Cell cycle control of endoplasmic reticulum structure and Ca^{2+}-release in the mouse oocyte and early embryo

Greg FitzHarris

Department of Physiology

University College London

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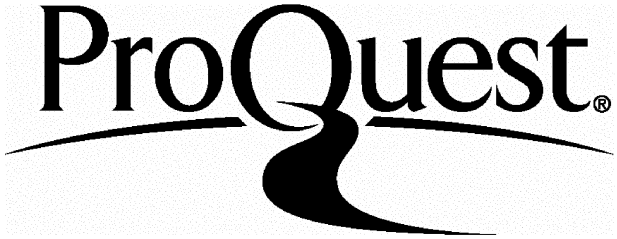

Abstract

In mammals, fertilisation triggers a series of intracellular Ca^{2+} transients which are responsible for egg activation and completion of meiosis. These oscillations are generated by $InsP_3$-induced release of Ca^{2+} from the endoplasmic reticulum (ER). Ca^{2+} oscillations last for 3-4 hours in mouse, ceasing at the time of pronucleus formation. The subsequent breakdown of the pronuclei (NEBD) at mitosis entry is accompanied by the resumption of Ca^{2+} oscillations. The experiments presented in this thesis examine the relationship between ER structure and Ca^{2+} release in the mouse oocyte and early embryo, and investigate the role of Ca^{2+} release in mitosis.

Using the ER-specific marker DiI, we report that germinal vesicle breakdown is associated with a dramatic microtubule-dependent redistribution of ER to the region surrounding the metaphase-I spindle. ER remains tightly packed around the spindle during centro-cortical migration. The formation of clusters of ER in the oocyte cortex occurs around the time of polar body formation, and coincides with increased responsiveness of $InsP_3$-mediated Ca^{2+} release. The decrease in cdk1-cyclin B activity which occurs following activation is both necessary and sufficient for the subsequent disappearance of ER clusters, and corresponds with diminished Ca^{2+} release in response to $InsP_3$. Cortical ER clusters do not re-appear following NEBD, rather ER accumulates around the mitotic spindle. NEBD is associated with increased responsiveness of Ca^{2+} release both in fertilised and parthenogenetic embryos.

The role of Ca^{2+} in mitosis was examined. Mitotic Ca^{2+} transients are dispensable since $InsP_3$-receptor-downregulation and Ca^{2+}-chelators prohibit mitotic Ca^{2+} transients without affecting the first embryonic division. Microinjection of a fluorescent marker into one pronucleus reveals that nuclear membrane permeablisation begins prior to initiation of mitotic Ca^{2+} signals. The subsequent cessation of mitotic oscillations precedes the formation of nuclei in the two-cell embryo. No Ca^{2+} transients are detected during the second mitotic division.

These data demonstrate dynamic microtubule and cell cycle dependent ER reorganisations in meiosis and mitosis, in which clustering of ER in the cortex or around the spindle is associated with increased responsiveness of $InsP_3$-releasable Ca^{2+} stores. Additionally, the results presented suggest that global mitotic Ca^{2+} transients are triggered by NEBD, rather than being the cause.

Publications containing work from this thesis

FitzHarris G., Marangos P. and Carroll J. (2003).
Cell Cycle-dependent Regulation of Structure of Endoplasmic Reticulum and Inositol 1,4,5-Trisphosphate-induced Ca^{2+} Release in Mouse Oocytes and Embryos.
Molecular Biology of the Cell. 2003 Jan;14(1):288-301.

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Carroll J., **FitzHarris G.**, Marangos P. and Halet G. (2003).
Ca^{2+} signalling and cortical re-organisation during the transition from meiosis to mitosis in mammalian oocytes.
Molecular Cellular Endocrinology. (In Press).

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1. Introduction

In mammals, fertilisation initiates a series of intracellular Ca^{2+} transients which trigger egg activation and the completion of meiosis. Changes in $[Ca^{2+}]_i$ are also known to regulate cell cycle progression in mitosis. In both cases, these Ca^{2+} transients are thought to be generated by release of Ca^{2+} ions from within the endoplasmic reticulum (ER). The experiments presented in this thesis have been designed to investigate the relationship between the cell cycle, Ca^{2+} release, and ER structure, and to examine the role of Ca^{2+} release in mitosis in the early mouse embryo. This introduction will therefore introduce topics relevant to the experiments presented. Following a brief overview of oogenesis and preimplantation development, the mechanisms of cell cycle control during oocyte maturation are considered. The function of the calcium ion as a second messenger will then be addressed prior to a review of the role of Ca^{2+} at mammalian fertilisation. This will be followed by an examination of the relationship between the cell cycle and Ca^{2+} release in the prelude to, and following fertilisation. Finally, the role of Ca^{2+} in mitotic cell cycles will be discussed.

1.1 A morphological description of early mammalian development

1.1.1 Oogenesis

The term oogenesis refers to the process of producing an egg capable of providing half the genome of the prospective offspring, and competent to be fertilised and subsequently support preimplantation development. In mammals, this process begins early in fetal life, and is not in effect completed until fertilisation itself.

Around five days after birth mammalian oocytes are arrested at prophase I of the first meiotic division, each oocyte contained within a layer of pregranulosa cells which form a primordial follicle. During this meiotic arrest, oocytes grow in size. In mouse, oocyte

growth takes around three weeks, during which the oocyte increases in size from about 12μm in diameter (0.9 pl in vol) to about 80μm (270pl). An accompanying nuclear growth ensues, such that fully grown oocytes contain a characteristically large nucleus termed the germinal vesicle (GV; Chouinard, 1975). Moreover, during this growth phase the oocyte manufactures a glycoprotein coat around 7μm thick called the zona-pellucida, which later serves to regulate penetration of the oocyte by fertilising sperm (Bleil and Wassarman, 1980a;Bleil and Wassarman, 1980b;Green, 1997). Growth of the oocyte is highly dependent upon oocyte-granulaosa cell communication, the rate of growth directly reflecting the number of granulosa cells contacting the oocyte (Brower and Schultz, 1982).

In a normal mouse reproductive cycle, 6-15 follicles respond to follicle stimulating hormone (FSH), causing follicular cells to secrete fluid within the follicle to form a Graafian, or antral follicle which migrates to the oocyte periphery. Ovulation is triggered by a surge in circulating levels of luteinising hormone (LH) which causes the rupture of (in mouse) several follicles, and the liberation of their contents. A normal mouse ovulation results in the release of 8-12 eggs over the course of two or three hours, each surrounded by a mass of follicular cells (termed cumulus cells) and proteoglycans. Administration of gonadotrophins causes superovulation, allowing the collection of an increased number of eggs or embryos (see Chapter 2). As the cells are released from the ovary, they are gathered in by the cilliated epithelium of the open end of the oviduct and swept along the oviduct towards the uterus (Hogan et al, 1994).

