscillations and formed pronuclei

The results were analysed and showed that even after high protein expression levels of PLCζ^{H435P} were achieved in the mouse eggs (41.7 c.p.s./egg), as tested on the luminescence system, there was no discernible effect on sperm-induced Ca^{2+} oscillations. The Ca^{2+} spike number in the first 3 hours are closely similar, with 33.4 spikes in the IVF control group and 32.9 spikes in the IVF + PLCζ^{H435P} group (Fig. 4.3 and Table 4.3).

Constructs Injected	no of Ca^{2+} spikes in 1st 3 hrs	Lum. Before (c.p.s.)	Time of 1st spike (min)	Total no of eggs
sperm	33.4 ± 3.8		**~30**	**7**
PLCζ^{H435P} & sperm	32.9 ± 4.5	41.7 ± 1.8	**~30**	**11**

Table 4.3 Properties of sperm PLCζ^{WT} with cRNA PLCζ^{H435P} in mouse eggs. Ca^{2+} oscillation-inducing activity by sperm alone and sperm + PLCζ^{H435P} are summarised in this table. The number of Ca^{2+} spikes in control eggs (sperm only) was 33.4±3.8 (mean±S.E.M., n=7), and the number of Ca^{2+} spikes in sperm + PLCζ^{H435P} cRNA-injected eggs was 32.9±4.5 (mean±S.E.M., n=11)

4.3 The role of the X-Y linker region of PLCζ in causing Ca^{2+}- oscillations in eggs

Each isoform of PLCs has a different way to access the target phospholipid substrate. For example, PLCδ1, the closest relative to PLCζ, binds strongly to membranes via its PH domain. Interestingly, PLCζ lacks a PH domain, making the exact mechanism of how PLCζ targets its membrane substrate unresolved. Previous study used Venus GFP–PLCζ claimed that PLCζ dispersed all over the cytoplasm and only a small fraction of PLCζ binds to PIP_2 in plasma membrane (Yoda *et al.,* 2004). This apparently suggests that PLCζ does not specifically targeting the plasma membrane and might interact with membranes of the cytoplasmic vesicles. Given that we already have presumed roles of the N-terminal EF hand and the catalytic X and Y domains, there are only two putative candidate domains that might be involved in targeting of PLCζ to PIP_2: the C-terminal C2 domain and the XYl region, which is the intervening region separating the catalytic X and Y domains.

The first putative candidate for targeting PLCζ to PIP_2 is the C2 domain which is similar to PH domain that comprising of over 120 amino acid residues and has been identified in numerous proteins, including all isoforms of protein kinase C, synaptotagmin, phospholipase A and PLC. First identified in protein kinase C, the C2 domain was functionally implicated in Ca^{2+}-dependent phospholipid interactions (Nalefski and Falke, 1996). The C2 domain has since been characterized as an intermolecular interaction and membrane-associating domain in a variety of proteins (Medkova and Cho, 1999). Most C2 domains bind to Ca^{2+}, a crucial determinant for the associated enzyme activity (Zheng *et al.,* 2000). The PLCζ C2 domain appears to have an essential role in cellular function because deletion of this domain leads to inability of the truncated PLCζ to cause Ca^{2+}-oscillations in intact mouse eggs, although enzyme activity is retained (Nomikos *et al.,* 2005; Kouchi *et al.,* 2005). There is currently no evidence for PLCζ C2 domain binding to membrane phospholipids through Ca^{2+}-dependent or -independent way, although screening phosphoinositides for interaction with the C2 domain of PLCζ revealed that it can bind to both phosphatidylinositol 3-phosphate (PtdIns3*P*) and phosphatidylinositol 5-phosphate (PtdIns5*P*) (Kouchi *et al.,* 2005).

The alternative putative candidate that might be involved in targeting of PLCζ to biological membranes PIP_2 is the PLCζ-XYl region. Notably, the sperm-specific PLCζ is unique in displaying extended XYl region compared to PLCδ1 (the closest isoform to PLCζ). Furthermore, this XYl region in PLCζ is abundant with positively charged basic amino acid residues (lysine) (Saunders *et al.,* 2002; Cox *et al.,* 2002), which is not the

case in the somatic cell PLCβ, δ and γ isoforms, where this region is negatively charged.

In this Chapter, I investigate the potential importance of the XYl region on PLCζ association with Ca^{2+} releasing activity in mouse eggs. This study was carried out by preparing a full-length $PLC\zeta^{WT}$ cRNA and series of site-directed mutagenesis to produce three cumulative mutations within the positively charged region (lysine amino acid) that clustered within the XYl of mouse PLCζ. The resultant mutants PLCζ produced by sequentially inserting an alanine neutral amino acid in positions 374, 374&5, 374,5&7 instead of the positively charged lysines, to create the single ($PLC\zeta^{K374A}$), double ($PLC\zeta^{K374,5AA}$) and triple ($PLC\zeta^{K374,5,7AAA}$) X-Y domain mutants (Fig. 4.4). I carried out the *in vivo* study, by comparing the Ca^{2+} oscillation-inducing properties of these lysine mutants with that of $PLC\zeta^{WT}$ by microinjecting them into unfertilized mouse eggs. In addition and in order to establish the degree of inhibition effected by the generated (K-to-A) mutations, a negative control was also injected into eggs, ($PLC\zeta^{D210R}$) the catalytically inactive PLCζ mutant (Saunders *et al.,* 2002; Nomikos *et al.,* 2011b). Other studies within our research team have also investigated the *in vitro* interaction of the PLCζ-XYl and C2 domain with PIP_2 by employing the protein-lipid overlay and a liposome-binding assay (Nomikos *et al.,* 2011b).

4.3.1 Methodology

In order to study the effects of the above mutation on Ca^{2+}-inducing activity of PLCζ, Nomikos and his colleagues introduced sequential mutations within the XYl region by

substituting lysine (positively charged) by alanine (neutral) to yield single ($PLC\zeta^{K374A}$), double ($PLC\zeta^{K374,5AA}$) and triple ($PLC\zeta^{K374,5,7AAA}$) $PLC\zeta^{H398P}$ mutants (Fig. 4.4).

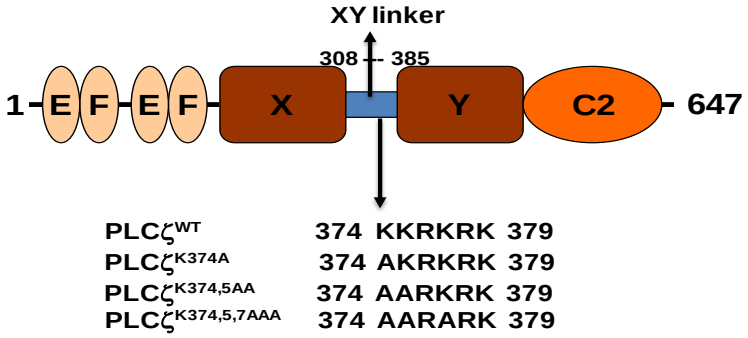


Figure 4.4 Schematic of mouse PLCζ domain structure and identifying the successive K-to-A mutations between residues 374 and 379 in the XY-linker region

The full details of how this mutation was made are given in (Nomikos *et al.,* 2011b). In brief, after the cloning of PLCζ-XYI, C2ζ and PLCδ1PH constructs from the original cDNA clone (GenBank™ accession number AF435950) (Saunders *et al.,* 2002), using Phusion polymerase (Finnzymes) and the appropriate primers, the pCR3 mouse PLCζ-luciferase construct (Nomikos *et al.,* 2005) was subjected to site-directed mutagenesis (QuikChange II, Stratagene) to sequentially generate the three substitutions of alanine for lysine at K374, K375 and K377, thus producing the PLCζK374A, PLCζK374,5AA and PLCζK374,5,7AAA mutants. Following linearization of wild-type and mutated PLCζ constructs, cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and then polyadenylated using the poly(A) tailing kit (Ambion), as per the manufacturer's instructions (Nomikos *et al.,* 2005 and Nomikos *et al.,* 2011b). Then I took the resultant constructs and test them individually *in vivo* by co-injecting them into unfertilized mouse eggs with Ca^{2+} dye (OGBD) as per usual protocol.

4.3.2 Results

To investigate the potential importance of the cluster of basic residues within the PLCζ XY linker region (Fig. 4.4), the three mutated PLCζ constructs; the single ($PLC\zeta^{K374A}$), double ($PLC\zeta^{K374,5AA}$) and triple ($PLC\zeta^{K374,5,7AAA}$) were tested by microinjection of the corresponding cRNA into eggs mouse eggs. This was to assess their ability to trigger Ca^{2+}-oscillations and in order to verify their expression.

Figure 4.5, 4.6 and Table 4.4 summarize the results of the wild-type and mutant PLCζ–luciferase cRNA microinjection experiments. In the $PLC\zeta^{WT}$ cRNA-injected eggs prominent Ca^{2+}-oscillations (19 spikes in the first 2 hours) were observed, with the first Ca^{2+} spike occurring ~30 minutes after microinjection at a luminescence level of (0.35 c.p.s./egg) (Fig. 4.5). Microinjection of cRNA encoding the PLCζK374A single mutant also caused prominent Ca^{2+}-oscillations in injected mouse eggs, exhibiting a similar potency to wild-type PLCζ (17 spikes in 2hrs) with the first Ca^{2+} spike detected at a expression level of (0.33 c.p.s./egg) (Fig. 4.6). On the other hand, microinjection of cRNA of either PLCζK374,5AA or PLCζK374,5,7AAA into mouse egg clearly resulted in a significant reduction in the frequency of Ca^{2+} oscillations and causing only (8.0 and 2.6 spikes in 2 hours) respectively (Fig. 4.6) compared with 19 spikes in case of $PLC\zeta^{WT}$. Notably, there was also a dramatic increase (more than twice) in the time required for initiation of Ca^{2+}-oscillations in both the double and triple PLCζ mutants, with the first Ca^{2+} spike appearing at ~65 minutes (Fig. 4.6 and Table 4.4). Moreover, the luminescence level required to produce the first Ca^{2+} spike has also significantly increased compared to the $PLC\zeta^{WT}$ (0.93 and 1.43 c.p.s./egg) for the double and triple

mutant, respectively. Finally, as reported previously (Saunders *et al.,* 2002) the microinjection of cRNA encoding the PLCζ^{D210R} mutant failed to cause any Ca^{2+}-oscillations even though it was expressed at much higher levels (14.5cps/egg) than wild-type PLCζ (7.3 c.p.s/egg) (Fig. 4.5 and Table 4.4) and PLCζ K-to-A mutants (3.1, 3.0 and 5.8 c.p.s/egg) for single, double and triple mutation respectively (Table 4.4).

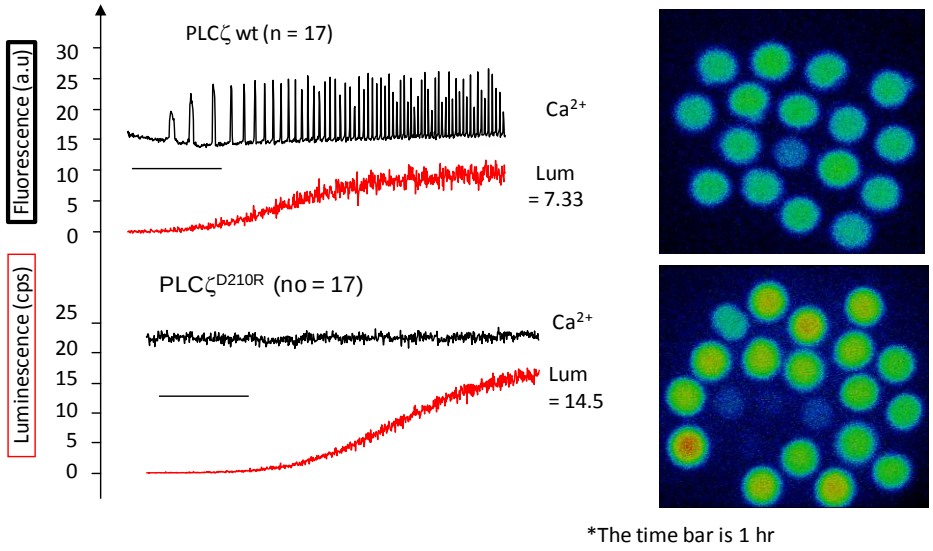


Figure 4.5 The effect of PLCζ^{WT} and PLCζ^{D210R} mutation on Ca^{2+} oscillation-inducing activity in mouse eggs. Fluorescence and luminescence recordings reporting the Ca^{2+} changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum, in counts per second; cps) in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged, wild-type PLCζ or D210R mutant of PLCζ (left panels). The average luminescence level (cps) achieved in mouse eggs is indicated for each microinjected cRNA, e.g. PLCζ^{WT} Lum=7.33 (top trace) and 14.5 cps for D210R. Panels on the right are the integrated luminescence images of individual mouse eggs following cRNA microinjection of either wild-type or mutant PLCζ (Table 4.4).

These data indicate that the substitution of two or more alanines for lysines within the positively charged cluster of the PLCζ XYI dramatically alters their Ca^{2+} oscillation-inducing activity in mouse eggs by reducing the Ca^{2+} spike frequency and increasing both the time and amount of mutant PLCζ expression required to initiate the first spike.

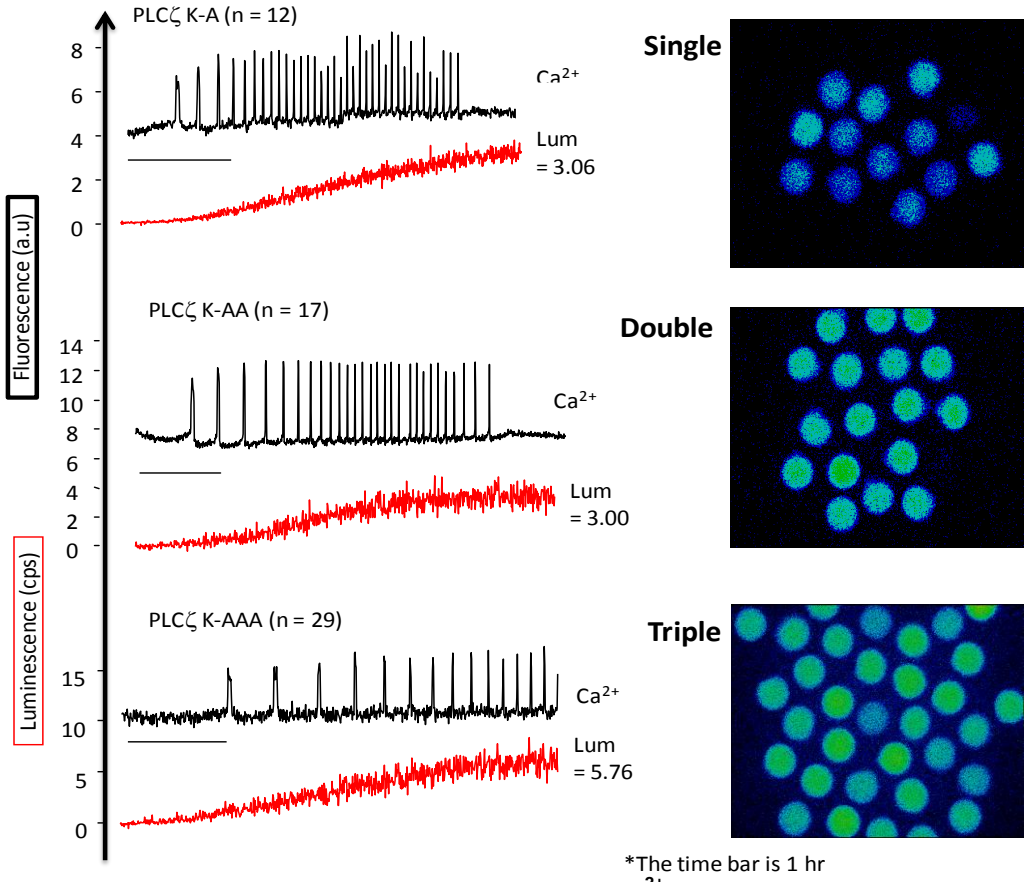


Figure 4.6 Effect of PLCζ XY-linker mutations on Ca^{2+} oscillation-inducing activity in mouse eggs. Fluorescence and luminescence recordings reporting the Ca^{2+} changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum, in counts per second; cps) in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged, wild-type PLCζ or the indicated single, double, triple K-to-A and D210R mutants of PLCζ (left panels). The average luminescence level (cps) achieved in mouse eggs is indicated for each microinjected cRNA, e.g. $PLC\zeta^{WT}$ Lum=7.33 (top trace). Panels on the right are the integrated luminescence images of individual mouse eggs following cRNA microinjection of either wild-type or mutant PLCζ (Table 4.4)

To sum up, the results from the *in vivo* experiments are consistent with the suggestion that the PLCζ XYI possesses significant affinity for PIP_2. This *in vivo* study showed the generated mutations (K-to-A) of sequential reduction of the XYI net positive charge significantly affects *in vivo* Ca^{2+} oscillation-inducing activity. From these results it appears that the PLCζ XYI region plays a major role in the binding of PLCζ to PIP_2-enriched membranes.

Luciferase-tagged PLCζ	1st 2 hrs Ca^{2+} spike no	Lum. peak (c.p.s.)	Time of 1st spike (min)	Lum at 1st spike (c.p.s.)	Protein expression = 1st spike (fg)	Total no of eggs
PLCζ	19 ± 0.14	7.3 ± 0.38	~30	0.35 ± 0.037	347.15	17
$PLC\zeta^{D210R}$	0	14.5± 0.87	0	0	0	17
$PLC\zeta^{K374A}$	17 ± 1.0	3.1 ± 0.23	~30	0.33 ± 0.03	343.77	12
$PLC\zeta^{K374,5AA}$	8.0 ± 0.30	3.0 ± 0.1	~65	0.93 ± 0.07	45.26	16
$PLC\zeta^{K374,5,\&AAA}$	2.6 ± 0.50	5.8 ± 1.5	~65	1.43 ± 0.30	537.32	29

Table 4.4 Summary of the properties of Ca^{2+}-oscillations observed in mouse eggs following cRNA microinjection of the luciferase-tagged $PLC\zeta^{WT}$ and various $PLC\zeta^{XYl\ K\text{-}to\text{-}A}$ mutants. See Fig. 4.5 and 4.6.

4.4 Novel regulation of PLCζ activity via the XY-linker

Since the discovery of PLCζ it has been recognised as the most likely physiological mammalian sperm factor by its recognized role in the fertilization process, however the exact mechanism/s that regulate/s PLCζ activity are still unknown. Sperm specific PLCζ is a unique PLC in different aspects compared to somatic PLCs. Notably it lacks a PH domain that present in most somatic PLCs. Also it possess unique positively charged XYl region compared to the negatively charged XYl region in all somatic PLCs. It has been shown that in the somatic PLCβ, δ and γ the XYl region mediates auto-inhibition of PIP_2 hydrolytic activity specifically. This has led to a proposed general mechanism of PLC auto-inhibition mediated by the XYl region (Gresset *et al.,* 2010). However, there is no report that has examined this proposed regulatory mechanism in PLCζ.

4.4.1 Objectives

The main objective in this section is to investigate the idea that the XYI region of PLCζ plays a role in inhibiting the catalytic activity of the enzyme, and hence to establish in this respect its similarity to other mammalian PLC isoforms.

4.4.2 Methodology

In order to establish whether the XYI-mediated auto-inhibition applies to PLCζ activity, we specifically removed the entire PLCζ XYI region. To create the XYI deletion constructs PLCζ$^{\Delta XYI}$ and PLCδ1$^{\Delta XYI}$ respectively, we excised XYI (amino acids 308–385) and (amino acids 441-490) from PLCζ and PLCδ1 respectively (Fig. 4.7). The generated constructs were examined and monitored for their consequent affects on the *in vivo* Ca^{2+} oscillation-inducing activity. There is a unique cluster of basic residues in the XYI region of PLCζ that may be involved in enzyme function (Swann *et al.,* 2006 and Saunders *et al.,* 2007). For further investigation about the potential role of this short positively charged XYI segment in the regulation of PLCζ activity, we generated chimaeric constructs between PLCζ and PLCδ1, in which the last 12 amino acids from the XYI region (residues 374-385, KKRKRKMKIAMA; +7 charged residues) were replaced with their correspondents stretch from PLCδ1 (residues 480–491, KPKEDKLKLVPE; +4/−3 charged residues) creating PLCζ-XYIδ1$^{480–491}$ (Fig. 4.7). All created constructs including wild-type PLCs, were each tagged at the C-terminus with luciferase to enable real-time monitoring of relative protein expression by luminescence

quantification (Swann *et al.,* 2009). Each constructs was injected separately into mouse eggs and monitor its *in vivo* Ca^{2+} oscillation-inducing activity.

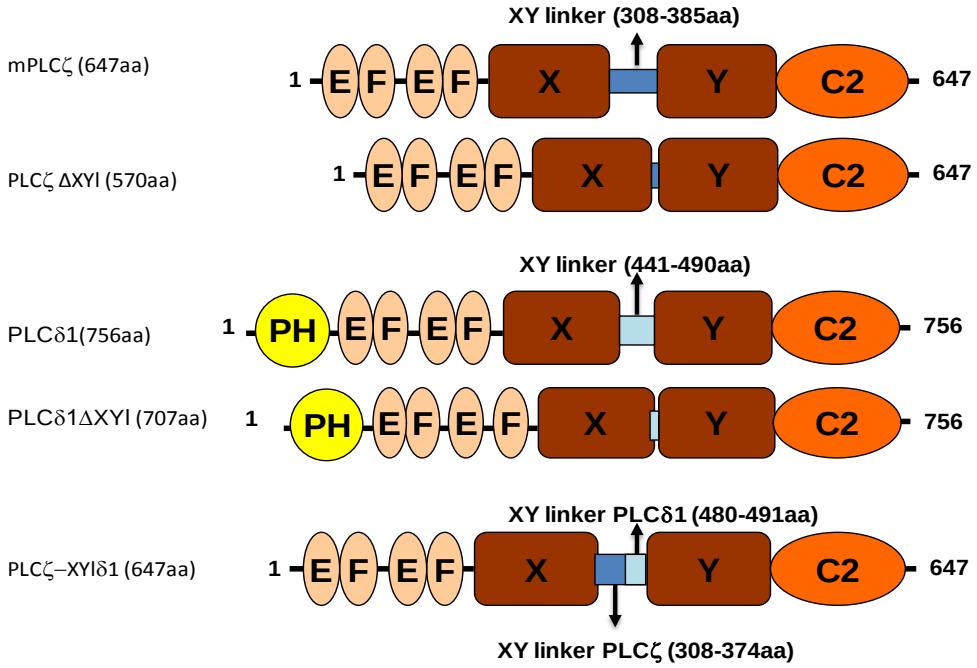


Figure 4.7 Domain organization of wild type PLCζ, PLCδ1 and the deletion/chimaera constructs. Schematic representation of the domain organization of wild-type PLCζ and PLCδ1 and their corresponding XYl deletions $PLC\zeta^{\Delta XYl}$ (PLCζΔXYlinker) and $PLC\delta1^{\Delta XY}$ (PLCδ1ΔXYlinker), and the $PLC\zeta/XYl\delta1^{480\text{-}491}$ (PLCζ/XYlinkerδ1) chimaera. Note the similar presence in PLCζ and PLCδ1 of the EF, X, XYl, Y and C2 domains, but the absence of the PH domain in PLCζ. The various amino acid (aa) lengths and respective XYl co-ordinates are also indicated for each construct.

4.4.3 Results

Consistent with the previous studies microinjection of PLCζ cRNA always gives prominent Ca^{2+} oscillations in unfertilized mouse eggs (25 spikes/2hrs) (Fig. 4.8), with the first Ca^{2+} spike starting within 30 minutes of recording and at a luminescence of (0.5 c.p.s./egg) for the expressed PLC-luciferase fusion protein (Table 4.5). In contrast, much lower frequency (3.4 spikes/2hrs) Ca^{2+}-oscillations were observed in mouse eggs microinjected with cRNA encoding the XYl-deletion construct $PLC\zeta^{\Delta XYl}$ compared to

wild-type PLCζ, and with the first Ca^{2+} spike only appearing after around 100 minutes of recording and when luminescence had reached (3.6 c.p.s./egg). These results show that the absence of the XYl region of PLCζ dramatically attenuated the Ca^{2+} oscillation-inducing activity, yielding a 7-fold reduction in spike frequency 3.4 spikes/2hrs compared to 25 spikes/2hrs in $PLC\zeta^{WT}$ and requiring a 7-fold increased level of $PLC\zeta^{\Delta XYl}$ expression (3.6 c.p.s./egg) compared with (0.52 c.p.s./egg) in $PLC\zeta^{WT}$ to initiate the first Ca^{2+} spike (Table 4.5). Similarly, microinjection of cRNA corresponding to the XYl chimaera PLCζ-XYlδ1$^{480–491}$ also triggered relatively low-frequency Ca^{2+} oscillations (5.3 spikes/2hrs), with the first Ca^{2+} spike appearing at a luminescence of (4.0 c.p.s./egg) but appearing after ~50 minutes of recording (Fig. 4.8 and Table 4.5). These molecular changes in $PLC\zeta^{WT}$ by replacing the cluster of basic residues in the XYl region (seven out of 12 residues are positively charged; overall +7) with the corresponding residues from the XYl of $PLC\delta1^{WT}$ (four positively charged residues and three negatively charged residues; overall charge +1), has also dramatically reduced the Ca^{2+} oscillation-inducing activity of PLCζ by 5-folds with a requirement for an 8-fold increased level of PLCζ-XYlδ1$^{480–491}$ expression to triggers the first spike 4.0 compared with (0.52 c.p.s./egg) in $PLC\zeta^{WT}$ (Table 4.5). Regarding the cRNA encoding $PLC\delta1^{WT}$ microinjection into mouse eggs, very low-frequency Ca^{2+}-oscillations (1.8 spikes/2hrs) were only observed after ~100minutes that commenced only when luminescence reaches a relatively large value (20.4 c.p.s./egg) of the expressed PLCδ1-luciferase protein. However, the cRNA $PLC\delta1^{\Delta XYl}$ deletion construct caused a ~2-fold increase in Ca^{2+}-oscillations frequency (3.3 spikes/2hrs) compared with (1.8 spikes/2hrs) in $PLC\delta1^{WT}$, with the first Ca^{2+} spike

appeared earlier at ~70 minutes with a reduced luminescence of (17.2 c.p.s./egg) compared with PLCδ1^{WT} (Fig. 4.8 and Table 4.5). These results were the converse of the PLCζ results, but they are consistent with the proposed regulatory auto-inhibition mechanisms of the XYl in the other somatic PLCs isoforms.

This suggests that XYl region presence in PLCζ is required to get higher catalytic activity of sperm PLCζ. These results together indicating that the presence of the XYl region and the highly positively charged cluster are necessary for maximal PIP_2 catalytic activity of PLCζ. In contrast, deleting the XYl from PLCδ1 (i.e. PLCδ1$^{\Delta XYl}$) enhanced the enzymatic activity by > 2.0-fold compared with PLCδ1^{WT}. Our studies show that, in contrast with somatic cell PLCs, the XYl of PLCζ does not confer enzymatic auto-inhibition. These differential results for XYl-deleted PLCs suggest that there are disparate regulatory roles for the XYl of PLCζ and this is a distinctive feature of the sperm-specific PLC isoenzyme (PLCζ) with respect to hydrolytic activity.