1.1.2 Oocyte maturation

Throughout the growth period the oocyte gradually acquires competence to resume meiosis and enter the final stages of oogenesis; mouse oocytes typically become competent to resume meiosis once 60μm in diameter (Wassarman and Albertini, 1994;Sorensen and Wassarman,

1976). *In vivo*, the completion of oogenesis is triggered by the LH surge. Within 2-3 hours germinal vesicle breakdown occurs (GVBD), chromosomes condense, and microtubules invade the nucleoplasm to begin associations with the chromosomes (Combelles and Albertini, 2001). A metaphase I (MI) spindle is first distinguishable after about 6h, and is fully formed (about 40μm in length) by 9-10 hours (Wassarman and Albertini, 1994). Around this time the spindle migrates across the ooplasm to the nearest part of the cortex (Verlhac *et al.*, 2000), providing the earliest known indication of the animal-vegetal axis (Zernicka-Goetz, 2002). Anaphase (sister chromosome disjunction) and telophase (movement of the chromosomes towards opposing spindle poles) are ultimately followed by cytokinesis, the process by which cell division takes place, some 10-12 hours after the LH surge (Wassarman and Albertini, 1994). Given appropriate culture conditions GV-stage oocytes removed from antral follicles will mature spontaneously, fertilise, and undergo normal preimplantation development in the absence of hormonal stimulation (Schroeder and Eppig, 1984).

The outcome of oocyte maturation is that an extraordinarily non-symmetrical cell division takes place, in which one of the daughter cells is around thirty times greater in volume than the other, despite equal division of the chromosomes. The smaller cell, termed the first polar body (Pb1), degenerates shortly after formation. The larger cell (the secondary oocyte), immediately re-enters meiosis and forms a second meiotic spindle in the oocyte cortex. The cell cycle then becomes arrested at metaphase of the second meiotic division (MII) pending successful fertilisation. This MII-arrested oocyte can be referred to as an egg.

1.1.3 Fertilisation

Following penetration of the surrounding cumulus cell layer, several hundred spermatazoa adhere to the zona pellucida proteins ZP2 and ZP3. Binding of the sperm to ZP3 triggers the

so-called acrosome reaction, resulting in the release of hydrolytic enzymes from the spermhead which permit zona-penetration (Wassarman, 2002). Reports vary as to the time taken for zona penetration, though it is known that this period is greatly reduced during *in vitro* fertilisation (IVF) if sperm are pre-incubated for a few hours to allow so-called 'capacitation', the molecular nature of which is unclear (Yanagimachi, 1994). Having passed through the zona, the successful sperm rapidly binds to, and fuses with the egg plasma membrane (PM). The molecular nature of the sperm-egg interaction is highly controversial, and beyond the scope of this review (but see Primakoff and Myles, 2002). In mammals, fertilisation is preferentially targeted to the vegetal hemisphere of the egg virtue of increased membrane convolutions, or microvilli to which the sperm bind (Evans *et al.*, 2000;Primakoff and Myles, 2002).

1.1.4 Egg activation and preimplantation development

Sperm-egg fusion initiates a series of events collectively termed oocyte activation. The first, exocytosis of the cortical granules (CGE), commences within minutes of sperm-egg fusion. CGE results in the release of a cocktail of enzymes into the sub-zonal (periviteline) space, which catalyse the partial hydrolysis of ZP2 and ZP3. Termed the zona reaction, these conversions prevent further penetration of the zona and thus form a block to polyspermy (Green, 1997).

Ultimately, egg activation stimulates the completion of meiosis. In mouse, exit from MII and the onset of anaphase occur around 90 minutes after activation, the second polar body forming immediately after. Individual nuclear membranes, or pronuclei, first become visible around the maternal and paternal chromosomes around four hours after sperm-egg fusion. Formation of the pronuclei is followed by their coordinated repositioning towards the centre of the cell (termed pronuclear syngamy) such that the two pronuclei

become closely apposed, but remain separate, pending the first embryonic cell division, 17-20 hours post activation (Howlett and Bolton, 1985;Yanagimachi, 1994; see figure 1.1).

In vivo, the second embryonic occurs 46-54 hours after fertilisation, and the embryo typically reaches the 8-cell stage by around 60 hours. The activation of intercellular adhesion at the 8-cell stage causes the embryonic cells (blastomeres) to become tightly packed, forming a compacted morula (Fleming *et al.*, 2000). Three to four days after fertilisation the embryo becomes differentiated into an outer cell layer, the trophoectoderm, enclosing an accumulation of cells at one pole termed the inner cell mass. It is this blastocyst-stage embryo which will ultimately hatch from the zona pellucida and implant in the uterine wall, some four to five days after conception (timings from Hogan et al, 1994).

1.2 Cell cycle control in early development

1.2.1 The discovery and role of maturation promoting factor (MPF)

Seminal work carried out on oocytes of the frog *Rana Pipiens* demonstrated that cytoplasm transferred from maturing (progesterone treated) frog oocytes causes recipient immature oocytes to undergo maturation. A similar result was not observed after transfer of cytoplasm from untreated oocytes (Smith and Ecker, 1971;Masui and Markert, 1971). Thus, release of oocytes from G2 entry into M-phase causes the acquisition of a cytosolic activity capable of driving recipient oocytes into M-phase. This cytosolic activity was dubbed maturation-promoting-factor, or MPF (Masui and Markert, 1971; see Masui, 2001 for review). MPF activity was subsequently found to be present in cleaving amphibian embryos, suggesting that MPF may play a role in cell cycle progression both in meiosis and mitosis (Wasserman and Smith, 1978).

The first indication as to the molecular identity of MPF came in the early 80s, when a novel family of proteins was described whose expression levels corresponded with

cell cycle stage. These proteins, which were synthesised in interphase and destroyed before cell division were named cyclins (Rosenthal *et al.*, 1980;Evans *et al.*, 1983). That the remarkable similarity between the patterns of MPF activity and cyclin expression might be more than a coincidence was confirmed when cyclin A from clam embryos was shown to trigger M-phase entry when expressed in *Xenopus* oocytes (Swenson *et al.*, 1986). Later, purification of MPF in *Xenopus* egg extracts revealed that it consisted of 32 and 45 kDa peptides, with kinase activity capable of phosphorylating both its own 45 kDa peptide, and histone H1 (Lohka *et al.*, 1988;Nurse, 1990). This purified form of MPF was able to initiate GVBD in recipient oocytes with similar efficiency as M-phase cytoplasm (within 2h; Lohka *et al.*, 1988). Several lines of evidence suggested that the 32kDa component may be the *Xenopus* homolog of cdc2, a protein kinase essential for mitosis in yeast (Lee and Nurse, 1987;Doree and Hunt, 2002). Crucially, an antibody raised against cdc2 both depleted MPF activity and immunoprecipitated a 32kD protein from *Xenopus* eggs (Gautier *et al.*, 1988). Cdc2 was subsequently found to co-purify with cyclin B, suggesting that cyclin B and cdc2 may together comprise MPF (Meijer *et al.*, 1989;Moreno *et al.*, 1989). This notion was confirmed when the M-phase specific kinase of starfish oocytes was purified and identified as a 1:1 stoichiometry of cdc2 and cyclin B (Labbe *et al.*, 1989). cdc2 was therefore renamed cyclin dependent kinase 1 (cdk1; Doree and Hunt, 2002).

It is now established that activation of cdk1-cyclin B is the major driving force for M-phase entry in all eukaryotic cells (Nurse, 1990). Cdk1 activation depends upon dephosphorylation and association with cyclin B (Nurse, 1990;Morgan, 1997). Cdk1 is maintained in an inactive state in interphase by phosphorylation of residues Tyr15 and Thr/Ser14 by the dual specific kinases Wee1 and Myt1 (Mueller *et al.*, 1995a;Mueller *et al.*, 1995b). This phosphorylated form takes the name pre-MPF (Wasserman and Masui, 1975). Inactivation of Wee1 and Myt1 and dephosphorylation of cdk1 by the phosphatase Cdc25

therefore activates MPF and triggers M-phase entry (Kumagai and Dunphy, 1992;Ohi and Gould, 1999).

There are a number of isoforms of B-type cyclins that may complex with cdk1. The first to be described were cyclins B1 and B2 (Minshull *et al.*, 1989; see Ohi and Gould, 1999, for review). Both isoforms are expressed in *Xenopus* and mouse oocytes and trigger GVBD when injected or expressed in oocytes or added to oocyte extracts (Minshull *et al.*, 1989;Ledan *et al.*, 2001). Cyclin B2 is most abundant in *Xenopus* (Kobayashi *et al.*, 1991) while B1 appears to be the predominant subtype in mouse oocytes. Both isoforms are also expressed in somatic cells and are targeted to different substrates by way of localisation (see below). In mice, cyclin B1 is essential for embryonic development since cyclin $B1^{-/-}$ mice die in utero (Brandeis *et al.*, 1998). In contrast, cyclin $B2^{-/-}$ mice develop normally suggesting functional compensation may be provided by other B-type cyclins (Brandeis *et al.*, 1998). Cyclin B3 has properties similar to other B-type cyclins (Gallant and Nigg, 1994;Lozano *et al.*, 2002) but appears to have no role in oocyte maturation, at least in Xenopus (Hochegger *et al.*, 2001). In drosophila, homozygous carriers of a cyclin B3 null allele reach adulthood but are sterile, suggesting that cyclin B3 is essential for reproduction, but dispensable in mitosis (Jacobs *et al.*, 1998). No cyclin $B3^{-/-}$ mouse has yet been generated. More recently, two further forms of cyclin B have been described in *Xenopus* oocytes and embryos, but not adult cells (Hochegger *et al.*, 2001). Cyclin B4 and B5 are similar to cyclin B1 and B2, respectively but, as yet, have not been described in other species.

Exit from M-phase is triggered by destruction of cyclin B, which is initiated when the last of the chromosomes line up at the metaphase plate (Clute and Pines, 1999). Cyclin destruction occurs by a process of ubiquitination by the anaphase promoting complex (APC, or cyclosome; Glotzer *et al.*, 1991;King *et al.*, 1995;Morgan, 1999). Ubiquitination of cyclin depends upon a short N-terminus sequence termed the destruction box. Addition of a

non-destructable cyclin B mutant deficient of the 90 N-terminal amino acids to interphase frog egg extracts generates a persistant mitotic-state (Holloway *et al.,* 1993). Moreover, studies using radiolabelled ubiquitin illustrated that ubiquitination is M-phase-specific, and prohibited by mutation of the destruction box sequence (Glotzer *et al.*, 1991). Ubiquitination of cyclin B confers the ability to undergo proteolysis (destruction) at the hands of the 26S proteosome (Townsley and Ruderman, 1998).

Recently, co-localisation of cdk1-cyclin B to the ER, golgi, cytoskeleton and, to the centromeres, have suggested that cdk1-cyclin B activity may have localised roles (Jackman *et al.*, 1995;Liu *et al.*, 1997;Ohi and Gould, 1999;Jackman *et al.*, 2003). One such example is the demonstration that cdk1 bound either to cyclin B1 or B2 can phosphorylate the golgi protein GM130, inhibiting its binding to the vesicle-docking protein P115, thus providing the mechanism of golgi fragmentation in mitosis (Lowe *et al.*, 1998). Whilst cdk1-cyclin B1 is able to cause reorganisation of the cytoskeleton and chromosomes, targeting of cyclin B2 to the golgi in CHO cells restricts its action to golgi disassembly (Draviam *et al.*, 2001). The ability of cdk1 to bind to cyclin B partners that may be spatially and developmentally requlated provide an additional level of control for the entry and exit from M-phase.

1.2.2 The role of cdk1-cyclin B activity in oocyte maturation in mouse

In several respects the role of cdk1-cyclin B activity in the control of the first meiotic division in mammals reflects that in mitosis. In mouse, GVBD is associated with an increase in cdk1-cyclin B activity thought to be attributable to dephosphorylation of cdk1 rather than cyclin synthesis (since inhibitors of protein synthesis fail to block GVBD; Clarke and Masui,;Hashimoto and Kishimoto, 1988;Ledan *et al.*, 2001). Cyclin B synthesis is subsequently upregulated following GVBD (Hashimoto and Kishimoto, 1988;Hampl and

Eppig, 1995a), the rate of production determining the timecourse of meiosis I (Polanski et al., 1998). Cdk1-cyclin B activity eventually reaches a plateau at the end of MI (Choi et al., 1991;Verlhac et al., 1994;Hampl and Eppig, 1995b;Ledan et al., 2001). A brief decline in cdk1-cyclin B activity which occurs at the end of MI is responsible for the onset of anaphase and extrusion of the first polar body (Ledan et al., 2001).

Recently it was reported that inhibition of the APC fails to block first polar body extrusion in *Xenopus* oocytes, suggesting that a drop in cdk1-cyclin B activity may not be an absolute requirement for the MI-MII transition in that species (Peter et al., 2001). Whilst similar experiments are yet to be carried out in mouse, it appears unlikely that the drop in cdk1-cyclin B activity will prove dispensable, since disruption of the MI spindle, which prevents cyclin proteolysis (see below), causes persistent MPF activity (as gauged by cytoplasmic transfer) and inhibits the MI to MII transition (Hashimoto and Kishimoto, 1988). A schematic diagram depicting the changing levels of cdk1-cyclin B activity during oocyte maturation and following egg activation is presented in figure 1.1.