Luciferase-tagged PLCζ	1st 2 hrs Ca^{2+} spike no	Lum. peak (c.p.s.)	Time of 1st spike (min)	Lum at 1st spike (c.p.s.)	Protein expression = 1st spike (fg)	T no of eggs
PLCζ^{WT}	24.58 ± 0.88	8.7± 1.16	~30	0.5 ± 0.06	372.5	13
PLCζ$^{\Delta XYl}$	3.4 ± 0.27	7.1 ± 0.20	~100	3.6 ± 0.20	896.4	20
PLCδ1^{WT}	1.8 ± 0.1	45.0 ± 1.7	>100	20.4 ± 3.00	3735.6	17
PLCδ1$^{\Delta XYl}$	3.30 ± 0.20	40.2 ± 1.7	~70	17.2 ± 0.35	3194.8	19
PLCζ-δ1XYl	5.3 ± 0.16	30.48 ± 2.0	~50	4.0 ± 0.39	964	9

Table 4.5 Properties of PLC-luciferase and deletion/chimaera constructs expressed in eggs. Ca^{2+} oscillation-inducing activity (Ca^{2+} spike number in 2hrs) and luciferase luminescence levels (peak luminescence and luminescence at first spike) are summarized for mouse eggs microinjected with each of the PLC–luciferase constructs, PLCζ, PLCζ$^{\Delta XYl}$, PLCδ1, PLCδ1$^{\Delta XYl}$ and PLCζ^{XYlδ1} (Fig. 4.7).

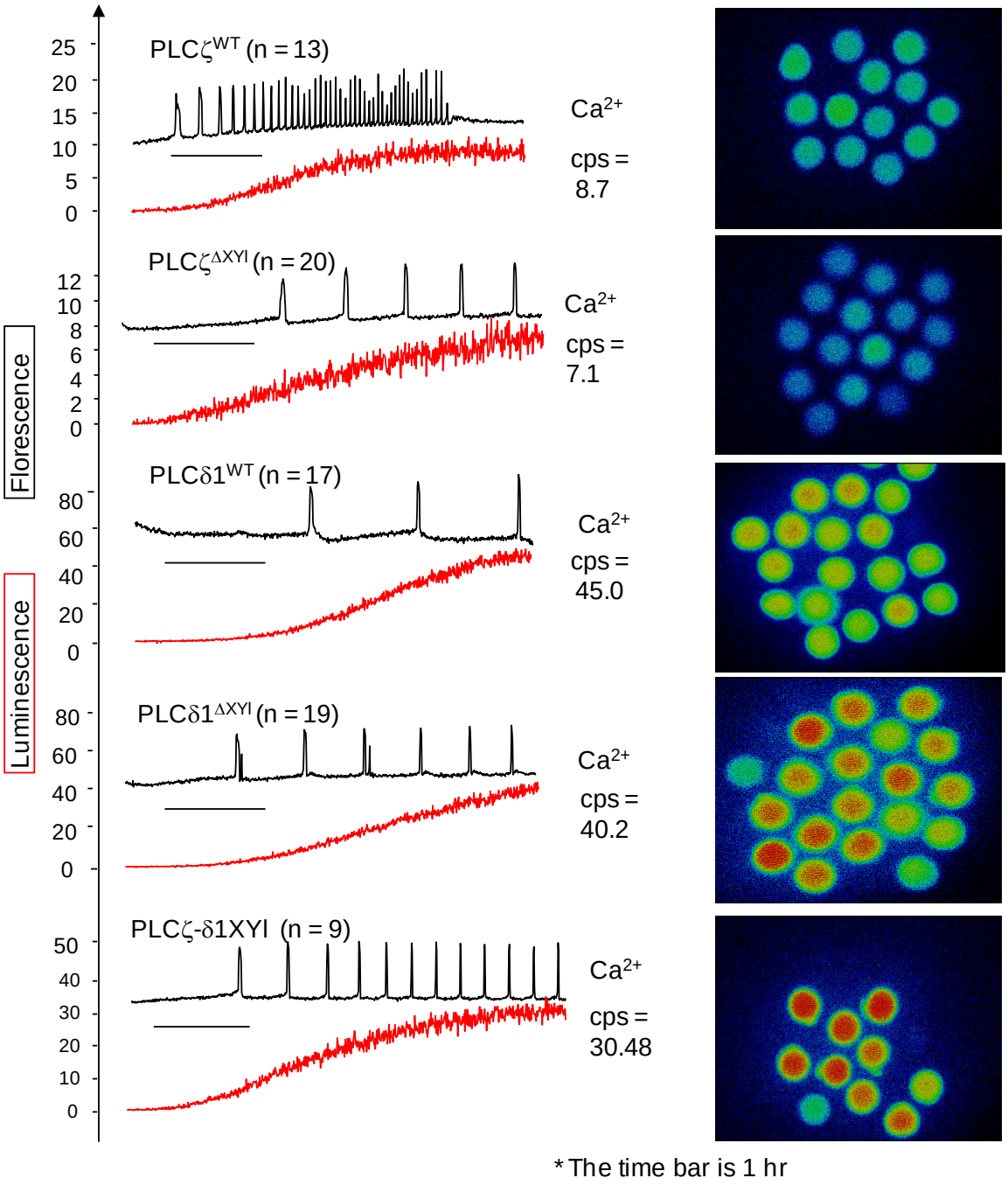


Figure 4.8 Ca^{2+} oscillation-inducing activity of the PLCs and XYl deletion or chimaera expressed in mouse eggs. Fluorescence and luminescence recordings reporting the Ca^{2+} changes [fluorescence (red traces), in arbitrary units (a.u.), and luciferase expression (black traces; luminescence) in c.p.s. respectively] in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged PLCζ, PLCδ1, their corresponding XYl deletions (PLCζ$^{\Delta XYl}$ and PLCδ1$^{\Delta XY}$) and chimaera [PLCζ/XYlδ1$^{480–491}$; (PLCζ/XYlinkδ1)] (left-hand panels). Right-hand panels show the integrated luminescence image of a field of mouse eggs following cRNA microinjection of each PLC construct (see Table 4.5).

4.5 The PLCζ-XY linker region has the similar role in different species

Further to our previous work on the novel regulation PLCζ by its XYl we created different constructs using human PLCζ as a framework and swapping its XYl region by those from mouse and rat PLCζs to generate hPLCζ^{-mXYl} and hPLCζ$^{-rXYl.}$ We then examined their Ca^{2+} releasing activities by injecting them individually into unfertilized mouse eggs. The same protocol was used to examine the two generated constructs by comparing their effects on the *in vivo* Ca^{2+} oscillation-inducing activity with that of hPLCζ^{WT}.

4.5.1 Results

Human PLCζ has to been shown to be the most potent PLCζ among all mammalian species tested. Microinjection of hPLCζ^{WT} cRNA into mouse eggs triggers prompt high frequency Ca^{2+} oscillations (39.2 spikes/2hrs) that are observed within 20 minutes post injection and it does this when the luminescence reaches only (0.16 c.p.s./egg) of the expressed hPLCζ–luciferase protein see (Fig. 4.9 and Table 4.6). On the other hand, hPLCζ^{-mXYl} injected eggs show only flat Ca^{2+} traces and in comparions to the case of hPLCζ^{-rXYl} injected eggs, only low frequency Ca^{2+} oscillations (6.74 spikes/2hrs) were noticed. It was noted that not only the Ca^{2+} oscillations activity of the newly created constructs (hPLCζ^{-mXYl} and hPLCζ^{-rXYl}) were completely abolished or reduced, but their protein expression levels were also significantly reduced and even when eggs were injected with the highest concentration of cRNA (0.95 and 1.96 c.p.s./egg). This contrasts with markedly with that of hPLCζ^{WT} (7.04 c.p.s./egg) (Fig. 4.9 and Table 4.6).

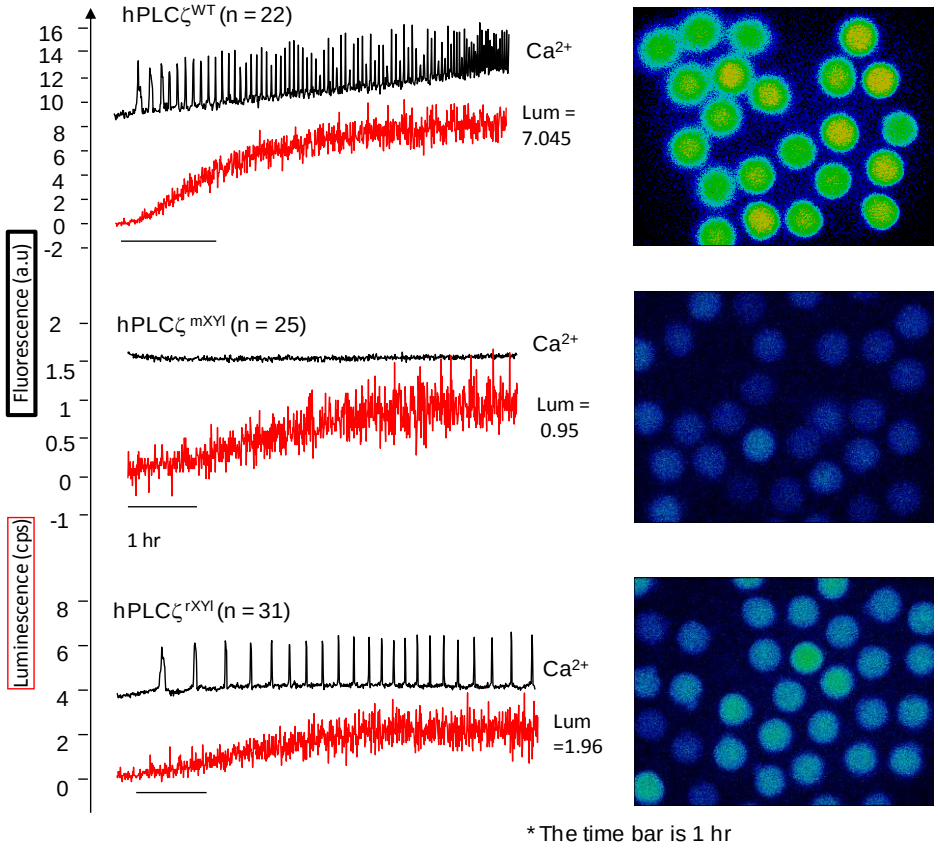


Figure 4.9 The effects of PLCζ species chimeras between hPLCζ and mouse or rat XY domains on Ca^{2+}-oscillations and expression levels in mouse eggs. Left-hand panels show representative fluorescence and luminescence recordings reporting Ca^{2+} concentration changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum) respectively in a mouse egg. The egg was microinjected with PLCζ-luciferase cRNA encoding hPLCζ^{-hXYl} (top panel), or hPLCζ^{mXYl} (mid panel) or hPLCζ^{-rXYl} (bottom panel). Right-hand panels show the integrated image of luciferase luminescence from eggs microinjected with these chimeras

These data suggest that the substitutions of XYl region in hPLCζ with its analogues from either mouse or rat PLCζ will dramatically reduce the protein stability and PIP_2 catalytic activity of hPLCζ.

Luciferase-tagged PLCζ	1st 2hrs spike no	Peak Lum cps	Time to 1st spike (min)	Lum at 1st spike (cps)	Protein1st spike (fg)	T no of eggs
hPLCζ^{WT}	39.2 ± 3.5	7.04 ± 0.43	~20	0.16 ± 0.02	315	22
hPLCζ ^{rXY}	6.74 ± 0.39	1.96 ± 0.15	~30	0.18 ± 0.01	318.4	31
hPLCζ^{mXY}	-	0.95 ± 0.06	-	-	-	25

Table 4.6 Summary of the properties of Ca^{2+}-oscillations observed in mouse eggs following cRNA microinjection of the luciferase-tagged hPLCζ wild type and species PLCζ chimeras between hPLCζ and both mouse or rat XYl domains (see Fig. 4.9)

4.6 PLCζ & PLCδ1 chimeras; the effect of swapping PLCδ1 domain(s) with PLCζ domain(s) upon PLCδ1 activity in mouse eggs

Based on sequence alignment analysis of all PLCs, PLCζ has the closest sequence homology to PLCδ1 (47% similarity, 33% identity) (Saunders *et al.,* 2002). On further sequence comparison between PLCζ and rat PLCδ1 catalytic domains it was notable that both share a similarly structured catalytic domain (Saunders *et al.,* 2007). Moreover, studies on structural analysis on PLCδ1catalytic domain has identified an active-site residue (Essen *et al.,* 1996) The mutation of this active-site residue analogous in PLCζ Asp343 created PLCζ^{D210R}, this was a completely inactive construct (Saunders *et al.,* 2002).

Based on the closer homology between PLCζ and PLCδ1 Ca^{2+}, we decided to remove PH domains from PLCδ1 and generate chimeras between the two. We used PLCδ1 as frame-work and replaced different domain(s) with their analogous domains from PLCζ to

create three different constructs. These were PLCδ1$^{\Delta PH\text{-}\zeta EF\&XYI}$, PLCδ1$^{\Delta PH\text{-}\zeta EF\&C2}$ and PLCδ1$^{\Delta PH\text{-}\zeta XYI}$ (Fig. 4.10). The generated constructs were examined by monitoring their effects on the *in vivo* Ca^{2+}-oscillation-inducing activity compared to PLCδ1^{WT}.

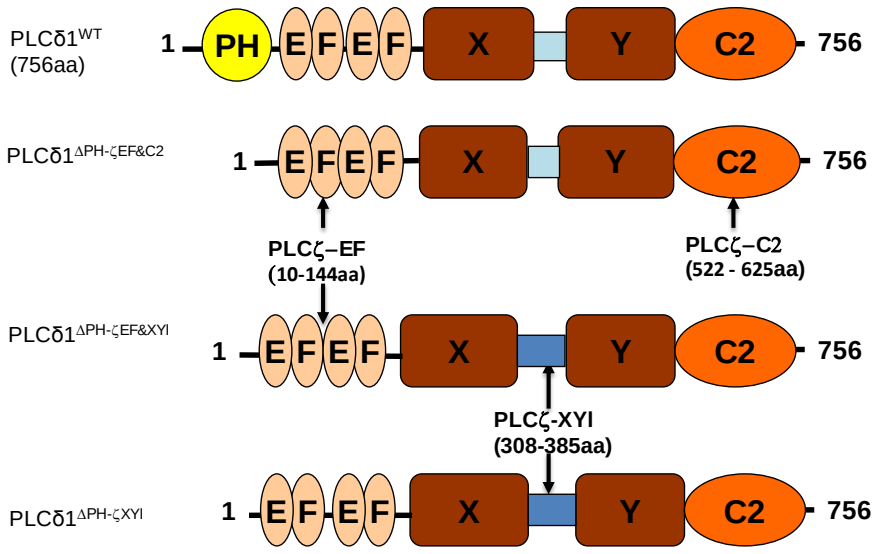


Figure 4.10 Domain organization of PH deletion from PLCδ1 and PLCζ domain(s) chimaera constructs. Schematic representation of the domain organization of PLCζ1^{WT}, PLCδ1$^{\Delta PH\text{-}\zeta EF\&C2}$, PLCδ1$^{\Delta PH\text{-}\zeta EF\&XYI}$, and PLCδ1$^{\Delta PH\text{-}\zeta XYI}$.

4.6.1 Objectives

We intended to cross match the different domains between the two PLCs (PLCζ and PLCδ1) in order to examine their effect on the catalytic activity *in vivo,* and whether any swapping could potentially augments the catalytic activity of that construct.

4.6.2 Results

As shown earlier PLCδ1^{WT} cRNA microinjection into mouse eggs triggers a very low-frequency Ca^{2+}-oscillations (3.2 spikes/2hrs) that were only observed after ~100minutes post injection, and which was when luminescence reached relatively large value (22.48

c.p.s./egg) of the expressed PLCδ1–luciferase protein see (Fig. 4.11 and Table 4.7). On the other hand, none of the chimeras between PLCδ1 and PLCζ domain(s) (PLCδ1$^{\Delta PH\text{-}\zeta EF\&C2}$, PLCδ1$^{\Delta PH\text{-}\zeta EF\&XYl}$, and PLCδ1$^{\Delta PH\text{-}\zeta XYl}$) showed any sign of Ca^{2+} releasing activity despite the relatively very high expression levels (84.56, 56.79 and 65.84 c.p.s./egg) respectively (Fig. 4.11 and Table 4.7).

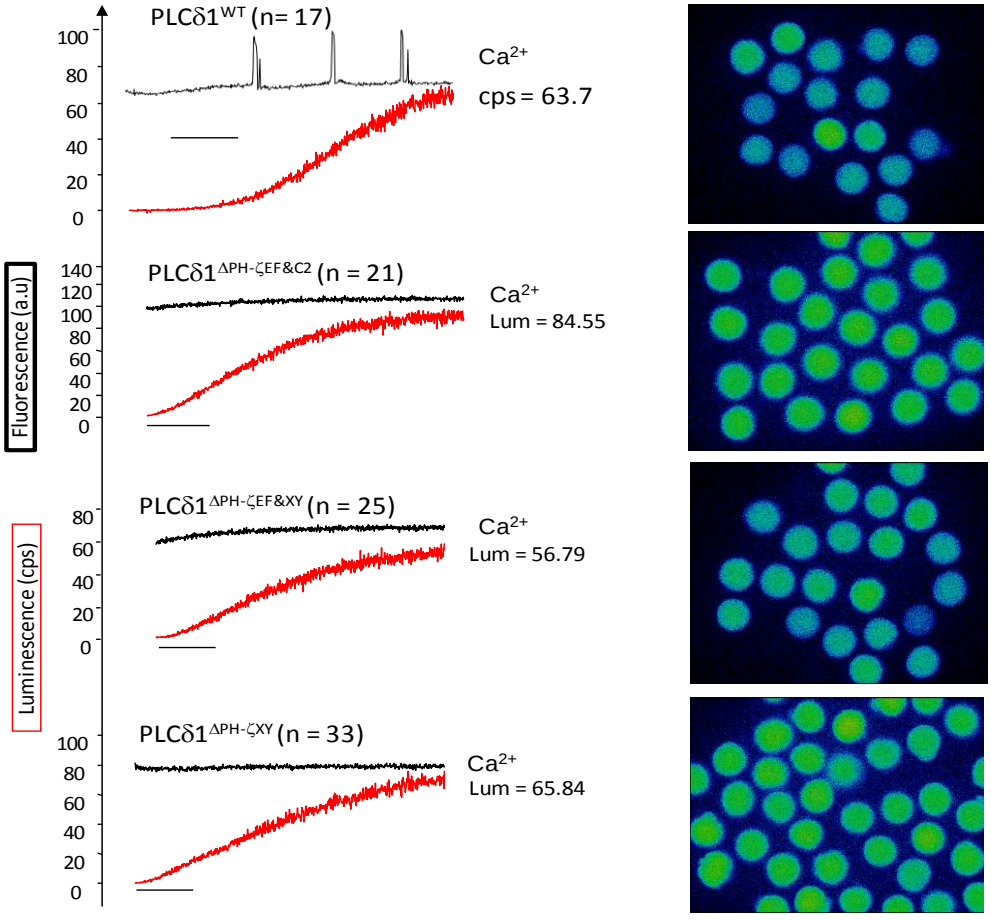


Figure 4.11 The effects of PLCδ1 chimeras with selected domain(s) from PLCζ on Ca^{2+}-inducing activity. Fluorescence and luminescence recordings reporting the Ca^{2+} changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum, in cps) in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged, PLCδ1^{WT} or PLCδ1&PLCζ chimeras (left panels). The average luminescence level (cps) achieved in mouse eggs (*n* is number of eggs) is indicated for each microinjected cRNA, e.g. PLCδ1^{WT} Lum=63.7 (top trace). Panels on the right are the integrated luminescence images of individual mouse eggs following cRNA microinjection of either wild-type or chimeras construct (see Table 4.7).

Luciferase-tagged PLCζ	1st 2 hr spike no	Peak Lum (cps)	Time to 1st spike	Lum at 1st spike (cps)	1st spike Protein (fg)	T no of eggs
PLCδ1^{WT}	2.3 ± 0.3	63.7 ± 4.0	>100	22.48 ± 0.9	4087.12	17
PLCδ1$^{\Delta PH\text{-}\zeta EF\ \&\ C2}$	-	84.56 ± 1.8	-	-	-	21
PLCδ1$^{\Delta PH\text{-}\zeta EF\ \&X\text{-}Y}$	-	56.79 ± 2.58	-	-	-	25
PLCδ1$^{\Delta PH\text{-}\zeta XYI}$	-	65.84 ± 2.73	-	-	-	33

Table 4.7 Summary of expression levels and the properties of Ca^{2+}-oscillations observed in mouse eggs following cRNA microinjection of the luciferase-tagged PLCδ1^{WT} and various PLCδ1 & PLCζ chimeras (see Fig.4.11).

These data suggest that with the deletion of PH domain and replacing PLCδ1 domain(s) with their analogue from PLCζ will completely disrupt PIP_2 catalytic activity of PLCδ1.

4.7 PLCζ species domain(s) swapping and their effects upon PLCζ-activity in mouse eggs

Human PLCζ is the most potent among all tested mammalian species is the PLCζ. To investigate why this may be we swapped domains between two species of PLCζ i.e. human and mouse. Based on closer number compositions of amino acid between hPLCζ^{WT} (608aa) and mPLCζ^{WT} (647aa), we made six different chimeras between the two. These constructs was created by swapping a selected domain from one species and replaced it with its analogue from the other, to end with three constructs from each one, i.e. for mouse (mPLCζ^{WT} mPLCζ^{hXYI} mPLCζ^{hEF} mPLCζ^{hC2}) and for human (hPLCζ^{WT}, hPLCζ^{mXYI}, hPLCζ^{mEF}, hPLCζ^{mC2}). All generated constructs were again tagged at the C-terminus with luciferase before examined *in vivo* by monitoring their

consequent effect on the Ca^{2+} oscillation-inducing activity in comparison with the correspondent wild type PLCζ.

4.7.1 Results

Microinjection $PLC\zeta^{WT}$ from any tested species into unfertilized mouse eggs always showed strong activity by triggering prominent Ca^{2+} oscillations. This was evident by the 15.7 spikes/2hrs (Table 4.8) and 46.2 spikes/2hrs (Table 4.9) for $mPLC\zeta^{WT}$ and $hPLC\zeta^{WT}$ respectively. These oscillations were initiated within the 1^{st} 30 minutes post injection, i.e. ~20minutes in $hPLC\zeta^{WT}$ when expression level reached (0.17c.p.s. see Table 4.9), and ~ 30 minutes in $mPLC\zeta^{WT}$ when the level of expression reaches (0.193 c.p.s. see Table 4.8).

In the case of the mPLCζ chimeras, I injected different concentrations of cRNA, i.e. in wild type it was 0.5µg/µl and for the chimera it was from 5.0 to 5.5µg/µl in the chimeras constructs. With the first replacement of the mouse PLCζ with a XYl from human PLCζ to yield $mPLC\zeta^{hXYl}$, a dramatic decline in activity noted since it only caused Ca^{2+}-oscillations 5 times lower frequency (3 spikes/2hrs) compare to wild type PLCζ (15.7 spikes/2hrs) see Table 4.8 & Fig. 4.12. Similarly the first Ca^{2+} spike was delayed up to 100 minutes and only occurred when the luminescence had reached level of 60 times higher than the $PLC\zeta^{WT}$ (11.98 c.p.s. compare to 0.193 c.p.s.) respectively, see Table 4.8. Similar results were obtained by substituting EF hand domains mouse PLCζ by its analogue from hPLCζ (i.e. $mPLC\zeta^{hEF}$). Again less frequent Ca^{2+}-oscillations (6.3

spikes/2hrs compared to wild type 15.7 spikes/2hrs) were recorded (Table 4.8 & Fig. 4.12).

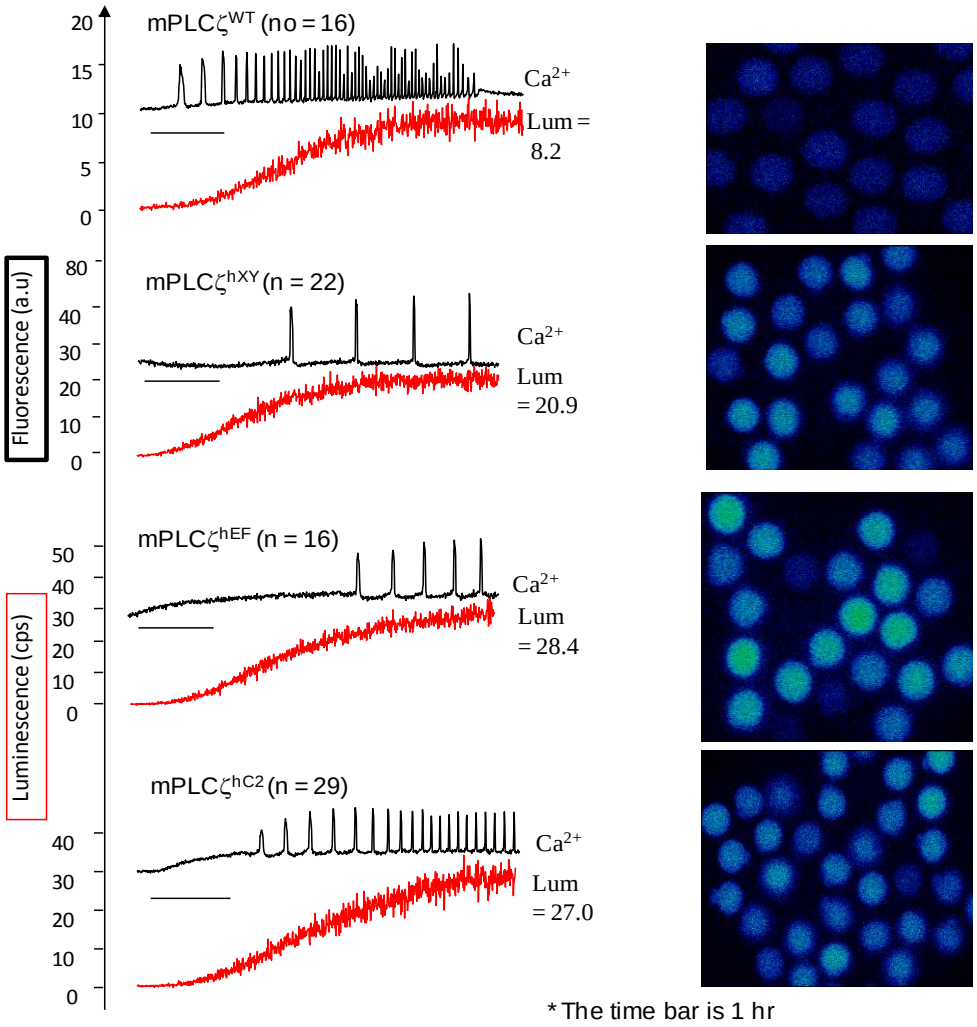


Figure 4.12 The effects of PLCζ species chimeras between mPLCζ and selected domains from hPLCζ on Ca^{2+}-oscillations and expression levels in mouse eggs. Left-hand panels show representative fluorescence and luminescence recordings reporting Ca^{2+} concentration changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum) respectively in a mouse egg. The egg was microinjected with PLCζ-luciferase cRNA encoding $mPLC\zeta^{WT}$ (top panel), or $mPLC\zeta^{hEF}$ or $mPLC\zeta^{hXYl}$ or $mPLC\zeta^{hC2}$ (bottom panel). Right-hand panels show the integrated image of luciferase luminescence from eggs microinjected with these chimeras. The peak luminescence (Lum) recorded is shown in c.p.s.