Extrusion of the first polar body is rapidly followed by reformation of a metaphase spindle in the oocyte cortex, at which time the cell cycle becomes arrested at metaphase of the second meiotic division.

1.2.3 Maintenance of the metaphase II arrest: cytostatic factor (CSF)

That a cytoplasmic factor might be responsible for arresting the cell cycle at MII was first illustrated by experiments in which transfer of cytoplasm from MI or MII, but not GV stage oocytes, was shown to cause cell cycle arrest at metaphase when injected into recipient blastomeres. This activity was named cytostatic factor, or CSF (Masui and Markert, 1971;Masui, 2001;Maller *et al.*, 2002).

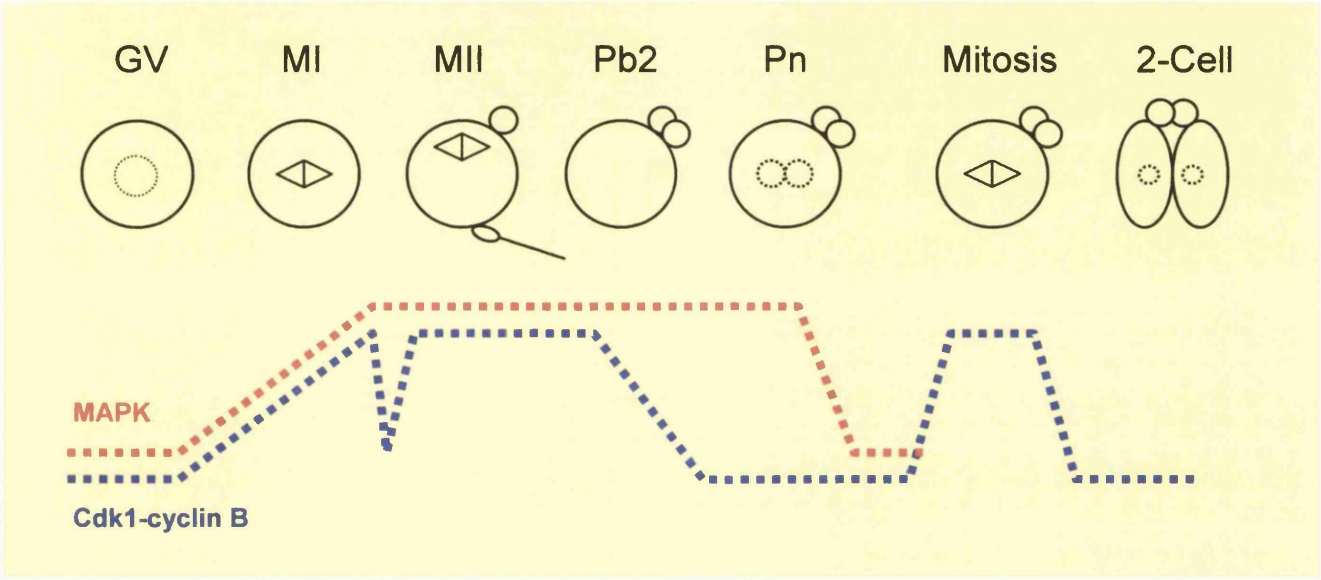


Figure 1.1. M-phase kinase levels during early development. Schematic representation of cdk1-cyclin B activity and MAPK activity levels during oocyte maturation and following fertilisation in mouse. Diagram adapted from Carroll 2001. Cdk1-cyclin B activity (blue) and MAPK activity (red) are initially low in the germinal vesicle stage (GV) oocyte. The activities of both increase following germinal vesicle breakdown, during metaphase I (MI). A brief decline in cdk1-cyclin B activity accompanies the transition from metaphase I to metaphase II (MII). Following fertilisation the level of cdk1-cyclin B activity decreases around the time of second polar body extrusion (Pb2), whereas the level of MAPK activity remains high until the time of pronucleus formation (Pn). The level of cdk1-cyclin B activity rises again during the first mitotic division. Dotted lines indicate the presence or absence of nuclei at each cell cycle stage. Meiotic and mitotic spindles are indicated by ◁▷. See text for further details.

The presence of CSF activity in mouse oocytes has since been demonstrated by electrofusion experiments. Fusion of MII oocytes with mitotic embryos results in a persistant (over 24 hours) metaphase arrest not observed following fusion of two mitotic cells (Kubiak *et al.*, 1993). The MII arrest in mouse oocytes is caused by persistantly high levels of cdk1-cyclin B activity. Cyclin turnover continues at MII, however, suggesting that the M-phase arrest involves an equilibrium between cyclin destruction and resynthesis (Kubiak *et al.*, 1993). Though it is thought that CSF stabilises cdk1-cyclin B activity and maintains the MII arrest by controlling the equilibrium between cyclin synthesis and destruction, the mechanism by which this is achieved is unclear. The remaining part of this section will therefore focus upon some of the candidate mechanisms which have been proposed to contribute to CSF activity.

The c-mos proto-oncogene provided the first candidate for CSF activity. Expression of mos was initially reported to be germ cell specific (Goldman *et al.*, 1987), though smaller amounts of mos RNA have also been detected in brain and kidney (Propst *et al.*, 1987). In mammalian oocyes, whilst Mos is detected at GV stage, expression is dramatically upregulated during oocyte maturation and is abundant in MII eggs. Expression is subsequently reduced following fertilisation, such that Mos is undetectable in pronucleate embryos (Paules *et al.*, 1989;Wu *et al.*, 1997). Mos posesses CSF activity, since microinjection of mos mRNA causes metaphase arrest in cleaving *Xenopus* blastomeres, and immunodepletion of the mos product from *Xenopus* extracts prevents them from causing cleavage arrest when microinjected into recipient cells (Sagata *et al.*, 1989). In mouse, microinjection of antisense Mos oligonucleotides prevents the MII arrest, causing the formation of a single pronucleus, and eventual cleavage to the two-cell stage (O'Keefe *et al.*, 1989). Oocytes of the $mos^{-/-}$ mouse exhibit a similar phenotype (Hashimoto *et al.*,

1994;Colledge *et al.*, 1994). Thus there is strong evidence that mos is a key player in CSF activity.