These oscillations were further delayed and only first noted after around 120 minutes , which is almost 4 times longer than with the wild type (30 minute), and at a significantly

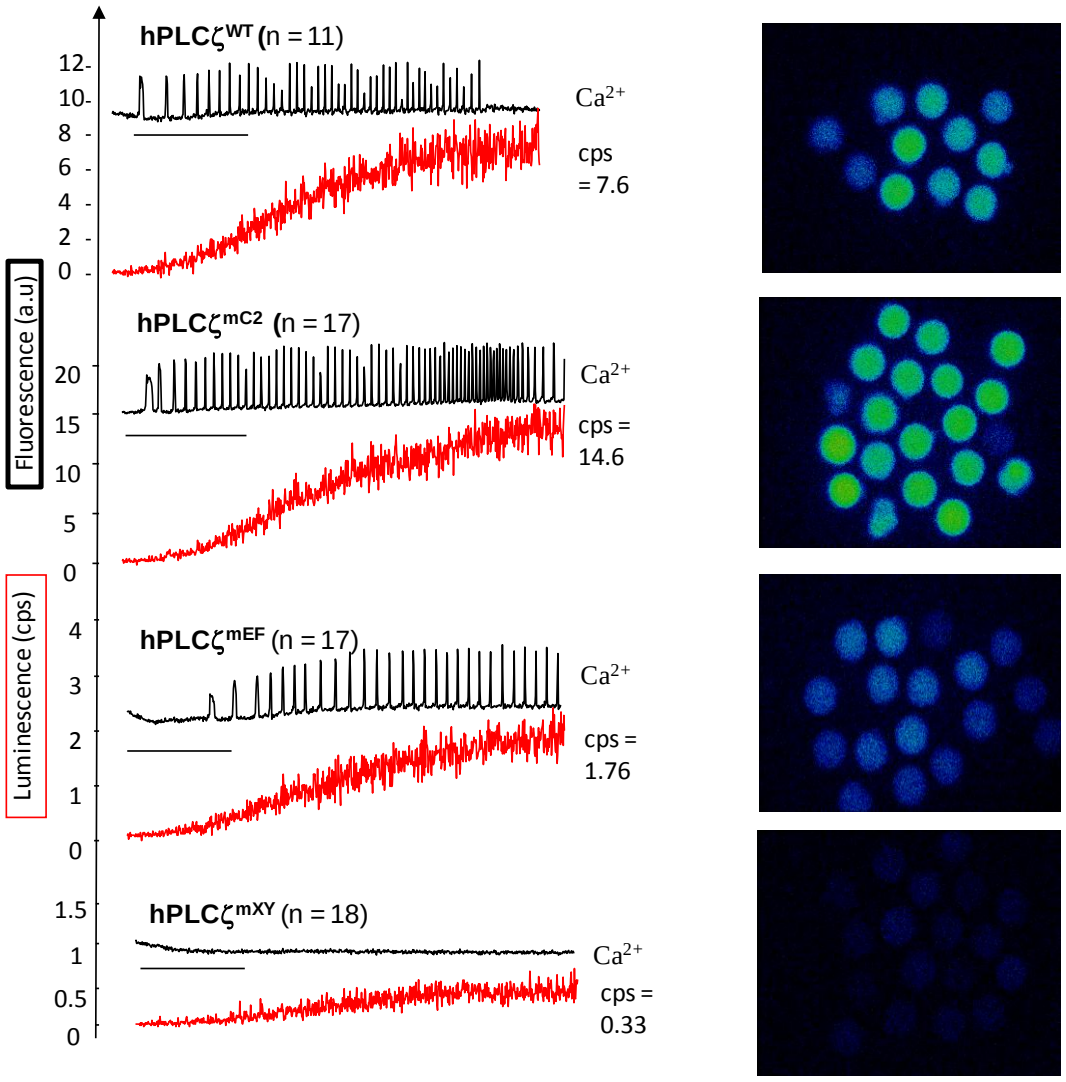
higher luminescence level (110 times higher) than that seen with the wild type PLCζ (21.9 c.p.s. compare to 0.193 c.p.s. respectively), see Table 4.8. Finally, when the mouse C2 domain of mPLCζ was substituted by its human equivalent (mPLCζ^{hC2}) there was also a negative effect on mPLCζ Ca^{2+}-releasing activity. In this case the severity of this decline was not as extensive as with the previous two chimeras. The time to first spike was delayed by 75 minutes which is 2.5 times longer than with the wild type (30 minute) mPLCζ , and Ca^{2+} release was only seen with a luminescence value of around 5.7 c.p.s., which is 28 times higher than with the wild type mPLCζ (0.193 c.p.s.). The frequencies of these oscillations were the highest among all three chimeras (8.27 spikes/2hrs) but still much lower than with the wild type mPLCζ (15.7 spikes/2hrs) (Table 4.8).

Luciferase-tagged PLCζ	**1st 2 hrs spike no**	**Peak Lum cps**	**Time to 1st spike (min)**	**Lum at 1st spike (cps)**	**Protein1st spike (fg)**	**T no of eggs**
mPLCζ^{WT}	15.7 ± 2.84	8.2 ± 0.65	~30	0.193 ± 0.05	32.0	16
mPLCζ^{hXY}	3.0 ± 0.15	20.9 ± 1.21	~100	11.98 ± 0.65	231.26	22
mPLCζ^{hEF}	6.30 ± 0.36	28.4 ± 2.04	~120	21.9 ± 1.61	398.9	16
mPLCζ^{hC2}	8.27 ± 0.26	27.0 ± 1.26	~75	5.70 ± 0.22	125.13	29

Table 4.8 Summary of the properties of Ca^{2+}-oscillations observed in mouse eggs following cRNA microinjection of the luciferase-tagged PLCζ^{WT} and species PLCζ chimeras between mPLCζ and selected hPLCζ domains (see Fig. 4.12)

In case of hPLCζ chimeras, all cRNA constructs including the wild type hPLCζ were injected into mouse eggs at the same concentrations (0.10µg/µl). With the replacement

of the human XYl by its mouse analogue (hPLCζ^{mXYl}), a complete loss of activity was noted in that there were no Ca^{2+} oscillations at all in recordings, This compared to wild type hPLCζ injected eggs that were injected in the same experiments where 15.7 spikes/2hrs were triggered, see Table 4.9 & Fig. 4.13. Similarly the peak expression levels were also significantly reduced (0.33 c.p.s.) with this chimera compared with that of wild type hPLCζ (7.6 c.p.s.) see Table 4.9. In contrast, the results obtained from substituting the human C2 domain with its mouse equivalent (hPLCζ^{mC2}) were completely different. In this chimera I noted that there were slightly higher Ca^{2+} spikes frequencies (49.1 spikes/2hrs) compared to (46.2 spikes/2hrs) in hPLCζ^{WT} (Table 4.9). However, it was noted that the expression level at the first Ca^{2+} spike and the maximum expression levels were also slightly higher (doubled) in hPLCζ^{mC2} (0.32 c.p.s) and (14.6 c.p.s.) compared to that of hPLCζ^{WT} (0.17 c.p.s) and (7.6 c.p.s.) respectively, (see Table 4.9 & Fig. 4.13). Finally, the effect of inserting of EF hand domains from mPLCζ in place of its equivalent in hPLCζ sequence (i.e. hPLCζ^{mEF}) showed lower frequency Ca^{2+}-oscillations (10.5 spikes/2hrs compared to wild type 46.2 spikes/2hrs) that were only seen at luminescence level of (0.25 c.p.s.) compared to 0.17 c.p.s in hPLCζ^{WT} (see Table 4.9 & Fig.4.13). These oscillations were delayed and first noted after around 45 minutes after microinjection which is more than twice the time seen with the hPLCζ^{WT} or indeed with hPLCζ^{mC2} (~20 minutes).



* The time bar is 1 hr

Figure 4.13 The effects of PLCζ species chimeras between hPLCζ and selected domains from mPLCζ. Left-hand panels show representative fluorescence and luminescence recordings reporting Ca^{2+} concentration changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum) respectively in a mouse egg. The egg was microinjected with PLCζ-luciferase cRNA encoding $hPLC\zeta^{WT}$ (top panel), or $hPLC\zeta^{mC2}$or $hPLC\zeta^{mEF}$ or $hPLC\zeta^{mXYI}$ (bottom panel). Right-hand panels show the integrated image of luciferase luminescence from eggs microinjected with these chimeras

To sum up, all these results with various chimeras suggest that any changes or swapping of the PLCζ domain(s) of human with mouse results in either a complete, or else a very significant loss of Ca^{2+} oscillation-activity.

Luciferase-tagged PLCζ	1st 2 hrs spike no	Peak Lum cps	Time to 1st spike (min)	Lum at 1st spike (cps)	Protein1st spike (fg)	T no of eggs
hPLCζ^{WT}	46.2 ± 5.4	7.6 ± 1.28	~ 20	0.17± 0.03	316.7	11
hPLCζ^{mXY}	0	0.33 ± 0.02	0	0	0	18
hPLCζ^{mC2}	49.1 ± 2.89	14.6 ± 0.89	~ 20	0.32 ± 0.033	342	17
hPLCζ^{mEF}	10.5 ± 0.47	1.76 ± 0.15	~ 45	0.25 0.02	330.2	17

Table 4.9 Summary of expression levels and the properties of Ca^{2+}-oscillations observed in mouse eggs following cRNA microinjection of the luciferase-tagged hPLCζ^{WT} and various chimeras between hPLCζ & selected domain from mPLCζ (Fig. 4.13)

Despite the apparent positive effect of inserting the mouse PLCζ-C2 domain in the place of human C2 domain in hPLCζ sequence (hPLCζ^{mC2}), this newly created construct was unable to triggers first Ca^{2+} spike until the luminescence has reached (0.32 c.p.s) which is an equivalent protein expression of around 56.96 fg compared to only 31.61 fg (or with 0.17 c.p.s) in hPLCζ^{WT}. These figures suggest that that the wild type hPLCζ is still more active by nearly two-fold compared with this newly created construct (hPLCζ^{mC2}).

4.8 Discussion

Since the discovery of PLCζ ten years ago (Saunders *et al.,* 2002) accumulating evidence suggests that sperm-specific PLCζ play a pivotal role at fertilization in mammals by triggering Ca^{2+}-oscillations and consequent egg activation. It appears that each domain of PLCζ structures has its specific role in generating Ca^{2+} oscillation-inducing activity.

4.8.1 Sperm PLCζ point mutation in the Y catalytic domain linked to male infertility

The PLCζ role in mammalian fertilization has been recently reinforced by two clinical studies that linked PLCζ with cases of male infertility after ICSI (Yoon *et al.,* 2008; Heytens *et al.*, 2009). ICSI is still the most successful IVF technique that is employed to solve many male infertility conditions such as severe oligospermia, asthenospermia and teratospermia. However, a significant ICSI failure rate occurs, and in some cases of repeat ICSI failure this could be attributed to either reduced or absent PLCζ expression in sperm from those patients whose sperm was also shown to be unable to initiate Ca^{2+}-oscillations in mouse eggs (Yoon *et al.,* 2008).

Further correlation between infertility and PLCζ came with a second clinical study that identified a point mutation at position 398 in the catalytic Y domain that involved a replacement of a histidine with a proline residue in the PLCζ gene of a patient with failed ICSI (Heytens *et al.,* 2009). This study also reported also that the injection of the mutant (H398P) human version of cRNA-PLCζ has failed to induce Ca^{2+}-oscillations in unfertilized mouse eggs.

In our collaborative study, I have taken this result further by studying this newly discovered mutation and its effects on Ca^{2+} oscillation-inducing activity using mouse PLCζ. To create the mouse PLCζ version of this infertility-linked human PLCζ^{H398P} mutation, the mouse PLCζ equivalent residue was identified to be histidine 435 and switching this to a proline generates mPLCζ^{H435P} (Fig. 4.1A). My study was involving the monitoring *in vivo* Ca^{2+} release-inducing activity of PLCζ^{H435P} after injection into mouse

eggs. Our results indicate that the H435P mutation totally abolishes the ability of PLCζ to trigger Ca^{2+}-oscillations in mouse eggs (Fig. 4.1B; Table 3). These results were corroborated with *in vitro* studies that showed complete abrogation of $PLC\zeta^{H435P}$ enzyme activity in a PIP_2 hydrolysis assay (Nomikos *et al.,* 2011a). We also employed an additional PLCζ mutation at the same residue (His^{435}) in order to see if the specificity of this position on the PLCζ might be related to a general helix-destabilizing effect on the protein structure. This was tested by replacing His^{435} with a neutral non-helix-destabilizing amino acid, alanine to create $PLC\zeta^{H435A}$. The injection of cRNA encoding $PLC\zeta^{H435A}$ into mouse eggs interestingly showed robust Ca^{2+}-oscillations with a similar potency to $PLC\zeta^{WT}$ in contrast to the inactive $PLC\zeta^{H435P}$ (Fig. 4.1B; Table 4.1). Consistent with that proposed explanation in the previous study (Heytens *et al.,* 2009) this result suggests that the H435P substitution may cause major protein structural changes that inactivate of PLCζ. In this study and in the others by our group we have also examined the effect of $PLC\zeta^{H435P}$ mutation on its equivalent in PLCδ1 ($PLC\delta1^{H542P}$) upon *in vitro* enzymatic properties on PIP_2 hydrolysis assay. Interestingly similar results were obtained from the *in vitro* studies of these mutants which were carried out by Nomikos and his colleagues. In brief, PIP_2 hydrolysis assays of H435P and D210R show completely inactive enzymes at both low (1 μM) and high (1 mM) Ca^{2+}. This is in contrast to $PLC\zeta^{WT}$ which showed a specific activity at low Ca^{2+} and slightly less activity at high Ca^{2+}. On the other hand $PLC\delta1^{WT}$ displayed high activity at higher Ca^{2+} level (1 mM) that is almost 3.5 folds higher than PIP_2 hydrolysis activity at low Ca^{2+} (1 μM), in contrast, $PLC\delta1^{H542P}$ retained only 16% of the $PLC\delta1^{WT}$ activity at 1 mM Ca^{2+} and was

completely inactive at 1 μM Ca^{2+}. Full details can be found in this study (Nomikos *et al.,* 2011a). Consistently, we found out that none of the *in vitro* results were contradicted with my results. These results altogether show that the above H435P mutation completely inactivates the PIP_2 hydrolysis activity of both PLCζ and PLCδ1 *in vivo* and *in vitro*, whereas the H435A substitution in PLCζ retains only *in vivo* functionality (Nomikos *et al.,* 2011a).

In the previous study based on the heterozygosity of identified sample it was suggested that human $PLC\zeta^{H398P}$ might have a dominant-negative effect on $PLC\zeta^{WT}$ (Heytens *et al.,* 2009). I performed two protocols using cRNA $PLC\zeta^{H435P}$ and $PLC\zeta^{WT}$ to investigate this. First by co-microinjection of both constructs together into mouse eggs, and the second by sequential injection of $PLC\zeta^{H435P}$ and $PLC\zeta^{WT}$ starting with $PLC\zeta^{H435P}$ 1 hr and 2 hrs before re-injecting those eggs with the $PLC\zeta^{WT}$. However, despite nearly 8 fold (7.06 c.p.s./egg) and 26 fold (18.68 c.p.s./egg) higher expression of $PLC\zeta^{H435P}$ in 1 hr and 2 hrs respectively compared with controls (0.68 c.p.s./egg), the co-injected experiment, $PLC\zeta^{H435P}$ did not block the $PLC\zeta^{WT}$ ability to trigger Ca^{2+} oscillations (Fig. 4.2 and Table 4.2). This suggests that, in the mouse, $PLC\zeta^{H435P}$ does not demonstrate any dominant-negative behaviour. Similar results were obtained after the exposing the unfertilized (post-injected with $PLC\zeta^{H435P}$) mouse eggs to *in vitro* fertilization by mouse sperm (Fig. 4.3 and Table 4.3). These results avoid the need for a dominant negative hypothesis of the H-to-P over the wild-type activity. In fact, an explanation of the infertility is gieven after a new point mutation was discovered in the same patient PLCζ sequence but in different allele. This new mutation involved replacing histidine 189 by

leucine at position 233 (H233L) (Kashir *et al.,* 2012). This newer mutation was shown to cause a dramatic loss of PLCζ activity (Kashir *et al.,* 2012).

My study is an extension of the previous work (Heytens *et al.* 2009) in displaying several facts. Firstly it reveals that a mouse PLCζ^{H435P} mutation which is the equivalent of the infertile human PLCζ^{H398P} is completely inactive both *in vivo* and *in vitro. S*econdly my study has enabled a quantitative analysis of their level of inactivity by monitoring *in vivo* expression of the luciferase-tagged mutant PLCs. Finally, this study has demonstrated that PLCζ^{H435P} has no role by any means as dominant-negative inhibition mechanism with respect to either cRNA PLCζ injection or with the sperm at fertilization.

4.8.2 PLCζ-PIP_2 binding specifically requires PLCζ XYl region

Despite the evidence for the role of PLCζ in fertilization, the exact way by which PLCζ targets the PIP_2 in eggs is still elusive. It has been suggested that the positively charged PLCζ residues within the XYl region potentially targeting the negatively charged PIP_2 of the biological membranes via electrostatic interactions (Nomikos *et al.,* 2007). To investigate this *in vivo* we employed different approach using site-directed mutagenesis to generate three cumulative mutations within the positively charged region of the mouse PLCζ XYl region (Fig. 4.4). A sequentially replacement of three lysine residues cluster (K374, K375 and K377) with the neutral amino acid alanine, to generate three mutant constructs of PLCζ, with single (K374A), double (K374,375AA) and triple (K374,375,377AAA) substitutions. The reason behind choosing the alanine to replace lysine is that it is less likely to cause large effects on PLCζs structure compared with a charge-reversed amino acid. The microinjection of the luciferase-tagged cRNA

encoding PLCζ^{WT}, PLCζ^{K374A}, PLCζ^{K374,5AA} or PLCζ^{K374,5,7AAA} individually into unfertilized mouse eggs all caused a robust recombinant protein expression followed by the initiation of a series of Ca^{2+}-oscillations with variable frequencies (Fig. 4.6). A reduction in the Ca^{2+}-oscillations frequency was observed significantly with the triple and to some extend with the double mutants, PLCζ^{K374,5,7AAA} (14% vs wild type) and PLCζ^{K374,5AA} (40% vs wild type) (Fig. 4.5, Fig. 4.6 and Table 4.4). In contrast, only a slight decrease was observed with the single PLCζ^{K374A} mutant (90% vs wild type). Compared with the rapid initiation of Ca^{2+}-oscillations (~30 minutes) in wild type PLCζ and the single K-to-A mutant there was a significant delay (~65 minutes) with the double and triple K-to-A mutants (Table 4.4). All together this suggests a significant change in PLCζ *in vivo* activity was mediated by the loss of two or more positive charges in the XYl. It is reasonable to suggest that his reduction of the net positive charge diminishes the affinity of PLCζ for its negatively charged, membrane-bound substrate, PIP_2.

A previous study showed that the XYl region is the most non-conserved region of PLCζ despite that fact that it connects the two highly conserved halves of the catalytic barrels (the X and Y domains) (Saunders *et al.*, 2007). The importance of this diversity structure is unclear, but it might explain the different relative potency in inducing Ca^{2+}-oscillations between PLCζ isoforms of different species. However, the XYl region retains a net positive charge. Notably, the XYls of PLCβ, δ and ε are also highly negatively charged. It has been suggested that these XYls might have auto-inhibitory effect on the PLC activity by preventing PIP_2 getting access to the active site. This could be mediated by a combination of steric exclusion and electrostatic repulsion of negatively charged

membranes (Hicks *et al.,* 2008). However, our observations suggest that the PLCζ has unique regulatory mechanism and the XYl region role might be distinct from other mammalian somatic cell PLCs pattern.

My collaboration group have examined the binding ability of the XYl and C2 domain of PLCζ to bind to PIP_2. This was done by employing the protein-lipid overlay and a liposome-binding assay to assess distinct GST fusion proteins of either the XYl or C2 domain of PLCζ and compare that with the PH domain of PLCδ1 as a positive control. The results obtained from *in vitro* experiments have provided congruent evidence that only the PLCζ XYl and not the C2 domain has the ability to bind to PIP_2 (Nomikos *et al.,* 2011b). Furthermore, these mutations also affect the *in vitro* interaction of PLCζ with PIP_2 (Nomikos *et al.,* 2011b).

4.8.3 XYl is the novel regulator of PLCζ activity

Although its precise regulatory mechanism remains unclear, PLCζ is the smallest mammalian PLC isoform and only one not found in mammalian somatic cells (Saunders *et al.,* 2002). PLCζ has a unique Ca^{2+} sensitivity that requires only the egg's basal cytosolic Ca^{2+} concentrations of ~100 nM to display strong enzymatic activity. It exhibits ~100-fold higher affinity for Ca^{2+} than the more structurally closest isoform, PLCδ1 (Fig. 4.7). The molecular determinants that confer the high Ca^{2+} sensitivity of PLCζ and how PLCζ activity is intrinsically regulated still need investigation. Previous studies on the negatively charged XYl regions in somatic PLCs suggest that are part of an auto-inhibition mechanism (Hicks *et al.,* 2008). It was therefore important to investigate whether this putative auto-inhibition also applies to the sperm-derived PLCζ.

In the present study, five different constructs were created, PLCζ^{WT}, PLCδ1^{WT}, PLCζ$^{\Delta XYl}$, PLCδ1$^{\Delta XYl}$ and PLCζ$^{\delta 1\text{-}XYl}$ (Fig. 4.7). The microinjection of the these constructs alongside the wild type enabled the examination of how these XYl changes might change the *in vivo* Ca^{2+} oscillation-inducing. Consistent with an auto-inhibition mechanism, the deletion of the XYl from PLCδ1 resulted in a 2-fold increase in Ca^{2+} oscillation-inducing activity in eggs relative to wild-type PLCδ1 (Fig. 4.8 and Table 4.5). In contrast, with PLCζ, the deletion of the XYl resulted in ~7-fold reduction in Ca^{2+} oscillation-inducing activity in eggs (Table 4.5). Significantly, the replacement of only the PLCζ XYl cluster of basic residues (overall charge +7) by the homologous 12 amino acids of the XYl region of PLCδ1 (overall charge +1) also resulted in 5-fold reduction in Ca^{2+} oscillation-inducing activity in eggs (Table 4.5).

These findings suggest that the XYl of PLCζ serves a completely different regulatory role to that of the XYl in PLCδ1. It could be the high density of basic amino acids in the XYl of PLCζ that causes this disparity since it is absent from PLCδ1 and other somatic PLC isoforms. In this case the XYl cluster of positively charged residues may perform a central role in the interaction of PLCζ with the substrate PIP_2. The presence of positively charged residues in XYl is a common feature in the PLCζ sequences across species. However, the specific sequence of the XYl in PLCζ is poorly conserved. This diversity sequence might explain the difference in PLCζ potencies from different species in regard to inducing Ca^{2+}-oscillations. For further understanding of XYl regulatory role across species we created three constructs using human PLCζ as a template. These included hPLCζ^{WT}, hPLCζ^{mXYl} and hPLCζ^{rXYl}. Interestingly the microinjection of

hPLCζ^{mXYl} demonstrates a total loss of the Ca^{2+}-inducing activity, whereas the microinjection of hPLCζ^{rXYl} shows a significant but not complete loss of the enzyme activity comparable to wild type hPLCζ (Fig. 4.9 and Table 4.6). Not only the enzyme activity was affected but also there was a dramatic decline in the protein expression levels in both generated constructs, for reasons that are not clear. Nevertheless, these results confirm the importance of the specific amino acid sequence of the XYl within the PLCζ of specific species.

Similar results were also obtained from *in vitro* study work by collaboration group. They examined the effect of these generated constructs (i.e. PLCζ$^{\Delta XYl}$, PLCδ1$^{\Delta XYl}$ and PLCζ/XYlδ1$^{480–491}$) on the *in vitro* PIP_2 hydrolysis activity of PLCζ or PLCδ1 constructs (Nomickos *et al.,* 2011c).

4.8.4 PLCζ domain(s) abolish PLCδ1 catalytic activity

PLCδ1 has the closest homology to PLCζ among all the mammalian PLCs. We created three chimaeric constructs using PLCδ1 as a template. This was done after removal of the PH domains, and replacing different domain(s) with their analogous from PLCζ i.e. PLCδ1$^{\Delta PH\text{-}\zeta EF\&XYl}$, PLCδ1$^{\Delta PH\text{-}\zeta EF\&C2}$ and PLCδ1$^{\Delta PH\text{-}\zeta XYl}$ (Fig. 4.10). We proposed these experiments to enable an understanding of the specificity of PLCζ domains Interestingly, the microinjection of all these construct PLCδ1$^{\Delta PH\text{-}\zeta EF\&XYl}$, PLCδ1$^{\Delta PH\text{-}\zeta EF\&C2}$ and PLCδ1$^{\Delta PH\text{-}\zeta XYl}$ individually into unfertilized mouse eggs failed to initiate any cytosolic Ca^{2+} changes despite a very high protein expression in all these newly generated constructs (Fig. 4.11 and Table 4.7).

These finding suggest that the enzymatic activity of PLCζ and PLCδ1 are require a specific set of domains acting together to enable full functionality. It was not considered necessary to carryout *in vitro* studies to investigate the effect of these constructs on PIP_2 hydrolysis to compare that with my *in vivo* study. The reason was because the *in vivo* study shows a complete lack of PLCδ1 enzymatic activity.

4.8.5 PLCζ species chimeras

Among the mammalian PLCζs, the human hPLCζ^{WT} (608aa) is the smallest in regard to the number of amino acids. The next smallest is the mouse PLCζ (mPLCζ^{WT}) (647aa) and for this reason we made six different swapping chimeras between the two, i.e. for mouse (mPLCζ^{WT}, mPLCζ^{hXYI}, mPLCζ^{hEF} and mPLCζ^{hC2}) and for human (hPLCζ^{WT}, hPLCζ^{mXYI}, hPLCζ^{mEF} and hPLCζ^{mC2}). All these constructs were tested *in vivo* by microinjection into mouse eggs to monitor their ability to trigger Ca^{2+}-release in comparison with their correspondent wild type. It was expected from the previous work that any changes in the amino acid sequence in any domain in PLCs would lead to a significant drop in the enzymatic activity of that particular PLC. The individual micro-injection of these constructs either from human or mouse showed either a complete (as in case of hPLCζ^{mXYI}) or else a significant decline in activity as in case of hPLCζ^{mEF} (Fig. 4.13 and Table 4.9) and in all mPLCζ originated constructs (Fig. 4.12 and Table 4.8). The exception was with the apparently positive results for activity augmentation noted in the case of hPLCζ^{mC2}, but even in this case the chimera does not initiate Ca^{2+}-oscillations until the luminescence has reached nearly 2-fold the wild type level of

hPLCζ (0.32 c.p.s/egg vs 0.17 c.p.s/egg) respectively (Fig. 4.13; Table 4.9). These findings suggest that the enzymatic activity of the wild type PLCζ of any species is superior to any newly created constructs.

Chapter 5

Ca^{2+}-OSCILLATIONS IN HUMAN EGGS

5.1 Introduction

Nowadays the importance of Ca^{2+} oscillations during mammalian fertilization has been established. Based on parthenogenetic activation studies from different stimuli, the free cytoplasmic Ca^{2+} increase has been shown to be a sufficient trigger for development in mouse, pig and hamster eggs (Steinhardt *et al.,* 1974; Fulton and Whittingham, 1978; Sun *et al,* 1992). In human eggs, Ca^{2+} ionophores have also been shown to be an effective parthenogenetic trigger (Winston *et al.*, 1991). However, almost all parthenogenetic activation protocols used are only trigger a monotonic increases in free Ca^{2+}, which is unlike fertilization where there are repetitive Ca^{2+} increases (Homa *et al.,* 1993). These monotonic increases produce significantly lower activation rates (Fulton and Whittingham, 1978; Vincent *et al.,* 1992). This is not the case with Sr^{2+}, where Sr^{2+} not only stimulates repeated Ca^{2+}-oscillations (Kline and Kline, 1992), but can also activate most of the freshly ovulated mouse eggs (Homa *et al.,* 1993).

One of the concerns facing human IVF programmes is the underlying reasons of failed fertilization. Although ICSI has overcome most of male infertility problem (Palermo *et al*., 1992), this technique does not work in every single case (Yoon *et al.,* 2012). In the literature, several clinical reports have suggested that, in cases of repeated failed ICSI, the application of assisted oocyte activation, such as Sr^{2+} treatment or other stimuli such as Ca^{2+} ionophore, post ICSI might improve the ICSI outcome (Yanagida *et al*., 2006; Kyono et al., 2008; Chen *et al*., 2010; Borges *et al*., 2009; Taylor *et al*., 2010; Check *et al*., 2010). Taking these studies into account, it appears that assisted egg activation procedures could help some infertile patients where the sperm may lack the

ability to triggers Ca^{2+} signal (Chen *et al*., 2010; Nasr-Esfahani *et al*., 2010). Unfortunately, the safety of such pharmacological agents has not been fully tested, and there is not a single report showing Ca^{2+} oscillations in human eggs triggered by Sr^{2+}.

Theoretically, once we understand the molecular mechanisms that are involved in successful fertilization, then we might be able to begin to tackle this issue of failed activation. Fertilization normally occurs when the egg is meiotically mature, and is initiated after the spermatozoon fuses with the plasma membrane of the egg. We now consider that upon sperm-egg fusion, sperm delivers sperm-specific phospholipase C (PLCζ) (Saunders *et al.,* 2002; Swann *et al.,* 2006), that persistently activating the phosphoinositide pathway and results in the IP_3 production, which then activate IP_3R located on ER and causing repeated episodes of Ca^{2+} release (Swann, 1990; Stricker, 1999; Rice *et al*., 2000). It has been shown that PLCζ not only activate eggs but also enhances embryo development up to the morula and blastocyst stages in mouse (Yu *et al.,* 2008), porcine (Yoneda *et al.,* 2006), cow (Ross *et al.,* 2008) and human oocytes (Rogers *et al.,* 2004).

Consequently, the most convenient human egg activator may well be sperm PLCζ. This notion has been further supported recently by two studies on hPLCζ from sperm of patient who had repeated failed ICSI. These studies suggested that sperm with low quantity (Yoon *et al*. 2008) or mutated (maternally inherited point mutation) hPLCζ (Heytens *et al*., 2009) are unable to induce Ca^{2+}-oscillations and fertilize the human eggs. Moreover, using mouse PLCζ, we have shown that injection of mRNA encoding

this mutation abolishs its ability to induce Ca^{2+}-oscillations and this further supports the suggestion that this is the cause of the infertility (Nomikos *et al*., 2011a).

PLCζ is known to be an effective egg activator in mammals. However, the most available form of this artificial human egg activator, and one that can cause fertilization-like Ca^{2+} oscillations, is the complementary RNA of sperm PLCζ (Kouchi *et al.,* 2004). Experimentally, once PLCζ cRNA introduced into eggs by the direct cytoplasmic microinjection it starts to translate into PLCζ protein that continues over a several hours period (Yu *et al.,* 2008), and in turn this PLCζ protein causes prolonged Ca^{2+}-oscillations.

However, there are legal issues with regards to the use of cRNA in human eggs. In addition the precise quantification and the translation ability of the injected cRNA is difficult in human eggs. For both of these reasons the use of cRNA in IVF clinics is unlikely. These obstacles have led researchers to look for a more usable and natural human egg activator, and this is recombinant PLCζ protein. Several attempts have been reported to find a way to prepare recombinant hPLCζ, but the purity and stability of the PLCζ protein are still unresolved. Recently, one report has been published on potential successful synthesis and purification of recombinant human PLCζ (hPLCζ) protein, which was shown to induce Ca^{2+}-oscillations in mouse and human eggs (Yoon *et al.,* 2012).

The human eggs used in this study were donated by patients attending the IVF Wales clinic at the University Hospital of Wales, Cardiff, UK. The current project and all associated procedures were approved by the local South East Wales Research Ethics

Committee and also by the UK Human Fertilisation and Embryology Authority (HFEA) (R0161). In this chapter I will show how human eggs respond to the Sr^{2+} protocols that were successfully applied on mouse eggs, and I show under which circumstances Sr^{2+} might trigger Ca^{2+}-oscillations in human eggs. In addition, I will demonstrate to what extend our research group has achieved the synthesis of active hPLCζ recombinant protein. In this regard, we have succeeded in producing a very stable partially purified recombinant hPLCζ protein that could be stored in fridge or in -80C freezer. The microinjection of either of these stored recombinant hPLCζ proteins has Ca^{2+} inducing activity in both mouse and human eggs. Finally, briefly some preliminary studies monitoring cytoplamic movements during human egg activation using hPLCζ cRNA.

5.2 Direct intra-cytoplasmic Sr^{2+} injection may not trigger Ca^{2+} release in human eggs

After the results of my experiments on Sr^{2+} injection in mouse eggs, I tried to apply the same protocol to human eggs done by injecting human eggs with the Ca^{2+} dye OGBD then transferred to the inverted fluorescent microscope for the next injection with 100mM Sr^{2+} (pipette concentration). This does not mean that cytoplasmic Sr^{2+} concentration is 100mM since it leaks slowly and dilutes within the egg. Due to the shortage in the human eggs, I ended up using only 5 human eggs for the Sr^{2+} injection protocol. Those eggs were from three different patients, 3 from failed IVF/ICSI (old eggs) and the other two were from follicular reduction (fresh eggs). After monitoring fluorescence for over five minutes, the injection pipette was introduced into the human egg and Sr^{2+} started to leak directly into the egg with continuous florescence (Ca^{2+})

monitoring. Sr^{2+} injection was continued for over half an hour by process called ionotophoresis (see material and methods chapter) and in some eggs the injection cycle was repeated twice with a new injection pipette. My results shows that none of those eggs demonstrate any Ca^{2+} rises in response to Sr^{2+} injection (Fig 5.1). The main obstacle for this experiment was the poor availability of human eggs. Hence, it is difficult to give a final conclusion for this kind of experiments, but it does suggest that human eggs may be less sensitive to Sr^{2+} even when it is present in the cytoplasm.

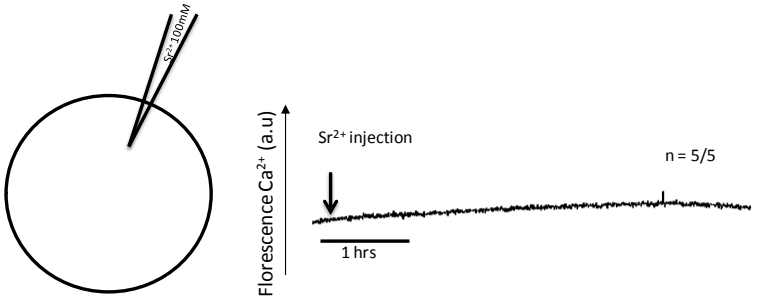


Figure 5.1 Schematic diagram of Sr^{2+} injection and example of Ca^{2+} trace in human eggs in response to injected Sr^{2+}

5.2.1 Do human eggs need enhancement to respond to Sr^{2+}?

Based on these similarities between the human and mouse egg IP_3R type, and after the negative results with Sr^{2+} injection, a modified technique was tried to try and enhance Ca^{2+}-oscillations. In brief, I thought if I could sensitize the human egg IP_3R with a small dose of the agonist adenophostine, that only triggers few Ca^{2+} spikes and then treat those eggs with Sr^{2+}, this could induce the egg to respond to Sr^{2+}. The injection protocol was carried out by mixing adenophostine with the Ca^{2+} dye RhD (50:50). Then the injected eggs were transferred to a prepared dish for recording on the florescent inverted microscope. In this experiment I used a total of 11 eggs, 5 were failed IVF from

one patient, and 6 eggs from another patient, 3 of them were fresh eggs and other 3 were failed ICSI. I injected the failed ICSI eggs with final concentration of 0.5µM adenophostine.

With continuous Ca^{2+} monitoring, the results was that no Ca^{2+} oscillations occurred even after co-treating eggs with 20mM Sr^{2+} $0Na^{+}$ high K^{+} media (Fig 5.2A). I then increased the adenophostine concentration to 1µM and I injected those IVF failed and the fresh eggs with the new higher concentration of adenophostine.

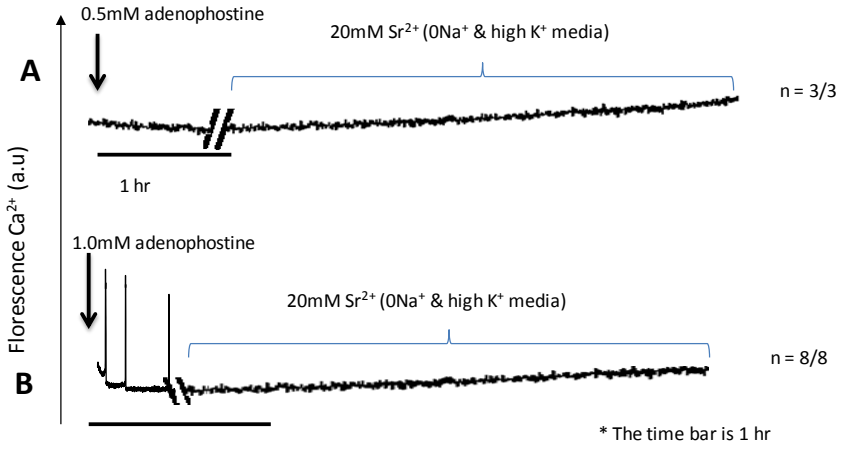


Figure 5.2 Human egg response to adenophostine and 20mM Sr^{2+} media (0 Na^{+}& high K^{+}). A) Representative figure of a failed ICSI human egg in response to the injection of 0.5mM adenophostine followed by 20mM Sr^{2+} media. B) Representative figure of failed IVF/ fresh human eggs in response to the injection of 1.0mM adenophostine followed by 20mM Sr^{2+} media

The results showed that all injected eggs responded to adenophostine with Ca^{2+} increases for around 20 minutes, and when I transferred them to 20mM Sr^{2+} media, no further Ca^{2+} oscillations were detected (Fig 5.2B). These results suggest that a difference in degree of IP_3R sensitization to Sr^{2+} between mouse and human eggs is not likely to account for the lack of response to the Sr^{2+} protocol in human eggs.

5.2.2 Sr^{2+} may trigger Ca^{2+}-oscillations in human eggs under certain conditions

It is been well known that Sr^{2+} works reliably in rodent eggs but not in human and domestic animals, and the reason behind this is still unclear, despite several clinical studies that reported higher rate of ICSI outcome after Sr^{2+} exposure of ICSI eggs (Yanagida *et al*., 2006; Kyono et al., 2008; Chen *et al*., 2010). The results on Sr^{2+} influx mechanisms in mouse eggs suggested that Sr^{2+} influx may occur mainly via the reverse NCX. Consequently in this study, I tried to activate the reverse mode of NCX using similar protocols that I used on mouse eggs. One of the most successful protocols is the totally Na^{+} free HKSOM media with high K^{+} i.e. $0Na^{+}$ with 10mM Sr^{2+} for 2-3 hours (see chapter 2). This was the most extreme media I prepared to activate the reverse NCX. When I used this protocol on human eggs, there were no Ca^{2+} rises in all of the eggs tested (10/10), from 5 patients (failed IVF/fresh eggs) (Fig 5.3A). Then I adjusted the protocol by increasing the time of exposure for up to 5 hrs and increasing the Sr^{2+} concentration to 20mM. Unfortunately, the results were still the same with no response in all tested eggs (16/16) (Fig 5.3 B), the eggs where donated from different patients that varies between fresh and failed IVF/ICSI.

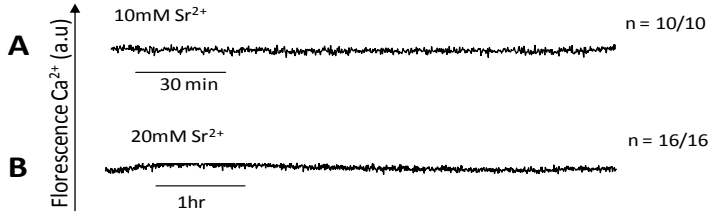


Figure 5.3 Human eggs response to two protocols of Sr^{2+} activation in NCX reversal media.

A) Represent the human egg response to 10mM Sr^{2+} for 2-3 hours. B) represents the response of human eggs to a higher Sr^{2+} concentration (20mM) with longer exposure time (5 hrs).

Unexpectedly, when I kept the human eggs overnight on the recording dish in the Sr^{2+} 20mM NCX reversal media (0mM Na^{+} & high K^{+}), the results were encouraging and showed that most surviving eggs responded to Sr^{2+} by showing repetitive Ca^{2+} rises.

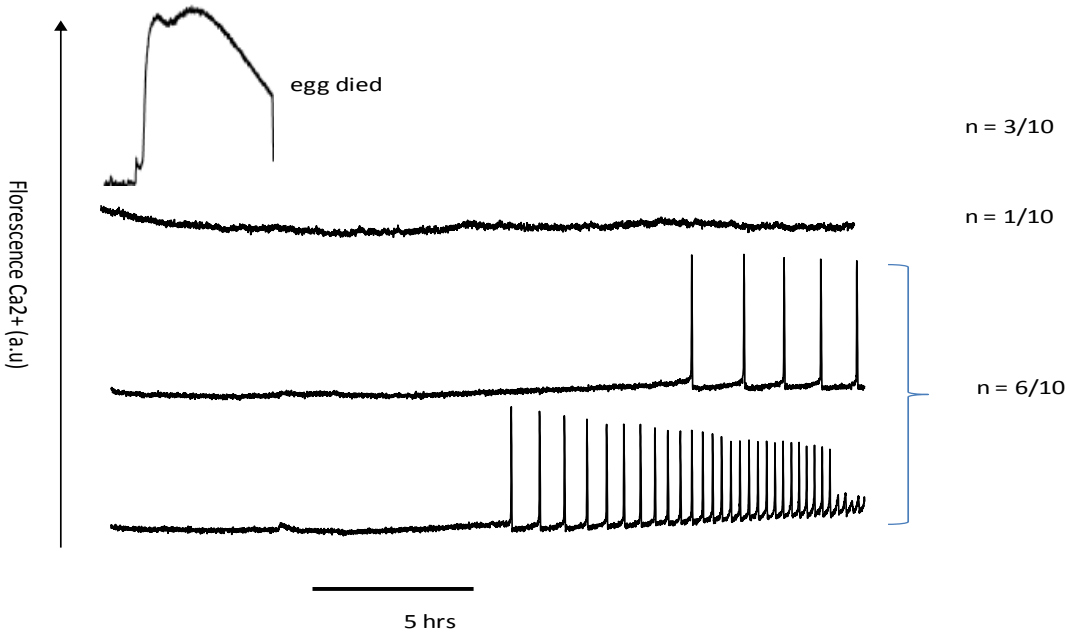


Figure 5.4 Ca^{2+}-oscillations in human (failed ICSI) eggs in response to overnight incubation in 20mM Sr^{2+} , 0Na^{+} & high K^{+} media

The interesting point is that all tested eggs were failed ICSI eggs. In total I tested 10 eggs, 7 from one patient, and 3 eggs from two other patients. From the 10 tested eggs, 7 survived eggs, and amongst them 6 displayed Ca^{2+}-oscillations (6/7) 85.7% (Fig. 5.4). However, the starting time and the oscillations frequencies were very variable. The earliest an egg that started oscillating was 10 hrs after recording. These data suggest that prolonged incubation in some Sr^{2+} media can cause some Ca^{2+}-oscillations in human eggs. However, all responded eggs looked unhealthy by the next morning, they never underwent pronuclear formation and eventually all died.

5.3 Recombinant PLCζ-protein and human eggs

It has been shown that all mammalian eggs including, those of humans, respond reliably to sperm specific PLC (PLCζ). However, due to the nature of the cRNA encoding PLCζ, and the difficulty to control the injection amount of the cRNA, the production of recombinant PLCζ protein has become desirable. In this regard Michael Nomikos from our group has succeeded in producing stable and partially purified (with NusA) human recombinant PLCζ protein (Nomikos *et al.,* 2013). This recombinant human PLCζ was expressed as a NusA hexahistidine fusion protein in E. coli and then purified by Ni-NTA affinity chromatography. Due to the potential inactivation issues, our human recombinant PLCζ protein was kept with the attached NusA, and tested in mouse as well as in human eggs (Nomikos *et al.,* 2013). Our results showed the presence of prompt Ca^{2+} oscillations in response to the injected recombinant hPLCζ protein in both mouse and human eggs indicate that we have made potent recombinant hPLCζ protein. We have also showed that purified recombinant human PLCζ protein is capable of hydrolyzing PIP_2 with a similar Ca^{2+} dependence to mouse PLCζ, and enhance early embryonic development in mouse eggs (Nomikos *et al.,* 2013).

Figure 5.5A, shows failed IVF human eggs from same patient showed Ca^{2+}-oscillations triggered by injected fresh partially purified hPLCζ recombinant protein with NusA attached. This stock was then divided and kept in two different conditions i.e. in fridge ~4C° and in -80C° freezer for over 10 months period. The injection of this concentrated stock, that was stored in -80C° freezer, into failed ICSI human eggs triggered Ca^{2+}-

oscillations for around 1 hour (5/5) (Fig. 5.5B). The injection of the concentrated stock, that was stored in fridge, into a (failed ICSI) human egg failed to trigger Ca^{2+} oscillations in 3 hours. However, the oscillations stimulated after re-injecting this egg with the (-80C°) freezer stock (1/1) (Fig. 5.5C).

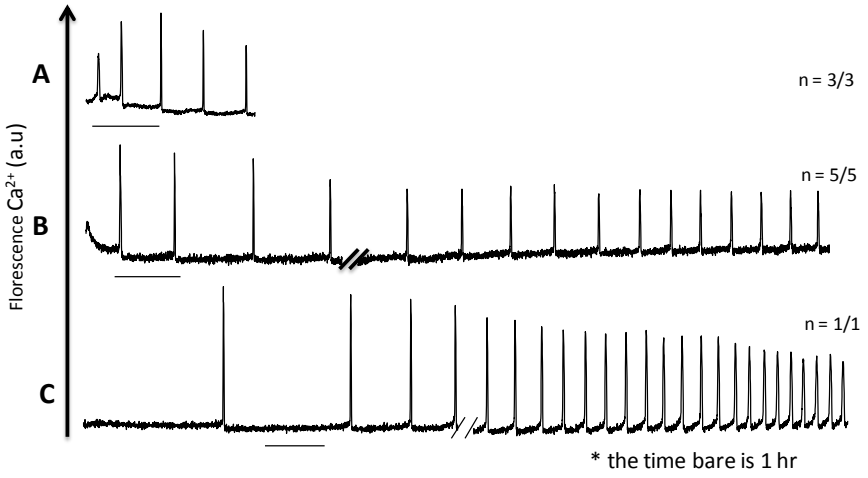


Figure 5.5 hPLCζ recombinant protein into failed IVF and failed ICSI human eggs

A) These eggs are from a failed IVF and they were injected with freshly partially purified recombinant hPLCζ protein. B) These eggs are failed ICSI and were injected with hPLCζ protein that was kept in -80C freezer for > 10 months .The oscillations starts after nearly 1 hr of recording. There is ~10 minutes gap. C) This egg was a failed ICSI case and was injected with hPLCζ protein that was kept in the fridge for >10 months and it showed, no oscillations for almost 3 hrs, but then when re-injected with the same preparation that was kept in -80C freezer for > 10 months, the oscillations started and continued for nearly another 3 hours of recording. There is 20 minutes gap (time to check the response of eggs)

Figure 5.6, is an illustrative trace showing Ca^{2+}-oscillations in mouse eggs in response to injected hPLCζ recombinant protein that was treated in different ways. The traces in (Fig. 5.6 A&B), shows prompt Ca^{2+}-oscillations in mouse eggs triggered by crude (with out purification) fresh, as well as 1week old (fridge stored) recombinant hPLCζ protein. There were 6/6 and 7/7 eggs respectively. However, the frequency of Ca^{2+}-oscillations is higher in the protein stored in the fridge by over 60% (24 spikes/1hr versus 15

spikes/1hr). The injection of partially purified hPLCζ recombinant protein into mouse eggs showed around a 50% reduction in the hPLCζ protein activity that was represented with lower frequency oscillations (13 spikes/1hr) (15/15) (Fig. 5.6C) compared to (24 spikes/1hr) that triggered by crude hPLCζ recombinant protein (7/7) (Fig. 5.6B).

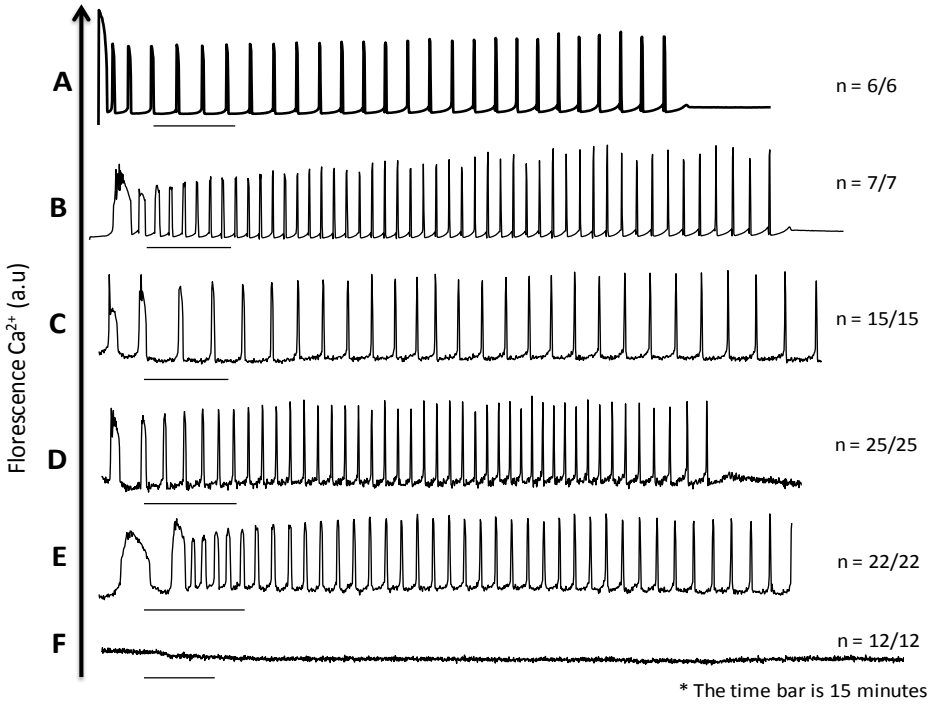


Figure 5.6 The effect of hPLCζ recombinant protein injection into mouse eggs.

A) Ca^{2+}-oscillations in mouse eggs in response to fresh crude hPLCζ recombinant protein B) Ca^{2+}-oscillations triggered by 1 week old (in fridge) crude hPLCζ recombinant protein In C) 1 week (in fridge) partially purified hPLCζ recombinant protein. In D) 10 months old (in fridge) partially purified hPLCζ recombinant protein. In E) >10 months old (in -80C) partially purified hPLCζ recombinant protein. In F) NusA purified hPLCζ recombinant protein into mouse eggs

Interestingly, after 10 months storage of this partially purified protein in the fridge or in -80C° freezer, the injection of this protein into mouse eggs still showed a significant Ca^{2+} releasing activity that looks similar to the activity of the one week old un-purified stock in

Figure 5.6B [21 spikes/1hr (25/25)] (Fig. 5.6D) & [19.5 spikes/1hr (22/22)] (Fig. 5.6E) respectively. However, further purification that removed the NusA completely abolished the protein enzymatic activity and showed completely flat traces in all injected mouse eggs (12/12) Fig.5.6F.

These data might be interpreted to suggest that the freshly made hPLCζ recombinant protein is less active than the stored protein. This in turn may lead to a concept that hPLCζ recombinant protein requires a period of time to 'mature' in order to get the highest PIP_2 hydrolytic activity. These data also suggest that preserving the NusA attached on the hPLCζ protein stabilizes the protein activity for longer periods.

5.4 A potential non-invasive method of calcium monitoring and possible predictive method for embryo selection

The importance Ca^{2+}-oscillations during fertilization in mammalian eggs are well known and extensively studied. Until recently, the usual method to detect Ca^{2+} changes is by using an invasive Ca^{2+}-sensitive fluorescent dye and then exposes eggs to excitation light at specific wavelengths. This light and dye combination can cause damage to the egg that consequently might interfere with the later development of the resulting embryo (Nematollahi-mahani *et al.,* 2009). It has been reported previously that during fertilization in mouse eggs the first few Ca^{2+} spikes are simultaneously accompanied by sudden cytoplasmic-movements (Deguchi *et al.* 2000). By using advanced image analysis based on particle image velocimetry (PIV) analysis, these cytoplasmic movements have been detected in fertilizing mouse zygotes (Ajduk *et al.,* 2011). This could be used as non-invasive technique to monitor the occurrence of Ca^{2+} oscillations

in the absence of fluorescent dyes. These cytoplasmic-movements have not been reported in any other mammalian species. In this study I was involved in a study of this phenomenon in human eggs. I co-injected human eggs that failed to fertilize after ICSI eggs with cRNA PLCζ mixed with Ca^{2+} sensitive dye (OGBD). These eggs were then monitored for both Ca^{2+}-oscillations and cytoplasmic-movements with PIV simultaneously. The PIV analysis was carried out in collaboration with Dr Chris Graham in Oxford University Department of Zoology where the PIV image analysis was carried out. In total, from 10 different zygotes a range between 95 and 102 cytoplasmic-movements were detected. After analyzing the results of the injected human eggs with cRNA PLCζ, it was clearly seen that Ca^{2+} oscillations were easily detectable with low and then higher frequency. In the meantime, cytoplasmic-movements accompanied the oscillations. They were more clearly detected after the first 1 or 2 Ca^{2+} spikes. Our study shows that small abrupt cytoplasmic-movements can be detected simultaneously with each Ca^{2+} transient in human failed ICSI eggs (Fig. 5.7).

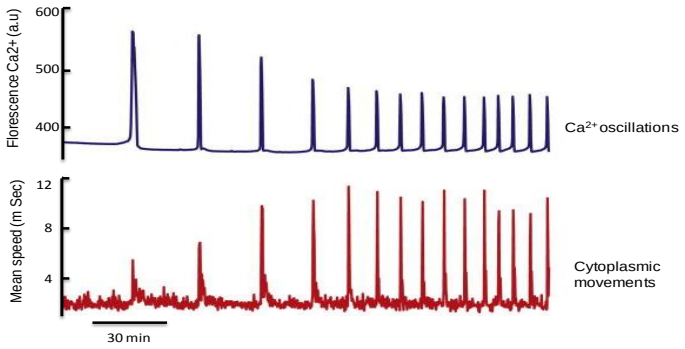


Figure 5.7 PLCζ- and ICSI-induced Ca^{2+} oscillations in human oocytes are accompanied by coincident transient movements in the oocyte cytoplasm. Recording of intracellular Ca^{2+} increases measured by OGBD fluorescence and the corresponding cytoplasmic movements as measured with PIV

We carried out a similar analysis of PLCζ cRNA-induced Ca^{2+}-oscillations and cytoplasmic-movements in seven unfertilized eggs that were obtained after follicle reduction mainly from women undergoing intra-uterine insemenation (IUI). Notably, we failed to detect any movements associated with the 41 Ca^{2+} spikes observed in these follicle reduction-derived eggs. This method can be regarded as potential non-invasive way for monitoring Ca^{2+}-oscillations occurrence and their patterns in human eggs in the absence of Ca^{2+} dyes and high intensity excitation light.

5.5 Discussion

5.5.1 Sr^{2+} injection may not provoke Ca^{2+} release in human eggs

Although it has previously been reported that Sr^{2+} can be injected into mammalian eggs (Georgiou *et al.,* 1987 and Okada *et al.,* 2003), these reports did not keep the injection pipette in the egg for prolonged times. Hence they did not see if oscillations in Ca^{2+} could be generated. The Sr^{2+} injection experiments were the most challenging experiments with regards to the survival of the injected egg. My protocol was planned using repeated electrical pulses to ensure continuous leaking of Sr^{2+} from the injection pipette that was kept in for long times (~30minutes) before withdrawal from the egg. It has been reported previously that Sr^{2+} works by activating the Ca^{2+} binding stimulatory site of the IP_3R (Marshall and Taylor, 1994). Amongst the three types of IP_3R that have been reported in mammalian cells 1, 2 and 3 (Patterson *et al.,* 2004), the IP_3R1 is the predominant type in mammalian eggs and is highly expressed at the MII stage in mouse (Mehlmann *et al.,* 1996) and human eggs (Goud *et al.,* 1999; Mann *et al*., 2010).