Downstream actions of Mos are most likely mediated by mitogen-activated-protein-kinase (MAPK). Mos activates MAPK both *in vivo* and *in vitro* (Shibuya and Ruderman, 1993;Posada *et al.*, 1993). Accordingly, the pattern of MAPK activation in mouse reflects the expression of mos; activation of MAPK follows GVBD, and persists through to MII (Verlhac *et al.*, 1993). More direct evidence for the involvement of MAPK is provided by the finding that MAPK fails to activate in oocytes of the $mos^{-/-}$ mouse (Verlhac *et al.*, 1996). Moreover, *in vitro* studies reveal that Mos can directly phosphorylate and activate an upstream regulator of MAPK (a MAP kinase kinase; Posada *et al.*, 1993). Thus, Mos acts as a MAP kinase kinase kinase (MAPKKK). In *Xenopus* oocytes, activation of MAPK is necessary for the CSF activity of Mos, since Mos fails to induce metaphase arrest when coinjected with an antibody specific for MAPKK (Kosako *et al.*, 1994). Finally, MAPK has itself been shown to posess CSF activity. Constitutively active MAPK induces a metaphase arrest when injected into *Xenopus* blastomeres (Haccard *et al.*, 1993), and the MAPK inhibitor UO126 decreases the half-life of cyclin B1 in *Xenopus* oocytes from over 30 minutes to around 15 (Gross *et al.*, 2000). Moreover, UO126 activates mouse eggs and produces parthenogenetic embryos with $mos^{-/-}$ phenotype (Phillips *et al.*, 2002). Thus the CSF-like properties of mos owe to its ability to activate MAPK. A schematic representation of the timecourse of MAPK activity during oocyte maturation and following egg activation is presented in figure 1.1.

Recent work has identified the protein kinase p90rsk as a potential downstream target of MAPK, which may mediate the CSF activity of MAPK. Immunodepletion of p90rsk from *Xenopus* extracts prevents MAPK-induced mitotic arrest (Bhatt and Ferrell, Jr., 1999). Microinjection of a constitutively active form of p90rsk into *Xenopus* oocytes treated with

the MAPK inhibitor UO126 causes the recovery of cyclin B levels (Gross *et al.*, 2000). Moreover, microinjection of the same molecule into *Xenopus* blastomeres results in a (CSF-like) metaphase arrest (Gross *et al.*, 1999). These observations suggest that p90rsk can alone support the CSF properties of MAPK (Maller *et al.*, 2002). The downstream action of p90rsk may involve inactivation of the APC. In vitro, p90rsk can phosphorylate and activate Bub1, a protein known to regulate the APC by inhibiting Fizzy/cdc20, a necessary activator of the APC (Schwab *et al.*, 1999;Lorca *et al.*, 1998;Amon, 1999). Moreover, activation of Bub1 can be inhibited by UO126, and then subsequently restored by constitutively active p90rsk (Schwab *et al.*,1999). Thus it appears, at least in *Xenopus* oocytes, that the CSF activity of mos/MAPK might maintain cdk1-cyclin B activity by inhibiting the APC in a pathway involving p90rsk, Bub1, and Fizzy/cdc20 (Tunquist and Maller, 2003).

Finally, a novel protein has recently been described which has been proposed to be a component of CSF. Emi1 (*E*arly *M*itotic *I*nhibitor) was initially described as a protein whose expression upregulates in interphase, and which prevents premature APC activation through interaction with Fizzy/cdc20 (Reimann *et al.*, 2001a;Reimann *et al.*, 2001b). A potential CSF role is suggested by the finding that injection of Emi1 into cleaving blastomeres causes cell-cycle arrest similar to that of mos, MAPK and p90rsk (Reimann *et al.*, 2001a). Moreover, addition of Emi1 to CSF extracts prevents Ca^{2+}-stimulated destruction of mos, MAPK or cyclin B, and immunodepletion of Emi1 causes cyclin B destruction and exit from the mitotic state (Reimann and Jackson, 2002). However, though it has been demonstrated that Emi1 accumulates during oocyte maturation in *Xenopus* (Reimann and Jackson, 2002), functional studies are yet to be carried out in intact oocytes.

Thus, there are several players thought to contribute to CSF and it is unclear how they interact and coordinate to maintain sufficiently high cdk1-cyclin B activity at MII to prevent premature egg activation. Breach of this metaphase II arrest occurs at fertilisation

and is achieved by a remarkable sequence of changes in free calcium ion concentration within the oocyte. The following sections will firstly review the role of Ca^{2+} as an intracellular second messenger, then examine the mechanisms by which Ca^{2+} release at fertilisation triggers resumption of the cell cycle

1.3 Ca^{2+}; a ubiqiutous second messenger responsible for egg activation

Increases in cytosolic free calcium ion concentration ($[Ca^{2+}]_i$) trigger a diverse array of processes in eukaryotic cells. Since the role of Ca^{2+} as a second messenger has been thoroughly reviewed (Berridge, 1993;Clapham, 1995;Berridge *et al.*, 2000;Bootman *et al.*, 2001a), the following sections will provide a brief summary of the topics pertinent to the the experiments presented. Generally, in resting cells $[Ca^{2+}]_i$ is maintained at around 100nM. On stimulation by an agonist $[Ca^{2+}]_i$ is elevated (to around 1μM). Unlike other second messengers, calcium ions cannot be degraded or metabolised, thus cells employ a wide range of Ca^{2+} binding and transporting molecules to control $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ can be modulated by accessing several different sources of Ca^{2+} ions including the endoplasmic reticulum (ER), nuclear envelope, mitochondria, golgi, acidic vesicles, and the extracellular media (Pozzan *et al.*, 1994;Gerasimenko *et al.*, 1995;Subramanian and Meyer, 1997;Ichas *et al.*, 1997;Pinton *et al.*, 1998;Churchill *et al.*, 2002).

1.3.1 Release of Ca^{2+} from the endoplasmic reticulum

In oocytes, the endoplasmic reticulum (ER) is the major source of Ca^{2+} ions for intracellular release. This notion is elegantly illustrated by demonstration that following stratification by centrifugation, $InsP_3$-induced Ca^{2+} release occurs in the layer consisting predominantly of ER (Eisen and Reynolds, 1985;Han and Nuccitelli, 1990). Key to the role of the ER as a Ca^{2+} store is that under resting conditions $[Ca^{2+}]_{ER}$ is in the range of a few hundred μM, some

three to four orders of magnitude greater than cytosolic [Ca^{2+}] (Pozzan *et al.*, 1994;Miyawaki *et al.*, 1997;Ashby and Tepikin, 2001). Within the ER lumen Ca^{2+} ions are sequestred by low affinity, high capacity Ca^{2+} binding proteins such as calsequestrin and calreticulin (see Meldolesi and Pozzan, 1998;Corbett and Michalak, 2000;Ashby and Tepikin, 2001 for review). Constitutive leak of Ca^{2+} down this steep concentration gradient is counterbalanced by Ca^{2+}-ATPases spanning the ER membrane (Ca^{2+} pumps) which reclaim Ca^{2+} ions for the ER lumen (Carafoli and Brini, 2000). Maintenance of this equilibrium provides an environment in which opening of Ca^{2+} channels in the ER membrane provokes rapid diffusion of Ca^{2+} ions into the cytosol. The channels responsible for release of Ca^{2+} from the ER to the cytosol fall into two families; inositol (1,4,5)-trisphosphate receptors ($InsP_3R$) and ryanodine receptors (RyR).

The $InsP_3R$ is a tetramer, four subunits born of three distinct genes (types I, II, and III) which together form a membrane-spanning gated pore (Berridge, 1993;Mikoshiba *et al.*, 1993;Patel *et al.*, 1999). Typically, these channels are opened as a result of cell stimulation by extracellular signals such as hormones and growth factors that activate of phospholipase C (PLC) enzymes. PLC hydrolyses the plasma membrane lipid phosphatidyl inositol (4,5) bisphosphate (PIP_2) to produce the intracellular second messengers $InsP_3$ and diacylglycerol (DAG). The hydrophilic nature of $InsP_3$ permits its diffusion to the ER and association with the $InsP_3R$, whilst a relatively short half-life ensures temporal fidelity of signal transduction (Berridge, 1993). Though $InsP_3R$ opening is dependent upon $InsP_3$ binding, its activity is also sensitive to $[Ca^{2+}]_i$; the role of $InsP_3$ in $InsP_3R$ opening has been said to be 'permissive', insofar that it potentiates channel activation by Ca^{2+} (Li *et al.*, 1995). Experiments performed on $InsP_3Rs$ in planar lipid bilayers revealed the Ca^{2+} dependency of opening to be 'bell shaped'; small increases in $[Ca^{2+}]_i$ (up to 0.2μM) stimulating channel opening, and larger increases inhibiting opening (Bezprozvanny *et al.*, 1991). Recent reports

have, however, suggested that the Ca^{2+}-dependency of the type III $InsP_3R$ might in fact be monotonic; bilayer recordings of cells expressing predominantly type III receptors suggest that the channel is activated, but not inhibited by Ca^{2+} (Hagar *et al.*, 1998), and similar results are generated by permeablised B-cells exclusively expressing type III receptors (Miyakawa *et al.*, 1999). Whether $InsP_3R$ III activation is monophasic in intact cells is unclear however, since biphasic regulation of by cytosolic Ca^{2+} was reported in single-channel recordings of recombinant $InsP_3R$ III expressed in *Xenopus* oocytes (Mak *et al.*, 2000). The reasons for these conflicting data are unclear, but have been discussed and reviewed elsewhere (Taylor and Laude, 2002).

RyRs share several structural and functional features of $InsP_3Rs$. Like $InsP_3Rs$ they are comprised of four membrane-spanning subunits from three different genes (Sorrentino, 1995;Franzini-Armstrong and Protasi, 1997). The bell-shaped Ca^{2+}-dependency of RyR channel opening is analogous to that of the $InsP_3R$, though RyRs are both activated and inhibited at higher $[Ca^{2+}]_i$ than $InsP_3Rs$ (Bezprozvanny *et al.*, 1991). Nevertheless, unlike the $InsP_3R$, the opening of which is critically dependent upon $InsP_3$, the RyR is Ca^{2+}-sensitive in the absence of other agonists (Bezprozvanny *et al.*, 1991). Hence, RyRs are thought to function primarily as Ca^{2+}-induced Ca^{2+}-release (CICR) channels.

The inhibitory action of high cytosolic $[Ca^{2+}]$ upon ER Ca^{2+}-release channels means that Ca^{2+} release channels have a short 'open time', providing an efficient mechanism for re-establishing resting $[Ca^{2+}]_i$; closure of $InsP_3Rs$ prevents further release, and causes $[Ca^{2+}]_i$ to be driven back to basal levels by the continued action of Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers. Thus elevations of $[Ca^{2+}]_i$ are typically short-lived, and are referred to as Ca^{2+} transients.

1.3.2 Ca^{2+} store refilling and Ca^{2+} influx

Following cell stimulation, basal $[Ca^{2+}]_i$ is re-established by extrusion of Ca^{2+} ions not only to the ER, but also to the extracellular space. Thus a mechanism is required whereby $[Ca^{2+}]_{ER}$ can be replenished following stimulation. Accordingly, trans-plasmalemmal Ca^{2+} influx has been described in a variety of cells which is triggered by ER Ca^{2+}-store depletion. Such Ca^{2+} entry has been referred to as store-operated, or 'capacitative' Ca^{2+} entry (see Berridge, 1995;Putney, Jr. and McKay, 1999;Putney, Jr. *et al.*, 2001). The nature of the signal by which Ca^{2+} store depletion triggers plasmalemmal Ca^{2+} flux is hotly debated. Proposed mechanisms include the diffusion of a soluble factor from the ER to the plasma membrane (PM; Putney, Jr., 1990;Randriamampita and Tsien, 1993), regulated exocytosis allowing insertion of Ca^{2+} channels in the PM (Fasolato *et al.*, 1993;Yao *et al.*, 1999), regulation of PM Ca^{2+} channels by the magnitude of ER Ca^{2+} leak (Putney, Jr., 1986), and direct coupling of the ER with the PM (Irvine, 1990) -possibly involving the $InsP_3R$ itself (Vaca and Kunze, 1995; see above reviews). In mouse eggs, a Ca^{2+} influx pathway has been demonstrated (using the manganese-quench technique) which may be activated in response to store depletion, though its mechanisms and regulation have not yet been addressed (Kline and Kline, 1992a;McGuinness *et al.*, 1996;Mohri *et al.*, 2001).

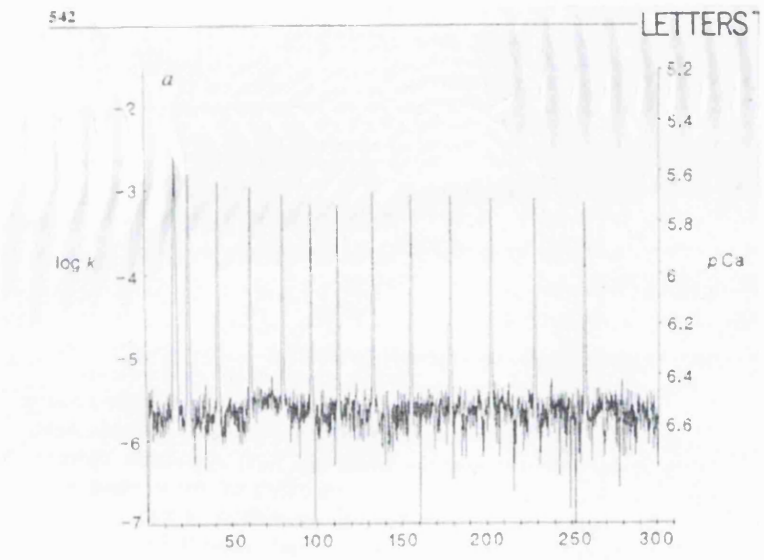
1.3.3 Temporal organisation of Ca^{2+} signals in cells: Ca^{2+} oscillations