The IP_3R1 has also been reported to be the receptor responsible for all of the of Ca^{2+} increases after fertilization or IP_3 injection in rodent (Xu *et al.,* 1994; Mehlmann and Kline, 1994; Yoshida *et al.,* 1998) and in human eggs (Goud *et al.,* 2002). In this project I ended with only 5 human eggs being injected which is a very small number. However, even in this small number of eggs, by bypassing the plasma membrane via direct Sr^{2+} injection, Sr^{2+} should be able to induce Ca^{2+} release as it does in mouse eggs. Despite this similarity between mouse and human eggs, Sr^{2+} injection did not show Ca^{2+} release in human eggs. It is difficult to give a reasonable explanation that could clarify this. One possibility, considering the egg's size, is the density of IP_3R1 in human egg could be less than that in mouse. The other possibility is the threshold sensitivity of IP_3R1 to Sr^{2+} in human eggs could be higher in comparison to that in mouse eggs. It might be due to other modifications of the IP_3 receptor such as different phosphorylations. Despite the fact that these are preliminary results, I suggest that the Sr^{2+} injection is not an effective way to activate or triggers Ca^{2+}-oscillations in human eggs. Nevertheless further injection experiments might still be needed to draw a precise and final conclusion.

5.5.2 Human egg response to Sr^{2+} is conditioned

Previous studies (Yanagida *et al*., 2006, Kyono *et al.,* 2008 and Chen *et al*., 2010) claimed that 10mM Sr^{2+} treatment for 1 hr after 30 minutes post ICSI on human eggs would be an effective method of assisted oocyte activation. This could improve fertilisation, embryo quality, pregnancy rates and successful live births, in cases of repeated ICSI failure. This is shorter exposure time than the used protocols in the

mouse, where it has been reported that the optimum Sr^{2+} activation protocol is the combination of 10mM Sr^{2+} for 2.5 hrs in Ca^{2+} free media (Ma *et al.,* 2005). These previous human egg studies have a couple of scientific based deficits. First, they lack the inclusion of a control group at the time of the study. Secondly, none of these reports has shown or reported Ca^{2+} monitoring during the activation process. These would be expected, since Ca^{2+}-oscillations are a physiological prerequisite step for human egg activation (Taylor *et al.,* 1993).The Ca^{2+}-oscillations in my experiments starts very late, ~10hrs into recording, which is roughly ~ 36 hrs after ICSI. In the end none of the eggs in my study showed pronuclear formation and eventually they all died. These results, despite being the first reported Ca^{2+}-oscillations in human eggs triggered by Sr^{2+}, is far from physiological conditions, and hence cannot be recommended to be used in IVF clinics. Hence my current results on Sr^{2+} treatments suggest it is too early to use Sr^{2+} is an agent for human egg activation.

One interesting point in my study is that all human eggs that showed some response to Sr^{2+} were failed ICSI eggs (i.e. containing sperm). Therefore, due to the short availability of human eggs, this overnight protocol that I used in these experiments should be tested in future on fresh and failed IVF human eggs before drawing too many conclusions. The other recommendation is with regards to the media used. It would be worthy testing the overnight protocol using the regular Ca^{2+} free Sr^{2+} media before we can say that the oscillations were definitely a result of the special NCX reversal media derived from studies in mouse eggs.

5.5.3 ICSI failure of male factor origin might be rescued with PLCζ recombinant protein

Despite the invention of the ICSI technique as a successful tool to solve most of male infertility problems in IVF clinics, there are still some cases of repeated failure in ICSI. The majority of these cases are caused by egg activation failure (Tesarik *et al.,* 1994; Nasr-Esfahani *et al.,* 2010). Following the identification of PLCζ as the sperm factor in mammals, there is a growing interest in the use of recombinant PLCζ protein in IVF clinics. This might be a means to solve the egg activation failure after ICSI. In principal the co-microinjection of PLCζ cRNA during ICSI could be used to rescue egg activation failure due to sperm PLCζ dysfunction.

In this study, I have focused on the fact that our lab has made an active recombinant hPLCζ protein. We prepared recombinant human PLCζ protein fused to NusA (Nomikos *et al.,* 2013). NusA is a fusion protein known to greatly enhance the stability and solubility of recombinant proteins (De Marco *et al.,* 2004). Our study has now showed that the microinjection of wild-type recombinant human PLCζ protein induced Ca^{2+}-oscillations in both human and mouse eggs. In the extension on this study my colleague (Yuansong Yu) has succeeded in successfully activating mouse early embryo development up to the blastocyst stage using recombinant human PLCζ protein (Nomikos *et al.,* 2013). The micro injection of recombinant human PLCζ protein into mouse eggs alone, without the fusion protein NusA showed a dramatic loss of PLCζ activity as represented by very poor Ca^{2+}-oscillations. This indicates the importance of the accompanying the fusion protein in stabilizing the recombinant PLCζ protein. The

production of active purified recombinant PLCζ protein promotes its potential application in IVF clinics as a therapeutic option for egg activation following failure caused by male factor related to PLCζ dysfunction.

5.5.4 Possible non-invasive method of Ca^{2+} monitoring and embryo selection

Cytoplasmic movement have been reported in mouse eggs after sperm entry (Ajduk *et al.,* 2011). In addition, the point of sperm entry in mammalian eggs is known to be linked the organization of the cytoplamic components (Ajduk et *al.,* 2011). In our studies the cytoplasmic movements were observed in 10 failed ICSI human eggs i.e. containing sperm, but not in the 7 follicular reduction or 'fresh' human eggs (Swann *et al.,* 2012). This suggests that the presence of the sperm is important for the effective observation of cytoplasmic movements in human eggs during fertilization.

To use this technique as a potential indicator of successful fertilization or embryo development, it is worthwhile to know to what extend the human embryo can tolerate light exposure. Previous studies have suggested that we can predict the ability of developing human embryo to the blastocyst by monitoring group of human IVF zygotes from day 2 after fertilization by automated image analysis system (Wong *et al.,* 2010). These and other studies reported that the exposing of light for <1 second every couple of minutes for several days on human embryos showed no harmful effect upon development (Nakahara *et al.,* 2010). Our method for monitoring the cytoplasmic movements was programmed to expose human eggs to light for <1 second every 10 seconds for several hours. It is unclear whether or not this level of light exposure will

have any damaging effect upon human embryo later development. However (Adjuk *et al.,* 2011) reported that there is no harmful effect upon mouse embryo development to term.

Previous mouse embryo studies results indicated that the pattern of cytoplasmic movements, that mirroring the Ca^{2+}-oscillations, provide a powerful indicator of embryo quality, and consequently provide a predictive measure of the quality of the pre-implantation embryo (Ajduk *et al.,* 2011). Extrapolating these finding to our study we can expect that this technique could be a novel, early and non-invasive method to determine the viability and potential for later human embryo development. This could then be helpful in raising the implantation success in IVF clinics.

Chapter 6

GENERAL DISCUSSION

6.1 Overview

In this Thesis I have presented data from several studies that have investigated the optimal mechanisms of triggering Ca^{2+}-oscillations artificially in mouse and human eggs. Several scientific facts have resulted from this work. I have shown that Sr^{2+}-triggers Ca^{2+}-oscillations in mouse eggs by an influx mechanism, in which the NCX seems to play the major role. I have also shown that human eggs, that had failed fertilization post ICSI, can respond to Sr^{2+}. However, this response is conditional and only occurs in particular circumstances, such as in a special media (0 Na^{+} and high K^{+}) and with a long Sr^{2+} exposure period.

The other part of my research concerned sperm specific PLCζ. In this I showed the impacts of some sequence mutations on PLCζ functional activity. Moreover, we have investigated the regulatory mechanism of PLCζ. Finally, we succeeded in making an active recombinant PLCζ protein. PLCζ is thought to be the sperm factor in all mammals. Following on the results from previous studies on human PLCζ (Yoon *et al*., 2008; Heytens *et al*., 2009), I have shown that the H398P single site mutations seen in human PLCζ works in exactly the same in mouse PLCζ in abolishing all PLCζ activity. This mutation is special since I showed also that the H398A mutation enables mPLCζ to retain most of its catalytic activity (Nomikos *et al.,* 2011a). I also showed that the PLCζ XYl region has a special cluster of amino acids that enables PLCζ to target PIP_2 (Nomikos *et al.,* 2011b). Beside the PIP_2 targeting role of the XYl in PLCζ, I have shown also that PLCζ is regulated by its XYl in a different way from the mechanism in somatic PLCs (Nomikos *et al.,* 2011c). A number of experiments on PLCs mutation chimeras

and their effect on Ca^{2+} release activity were also carried out and they are included in this Thesis. These experiments are mainly comparisons between PLCζ and its closest homolog PLCδ1, and with sperm specific PLCζ between species; mainly human and mouse. These PLCζ studies, including the chimeras work, suggest that any change in the nature of the domain structure of PLCζ will result in a significant impact on PLCζ Ca^{2+}-releasing activity. In this chapter I will discuss the pathenogenetic stimuli causing Ca^{2+} release in mammalian eggs, including mouse and human, for a general comparison. Similarly I will include in my discussion other PLCs (somatic), and compare them with the sperm specific PLCζ.

6.2 Parthenogenetic mammalian egg activation via Sr^{2+} application

For decades it is been known that egg activation can be achieved even in absence of sperm (Swann and Ozil., 1994). Previously the lack of knowledge that surrounds egg activation events presented challenges for artificial oocyte activation methods. Many approaches been used to artificially induce oocyte activation and they have focused on triggering in intracellular Ca^{2+} increases (Loren and Lacham-Kaplan, 2006). A few successful candidates have been shown to release the MII arrested eggs from the meiotic arrest by promoting of extracelluar Ca^{2+} entry or release to increase free intracellular Ca^{2+}. These include Ca^{2+} ionophores, 7% ethanol (Cuthbertson & Cobbold 1985), and the divalent cation strontium (Sr^{2+}) (Kline & Kline 1992). While these methods may be credible for artificial egg activation, none are is able to achieve the

efficiency provided by spermatozoa. For example, Ca^{2+} ionophores (A23187) have been reported in several studies to activate mammalian eggs including those of humans (Borges *et al.,* 2009; Nasr-Esfahani *et al.,* 2010). However only Sr^{2+} has the ability to produce Ca^{2+}-oscillations and it appears to be most physiological compared with methods of egg activation that trigger a monotonic rise in cytosolic Ca^{2+}. The optimum parthenogenetic stimulus in rodent eggs is Sr^{2+} and it has been successfully employed in many studies (Miyazaki and Ito, 2006; Loren and Lacham-Kaplan, 2006; Kyono *et al.,* 2008)**.** In fact Sr^{2+} is widely used in research labs as an egg activator in rodent eggs as it is the only cation that provokes Ca^{2+} oscillations that are similar to those seen in normal fertilization (Kono *et al.,* 1996).

Sr^{2+} exposure times have been shown to support and influence blastocyst composition and early embryonic development (Bos-Mikich *et al.,* 1997; Lacham-Kaplan *et al.,* 2003). However, Sr^{2+} alone cannot produce live offspring since there are imprinted genes that are switched off in the egg and only switched on in sperm. A single report has shown that Sr^{2+} can lead to production of normal mice offspring in conjunction with ICSI of sperm collected from male mice treated with alkylated iminosugars (Suganuma *et al.,* 2005). Alkylated iminosugars act as reversible male infertility-inducing agents during short-term treatment by inducing sperm deformities that include irregular mitochondrial sheaths, aberrant attachment of tails, and deviant head shapes that impair sperm fertilization ability (Van der Spoel *et al.,* 2002). They do this without altering the genetic integrity of male mouse germ cells. When mouse eggs were treated with Sr^{2+} as per their protocol after ICSI of misshapen epididymal spermatozoa, the

majority of these eggs were activated and a normal generation of mice produced (Suganuma *et al.,* 2005).

In humans, a few clinical reports have shown that Sr^{2+} may enhance IVF and ICSI success rates in cases of repeated IVF/ICSI failure (Yanagida *et al.,* 2006; Kyono *et al.,* 2008). In large mammals like bovine eggs, the combination of electrical and chemical 'Sr^{2+}' parthenogenetic activation protocols have been shown to be able to compete with the rate of development seen in IVF/ICSI at the early cleavage stage, but not at the blastocyst stage (Hosseini *et al.,* 2008). However, other studies have failed to show Ca^{2+} transients in human eggs treated with Sr^{2+} (Rogers *et al.,* 2004; Heindryckx *et al.,* 2009). It is of note that no other groups are using Sr^{2+} to activate human, pig or cow eggs. So it is has remained unclear why Sr^{2+} only works reliably in rodent eggs, but not in eggs of other species including human.

It has been suggested that Sr^{2+} works through the IP_3R to release Ca^{2+} from the internal stores, so the IP_3R is the likely mediator of Sr^{2+} effects on eggs (Zhang *et al.,* 2005). These IP_3R are well known to be present in all mammalian eggs. Sr^{2+} is divalent cation that is very similar to Ca^{2+} (Whittingham and Siracusa, 1978; Kline and Kline 1992; Swann and Ozil, 1994; Rogers *et al.,* 2004). To this end, I have worked on the hypothesis that Sr^{2+} needs to cross the plasma membrane to triggers the IP_3R on internal store 'ER' and to commence Ca^{2+}-oscillations. In doing so, and due to the similarity between Sr^{2+} and Ca^{2+} ions, Sr^{2+} might use the same channels that Ca^{2+} normally use to enter the egg. This idea was supported with Sr^{2+} injection experiments in which I have shown that by bypassing the plasma membrane, Sr^{2+} is able to induce

Ca^{2+} rises in mouse eggs in normal Ca^{2+} free media (Fig 3.3A). In mammals, this is the first successful study to show Ca^{2+}-oscillations triggered by Sr^{2+} injection in mouse eggs. Although, it has been reported previously in two different studies that Sr^{2+} can be injected into mammalian eggs such as in hamster eggs (Georgiou *et al.,* 1987) and in pig eggs (Okada *et al.,* 2003). Interestingly both reports showed a single Ca^{2+} transient that is different from the physiological pattern of Ca^{2+} changes during fertilizations. In my protocol in which Sr^{2+} injected directly into the mouse egg cytoplasm using micropipette with sequential electrical pulses (see material and methods) to ensure a continuous leaking of Sr^{2+} from the injection pipette. The results were interesting and showed a succession of Ca^{2+}-oscillations within the first few minutes in all tested eggs. These Ca^{2+}-oscillations were promptly stopped after Sr^{2+} leakage was ceased and further resumed by resuming the Sr^{2+} injection (Fig. 3.3A). Egg survival was a challenging point here since the injection pipette has to be kept in continuously during the injection protocol running time. The overall results of this experiment favour the influx theory and suggest that Sr^{2+} has no particular interaction with plasma membrane except to cross it. The question remains about the nature and the type of the mediators that Sr^{2+} uses to cross the PM and reach the IP_3R on ER and subsequently provoke Ca^{2+}-oscillations.

Different kinds of mediators of Ca^{2+} influx, including the NCX, have been expressed in mammalian eggs. Any cytosolic Ca^{2+} load can be removed from the cytosol by using two main transport processes, the Ca^{2+}-ATPase (that actively pumps Ca^{2+} against its gradient mainly into internal store) and the NCX. The later has been found in many mammalian tissues including eggs in hamster (Igusa and Miyazaki, 1983; Georgiou *et*

al., 1988) and mouse (Pepperell *et al*., 1999). NCX can lead to changes in membrane potential (electrogenic) due to the cytosolic accumulation of the positive charges (Na^+) (Carroll, 2000). The NCX is driven by the electrochemical gradient, therefore, any manoeuvres that would minimise the extracellular Na^+ would activate the reverse mode and promote Ca^{2+} influx and in my experiments' protocol these should promote Sr^{2+} influx in exchange with Na^+ extrusion.

In my experiments I used either a low or zero Na^+ media to enhance the reverse NCX using as an osmolarity stabilizer either choline or K^+. Based upon my results of Sr^{2+} experiments on mouse eggs, I found out that NCX is the main Sr^{2+} influx mediator. This is because the activation of the reverse mode of NCX in normal osmolarity Sr^{2+} media allowed even 0.5mM Sr^{2+} to trigger Ca^{2+} oscillations in mouse eggs. This is bearing in mind that this Sr^{2+} concentration is unable to produce Ca^{2+} rises in normal situation. Interestingly, these Ca^{2+}-oscillations are completely and reversibly abolished by using 20µM of specific NCX reverse mode inhibitor (KB-R7943) see Fig. 3.21. This suggests the importance of the NCX in Sr^{2+} influx and could explain how Sr^{2+} cross the membrane to triggers Ca^{2+} release.

The effect of Na^+ on the direction of NCX was also further examined using the Na^+ ionophore (monensin). The addition of monensin to eggs placed in low Na^+ (~30mM) and low Sr^{2+} (0.5mM) media, demonstrated that Sr^{2+} able to triggered Ca^{2+}-oscillations in non-oscillating eggs (>50%) and increased the frequency of oscillating ones (>45%), see Fig. 3.23. These findings are consistent with the results from previous study on mouse eggs (Carroll, 2000). This again suggests that Sr^{2+} most likely substitutes for

Ca^{2+} in the reverse mode of NCX. Whilst clear cut these results cannot exclude the idea that other channels might also be involved in Sr^{2+} influx, such as SAC and TRPC channels. However, it give a firm hypotheses of how Sr^{2+} influx through the plasma membrane.

A previous study reported the potential mechanism of NCX switching between the two modes by manipulating the Na^+ gradient. The reverse mode is more likely to take place if the reversal potential of the NCX is similar to the resting membrane potential (Carroll, 2000). In order to measure the reversal potential of the NCX accurately, a calculation of the intracellular Na^+ is required. Estimated values for the reversal potential can be obtained using the equation $\boldsymbol{E_R = (nE\text{Na} - 2E\text{Ca})/(n - 2)}$ (Carroll, 2000) full details in page 53. Carroll (2000) estimated that the E_R for eggs is around –40 mV. This value is close to reports that showed the resting membrane potential of the mouse egg is around (- 30 to - 40mV) (Peres, 1986 and Carroll, 2000). At this level the reverse NCX is activated which should lead to Ca^{2+} influx into the egg. In my experiment Sr^{2+} is used instead of Ca^{2+}. However, it should be recognized that this type of calculation has to make an assumption about the intracellular free Na^+ concentration, and the behaviour of the NCX is very sensitive to the value of this parameter. Any minimal increase in the Na^+ concentration gradient across the plasma membrane, which largely driven by the intracellular values, will be enough to promote the reversal of the NCX. I used mainly two different concentrations of Sr^{2+} (10mM and 0.5mM) and have used the previous equation to calculate the effects of different values of intracellular Na^+ from 0 to 25mM. The results are summarised in the graph below that showed two curves that represent

the relation between the reversal potential of the NCX with different concentrations of intracellular Na^+. This is compared to the membrane potential of the egg, which is taken as – 40 mV (Fig. 6.1). I have assumed that Sr^{2+} and Ca^{2+} are equivalent to each other as divalent ions. The interpretation of this figure is that when the reversal potential value is above the membrane potential (taken as -40mV) the NCX works in forward direction to pump Ca^{2+}, or Sr^{2+} in case of Sr^{2+} media, out of the egg. However, when the NCX reversal potential value is equal to or less than the membrane potential the NCX will switch into the reverse mode and brings Sr^{2+} into the egg.

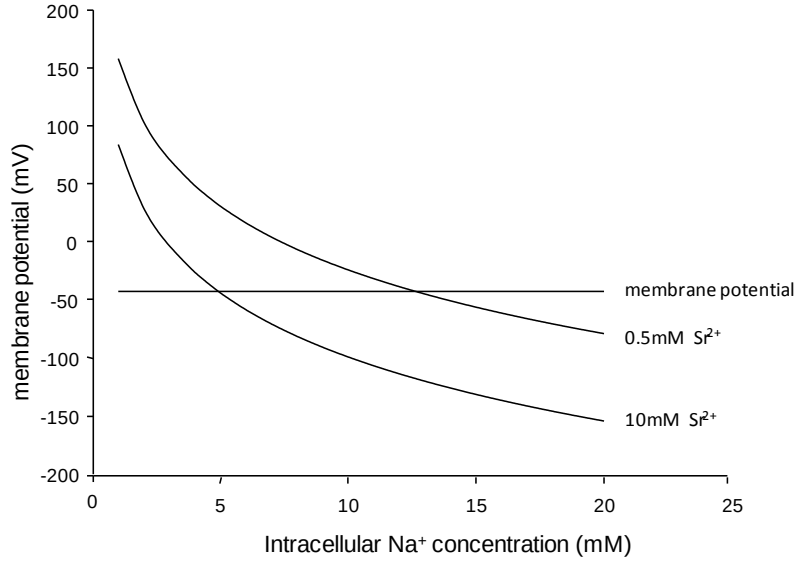


Figure 6.1 The relation between the Sr^{2+} influx and intracellular Na^+ concentration in correlation with the membrane reversal potential. Each curve represents the relation between the reversal potential of the NCX and the intracellular Na^+ concentration. This relationship is plotted for either 0.5mM or 10mM extracellular Sr^{2+} ions.

It is notable from the figure above that in the case of 10mM Sr^{2+} the NCX works normally in forward direction at any intracellular Na^+ level below 5mM, and will switch to the reverse mode when the reversal potential equal to or less that -40mV which is when the intracellular Na^+ increases over 5mM. So the Na^+ concentration only has to be over

5mM for Sr^{2+} to enter the egg. However, in the case of 0.5mM Sr^{2+} the forward mode NCX continues until intracellular Na^{+} level increases and reaches over 12mM. Only at that point will Sr^{2+} enter the eggs, and this is why 0.5mM Sr^{2+} does not cause Ca^{2+} oscillations unless the Na^{+} is manipulated. Notably, the Na^{+} concentration in cells is thought to be in the range from 4 to 12mM (Sonn and Lee, 1988; Johnson *et al*., 1991), hence the NCX is carefully balanced between its two modes of action. It has been reported previously that the membrane potential in human eggs is ~ -30 mV (Feichtinger *et al.,* 1988). Using this value in the (Fig. 6.1) might help understand one of the reasons for the inability of Sr^{2+} to trigger Ca^{2+} releases in human eggs. It could possibly be that in human eggs the intracellular Na^{+} concentration is maintained in very low (< 4mM) levels compared with that in rodent eggs. This would make the NCX always works in the forward direction in human eggs.

6.3 Can Sr^{2+} induce Ca^{2+}-oscillations in human eggs?

Taking the protocols that I used on mouse eggs, I applied these to human (failed ICSI) eggs donated from couples who treated in IVF Wales. I have found that there is still little ability to trigger either Ca^{2+} oscillations or egg activation. I have also tried the Sr^{2+} injection protocol on human eggs, unfortunately none of the injected eggs responded to Sr^{2+} leakage. In addition, treating human eggs with Sr^{2+} media in different circumstances by changing media osmolarity, Sr^{2+} concentration and time exposure, shows that human eggs may only respond to Sr^{2+} in very extreme situations. This involves overnight 20mM Sr^{2+} exposure in media that was prepared to maximize the

reverse mode of NCX. Interestingly, when failed ICSI human eggs were kept overnight in 20mM Sr^{2+} and 0Na^{+} media, despite a long delay of >10 hrs, there was clear Ca^{2+} oscillations in the majority of those eggs (7/10). Unfortunately, none of these eggs developed further, or even formed pronuclei, and eventually all died within next few hours. These results are interesting in showing that even human eggs can respond to Sr^{2+} and generate Ca^{2+} oscillations activations given that the appropriate conditions are provided.

Therefore, the unsolved question is why Sr^{2+} works reliably in rodent eggs, such as those of mouse, but not in eggs of humans and domestic mammals. One part of the explanation could be related to the intracellular Na^{+} level which could behave in a similar way to that found in cardiac cells. It has been suggested previously that in cardiac cells the intracellular Na^{+} ($[Na^{+}]_{in}$) is maintained at lower levels in larger mammals than that in the smaller mammals (Pieske and Houser, 2003). If this is the case in mammalian eggs, Na^{+} could be maintained at lower levels in the large eggs from large mammals such as human and pigs, compared to the Na^{+} values in smaller mammalian eggs such as mouse and rat. In this case the forward direction of NCX would dominate in the large mammalian eggs, which makes Sr^{2+} influx unlikely to happen in situations of the Sr^{2+} triggers egg activation protocols. Still, why Sr^{2+} does not work in human eggs? Gathering this with the previous assumption from Pieske and Houser (2003) regarding the intracellular Na^{+} in human eggs, the only possibility is that the intracellular Na^{+} in human eggs is maintained in its lowest values (i.e. below 4mM). This suggests that the forward NCX would dominate the situation. This consequently could partially explain

why Sr^{2+} unable to trigger Ca^{2+} release in human eggs at normal Sr^{2+} activation conditions.

Nevertheless, my experiments using $0Na^{+}$, high K^{+} media with 20mM Sr^{2+} have also failed to provoke Ca^{2+} release in human eggs. Significantly, by applying the same components' concentration in the Sr^{2+} activation media but using an overnight exposure I finally succeeded in stimulating Ca^{2+}-oscillations in failed ICSI human eggs. However, despite these promising results, which involve the first reported Ca^{2+}-oscillations in human eggs triggered by Sr^{2+}, it is far from the physiological conditions, and is apparently toxic to eggs. Therefore we cannot yet recommend a media to be used in IVF clinics. It is also difficult to draw a final conclusion not least because of the limited availability of the human eggs which meant that all tested eggs were from failed ICSI group. Because of this, a full comprehensive study is recommended to examine the overnight protocol on the fresh and failed IVF eggs in the extreme media, as well as in normal Ca^{2+}-free Sr^{2+}-media, that is routinely used for mouse eggs. Only then we can reach a final conclusion in this matter. It may be of interest to try some of the low Na^{+} protocols I used in eggs of domestic animals, such as pigs and cows, since they have also proved difficult to activate with normal containing Sr^{2+} media. They are also available for experiments in larger numbers than human eggs.

6.4 Sperm specific PLCζ

It has been suggested that during fertilization sperm convey a message to the fertilized egg in order to provoke the egg activation events. Later it was suggested that this message has to be introduced into the egg, since the injection of sperm extracts can trigger Ca^{2+} oscillations and egg activation events in mouse, hamster and human eggs (Swann, 1990; Swann, 1994; Homa and Swann 1994). Substantial evidence suggests that the Ca^{2+} oscillations at fertilization are triggered after the sperm fuses with the egg, and this leads to the introduction of a sperm-specific phospholipase C (PLCζ) into the oocyte (Saunders *et al.,* 2002; Swann *et al.,* 2006; Yu *et la.,* 2008). PLCζ then generates repeated cycles of IP_3 production within the ooplasm that cause the repetitive Ca^{2+} release phenomenon of Ca^{2+}-oscillations (Swann *et al.,* 2006; Yu *et al.,* 2008). However, a recent report criticised this and showed through IVF with indirect immunofluorescence analysis study that PLCζ is no longer detectable after sperm-egg membrane fusion (Aarabi *et al.,* 2012). They also showed evidence suggesting that PLCζ is produced during, and involved in, spermatogeneses in human and mouse and it is reduced in levels after acrosomal formation (Aarabi *et al.,* 2012). The specificity of the PLCζ antibody used in this study is, however, dubious.

An interesting study by (Borges *et al.,* 2009) suggests that it is most probably that PLCζ is expressed only in epididymal sperm and not in testicular correspondents, since they found that the artificial egg activation improves only ICSI cases with epididymal sperm injection and not those injected with testicular retrieved sperm (Borges *et al.,* 2009). This study is consistent with previous work that found out by reverse transcriptase PCR

(rtPCR) experiments that PLCζ appears only in spermatids but not in the earlier steps of spermatogeneses (Saunders *et al.,* 2002).