Since sustained elevations of $[Ca^{2+}]_i$ are cytotoxic, many cell types respond to stimulation by producing repetitive Ca^{2+} transients, or Ca^{2+} oscillations. The first direct measurements of agonist induced Ca^{2+} oscillations were performed in mouse oocytes. Using the Ca^{2+} sensitive luminescent protein aequorin, it was reported that fertilisation triggers 13-15 brief (~2mins) increases in $[Ca^{2+}]_i$ spread over the course of 4 hours (Cuthbertson and Cobbold, 1985; see figure 1.2a). Ca^{2+} oscillations were subsequently found to be triggered in somatic cells in

response to extracellular agonist. Hepatocytes, for example, produce Ca^{2+} oscillations which persist as long as the agonist is applied (Woods *et al.*, 1986; see figure 1.2b). Whilst the dynamics of Ca^{2+} waves (see below), and maximal $[Ca^{2+}]_i$ reached by each oscillation are independent of agonist concentration within a given cell, the rate of oscillation is dose dependent (Woods *et al.*, 1986;Jacob *et al.*, 1988; Rooney *et al.*, 1989;Bootman *et al.*, 2001a).

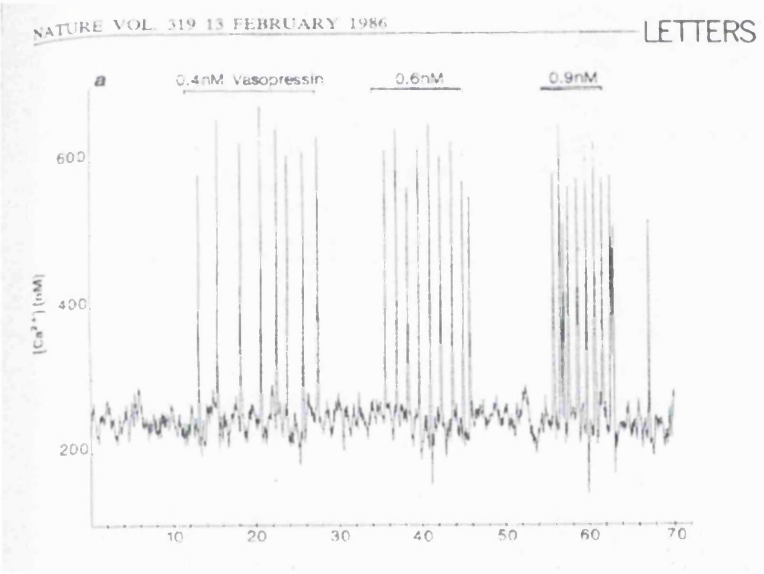
A number of models have been proposed to explain the generation of Ca^{2+} oscillations (Berridge, 1990). The first is that constant $InsP_3$ levels cause Ca^{2+}-oscillations, by a process of feedback inhibition of Ca^{2+} upon the Ca^{2+}-binding site of the $InsP_3R$ (Gray, 1988;Prentki *et al.*, 1988;Hajnoczky and Thomas, 1997). Within such a model the period of oscillation may be governed by the rate of store refilling by capacitative Ca^{2+} entry (Berridge, 1990) since it is well known that the sensitivity of $InsP_3$-induced Ca^{2+}-release is influenced by the degree of Ca^{2+}-store loading (Iino and Endo, 1992;Ashby and Tepikin, 2001). The second model proposes that a negative feedback loop may arise whereby each Ca^{2+} transient supresses the rate of PIP_2 hydrolysis, causing the level of $InsP_3$ to oscillate. A putative feedback loop may involve activation of protein kinase C (PKC) by DAG and Ca^{2+}, causing phosphorylation of membrane receptors and inhibiton of PLC (Woods *et al.*, 1987;Berridge and Irvine, 1989;Harootunian *et al.*, 1991). Support for the latter model comes from a recent report demonstrating an oscillatory translocation of an $InsP_3/PIP_2$-binding pleckstrin-homology (PH) domain from the cell periphery to the cytosol mirroring Ca^{2+} oscillations in epithelial cells following application of ATP (Hirose *et al.*, 1999). However, it seems unlikely that the PH-domain provides a faithful measure of $[InsP_3]$, since a subsequent study has reported that unphysiologically high concentrations of $InsP_3$ (introduced by photorelease of 'caged' $InsP_3$) are necessary to stimulate translocation of the PH-domain from the PM to the cytosol, though translocation can be triggered by physiological levels of

a



Cuthbertson R.K.S. & Cobbold P.H. (1985). Nature 316 p541-2

b



Woods N.M., Cuthbertson R.K.S. & Cobbold P.H. (1986). Nature 319 p600-2

Figure 1.2. Agonist-induced Ca^{2+} oscillations. (a) Sperm-induced Ca^{2+} transients in the mouse oocyte monitored using aequorin. (b) Ca^{2+} oscillations in a single hepatocyte in response to vasopressin. Notice that Ca^{2+} oscillations last for as long as the agonist is applied, and that the rate of oscillation increases with increasing concentration. Time in minutes.

bradykinin (van der Wal *et al.*, 2001). Such a result suggests that the PH-domain reports changes in plasmalemmal PIP_2 levels, rather than cytosolic $InsP_3$ (van der Wal *et al.*, 2001;Halet *et al.*, 2002). Development of a reliable reporter of $[InsP_3]_i$ in intact cells will be key in determining the role of $InsP_3$ metabolism in the generation of Ca^{2+} oscillations.

Specific patterns of oscillations can influence the downstream effects of Ca^{2+} release. By imposing various periods of oscillation upon jurkat T-cells using a Ca^{2+} clamp technique, one group demonstrated that whilst three Ca^{2+} sensitive transcription factors (NF-AT, NF-KB, and Oct/OAP) were all activated by high frequency (100s) oscillations, only NF-KB was still activated at oscillation periods of over 400 seconds. Since all three transcription factors were equally responsive to tonic changes in $[Ca^{2+}]_i$, this result demonstrates the ability of the frequency of Ca^{2+} oscillations to specifically activate downstream pathways (Dolmetsch *et al.*, 1998). Ca^{2+} oscillation frequency is also pertinent in mouse oocytes, since a critical number of transients is required to permit egg activation (Lawrence *et al.*, 1998;Ducibella *et al.*, 2002). The role of Ca^{2+} oscillations in oocytes is discussed at length below.