Sperm PLCζ is most conveniently introduced into oocytes by microinjection of its complementary RNA (cRNA) which is translated into PLCζ protein within the oocytes over a period of several hours until pronucleus formation (Jones *et al.* 1995). Hence, the injection of PLCζ cRNA into oocytes produces the PLCζ protein that causes prolonged Ca^{2+} oscillations, and in parallel experiments PLCζ has been shown to activate embryonic development of mouse, pig, cow and human oocytes up to the morula and blastocyst stages (Rogers *et al*., 2004; Yu *et al.,* 2008).

In this thesis I reported several studies on different aspects of the structural point of view and their effects on the functional activities of PLCζ. In addition, I reported differences in PLCζ regulation compared to other PLCs. I started with mutations studies and compared their effects on the Ca^{2+} releasing potency with that of the wild type PLCζ. It has been suggested that even though the sperm contains PLCζ, it is inactive until it is introduced into the egg cytoplasm (Swann *et al.,* 2004). Consequently, this could mean that there is some unrevealed intrinsic mechanism within the egg that switches on the activity of PLCζ.

In principle, any disruption of the PLCζ code of amino acids sequence could alter the protein structure and subsequent functional activity. The first direct link between male infertility and a defective PLCζ gene was made after identification of a PLCζ point mutation in an infertile oligospermic patient after ICSI treatment that had repeated failed fertilization due to egg activation failure. The problem with PLCζ was reported to involve

a single point mutation (i.e PLCζ^{H398P}) found after a genomic search of PLCζ sequence in this patient. This proline residue, which in protein structure prediction is known to have helix-destabilizing ability, specifically causes a perturbation of the catalytic Y domain that annuls PLCζ enzymatic activity. We studied this mutation using an equivalent site in mouse PLCζ (i.e. PLCζ^{H435P}). This could then be the monitoring for its ability to cause Ca^{2+}-oscillations after PLCζ expression in individual mouse eggs. Our study 'Nomikos *et al*., 2011a' showed a complete abolishing effect of this mutation on the mouse PLCζ activity, despite high expression values of this mutated PLCζ. This provides a clear evidence of the effect of this mutation This could be due to the introduction of major protein structural changes, consistent with that proposed previously (Heytens *et al.,* 2009). For a comparative study, we employed an additional PLCζ mutant (i.e. PLCζ^{H435A}) in which a histidine residue was replaced with a neutral non-helix-destabilizing amino acid, alanine. This mutation does not abolish PLCζ activity but reduces its catalytic activity by 20-30% (Nomikos *et al.,* 2011a). This H398P infertility linked point mutation was also examined for *in vitro* enzymatic properties of a closely related PLC isoform PLCδ1 (PLCδ1^{H542P}). Very similar results were observed in which only 16% of the PIP_2 hydrolysis activity of the PLCδ1^{WT} was retained at high Ca^{2+} levels, in which PLCδ1^{WT} is known to be fully enzymatically active (Nomikos *et al.,* 2005).

To determine whether an equivalent or excess level of PLCζ^{H435P} protein expressed in mouse eggs can block the Ca^{2+} oscillation-inducing activity of PLCζ^{WT,} as suggested by a previous study (Heytens *et al.,* 2009), we examined the dominant-negative effect of

PLCζ^{H435P} on PLCζ^{WT}. Our results on cRNA PLCζ^{WT} and in mouse IVF (on sperm PLCζ) suggest that mouse PLCζ^{H435P} does not appear to operate on a dominant negative manner. Our results that argued against the previous assumption of a dominant negative effect were later explained by the discovery of another point mutation within the PLCζ sequence from the same patient that was firstly found to have the PLCζH435P mutation. The newer mutation was found with a substitution of histidine with lucine a.a. in different 'mother-inherited' allele at position 233 in the PLCζ sequence (PLCζ^{H233L}). This mutation was found to dramatically reduce PLCζ activity (Kashir *et al.,* 2012).

Despite the basic knowledge of PIP_2 hydrolysis by PLCζ at fertilization, the exact mechanism by which PLCζ locates and binds the substrate in eggs remains unclear. It has been suggested that electrostatic interactions the positively charged PLCζ residues might interact with the negatively charged PIP_2 (Nomikos *et al.,* 2007). Experiments using a synthetic peptide corresponding to a portion of the XYI in a fluorescence resonance energy transfer (FRET) assay have suggested that basic amino acids could help anchor PLCζ to the membrane and facilitate local PIP_2 concentration adjacent to the catalytic domain.

There are also promising results from protein-lipid overlay and liposome-binding assay experiments on the ability of the mouse PLCζ XYI and C2 domain to interact with membrane PIP_2. These data suggest that only the XYI and not the C2 domain interact preferably with PIP_2 (Nomikos *et al.,* 2011b). We aimed to rule out any additional role of other PLCζ domains on this interaction by preparing sequential mutations using site-directed mutagenesis on positively charged region of the mouse PLCζ XYI. This

resulted in the generation of three cumulative mutations; single (K374A), double (K374,375AA) and triple (K374,375,377AAA) mutant constructs of PLCζ. The selection of lysine to alanine change was chosen as it is likely to bring about relatively small changes compared with charge-reversed amino acid substitution effects on PLCζ structure. In this study it was clearly reported that both PLCζ activity and expression levels declined by sequential increase numbers of the mutated lysine to alanine. However, the enzymatic properties of PLCζ were not clearly affected with these mutations (Nomikos *et al.,* 2011b). This result suggests that the sequential cumulative neutralization of these basic residues within the XYl region substantially reduces the in vitro affinity of PLCζ for PIP_2 without affecting the Ca^{2+} sensitivity of this enzyme.

All somatic PLCs such as PLCβ, δ and ε have highly negatively charged X-Y linker. Interestingly PLCζ is the only mammalian PLC that possesses a highly positively charged XYl region. It has been proposed that these XYls might mediate auto-inhibition of PLC activity (Hicks *et al.,* 2008). This was suggested after results that showed that the deletion of the XYl from PLCβ2 consistently increases PLCβ2 activity *in vitro* and *in vivo* (cells). Similar results were detected by the deletion of the XYl from PLCβ1, δ1, and ε. This suggests a common auto-inhibition mechanism for PLCs regulation (Hicks *et al.,* 2008). The suggested mechanism of this action is by prevention of PIP_2 gaining access to the active site by a combination of steric exclusion and electrostatic repulsion of negatively charged membranes with the negatively charged XYls of these PLCs (Hicks *et al.,* 2008). Beside the tyrosine phosphorylation activation process of XYl region, PLCγ is regulated by additional regulatory domains: a PH domain, two SH2

domains and an SH3 domain (Rodriguez *et al.,* 2001; Ozdener *et al.,* 2002; Sekiya *et al.*, 2004). Recently it was suggested that the auto-inhibition mediated by the XYl region also applies to the PLCγ isozyme. However, the crucial determinant for the auto-inhibition is the C-terminal SH2 domain (Gresset *et al.,* 2010). In contrast, the XYls of PLCη isozymes are not negatively charged overall but contain clusters of acidic residues near their ends. It is unclear whether there is a role of this region in the regulation of PLCη activity (Fukami *et al.,* 2010). By comparing the structural amino acid sequence of different domains amongst all PLCs, the XYl region sequence is clearly the most divergent. Herein, and as a consequent of this study we have tested the effects of XYl region deletion on PLCζ *in vitro* and *in vivo* (in eggs) in comparison with PLCδ1 as a control (Nomikos *et al.,* 2011c). As mentioned previously PLCδ1 was chosen based on its closer similarity with PLCζ and because it has been shown to trigger infrequent Ca^{2+}-oscillations in mouse eggs with high doses. Significantly, the important and unique functional feature of PLCζ is the relatively high Ca^{2+} sensitivity that results in ~100-fold higher Ca^{2+} sensitivity than PLCδ1. In other words, low Ca^{2+} concentrations (nanomolar) are needed for enzymatic activity of PLCζ compared to micromolar Ca^{2+} concentrations for optimal PIP_2 hydrolysis with the PLCδ1 isoform. Therefore, within eggs, with a basal cytosolic Ca^{2+} concentration of 50-100 nM, PLCζ but not PLCδ1 would be strongly activated.

Although previous studies suggest that EF hand domains are required for a functional PLCζ in the egg (Nomikos *et al.,* 2005). The molecular determinants that confer the high Ca^{2+} sensitivity of PLCζ were unknown. A recent study in our lab by my colleague Dr Yu

on the localization of PLCζ and whether and how PLCζ can bind to the plasma membrane, has showed that PLCζ targets PIP_2 on intracellular vesicles and not the plasma membrane PIP_2 (Yu *et al.,* 2012). This idea was based on several results. Firstly, yellow fluorescent protein (YFP)-tagged constructs of $PLC\zeta^{WT}$, the $PLC\zeta^{D210R}$, the XYI, the XY domain, and the C2 domain were all expressed in mouse eggs. Regardless of the stage that eggs were held in, a uniform fluorescence in the cytoplasm was noted in all. The only exception was the localization of PLCζ in the nucleus of the second polar body. This finding of unspecific localization of none of these PLCζ constructs was totally the opposite to that seen with distinctive plasma membrane localization of PLCδ1 using GFP-tagged PH domain (Yu *et al.,* 2012). Secondly, the changes in plasma membrane PIP_2 using GFP-tagged PH were used to record the relative changes of PIP_2 in the fertilizing mouse eggs (Halet *et al.*, 2002). This showed even though PLCζ is far more potent 'at causing Ca^{2+} oscillations' than PLCδ1, the decreases in plasma membrane PIP_2 was only recorded after PLCδ1 injection and not after PLCζ injection. Finally, it was found that by using PLCζ antibodies and immunocytochemistry PLCζ could be localized to small vesicles in the egg cytoplasm. It remains to be shown which PLCζ domains might be involved in binding to such vesicles (Yu *et al.,* 2012).

It was worth examining the general auto inhibitory mechanism of somatic PLCs regulation on sperm specific PLCζ. This is because PLCζ is very distinct from somatic PLCs in possessing a positively charged XYI region. In order to enable us to understand the regulatory role of the short linker region separating the catalytic X and Y domain,

the XYl of both PLCζ (amino acids 308–385) and PLCδ1 (amino acids 441-490) were excised from both $PLC\zeta^{WT}$ and $PLC\delta1^{WT}$ respectively to create $PLC\zeta^{\Delta XYl}$ and $PLC\delta1^{\Delta XYl}$ (Nomikos *et al.,* 2011c). Interestingly, the deletion of the XYl from PLCζ negatively affected the *in vivo enz*ymatic activity, which translated into an observed 7-fold reduction in Ca^{2+}-oscillation-inducing activity in mouse eggs. In contrast, deletion of the XYl from PLCδ1 resulted in a 2-fold increase in Ca^{2+}-oscillation-inducing activity in eggs. These results clearly support the general autoinhibitory mechanism suggested for somatic PLCs like PLCδ1. This result also correlates with the *in vitro* PIP_2 hydrolysis assays showing an ~2.3-fold increased enzymatic activity relative to wild-type PLCδ1 (Nomikos *et al.,* 2011c). Finally, the replacement of only the PLCζ XYl cluster of basic residues (12 a.a) by its homologous XYl region of PLCδ1 also resulted in 5-fold reduction in Ca^{2+}-oscillation-inducing activity in eggs with this chimeric PLCζ. The potential interpretation of our findings in these experiments is that the XYl of PLCζ has the opposite regulatory role to that of the XYl in PLCδ1. This could be due to the high density of basic amino acids (positively charged) in the XYl of PLCζ that is absent from PLCδ1 and other somatic PLC isoforms. These results are consisted with our previous findings on the sequential mutations (K→A) where we introduced the specific high density cluster of positively charged a.a. into the XYl of PLCζ. Both studies suggest the important role of XYl in PLCζ regulation and activity. In either case the excision of the complete XYl or exchanging positively charged cluster with neutral residues causes a significant reduction in PLCζ functional properties regarding PIP_2 targeting and hydrolysing activity.

6.5 Non-invasive potential method for embryo selection

The basic role of Ca^{2+}-oscillations during egg activation in mammals suggests the importance of developing a potential non-invasive monitoring technique of these cytoplasmic Ca^{2+} changes. Any success in this direction might be implemented in future for embryo selection techniques in IVF clinics. In this regard, our studies investigated monitoring the cytoplasmic movements in human eggs during activation that was previously first noticed in mouse eggs (Deguchi *et al.,* 2000). They were also reported in fertilizing mouse eggs using PIV analysis (Ajduk *et al.,* 2011). The implementation of this technique on activating human eggs injected by cRNA encoding hPLCζ, showed significant cytoplasmic-movements that correlated with each Ca^{2+} spike. The degree of synchrony between Ca^{2+} elevation and movement was the common finding in all of these human eggs. This suggests that the cytoplasmic-movements in human eggs are directly induced by elevated free Ca^{2+} ions. On detailed analysis of this relation, we found that distinct cytoplasmic movements occurred just after each Ca^{2+} transient, or within the same 10-second interval. In addition, we noticed either coincidence between the maximum mean PIV speed with the maximum of the Ca^{2+} spike or it occurred within the 50 seconds after the maximum of the Ca^{2+} transient. In a total from 10 different zygotes, 95/102 cytoplasmic-movements were detected within this range. The mean lag of the PIV speed peak was 18 seconds after the Ca^{2+} peak (with a range of 10-50 sec).

In mouse eggs it was suggested that such cytoplasmic-movements are dependent on the actin cytoskeleton and are greatly influenced by the presence of the sperm and fertilization cone ‘zygote shape changes’ (Ajduk *et al.,* 2011). Since we recruited only

failed ICSI human eggs for this study, it was difficult to identify the fertilization cone or region that exhibit shape changes, although there were slow progressive changes of cell profile detected during the recordings. However, in some records it was possible to notice a thick granular crescent formed beneath the cell membrane, which moved towards the position of the first polar body. The presence of sperm in these eggs could play a specific role in cytoplasmic-movements, as suggested in mouse zygotes. Moreover, the failure to detect any cytoplasmic-movement on 7 follicular reduction human eggs that were injected with cRNA encoded PLCζ to induce Ca^{2+}-oscillations, further suggest the important role of sperm presence for optimum fertilization in mammals.

6.6 Recombinant hPLCζ protein is a potential solution in some ICSI failure cases of paternal cause

One of the main goals of this particular research is to find an acceptable, optimum and ethically approvable way to activate human eggs that can be used safely in repeated male factor related IVF/ICSI failure. Recombinant PLCζ might represent a potential alternative physiological therapeutic agent that can overcome certain cases of failed fertilization after ICSI.

Our success in producing human recombinant PLCζ protein came from the expression of this protein as a NusA-hexa-histidine fusion protein in E. coli that was purified by affinity chromatography on Ni-NTA resin. The eluted proteins were dialyzed overnight and concentrated with centrifugal concentrators (Nomikos *et al.,* 2013). The selection of a fusion protein with NusA was chosen after instability issues with previous constructs in

our laboratory. One of these earlier studies used a hexa-histidine tag, and even though we succeeded in obtaining expression of some recombinant PLCζ protein, the product was functionally inactive. Recently, a promising study was reported that showed active recombinant hPLCζ protein with a hexa-histidine tag (Yoon *et al.,* 2012). Looking into this report, it is noticeable that it was consistent with our lab previous studies using expression of recombinant hPLCζ without a fusion protein partner. This is because their hPLCζ protein was of very low activity and in order to show some activity for this protein they have to inject as much as 5,000 to 10,000 fg into a an egg to cause Ca^{2+}-oscillations. Moreover, it did not result in further embryo development to the blastocyst stage. On contrary, in our study a significant expression of soluble NusA-PLCζ was observed, and displayed the predicted ~130 kDa molecular mass (70 kDa hPLCζ & NusA ~60 kDa) (NomiKos *et al.,* 2013). Prompt fertilization like pattern of Ca^{2+}-oscillations were observed after the microinjection of recombinant wild-type human PLCζ into mouse and partially delayed ones in human eggs. In addition, the estimated amount of human PLCζ protein in mouse eggs that was required to efficiently induce Ca^{2+}-oscillations and embryogenesis was ~80 fg/egg compare to 5,000 to 10,000 fg/egg in the previous report (Yoon *et al.,* 2012). This means our activity is about 100 times stronger than the recently published report. To rule out the possible effect of NusA tag, this protein was microinjected alone and failed to cause any Ca^{2+} changes. This revealed that the expressed recombinant PLCζ protein possesses a potent ability to hydrolyse PIP_2. Not only that, but it triggered, with high efficiency, early embryo

development, from pronuclei formation up to the multicellular blastocyst stage (Nomikos *et al.,* 2013).

The activity of our recombinant PLCζ protein was further tested by my colleagues in order to investigate whether injection of recombinant human PLCζ protein would be able to rescue the failed egg activation caused by infertility-linked PLCζ mutants. They produced two mutants of PLCζ encoding the two novel point mutations that previously reported so far in the PLCζ gene and linked specifically to male infertility PLCζ^{H398P} and PLCζ^{H233L} mutations (Heytens *et al.,* 2009; Kashir *et al.,* 2012) respectively. Both of these mutations have been found to dramatically abolish PLCζ activity and were unable to produce Ca^{2+}-oscillations in mouse eggs (Nomikos *et al.,* 2011a; Nomikos *et al.,* 2013). When they microinjected the wild-type human PLCζ protein into mouse eggs that were expressing these infertility-linked PLCζ mutants (H398P and H233L), the results demonstrate an effectively rescue the failure of egg activation by inducing a normal pattern of Ca^{2+}-oscillations. This finally led to successful early embryo development up to the blastocyst stage. These findings promote the potential application of PLCζ protein into IVF clinics as an effective therapeutic option for egg activation failure due to male factor deficiencies related to PLCζ dysfunction.

6.7 Clinical implications

Infertility is a major issue that faces many couples who want to start a family. Tremendous improves designed to tackle this issue have been made in the past few decades. Firstly the invention of conventional IVF solved a significant percentage of

both maternal and paternal sub-fertility dilemmas. This success was however followed by more advanced technique i.e. 'ICSI' which is predominantly used to treat male factor infertility problems following failure of conventional IVF. It is a highly successful technique that, on average, results in normal fertilization in 70% of cases (Nasr-Esfahani *et al.,* 2010). Although, these techniques and their derivatives have benefited a large number of infertile couples, there are still between 2% to 3% of infertile couples who have not benefited from these contemporary solutions in their infertility treatment (Nasr-Esfahani *et al.,* 2010; Vanden *et al.,* 2012). Another review study found out between 1 - 5% with complete or virtually complete fertilization failure still occurs after ICSI cycles (Kashir *et al.,* 2010). ART now accounts for 7% of all births in some developed countries (Nasr-Esfahani *et al.,* 2010). Scientists and clinicians have looked at this issue and found out that some cases of failed fertilization can be because of egg activation failure. This can be the case for approximately 70-80% of cases after ICSI (Tesarik *et al.,* 1994; Palermo *et al.,* 2009; Nasr-Esfahani *et al.,* 2010; Kashir *et al.,* 2010). For this reasons researchers have focused on solving this issue by finding an artificial way, by any possible means, to activate human eggs that have failed to fertilize after ICSI. Ca^{2+} ionophore has been reported to improve the fertilization and embryo quality after ICSI in human eggs, mainly if epidydemal sperm was injected compare to testicular sperm (Borges *et al.,* 2009). Amongst the same number of human eggs (n = 49) injected with epididymal sperm only, or injected with epididymal sperm and then treated with Ca^{2+} ionophore, the fertilization, high-quality embryo, implantation and pregnancy rates rose by 3.2%, 16.8%, 5.2% and 6.8% respectively. The interesting

point here is the increase in the high-quality embryos by nearly 17%, which suggests the beneficial effect of Ca^{2+} ionophores on development after activation. However, this was not the case with testicular sperm origin since no significant difference reported. More recent report showed that although the artificial egg activation is highly efficient (74.2% compare with 43.7%) in patients with a suspected egg related activation deficiency with previous 'post ICSI' total fertilization failure, it is not always beneficial (75.0% compared with 60.4%) for patients with previous low conventional fertilization rates and a suspected egg-related activation deficiency (Vanden *et al.,* 2012). A recent interesting study looked at whether PLCζ levels from fertile men sperm (control group) are similar, and whether a predominant localization pattern of PLCζ within sperm is related to better egg activation rates. The results showed marked variance in both, the PLCζ levels within the control group and in the predominant pattern of PLCζ localization in relation to normal egg activation. The diagnostic application of quantitative PLCζ immunofluorescent analysis might be prevented with such variability (Kashir *et al.,* 2013).

My study on Sr^{2+} activation protocols on human eggs may be one of the first serious attempts to activate human egg with Sr^{2+}. The safety of Sr^{2+} usage might be not present major issues since it has already been used in human ICSI in several clinical reports (Kashir *et al.,* 2010). For example, a previous report with low fertilization rate (17%) couples with no pregnancy in eight cycles was successfully treated in with Sr^{2+} post ICSI. This resulted in a 100% fertilization rate, with pregnancies from fresh and frozen embryos (Yanagida *et al.,* 2006). Similarly, the fertilization and pregnancy rates were

reported markedly improved following Sr^{2+} use (22%-65% and 0%-40%) respectively (Kyono *et al*. 2008). However, these studies lack the evidence to reveal whether the previous low fertilization rates were sperm related activation deficiencies or not. There were no controls done alongside the S^{2+} activation treatments. In addition, there is no clear evidence confirming Sr^{2+} action in these reports by showing Ca^{2+}-oscillations. The issue here is the mechanism by which of Sr^{2+} can work on human eggs, and the scientific proof of an effective action. As I reported in the human egg's results chapter, Sr^{2+} may only provoke Ca^{2+} release in human eggs in special conditions. These conditions were not provided in the previous reported clinical studies. It has yet to be elucidated whether Sr^{2+} is an efficient candidate for artificial human egg activation since my results are not yet conclusive and my protocol cannot be recommended for application in those cases with repeated failed ICSI. However, my results might be the preliminary steps towards future protocols.

PLCζ identification is a novel discovery that has implications in infertility and is a possible treatment option in future. Principally, the potential application, by co-microinjection, of PLCζ cRNA during ICSI could be used to rescue egg activation failure due to PLCζ-deficient/mutated sperm. Yoon *et al.,* (2008) demonstrate this by rescuing the egg activating capability of globozoospermic sperm by the co-injection of PLCζ cRNA into mouse eggs. However, some basic ethical and practical difficulties would face this application. These include the safety, the nature of cRNA PLCζ since the RNA would be regarded as a genetic material and hence its injection could be seen as a genetic modification that could incorporate into the embryo genome (Kashir *et al.,*

2010), and inability to control the bolus size of microinjected cRNA. The latter would affect significantly the rate of PLCζ protein synthesis and the total amount expressed within the egg. However, even if the same amount of cRNA was injected the protein synthesis can vary from one egg to another. This then leads to inappropriate condition for further embryo development. In this regard, previous report suggested that for a successful embryo development, PLCζ has to be available within a narrow window of relatively precise concentration range in the egg. That is closely similar to the physiologically relevant amount of PLCζ that would be provided by a single mature sperm at fertilization (Yu *et al.,* 2008). The availability of purified, active recombinant human PLCζ protein appears to represent both a highly practical and the most physiologic therapeutic agent for overcoming failed ICSI cases resulting from aberrant sperm PLCζ. In other words, artificial activation by recombinant protein may be useful when there is no or low fertilization potential in male patient such as globozoospermia, severe teratozoospermia, and nonobstructive azoospermia. Recombinant human PLCζ protein could potentially also be used in regenerative medicine approaches to aid the generation of parthenogenetic embryos and blastocysts that may facilitate stem cell derivation.

REFERENCES

Abou-Haila A and Tulsiani DR (2000) Mammalian sperm acrosome: formation, contents, and function. *Arch Biochem Biophys.* 2; 173-82

Allue I, Gandelman O, Dementieva E, Ugarova N and Cobbold P (1996) Evidence for rapid consumption of millimolar concentrations of cytoplasmic ATP during rigor-contracture of metabolically compromised single cardiomyocytes. *Biochem J.* 2; 463-9

Ahmady A and Michael E (2007) Case Report: Successful Pregnancy and Delivery Following Intracytoplasmic Injection of Frozen-Thawed Nonviable Testicular Sperm and Oocyte Activation with Calcium Ionophore. *Journal of Andrology* 28; 1

Aitken RJ (1989). The role of free oxygen radicals and sperm function. *Int J Androl* 12; 95-97

Alessandri-Haber N, Dina O A., Chen X, and Levine J D. (2009) TRPC1 and TRPC6 channels cooperate with TRPV4 to mediate mechanical hyperalgesia and nociceptor sensitization. *J Neurosci.* 19; 6217-6228

Ajduk A, Ilozue T, Windsor S, Yu Y, Seres KB, Bomphrey RJ, Tom BD, Swann K, Thomas A, Graham C, and Magdalena Zernicka-Goetz (2011) Rhythmic actomyosin-driven contractions induced by sperm entry predict mammalian embryo viability. *Nat Commun*. 2; 417

Aarabi M, Yu Y, Xu W, Tse MY, Pang SC, Yi YJ, Sutovsky P and Oko R (2012) The testicular and epididymal expression profile of PLCζ in mouse and human does not support its role as a sperm-borne oocyte activating factor. *PLoS One.* 3; e33496

Austin CR and Short RV (1982) Reproduction in Mammals, 2nd edn. Cambridge: Cambridge University Press.

Biagi BA and Enyeart JJ (1990) Gadolinium blocks low- and high-threshold calcium currents in pituitary cells, *Am. J. Physiol.* 259; 515-520

Barrientos G, Bose DD, Feng W, Padilla I, and Pessah IN (2009) The Na^{+}/Ca^{2+} Exchange Inhibitor 2-(2-(4-(4 Nitrobenzyloxy) phenyl) ethyl) isothiourea Methane sulfonate (KBR7943) Also Blocks Ryanodine Receptors Type 1 (RyR1) and Type 2 (RyR2) Channels. *Mol Pharmacol* 76; 560–568

Bae YS, Cantley LG, Chen CS, Kim SR, Kwon KS and Rhee SG (1998) Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 273; 4465-4469

Bunney TD, Harris R, Gandarillas NL, Josephs MB, Roe SM, Sorli SC, Paterson HF, Rodrigues-Lima F, Esposito D, Ponting CP, Gierschik P, Pearl LH, Driscoll PC and Katan M (2006) Structural and mechanistic insights into ras association domains of phospholipase Cε. *Mol Cell.* 21; 495-507

Beierlein M, Gee KR, Martin VV and Regehr WG (2004) Presynaptic calcium measurements at physiological temperatures using a new class of dextran-conjugated indicators. *J Neurophysiol.* 1; 591-599

Breitbart H (2002) Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Mol Cell Endocrinol.* 187; 139-44

Baldi, E., Luconi, M., Bonaccorsi, L., Krausz, C. and Forti, G. (1996) Human sperm activation during capacitation and acrosome reaction: role of calcium, protein phosphorylation and lipid remodelling pathways. *Front. Biosci.* 1; 189-205

Billig, H., Chun, S.Y., Eisenhauer, K. and Hsueh AJ. (1996) Gonadal cell apoptosis: hormone-regulated cell demise. Hum. Reprod. Update. 2; 103-107

Beech DJ (2007) Canonical transient receptor potential 5. Handb*. Exp. Pharmacol*. 179; 109-123

Berridge MJ (1995) Capacitative calcium entry. *Biochem. J.* 312; 1-11

Blaustein MP and Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. *Physiol. Rev.* 79; 763 - 854

Borges E Jr, de Almeida Ferreira Braga DP, de Sousa Bonetti TC, Iaconelli A Jr and Franco JG Jr. (2009) Artificial oocyte activation with calcium ionophore A23187 in intracytoplasmic sperm injection cycles using surgically retrieved spermatozoa. *Fertil Steril.* 92; 131-136

Bos-Mikich A., Wood M.J., Candy C.J., and Whittingham D.G. (1995) Cytogenetical Analysis and Developmental Potential of Vitrified Mouse Oocytes. Biology of Reproduction 53; 780-785

Bos-Mikich A, Whittingham D and Jones K (1997) Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Developmental Biology.* 182; 172-179

Bers DM. (2001) Excitation-contraction coupling and cardiac contractile force, 2nd ed. Dordrecht: Kluwer

Bers DM, Barry WH and Despa S (2003) Review: Intracellular Na^{+} regulation in cardiac myocytes. *Cardiovascular Research* 57; 897–912

Berridge, M. J. (1993) Inositol trisphosphate and calcium signaling. *Nature* 361; 315-325

Cuthbertson K and Cobbold P (1985) Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. *Nature,* 326; 541-542

Carafoli, E. (1987) Intracellular calcium homeostasis. *Annu. Rev. Biochem.*56; 395-433

Cahalan M D. (2009) STIMulating store-operated Ca^{2+}-entry Ca^{2+}-haemostasis. *Nat Cell Biol.* 6; 669-677

Conti, M. Andersen, C. B. Richard, F. Mehats, C. Chun, S. Y. Horner, K. Jin, C. and Tsafriri, A. (2002) Role of cyclic nucleotide signaling in oocyte maturation. *Mol Cell Endocrin* 187; 153-159

Campbell K and Swann K (2006) Ca^{2+} oscillations stimulate an ATP increase during fertilization of mouse eggs. *Dev Biol.* 298; 225-233

Coward K, Ponting CP, Zhang N, Young C, Huang CJ, Chou CM, Kashir J, Fissore RA and Parrington J. (2011) Identification and functional analysis of an ovarian form of the egg activation factor phospholipase C zeta (PLCζ) in pufferfish. *Mol Reprod Dev.* 78; 48-56

Cobbold PH and Rink TJ. (1987) Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem J.* 248; 313-328

Carroll J. (2000) N^{a+}/Ca^{2+} exchange in mouse oocytes: modifications in the regulation of intracellular free Ca^{2+} during oocyte maturation. *J of Reprod and Fertility* 118; 337-42

Cox L. J., Larman M. G., Saunders C. M., Hashimoto K., Swann K. and Lai F. A. (2002) Sperm phospholipase Czeta from humans and cynomolgus monkeys triggers Ca^{2+} oscillations, activation and development of mouse oocytes. *Reproduction* 124; 611-623

Check JH, Summers-Chase D, Cohen R and Brasile D (2010) Artificial oocyte activation with calcium ionophore allowed fertilization and pregnancy in a couple with long-term unexplained infertility where the female partner had diminished EGG reserve and failure to fertilize oocytes despite intracytoplasmic sperm injection. *Clin Exp Obstet Gynecol.* 37; 263-265

Chen J, Qian Y, Tan Y, and Mima H. (2010) Successful pregnancy following oocyte activation by strontium in normozoospermic patients of unexplained infertility with fertilisation failures during previous intracytoplasmic sperm injection treatment. *Reprod Fertil Dev*. 5; 852-5

De Marco V, Stier G, Blandin S and de Marco A (2004) The solubility and stability of recombinant proteins are increased by their fusion to NusA. *Biochem Biophys Res Commun.* 322; 766-71

Despa S, Vecer J, Steels P, and Ameloot M (2000) Fluorescence Lifetime Microscopy of the Na^{+} Indicator Sodium Green in HeLa Cells. *Analytical Biochemistry* 281; 159–175

Denburg, J. L. and McElroy, W. D. (1970) Anion inhibition of firefly luciferase. *Arch Biochem Biophys* 141; 668-75

Donovan PJ (1998) The germ cell-the mother of all stem cells. *Int J Dev Biol* 42; 1043-1050

De Pol1A., Vaccina F., Forabosco A., Cavazzuti E. and Marzona L. (1997) Apoptosis of germ cells during human prenatal oogenesis. *Hum Reprod* 10; 2235-2241

Dym, M. (1994) Spermatogonial stem cells of the testis. *Proc Natl Acad Sci US*A 91; 11287-89

DeHaven WI, Jones BF, Petranka JG, Smyth JT, Tomita T, Bird GS, and Putney JW Jr. (2009) TRPC channels function independently of STIM1 and Orai1. *J Physiol* 10; 2275-2298

Downs SM and Utecht AM (1999) Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes. *Biol Reprod.* 60

Dalit Ben-Yosef and Ruth Shalgi (1998) Early ionic events in activation of the mammalian egg *Reviews of Reproduction* 3; 96-103

Dirican EK, Isik A, Vicdan K, Sozen E and Suludere Z. (2008) Clinical pregnancies and live births achieved by intracytoplasmic injection of round headed acrosomeless spermatozoa with and without oocyte activation in familial globozoospermia: case report. *Asian J Androl.* 10; 332-6

Ducibella T, Duffy P, Reindollar R and Su B. (1990) Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging in vivo. *Biol Reprod.* 5; 870-876

Ducibella T (1991) Mammalian egg cortical granules and the cortical reaction. *In Elements of Mammalian Fertilization* 1; 205-321 Ed. PM Wassarman. Boca Raton, Boston: CRC Press.

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, et al. (2002) Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Dev Biol.* 250; 280-91

Ducibella T, Schultz RM, Ozil JP. (2006) Role of calcium signals in early development. *Semin Cell Dev Biol.* 17; 324-332

Ducibella T and Fissore R (2008) Review: The role of Ca^{2+}, downstream protein kinases, and oscillatory signalling in regulating fertilization and the activation of developments. *Dev Biol.* 315; 257-279

Dumollard R., Marangos P., Fitzharris G., Swann K., Duchen M. and Carroll J. (2004) Sperm-triggered Ca^{2+} oscillations and Ca^{2+} homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. *Development* 131; 3057-3067

Estacion M, Sinkins WG and Schilling WP. (2001) Regulation of Drosophila transient receptor potential-like (TrpL) channels by phospholipase Cdependent mechanisms. *J Physiol.* 530; 1-19

Essen, L.O., Perisic, O., Cheung, R., Katan, M. and Williams, R.L. (1996) Crystal structure of a mammalian phosphoinositide-specific phospholipase C delta *Nature* 380; 595-602

Ellis, M.V., James, S.R., Perisic, O., Downes, C.P., Williams, R.L. and Katan, M. (1998) Catalytic domain of phosphoinositide-specific phospholipase C (PLC). Mutational analysis of residues within the active site and hydrophobic ridge of PLCdelta1. *J. Biol. Chem* 273;11650-59

Erbach G. T., Lawitts JA, Papaioannou VE and Biggers JD (1994) Differential Growth of the Mouse Pre-implantation Embryo in Chemically Defined Media., *Biol of Reprod* 50; 1027-1033

Evsikov AV., Graber JH., Brockman JM, Hampl A, Holbrook AE., Singh P, Eppig JJ., Solter D, and Knowles BB. (2006) Cracking the egg: molecular dynamics and evolutionary aspects of the transition from the fully grown oocyte to embryo. *Genes & Development* 20; 2713-2727

Falasca M., Logan S. K., Lehto V. P., Baccante G., Lemmon M. A. and Schlessinger J. (1998) Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J* 17; 414-422

Feichtinger W. Osterode W. and Hoyer J. (1988) Membrane potential measurements in human oocytes. *Arch Gynecol Obstet* 3; 123-129

Fukami K, Inanobe S, Kanemaru K, and Nakamura Y (2010) Review: Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. *ScienceDirect. Prog Lipid Res* 4; 429-37

Fissore R A, and Robl J M. (1993) Sperm inositol triphosphate, and thimerosal-induced intracellular Ca^{2+} elevation in rabbit eggs. *Dev Biol* 159; 122-30

Funayama M, Goto K, and Kondo H. (1996) Cloning and expression localization of cDNA for rat homolog of TRP protein, a possible store-operated calcium (Ca^{2+}) channel. *Mol Brain Res* 43; 259-266

Faure J-E, Myles D G. and Primakoff P. (1999) The frequency of calcium oscillations in mouse eggs at fertilization Is modulated by the number of fused sperm. *Developmental Biology* 213; 370-377

Fitzharris G, Larman M, Richards C and Carroll J (2005) An increase in Ca^{2+} is sufficient but not necessary for driving mitosis in early mouse embryos. *J of Cell Science* 118; 4563-4575

Falin LI (1969) The development of genital glands and the origin of germ cells in human embryogenesis. *Acta Anat (Basel)* 72; 195-232

Fulton BP and Whittingham DG (1978) Activation of mammalian eggs by intracellular injection of calcium. *Nature* 273; 149-150.

Goud P.T., Goud A.P., Van Oostveldt P. and Dhont M. (1999) Presence and dynamic redistribution of type I inositol 1,4,5-trisphosphate receptors in human oocytes and embryos during in-vitro maturation, fertilization and early cleavage divisions. *Mol Hum Reprod* 5; 441-451

Goud PT, Goud AP, Leybaert L, Van Oostveldt P, Mikoshiba K, Diamond MP and Dhont M. (2002) Inositol 1,4,5-trisphosphate receptor function in human oocytes: calcium responses and oocyte activation-related phenomena induced by photolytic release of InsP(3) are blocked by a specific antibody to the type I receptor. *Mol Hum Reprod* 8; 912-918

Gandelman, O., Allue, I., Bowers, K. and Cobbold, P. (1994) Cytoplasmic factors that affect the intensity and stability of bioluminescence from firefly luciferase in living mammalian cells. *J Biolumin Chemilumin.* 6; 363-71

Gondos B (1985) Development of the reproductive organs. *Ann Clin Lab Sci* 15; 363-373

Guerini D (1998) The Ca^{2+} pumps and the Na^{+}/Ca^{2+} exchangers. *Biometals* 11; 319 -330

Gomis A, Soriano S, Belmonte C and F´elix Viana (2008) Hypoosmotic- and pressure-induced membrane stretch activate TRPC5 channels. *J Physiol* 23; 5633-5649

Georgiou P., Bountra C., McNiven A. and House CR. (1987) The Effect of Lanthanum, Quetcetin and Dinitrophenol on Calcium-Evoked Electrical Responses in Hamster Eggs. *Quarterly Journal of Experimental Physiology* 72; 227-241

Gresset A., Hicks S. N., Harden T. K. and Sondek J. (2010) Mechanism of phosphorylation-induced activation of phospholipase C-γ isozymes. *J. Biol. Chem* 285; 35836-47

Georgiou P, House CR, McNiven AI and Yoshida S (1988) On the mechanism of a pH-induced rise in membrane potassium conductance in hamster eggs. *Journal of Physiology* 402; 121-38

Hafner M, Petzelt C, Nobiling R, Pawley JB, Kramp D and Schatten G. (1988) Wave of free calcium at fertilization in the sea urchin egg visualized with fura-2. *Cell Motil Cytoskeleton.* 9; 271-7

Harrison PK, Falugi C, Angelini C and Whitaker MJ (2002) Muscarinic signalling affects intracellular calcium concentration during the first cell cycle of sea urchin embryos. *Cell Calcium.* 31; 289-97

Hosseini SM, Hajian M, Moulavi F, Shahverdi AH and Nasr-Esfahani MH. (2008) Optimized combined electrical-chemical parthenogenetic activation for in vitro matured bovine oocytes. *Anim Reprod Sci.* 108; 122-33

Hicks S. N., Jezyk M. R., Gershburg S., Seifert J. P., Harden T. K. and Sondek J. (2008) General and versatile autoinhibition of PLC isozymes. *Mol. Cell.* 31; 383-394

Heytens E, Parrington J, Coward K, Yoon SY, Lambrecht S, Young C, Grasa P, Ruas M, Fissore RA, Hamer R, Deane CM, Soleimani R, Cuvelier CA, Gerris J, Dhont M, Deforce D, Leybaert L and De Sutter P (2009) Reduced amounts and abnormal forms of phospholipase C zeta (PLCζ) in spermatozoa from infertile men. *Human Reprod*. 10; 2417-28

Harvey E N (1926) Oxygen and luminescence, with a description of methods for removing oxygen from cells and fluids. *Biol. Bull ,* 51; 89-97

Harlan J. E., Hajduk P. J., Yoon H. S., and Fesik S. W. (1994) Pleckstrin homology domains bind to phosphatidylinositol- 4,5-bisphosphate. *Nature* 371; 168-170

Hardie RC (2007). TRP channels and lipids: from Drosophila to mammalian physiology. *J Physiol*. 1; 9-24

Hardie RC and Minke B. (1993) Novel Ca^{2+} channels underlying transduction in Drosophila photoreceptors: implications for phosphoinositide-mediated Ca^{2+} mobilization. *Trends Neurosci*. 16; 371-376

Hofmann, T., A. G. Obukhov, M. Schaefer, C. Harteneck, T. Gudermann, and G. Schultz. (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature* 397; 259-63

Hyslop LA, Carroll M, Nixon VL, McDougall A and Jones KT (2001) Simultaneous measurement of intracellular nitric oxide and free calcium levels in chordate eggs demonstrates that nitric oxide has no role at fertilization. *Dev. Biol* 234; 216-230

Hong-Tao Ma, Ze Peng, Takaaki Hiragun, Shoko Iwaki, Alasdair M. Gilfillan, and Michael A. Beaven (2008) Canonical Transient Receptor Potential 5 Channel in Conjunction with Orai1 and STIM1 Allows Sr^{2+} Entry, Optimal Influx of Ca^{2+}, and Degranulation in a Rat Mast Cell Line. *The Journal of Immunology* 180; 2233-2239

Heller CG and Clermont Y (1963) Spermatogenesis in man: an estimate of its duration. *Science* 140; 184 -186

Halet G, Tunwell R, Balla T, Swann K and Carroll J (2002) The dynamics of plasma membrane PtdIns(4,5)P(2) at fertilization of mouse eggs. *J. Cell Sci* 115; 2139 -2149

Hirota J, Michikawa T, Miyawaki A, Takahashi M, Tanzawa K, Okura I, Furuichi T and Mikoshiba K. (1995) Adenophostin-medicated quantal Ca^{2+} release in the purified and reconstituted inositol 1,4,5-trisphosphate receptor type 1. *FEBS Lett* 368; 248-252

Hiromitsu T, Mika H, Keizo T, Hitoshi T, Yasushi M, Akira T, Akihiko O and Yoshitake N (2007) Molecular biological features of male germ cell differentiation. *Reprod Medicine and Biol* 1; 1-9

Hicks SN, Jezyk MR, Gershburg S, Seifert JP, Harden TK and Sondek J. (2008) General and versatile autoinhibition of PLC isozymes. *Mol Cell*. 31; 383-94

Homa, S.T., Carroll J. and Swann K. (1993) The role of calcium in mammalian oocyte maturation and egg activation. *Human Reproduction* 8; 1274-1281

Homa S T and K Swann (1994) A cytosolic sperm factor triggers calcium oscillations and membrane hyperpolarizations in human oocytes. *Human Reproduction* 9; 2356-2361

Isotani A, Nakanishi T, Kobayashi S, Lee J, Chuma S, Nakatsuji N, Ishino F and Okabe M (2005) Genomic imprinting of XX spermatogonia and XX oocytes recovered from XX↔XY chimeric testes. *Proc Natl Acad Sci USA*. 102; 4039-4044

Igusa Y and Miyazaki SI (1983) Effects of altered extracellular and intracellular Ca^{2+} concentration on hyperpolarizing responses of the hamster egg. *J Physiology* 340; 611-632

Jones K, Carroll J, Merriman J, Whittingham D and Kono T (1995) Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependent. *Development* 10; 3259-3266

Jones, KT, Soeller, C., and Cannell, MB. (1998). The passage of Ca^{2+} and fluorescent markers between the sperm and egg after fusion in the mouse. *Development* 125; 4627-4635

Johnson EM, Theler JM, Capponi AM and Vallotton MB (1991) Characterization of oscillations in cytosolic free Ca^{2+} concentration and measurement of cytosolic Na^{+} concentration changes evoked by angiotensin II and vasopressin in individual rat aortic smooth muscle cells. *J of Biol Chemistry* 266; 12618-12626

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K and Hirohashi N. (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proc Natl Acad Sci USA*. 108; 4892-4896

Kashir J, Konstantinidis M, Jones C, Lemmon B, Lee HC, Hamer R, Heindryckx B, Deane CM, De Sutter P, Fissore RA, Parrington J, Wells D and Coward K (2012) A maternally inherited autosomal point mutation in human phospholipaseC zeta (PLCζ) leads to male infertility. *Hum Reprod*. 27; 222-231

Kashir J, Jones C, Mounce G, Ramadan WM, Lemmon B, Heindryckx B, de-Sutter P, Parrington J, Turner K, Child T, McVeigh E and Coward K (2013) Variance in total levels of phospholipase C zeta (PLCζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. *Fertility and Sterility* 99; 107-117

Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J and K. Coward (2010) Oocyte activation, phospholipase C zeta and human infertility. *Hum. Reprod. Update* 16; 690-703

Kono T, Jones KT, Mikich AB, Whittingham DG and Carroll J (1996) A cell cycle-associated change in Ca^{2+} releasing activity leads to the generation of Ca^{2+} transients in mouse embryos during the first mitotic division. *J Cell Biol.* 132; 912-923

Kline D. and Kline JT. (1992) Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouseegg. *Dev. Biol,* 149; 80-89

Kyono K, Kumagai S, Nishinaka C, Nakajo Y, Uto H, Toya M, Sugawara J and Araki Y (2008) Case report: Birth and follow-up of babies born following ICSI using strontium oocyte activation. *Reprod Biomedicine online*. 17; 53-58

Kishigami S and Wakayama T (2007) Efficient Strontium-induced Activation of mouse Oocyte in Standard Culture Media by Chelating Callcium. *J. Reprod and Dev.* 53; 1207-1215

Kline D (2000) Attributes and dynamics of the endoplasmic reticulum in mammalian eggs. *Curr Topics Dev Biol*. 50; 125-154

Kuo RC., Baxter GT., Thompson SH., Stricker SA., Patton C, Bonaventura J and Epel D (2000) NO is necessary and sufficient for egg activation at fertilization. *Nature* 406; 633-636

Konishi I, Fujii S, Okamura H, Parmley T and Mori T (1986) Development of interstitial cells and ovigerous cords in the human fetal ovary: an ultrastructural study. *J Anat.* 148; 121-135

Kimura, Y., and Yanagimachi, R. (1995) Intracytoplasmic sperm injection in the mouse. *Biol. Reprod*. 52; 709-720

Kouchi Z, Fukami K, Shikano T, Oda S, Nakamura Y, Takenawa T and Miyazaki S (2004) Recombinant phospholipase Czeta has high Ca^{2+} sensitivity and induces Ca^{2+} oscillations in mouse eggs. *J Biol Chem.* 11; 10408-12.

Kim YH, Park TJ, Lee YH, Baek KJ, Suh PG, Ryu SH and Kim KT (1999) Phospholipase C-δ1 is activated by capacitative calcium entry that follows phospholipase C-β activation upon bradykinin stimulation. *J Biol Chem.* 274; 26127-34

Keenan M J. and R. Niedergerke (1967) Intracellular sodium concentration and resting sodium fluxes of the frog heart ventricle. *J Physiol*. 2; 235-260

Kouchi Z., Shikano T., Nakamura Y., Shirakawa H., Fukami K. and Miyazaki S. (2005) The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase Czeta. *J. Biol. Chem.* 280; 21015-21021

Li ST, Huang XY and Sun FZ. (2001) Flowering plant sperm contains a cytosolic soluble protein factor which can trigger calcium oscillations in mouse eggs. *Biochem Biophys Res Commun.* 1; 56-9

Liu C-M and Hermann TE. (1978) Characterization of Ionomycin as a Calcium Ionophore. *The Journal of Biological Chemistry*. 17; 5892-5894

Lomasney JW., Cheng HF., Wang LP., Kuan Y., Liu S., Fesik SW. and King K. (1996). Phosphatidylinositol 4,5-bisphosphate binding to the pleckstrin homology domain of phospholipase C-δ1 enhances enzyme activity. *J. Biol. Chem.* 271; 25316-25326

Linda L. Runft, Laurinda A. Jaffe, and Lisa M. Mehlmann (2002) REVIEW: Egg Activation at Fertilization: Where It All Begins Normal activation. *Developmental Biology* 245; 237-254

Leckie C, Empson R, Becchetti A, Thomas J, Galione A and M Whitaker (2003) The NO pathway acts late during the fertilization response in sea urchin eggs. *J. Biol. Chem.* 278: 12247- 54

Lai FA., Misra M., Xu L., Smith, HA. and Meissner G. (1989) The ryanodine receptor-Ca^{2+} release channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a cooperatively coupled, negatively charged homotetramer. *J. Biol. Chem.* 264; 16776-85

Leguia M and Wessel G M. (2007) The many faces of egg activation at fertilization. *Signal Transduction.* 7; 118 -141

Liao, Y., Erxleben C., Yildirim E., Abramowitz J., Armstrong D. L., and Birnbaumer L. (2007) Orai proteins interact with TRPC channels and confer responsiveness to store depletion. *Proc. Natl. Acad. Sci*. USA 104; 4682-4687

Liu L., Hammar K., Smith P. J. S., Inoue S. and Keefe D. L. (2001) Mitochondrial modulation of calcium signaling at the initiation of development. *Cell Calcium* 30; 423-433

Larman M G., Saunders C M., Carroll J, Lai F A and Swann K (2004) Cell cycle-dependent Ca^{2+} oscillations in mouse embryos are regulated by nuclear targeting of PLCζ. *J Cell Science* 117; 2513-2521

Lawrence Y., Whitaker M. and Swann K. (1997) Sperm-egg fusion is the prelude to the initial Ca^{2+} increase at fertilisation in the mouse. *Development* 124; 233-241

Lawitts JA and Biggers JD (1993) Culture of pre-implantation embryos. *The Methods in Enzymology* 225; 153-165

Liu Y, Cao YX, Zhang ZG and Xing Q. (2011) Artificial oocyte activation and human failed-matured oocyte vitrification followed by in vitro maturation. *Zygote.* 1-6

Lacham-Kaplan O, Shaw J, Sanchez-Partida L and Trounson A (2003) Oocyte activation after intracytoplasmic injection with sperm frozen without cryoprotectants results in live offspring from inbred and hybrid mouse strains. *Biology of Reproduction*. 69; 1683-1689

Loren J and Lacham-Kaplan O. (2006) The employment of strontium to activate mouse oocytes: effects on spermatid-injection outcome. *Reproduction*. 2; 259-67

Lorca T, Cruzalegui FH, Fesquet D, Cavadore JC, Mery J, Means A and Dorée M (1993) Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* 366; 270-273

Mann J S., Lowther K M., and Mehlmann L M. (2010) Reorganization of the endoplasmic reticulum and development of Ca^{2+} release mechanisms during meiotic maturation of human Oocytes. *Biology of Reproduction* 83; 578-583

Marangos, P., FitzHarris, G. and Carroll, J. (2003) Ca^{2+} oscillations at fertilisation in mammals are regulated by the formation of pronuclei. *Development* 130; 1461-1472;

Makabe, S., Tomonori, N., Nottola, S. et al. (1991) Migration of germ cells, development of the ovary and folliculogenesis. In Familiari, G., Makabi, S. and Motta, P.H. (eds) Ultrastructure of the Ovary. *Kluwer Acad. Publ, Boston*, 1-27

McLay, D. W. and Clarke, H. J. (2003) Remodelling the paternal chromatin at fertilization in mammals. *Reproduction* 125; 625-33

Moses, R. M. and Kline, D. (1995) Release of mouse eggs from metaphase arrest by protein synthesis inhibition in the absence of a calcium signal or microtubule assembly. *Mol Reprod Dev.* 41; 264-73

McGrath, J. and Solter, D. (1984) Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell.* 37; 179-83

Miyazaki S and Ito M (2006) Calcium Signals for Egg Activation in Mammals. *J Pharmacol Sci.* 100; 545-552

Mercer, J. C., W. I. Dehaven, J. T. Smyth, B. Wedel, R. R. Boyles, G. S. Bird, and J. W. Putney, Jr. (2006) Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1. *J. Biol. Chem.* 281; 24979-90

Miyazaki S, Hashimoto N, Yoshimoto Y, Kishimoto T, Igusa Y and Hiramoto Y. (1986) Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. *Dev Biol.* 118; 259-267

Miyazaki S, Yuzaki M, Nakada K, Shirakawa H, Nakade S, Nakanishi S and Mikoshiba K. (1992) Block of Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science* 257; 251-255

Miyazaki, S. (1988) Inositol 1,4,5-trisphosphate-induced Ca^{2+} release and guanine nucleotide-binding proteinmediated periodic calcium rises in golden hamster eggs. *J. Cell Biol.* 106; 345-353

Miyazaki S, Shirakawa H, Nakada K and Honda Y. (1993) Essential role of the inositol 1,4,5-trisphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. *Dev Biol.* 158; 62-78

Macha'ty Z, Funahashi H, Mayes MA, Day BN and Prather RS (1996) Effects of injecting calcium chloride into in vitro–matured porcine oocytes. *Biol Reprod*. 54; 316-322

Macha´ty Z, Jagdeece J. Ramsoondar, Aaron J. Bonk, Kenneth R. Bondioli, and Randall S. Prather (2002a) Capacitative Calcium Entry Mechanism in Porcine Oocytes. *Biol of Reprod.* 66; 667-674

Macha´ty Z, Jagdeece J. Ramsoondar, Aaron J. Bonk, Randall S. Prather, and Kenneth R. Bondioli (2002b) Na^{+}/Ca^{2+} Exchanger in Porcine Oocytes. *Biol of Reprod* 67; 1133-1139

Moos J, Schultz RM and Kopf GS (1996) Regulation of nuclear envelope disassembly by MAP kinase. *Dev Biol.* 175; 358-361

Motta P M, Nottola S A, Makabe S and Heyn R. (2000) Mitochondrial morphology in human fetal and adult germ cells. *Hum. Reprod.* 15.129-147

Mehlmann LM and Kline D. (1994) Regulation of intracellular calcium in the mouse egg: Ca^{2+} release in response to sperm or inositol trisphosphate is enhanced after meiotic maturation. *Biol Reprod.* 51; 1088-1098

Mehlmann LM, Mikoshiba K and Kline D. (1996) Redistribution and increase in cortical inositol 1,4,5-trisphosphate receptors after meiotic maturation of the mouse oocyte. *Dev Biol.* 180; 489-498

Mehlmann LM, Carpenter G, Rhee, SG and Jaffe LA (1998) SH2 domain-mediated activation of phospholipase Cgamma is not required to initiate Ca^{2+} release at fertilization of mouse eggs. *Dev. Biol*. 203; 221-32

Mehlmann LM, Chattopadhyay A, Carpenter G and Jaffe LA. (2001) Evidence That Phospholipase C from the Sperm Is Not Responsible for Initiating Ca^{2+} Release at Fertilization in Mouse Eggs. *Dev Biol*. 236; 492-501

Ma S-F, Liu X-Y, Miao D-Q, Han Z-B, Zhang X, Miao Y-L, Yanagimachi R, and Tan J-H (2005) Parthenogenetic activation of mouse oocytes by strontium chloride: A search for the best conditions. *Theriogenology* 64; 1142-1157

Morgan AJ. and Jacob R (1994) Ionomycin enhances Ca^{2+} influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. *Biochem. J.* 300; 665-672

Maroto R, Raso A, Wood TG, Kurosky A, Martinac B and Hamill OP. (2005) TRPC1 forms the stretch-activated cation channel in vertebrate cells. *Nature Cell Biology* 2; 179-85

Ma H-T, Peng Z, Hiragun T, Iwaki S, Gilfillan A M., and Beaven M A. (2008) Canonical Transient Receptor Potential 5 Channel in Conjunction with Orai1 and STIM1 Allows Sr^{2+} Entry, Optimal Influx of Ca^{2+}, and Degranulation in a Rat Mast Cell Line. *The Journal of Immunology* 180; 2233-39

Marshall ICB and CW Taylor (1994) Two calcium-binding sites mediate the interconversion of liver inositol 1,4,5-trisphosphate receptors between three conformational states. *Biochem. J.* 301; 591-598

Méo SC, Yamazaki W, Leal CL, de Oliveira JA and Garcia JM. (2005) Use of strontium for bovine oocyte activation. *Theriogenology* 8; 2089-2102

Medkova M. and Cho W. (1999). Interplay of C1 and C2 domains of protein kinase C-alpha in its membrane binding and activation. *J. Biol. Chem.* 274; 19852-61

Mordecai PB and WJ Lederer (1999) Na^{+}/Ca^{2+} Exchange: Its Physiological Implications. *Physiological Reviews* 79; 3

Nasr-Esfahani MH, Deemeh MR and Tavalaee M (2010) Artificial oocyte activation and intra-cytoplasmic sperm injection. *Fertil Steril.* 94; 520-526

Nomikos M, Blayney LM, Larman MG, Campbell K, Rossbach A, Saunders CM, Swann K and Lai FA. (2005) Role of phospholipase C-ζ domains in Ca^{2+}-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca^{2+} oscillations. *J Biol Chem*. 280; 31011-18

Nomikos M, Mulgrew-Nesbitt A, Pallavi P, Mihalyne G, Zaitseva I, Swann K, Lai FA, Murray D and McLaughlin S (2007) Binding of Phosphoinositide-specific Phospholipase C-ζ (PLCζ) to Phospholipid Membranes. *JBC*. 22; 16644-53

Nomikos M, Elgmati K, Theodoridou M, Calver BL, Cumbes B, Nounesis G, Swann K and Lai FA. (2011a) Male infertility-linked point mutation disrupts the Ca^{2+} oscillation-inducing and PIP(2) hydrolysis activity of sperm PLCζ. *Biochem J*. 2; 211-7

Nomikos M, Elgmati K, Theodoridou M, Calver B L., Nounesis G, Swann K and Lai F. A (2011b) Phospholipase Cζ binding to PtdIns(4,5)P2 requires the XY-linker region. *Journal of Cell Science* 124; 2582-2590

Nomikos M, Elgmati K, Theodoridou M, Georgilis A, Gonzalez-Garcia R, Nounesis G, Swann K and Lai FA. (2011c) Novel regulation of PLCζ activity via its XY-linker. *Biochem J.* 3; 427-32

Nomikos M, Yu Y, Elgmati K, Theodoridou M, Campbell K, Vassilakopoulou V, Zikos C, Livaniou E, Amso N, Nounesis G, Swann K and Lai FA. (2013) Phospholipase Cζ rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril.* 1; 76-85

Nakahara T., Iwase A., Goto M., Harata T., Suzuki M. and Ienaga M. (2010) Evaluation of the safety of time-lapse observations for human embryos. *J Assist Reprod Genet.* 27; 93–96

Niggli E (1989) Strontium induced creep currents associated with tonic contractions in cardiac myocytes isolated from Guinea pigs. *Journal of Physiology*. 414; 549-568

Nagy ZP, Liu J, Joris H, Devroey P and Van Steirteghem A. (1994) Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. *Hum Reprod*. 9; 1743-1748

Naokazu I, Msahito I, Ayako I, and Masaru O. (2005) The immunoglobin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature* 434; 234-238.

Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwabara, Y., and Miyazaki, S. (1997) Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. *Mol. Hum. Reprod*. 3; 1087-1093

Nakagawa K, Yamano S, Nakasaka H, Honokio M and Aano T (2001) A combination of calcium ionophore and puromycin effectively produces human parthenogenomes with one haploid pronucleus. *Zygote* 9; 83-88

Nalefski E A. and Falke J J. (1996) The C2 domain calcium-binding motif: Structural and functional diversity. *Protein Science*. 5; 2375-90

Obenauer JC., Cantley LC. and Yaffe MB. (2003) Scansite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic acids res* 31; 3635-3641

Ozil J-P and Swann K. (1995) Stimulation of repetitive calcium transients in mouse eggs. *J Physiol.* 2; 331-346

Ozil JP. (1990) The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development*.109; 117-127

Offermanns S, Toombs CF, Hu YH and Simon MI. (1997) Defective platelet activation in Gaq-deficient mice. *Nature*. 389; 183-186

Ozdener F, Dangelmaier C, Ashby B, Kunapuli SP and Daniel JL. (2002) Activation of phospholipase C-gamma2 by tyrosine phosphorylation. *Mol Pharmacol.* 3; 672-9

Okada K, Miyano T and Miyake M (2003) Activation of pig oocytes by intra- cytoplasmic injection of strontium and barium. *Zygote* 11; 159-165

Philipson KD and Nicoll DA. (1992) Sodium-calcium exchange. *Curr Opin Cell Biol.* 4; 678-83

Pepperell J.R., K. Kommineni, S. Buradagunta, P.J.S. Smith, and D.L. Keefe (1999) Transmembrane regulation of intracellular calcium by a plasma membrane Na^{+}/Ca^{2+} Exchanger in mouse ova. *Biology of Reproduction* 60; 1137-1143

Plant, T. D. and Schaefer, M. (2003) TRPC4 and TRPC5: receptor-operated Ca^{2+}-permeable nonselective cation channels. *Cell Calcium* 33; 441-450

Pirino G, Wescott MP and Donovan PJ (2009) Protein kinase A regulates resumption of meiosis by phosphorylation of Cdc25B in mammalian oocytes. *Cell Cycle* 4; 665-70

Palmer A and Nebreda AR (2000) The activation of MAP kinase and p34cdc2/cyclin B during the meiotic maturation of Xenopus oocytes. *Prog Cell Cycle* Res. 4; 131-143

Palermo G, Joris H, Devroey P and Van Steirteghem AC (1992) Pregnancies after intra-cytoplasmic injection of single spermatozoon into an oocyte. *Lancet.* 340; 17-18

Palermo G. D., Cohen J., and Rosenwaks Z. (1996) Intracytoplasmic sperm injection: a powerful tool to overcome fertilization failure. *Fertil. Steril.* 65; 899-908

Palermo GD, Neri QV, Takeuchi T and Rosenwaks Z. (2009) ICSI: where we have been and where we are going. *Semin Reprod Med.* 27; 191-201

Parekh AB and Putney JW Jr. (2005) Store-operated calcium channels. *Physiol. Rev.* 85; 757-810

Peitsch MC, Polzar B, Stephan H, Crompton T , MacDonald HR , Mannherz HG , and Tschopp J (1993) Characterization of endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *EMBO J.* 12; 371-377

Peter N. Schlegel and Sarah K. Giradi (1997) In-Vitro Fertilization for male factor infertility. Clinical Review 87: *Journal of Clinical Endocrinology and Metabolism* 3; 709-716

Perry AC, Wakayama T and Yanagimachi R. (1999) A novel trans-complementation assay suggests full mammalian oocyte activation is coordinately initiated by multiple, submembrane sperm components. *Biol Reprod*. 3; 747-55

Presicce, G. A. and Yang, X. (1994) Parthenogenetic development of bovine oocytes matured in vitro for 24 hr and activated by ethanol and cycloheximide. *Mol Reprod Dev*. 38; 380-5

Parrington J, Jones ML, Tunwell R, Devader C, Katan M and Swann K (2002) Phospholipase C isoforms in mammalian spermatozoa: potential components of the sperm factor that causes Ca^{2+} release in eggs. *Reproduction* 123; 31-39

Pedersen SF and Nilius B. (2007) Transient receptor potential channels in mechanosensing and cell volume regulation. *Methods Enzymol*. 428;183-207

Parnas M, Peters M, Dadon D, Lev S, Vertkin I, Slutsky I and Minke B. (2009) Carvacrol is a novel inhibitor of drosophila TRPL and mammalian TRPM7 channels. *Cell Calcium.* 3; 300-309

Penner R and Fleig A. (2007) The Mg^{2+} and Mg^{2+}-nucleotide-regulated channel kinase TRPM7. *Handb Exp Pharmacol.* 179; 313-28

Pawelczyk T. and Lowenstein J. M. (1993) Binding of phospholipase C delta1 to phospholipid vesicles. *Biochem. J.* 291; 693-696

Patterson RL, Boehning D and Snyder SH (2004) Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annual Review of Biochemistry* 73; 437-465

Pieske B and S R. Houser (2003) Na^{+} handling in the failing human heart. *Cardiovascular Research* 57; 874-886

Peres A (1986) Resting membrane potential and inward current properties of mouse oocytes and eggs. *Pflugers Archives* 407; 534-540

Rice A, Parrington J, Jones KT and Swann K (2000) Mammalian sperm contain a Ca^{2+}-sensitive phospholipase C activity that can generate $InsP_3$ from PIP_2 associated with intracellular organelles. *Dev Biol.* 228:125-135.

Ross PJ, Beyhan Z, Iager AE, Yoon SY, Schellander K, Fissore RA and Cibelli JB. (2008) Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C zeta. *BMC Dev Biol* 8-16.

Rebecchi M. J. and Pentyala S. N. (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol. Rev.* 80; 1291-1335

Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, Chapelle A de-la, Silber S and Page DC (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat Genet.* 10; 383-93

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y and Jacobson MD (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* 262; 659-700

Rogers NT, Hobson E, Pickering S, Lai FA, Braude P and Swann K (2004) Phospholipase Czeta causes Ca^{2+} oscillations and parthenogenetic activation of human oocytes. *Reproduction* 128; 697-702

Rabinovici J and Jaffe RB (1990) Development and regulation of growth and differentiated function in human and subhuman primate fetal gonads. *Endocr Rev* 11; 532–557

Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Veliçelebi G and Stauderman KA. (2005). STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. *J. Cell Biol.* 169; 435-445

Raz T and Shalgi R. (1998) Early events in mammalian egg activation. *Hum Reprod.* 13; 133-45

Runft L L., Jaffe LA. and Mehlmann LM. (2002) Egg activation at fertilization: Where it all begins normal activation. *Developmental Biology* 245; 237-254

Rhee, S. G. (2001) Regulation of phosphoinositide-specific phospholipase C. Annu. *Rev. Biochem*. 70; 281-312

Ridgway E B. and Gordon A M. (1984) Muscle calcium transient: Effect of post-stimulus length changes in single fibers. *J. Gen. Physiol.* 83; 75-108

Ridgway E B. and Ashley C C. (1967) Calcium transients in single muscle fibers. *Biochem. Biophys. Res. Commun*. 29; 229-234

Ridgway EB, Gilkey JC and Jaffe LF. (1977) Free calcium increases explosively in activating medaka eggs. *Proc Natl Acad Sci USA*. 2; 623-627

Suh PG, Park JI, Manzoli L, Cocco L, Peak JC, Katan M, Fukami K, Kataoka T, Yun S and Ryu SH (2008) Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep*. 41; 415-34

Summers, M. C., McGinnis, L. K. M., Lawitts, J. A., Raffin, M. and Biggers, J. D. (2000) IVF of mouse ova in a simple optimized medium supplemented with amino acids. *Hum Reprod.* 15; 1791-1801

Steinhardt R, Zucker R and Schatten G. (1977) Intracellular calcium release at fertilization in the sea urchin egg. *Dev Biol.* 1; 185-96

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K and Lai FA (2002) PLCζ: a sperm-specific trigger of oscillations in eggs and embryo development. *Development* 129; 3533-44

Saunders CM, Swann K and Lai FA. (2007) PLCzeta, a sperm-specific PLC and its potential role in fertilization. *Biochem Soc Symp*. 74;23-36

Sheu S. S. and Blaustein M. P. (1986) Na^{+}/Ca^{2+} exchange and regulation of cell Ca^{2+} and contractility in cardiac muscle, with a note about vascular smooth muscle in the heart and cardiovascular system. *New York: Raven Press.*1; 509-535

Swann K. and Whitaker M. (1986) The part played by inositol trisphosphate and calcium in the propagation of the fertilization wave in sea urchin eggs*. J. Cell Biol.* 103; 2333-2342

Swann,K. (1990) A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development,* 110; 1295-1302

Swann K (1992) Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store. *Biochem. J.* 287; 79-84

Swann,K. (1994) Ca^{2+} oscillations and sensitization of Ca^{2+} release in unfertilized mouse eggs injected with a sperm factor. *Cell. Calcium* 15; 331-339

Swann K and JP Ozil (1994) Dynamics of calcium signal that triggers mammalian egg activation. *International Review of Cytology* 152; 183-222

Swann K, Larman MG, Saunders CM and Lai FA. (2004) The cytosolic sperm factor that triggers Ca^{2+} oscillations and egg activation in mammals is a novel phospholipase C: PLCzeta. *Reproduction* 127; 431-439

Swann K, Saunders CM, Rogers N and Lai FA. (2006) PLC(zeta): a sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. *Semin Cell Dev Biol.* 17; 264-273

Swann K. and Yu Y. (2008) The dynamics of calcium oscillations that activate mammalian eggs. *Int. J. Dev. Biol.* 52; 585-594

Swann K, Campbell K, Yu Y, Saunders C and Lai FA. (2009) Use of luciferase chimaera to monitor PLCzeta expression in mouse eggs. *Methods Mol Biol.* 518; 17-29

Swann K, Windsor S, Campbell K, Elgmati K, Nomikos M, Zernicka-Goetz M, Amso N, Lai FA, Thomas A and Graham C (2012) Phospholipase C-ζ-induced $^{Ca2+}$ oscillations cause coincident cytoplasmic movements in human oocytes that failed to fertilize after intracytoplasmic sperm injection. *Fertil Steril*. 3; 742-747

Sun, F. Z., Hoyland, J., Huang, X., Mason, W., and Moor, R. M. (1992) A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. *Development* 115, 947–956.

Smyth, J. T., W. I. Dehaven, B. F. Jones, J. C. Mercer, M. Trebak, G. Vazquez, and J. W. Putney, Jr. (2006) Emerging perspectives in store-operated Ca^{2+} entry: roles of Orai, Stim and TRP. *Biochim. Biophys. Acta.* 1763; 1147-1160

Strubing, C., Krapivinsky, G., Krapivinsky, L. and Clapham, D. E. (2001) TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* 29; 645-655

Swenson.C.E. and Dunbar,B.S. (1982) Specificity of sperm-zona interaction. *J. Exp. Zool.* 219; 97-104.

Sathananthan.A.H. and Trounson,A.O. (1985) The human pronuclear ovum: fine structure of monospermic and polyspermic fertilization in vitro. *Gamete Res.* 12; 385-398

Shiraishi K, Okada A, Shirakawa H, Nakanishi S, Mikoshiba M and Miyazaki S. (1995) Developmental changes in the distribution of the endoplasmic reticulum and inositol 1,4,5-trisphosphate receptors and the spatial pattern of Ca^{2+} release during maturation of hamster oocytes. *Dev Biol.* 170; 594-606

Stoop H., Honecker F., Cools M., de Krijger R., Bokemeyer C. and Looijenga L.H.J. (2005) Differentiation and development of human female germ cells during prenatal gonadogenesis: an immunohistochemical study. *Hum Reprod*. 6; 1466-1476

Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T, Chinwalla A, Delehaunty A, Delehaunty K, Du H, Fewell G, Fulton L, Fulton R, Graves T, Hou S-F, Latrielle P, Leonard S, Mardis E, Maupin R, McPherson J, Miner T, Nash W, Nguyen C, Ozersky P, Pepin K, Rock S, Rohling T, Scott K, Schultz B, Strong C, Tin-Wollam A, Yang S-P, Waterston RH, Wilson RK, Rozen S and Page DC (2003) The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* 423; 825-837

Surani M. A., Barton S. C. and Norris M. L. (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* 308; 548-50

Sutovsky P and Schatten G (1997) Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biol of Reprod.* 56; 1503-1512

Song, C., Hu, C. D., Masago, M., Kariya, K., Yamawaki-Kataoka, Y., Shibatoghe, M., Wu, D., Satoh, T. and Kataoka, T. (2001) Regulation of a novel human phospholipase C, PLCε, through membrane targeting by Ras. *J. Biol. Chem.* 276; 2752-2757

Stricker SA. (1999) Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol*. 2; 157-76

Shimomura O (1985). "Bioluminescence in the sea: photoprotein systems". *Symp. Soc. Exp. Biol.* 39; 351-72

Shimomura O (2006) Bioluminescence: chemical principles and methods. *World Scientific Publishing Co Pte Ltd. Singapore* 47-138

Steghens JP, Min KL and Bernengo JC. (1998) Firefly luciferase has two nucleotide binding sites: effect of nucleoside monophosphate and CoA on the light-emission spectra. *Biochem. J. 336*; 109-113

Seliger HH. and Mc, EW (1960) Spectral emission and quantum yield of firefly bioluminescence. *Arch Biochem Biophys* 88; 136-41

Singh A, Hildebrand ME, Garcia E, and Snutch TP (2010) The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channels. *Br J Pharmacol.* 6; 1464–1475.

Singh SM. and Murray D. (2003) Molecular modeling of the membrane targeting of phospholipase C pleckstrin homology domains. *Protein Sci.* 12; 1934-1953

Suchyna TM, Johnson JH, Hamer K, Leykam JF, Gage DA, Clemo HF, Baumgarten CM, and Sachs F. (2000) dentification of a peptide toxin from Grammostola spatulata spider venom that blocks cation-selective stretch-activated channels. *J Gen Physiol.* 5; 583-98

Stachecki J J., Cohen J, and Willadsen S (1998) Detrimental effects of sodium during mouse oocyte cryopreservation. *Biol of Reprod.* 59; 395-400

Suganuma R, Walden CM, Butters TD, Platt FM, Dwek RA, Yanagimachi R, and van der Spoel AC. (2005) Alkylated imino sugars, reversible male infertility-inducing agents, do not affect the genetic integrity of male mouse germ cells during short-term treatment despite induction of sperm deformities. *Biol Reprod*. 4; 805-13

Steinhardt R., Epel D., Carroll E.S. and Yanagimachi, R. (1974) Is calcium ionophore a universal activator for unfertilized eggs? *Nature* 252; 41-43

Sonn JK and Lee CO (1988) Na^{+}/Ca^{2+} exchange in regulation of contractility in canine cardiac purkinje fibres. *American Journal of Physiology* 255; 278-290

Taylor SL, Yoon SY, Morshedi MS, Lacey DR, Jellerette T, Fissore RA and Oehninger S (2010) Complete globozoospermia associated with PLCzeta deficiency treated with calcium ionophore and ICSI results in pregnancy. *Reprod Biomed Online*. 20; 559-564

Taylor CT, Lawrence YM, Kingsland CR, Biljan MM and Cuthbertson KS. (1993) Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. *Hum Reprod*. 8; 2174-2179

Toner M, Cravalho EG, Stachecki J, Fitzgerald T, Tompkins RG, Yarmush ML and Armant DR. (1993) Nonequilibrium freezing of one-cell mouse embryos. Membrane integrity and developmental potential. *Biophys J*. 64; 1908-1921

Tulsiani DR, Abou-Haila A, Loeser CR and Pereira BM. (1998) The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. *Exp Cell Res*. 2; 151-64

Tulsiani DR, Zeng HT and Abou-Haila A. (2007) Biology of sperm capacitation: evidence for multiple signalling pathways. *Soc Reprod Fertil Suppl*. 63; 257-72

Takahashi M, Kagasaki T, Hosoya T and Takahashi S. (1993) Adenophostins A and B: potent agonists of inositol 1,4,5-trisphosphate receptor produced by Penicillium brevicompactum. Taxonomy, fermentation, isolation, physico-chemical and biological properties. *J Antibiot (Tokyo)* 46; 1643-1647

Takahashi M, Tanzawa K and Takahashi S. (1994) Adenophostins, newly discovered metabolites of Penicillium brevicompactum, act as potent agonists of the inositol 1,4,5-trisphosphate receptor. *J Biol Chem*. 269; 369-372

Tesarik J, Sousa M and Testart J. (1994) Human oocyte activation after intracytoplasmic sperm injection. *Hum Reprod.* 9; 511–8

Ugarova NN., Filippova N. and Berezin IV. (1981) Inhibition of luciferase from fireflies Luciola mingrelica by inorganic salts. *Biokhimiia* 46; 851-858

Vincent C., Cheek T.R. and Johnson MH. (1992) Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. *J. Cell Sci.* 103; 389-396

Vanden MF, Nikiforaki D, De Gheselle S, Dullaerts V, Van den Abbeel E, Gerris J, Heindryckx B, and De Sutter P. (2012) Assisted oocyte activation is not beneficial for all patients with a suspected oocyte-related activation deficiency. *Hum Reprod.* 7; 1977-84

Van der Spoel AC, Jeyakumar M, Butters TD, Charlton HM, Moore HD, Dwek RA, and Platt FM. (2002) Reversible infertility in male mice after oral administration of alkylated imino sugars: a nonhormonal approach to male contraception. *Proc Natl Acad Sci USA*. 26; 17173-8

Winston N., Johnson M., Pickering S. and Braude P. (1991) Parthenogenetic activation and development of fresh and aged human oocytes. *Fertil. Steril.* 56; 904-912

Wong C.C., Loewke K.E., Bossert N.L., Behr B., De Jonge C.J., and Baer T.M. (2010) Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol*. 28; 1115-1121

Wilson, T. and Hastings, J. W. (1998) Bioluminescence. *Annu Rev Cell Dev Biol* 14; 197-230

Whitman M, Downes CP, Keeler M, Keller T and Cantley L. (1988) Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* 332; 644-646

Whalley T., McDougall A., Crossley I., Swann K., and Whitaker M. (1992) Internal calcium release and activation of sea urchin eggs by cGMP are independent of the phosphoinositide signaling pathway. *Mol. Biol. Cell* 3; 373- 383

Wang S., Gebre-Medhin S., Betsholtz C., Stalberg P., Zhou Y., Larsson C., Weber G., Feinstein R., Oberg K., Gobl A. and Skogseid B. (1998) Targeted disruption of the mouse phospholipase C β3 gene results in early embryonic lethality. *FEBS Lett*. 441; 261-265

Wu H., Smyth J., Luzzi V., Fukami K., Takenawa T., Black SL., Allbritton NL. and RA Fissore (2001) Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide pathway. *Biol. Reprod.* 64: 1338-1349

Wu H., He CL. and Fissore RA. (1997) Injection of a porcine sperm factor triggers calcium oscillations in mouse oocytes and bovine eggs. *Mol. Reprod. Dev.* 46; 176-189

Whitaker M. (2006) Calcium at fertilization and in early development. *Physiol Rev.* 1; 25-88

Wassarman PM, Jovine L and Litscher ES. (2001) A profile of fertilization in mammals. *Nat Cell Biol.* 3; 59-64

Xu SZ, Sukumar P, Zeng F, Li J, Jairaman A, English A, Naylor J, Ciurtin C, Majeed Y, Milligan CJ., Bahnasi YM., Al-Shawaf E, Porter KE., Jiang LH, Emery P, Sivaprasadarao A and Beech D J. (2008) TRPC channel activation by extracellular thioredoxin. *Nature* 451; 69-72

Xu Z, Kopf GS and Schultz RM. (1994) Involvement of inositol 1,4,5-trisphosphatemediated Ca^{2+} release in early and late events of mouse egg activation. *Development* 120; 1851-1859

Yoneda A, Kashima M, Yoshida S, Terada K, Nakagwa S, Sakamoto A, Hayakawa K, Suzuki K, Ueda J and Watanabe,T. (2006) Molecular cloning, testicular expression and oocyte activation potential of porcine phospholipase C zeta. *Reproduction* 132; 393-401

Yamazaki W, Ferreira CR, Méo SC, Leal CL, Meirelles FV and Garcia JM. (2005) Use of strontium in the activation of bovine oocytes reconstructed by somatic cell nuclear transfer. *Zygote* 13; 295-302

Yoda A., Oda S., Shikano T., Kouchi Z., Awaji T., Shirakawa H., Kinoshita K. and Miyazaki S. (2004) Ca^{2+} oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation. *Dev. Biol.* 268; 245-257

Yanagimachi, R. (1994) Mammalian fertilization. In Knobil, E. and Neil, J.D. (ed.). *The Physiology of Reproduction*. *Raven Press, New York*, 189-317

Yuan JP., W. Zeng, GN. Huang, PF. Worley, and S. Muallem (2007) STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat. Cell Biol*. 9; 636-645

Yang C H, Yanagimarchi R and Yanagimarchi H. (1989) Morphology and fertilizability of zona-free hamster eggs separated into halves and quarters by centrifugation. *Biol Reprod* 41; 741-752

Yamano S, Nakagawa K, Nakasaka H and Aono T. (2000) Fertilization failure and oocyte activation. *J Med Invest.* 47; 1-8

Yao A, Su Z, Nonaka A et al. (1998a) Effects of overexpression of the Na^{+}/Ca^{2+} exchanger on Ca^{2+} transients in murine ventricular myocytes. *Circulation Research* 82; 657-665

Yao A, Su Z, Nonaka A, Zubair I Spitzer KW, Bridge JH, Muelheims G, Ross J.Jr and Barry WH (1998b) Abnormal Ca^{2+} homeostasis in rabbit-s with pacing-induced heart failure. *Am J Physiol*. 275; 1441-1448

Yu Y, Saunders CM, Lai FA and Swann K. (2008) Preimplantation development of mouse oocytes activated by different levels of human phospholipase C zeta. *Hum Reprod.* 23; 365-373

Yanagida K, Katayose H, Yazawa H, Kimura Y, Sato A, Yanagimachi H and Yanagimachi R (1999) Successful fertilization and pregnancy following ICSI and electrical oocyte activation. *Hum Reprod*.14; 1307-1311

Yanagida K, Katayose H, Hirata S, Yazawa H, Hayashi S and Sato A. (2001) Influence of sperm immobilization on onset of Ca^{2+} oscillations after ICSI. *Hum Reprod*. 1; 148-152

Yanagida K, Morozumi K, Katayose H, Hayashi S and Sato A. (2006) Successful pregnancy after ICSI with strontium oocyte activation in low rates of fertilization. *Reprod Biomed Online* 13; 801-806

Yoon SY, Jellerette T, Salicioni AM, Lee HC, Yoo MS, Coward K, Parrington J, Grow D, Cibelli JB, Visconti PE, Mager J and Fissore RA. (2008) Human sperm devoid of PLCzeta1 fail to induce Ca^{2+} release and are unable to initiate the first step of embryo development. *J Clin Invest.* 11; 3671-81

Yoon SY, Eum JH, Lee JE, Lee HC, Kim YS, Han JE, Won HJ, Park SH, Shim SH, Lee WS, Fissore RA, Lee DR and Yoon TK. (2012) Recombinant human phospholipase C zeta 1 induces intra-cellular calcium oscillations and oocyte activation in mouse and human oocytes. *Hum Reprod.* 6; 1768-80

Zhang J, Wang CW, Blaszcyzk A, Grifo JA, Ozil J, Haberman E, Adler A and Krey LC (1999) Electrical activation and in vitro development of human oocytes that fail to fertilize after intracytoplasmic sperm injection. *Fertil Steril.* 72; 509-12

Zhang Di, Lei Pan, Ling-Hai Yang, Xiao-Ke He, Xiu-Ying Huang and Fang-Zhen Sun (2005) Strontium promotes calcium oscillations in mouse meiotic oocytes and early embryos through InsP3 receptors, and requires activation of phospholipase and the synergitic action of InsP3. *Hum. Reprod.* 1-9.

Zhu X, Chu PB, Peyton M and Birnbaumer L (1995) Molecular cloning of a widely expressed human homologue for the Drosophila trp gene. *FEBS Lett.* 373; 193-198.

Zhu X, Jiang MS, Peyton M, Boulay G, Hurst R, Stefani E and Birnbaumer L (1996) Trp, a novel mammalian gene family essential for agonistactivated capacitative Ca^{2+} entry. *Cell*. 85; 661-671

Zhou Y,WingMR, Sondek J, Harden TK. (2005) Molecular cloning and characterization of phospholipase C-η2. *Biochem J.* 391; 667-76

Zheng L., Krishnamoorthi R., Zolkiewski M. and Wang X. (2000) Distinct Ca^{2+} binding properties of novel C2 domains of plant phospholipase dalpha and beta. *J. Biol. Chem.* 275; 19700-19706