The ability to decode Ca^{2+} oscillation rate may be provided by Ca^{2+}-binding molecules, so called 'Ca^{2+} sensors'. One such example is the small Ca^{2+}-binding protein calmodulin (CaM). Binding of Ca^{2+} causes calmodulin to undergo conformational changes such that the Ca^{2+}-CaM complex binds and activates a variety of downstream effectors, such as protein kinases (Hoeflich and Ikura, 2002). One of the best characterised downstream targets of calmodulin is calmodulin-dependent kinase II (CaMKII), which in turn phosphorylates and activates downstream proteins with broad substrate specificity. Autophosphorylation of CaMKII at Thr286 is Ca^{2+}-CaM dependent and causes the activity of CaMKII to become independent of Ca^{2+}-CaM (Hudmon and Schulman, 2002). One recent study demonstrated that increasing the frequency of pulses of Ca^{2+} and calmodulin increases

the proportion of autonomous CaMKII in vitro (De Koninck and Schulman, 1998). Thus, in effect, high frequency Ca^{2+} oscillations cause CaMKII to be maintained in an active state. CaMKII is thought to be one of the principal effectors of $[Ca^{2+}]_i$ changes in oocytes (Lorca *et al.*, 1993), and its role in cell cycle progression will therefore be discussed in a later section.

1.3.4 Spatial organisation of Ca^{2+} signals: elementary Ca^{2+}-release events and Ca^{2+} waves

In addition to being temporally organised in the form of oscillations, recent experiments have revealed that Ca^{2+} transients can also have spatial organisation within a cell. This notion was first illustrated using the Ca^{2+}-sensitive luminescent protein aequorin to monitor the sperm-induced Ca^{2+} transient in oocytes of the medaka fish. Fertilisation of the medaka fish egg stimulates a wavefront of Ca^{2+} release which emanates from the point of sperm-entry and traverses the cell at a rate of around 10μm/second (Gilkey *et al.*, 1978). Use of confocal microscopy and fluorescent Ca^{2+} indicators has subsequently revealed that Ca^{2+} transients frequently progress as waves both in oocytes and in somatic cells (Berridge, 1997;Berridge *et al.*, 2000;Jaffe, 2002).

Key to the generation of a Ca^{2+} wave is the sensitivity of Ca^{2+} release channels to Ca^{2+} itself (see above). As a result, Ca^{2+} released from one receptor has the potential to activate its neighbours, triggering a regenerative wave capable of spreading throughout the cell (Berridge, 1997). In this respect the cytoplasm has been considered an 'excitable medium'; second messengers such as $InsP_3$ cause the Ca^{2+}-sensitivity of Ca^{2+}-channels to rise, permitting the generation of repetitive Ca^{2+} waves (Lechleiter *et al.*, 1991;Lechleiter and Clapham, 1992;Berridge, 1997). Recently, high resolution confocal microscopy has revealed smaller localised Ca^{2+} release events, so-called 'elementary' events, which have been proposed to underpin the generation of global Ca^{2+} waves (for review see Bootman *et al.*,

2001b). For example, release of low levels of $InsP_3$ (by photolysis of 'caged' $InsP_3$) in *Xenopus* oocytes triggers spatially restricted bursts of Ca^{2+}, termed Ca^{2+} 'puffs', which are thought to represent Ca^{2+} release from discrete clusters of $InsP_3Rs$ (Yao *et al.*, 1995; Parker and Yao,1996). Analogous elementary release events from ryanodine receptors have been described as Ca^{2+} 'sparks' (Cheng *et al.*, 1993;Klein *et al.*, 1996). In HeLa cells, increasing agonist (histamine) concentration causes elementary Ca^{2+} release events to become more frequent, greater in magnitude, and closer in the spatial domain (Bootman *et al.*, 1997). However, with increasing agonist concentrations a threshold is reached above which the cytoplasm becomes sufficiently excitable for elementary release events to trigger global Ca^{2+} waves (Bootman *et al.*, 1997). Thus, global Ca^{2+} waves occur as a result of coordinated recruitment of Ca^{2+} release channels by the process of Ca^{2+}-induced Ca^{2+}-release in a sufficiently excitable medium (Berridge, 1997).

Given that Ca^{2+} wave propagation occurs by a process diffusion of Ca^{2+} ions from one release site to the next, the rate of propogation of Ca^{2+} waves is independent of the extracellular agonist, and are determined by the properties of the cell, such as the distribution of Ca^{2+} release channels (Bugrim *et al.*, 1997). In the hepatocyte for example, though different concentrations of phenylephrine and vasopressin generate Ca^{2+} oscillations of varying frequencies, the velocity of the initial Ca^{2+} wave generated is unaffected by the magnitude of the agonist (Rooney *et al.*, 1990).

Whether an elementary Ca^{2+} release event remains localised or becomes global depends upon several factors, including the sensitivity and spatial distribution of release channels, luminal and cytosolic Ca^{2+} buffering, and the magnitude of the initial elementary events (Marchant *et al.*,1999;Marchant and Parker, 2001;Bootman *et al.*, 2001b;Dumollard *et al.*, 2002). These same factors can lead to areas of elevated sensitivity within a cell in which elementary Ca^{2+} release events preferentially occur (Petersen *et al.*, 1999;Dumollard *et al.*,

2002). This notion is elegantly demonstrated by the *Xenopus* oocyte in that global photorelease of 'caged' $InsP_3$ consistently provokes Ca^{2+} release in the ER and $InsP_3R$-rich oocyte cortex (Kume *et al.*, 1993;Kume *et al.*, 1997;Callamaras and Parker, 1999;Terasaki *et al.*, 2001). Similarly, stimulation of HeLa cells with histamine or ATP consistently evokes Ca^{2+} release in the (ER rich) perinuclear region (Lipp *et al.*, 1997;Thomas *et al.*, 2000). Such areas of elevated sensitivity to $InsP_3$ frequently serve as initiation sites for Ca^{2+} waves; in which case they can be referred to as Ca^{2+} pacemaker regions (Speksnijder, 1992;Dumollard *et al.*, 2002). The role of Ca^{2+} pacemakers in the mammalian oocyte is discussed later in this Introduction, and in Chapter 3.

In addition to triggering global Ca^{2+} transients, individual elementary Ca^{2+} release events have been shown to alter cell function locally. For example, in vascular smooth muscle cells Ca^{2+} sparks directly influence K^+ channel permeability and thus cell excitability (Perez *et al.*, 2001;Jaggar *et al.*, 2002). In HeLa cells, individual Ca^{2+} puffs in the peri-nuclear region have the potential to trigger sustained elevations of nuclear $[Ca^{2+}]$ ($[Ca^{2+}]_{Nu}$), which have been proposed to influence Ca^{2+}-sensitive processes within the nucleus (Lipp *et al.*, 1997). This $[Ca^{2+}]_{Nu}$ increase may be a result of diffusion from the peri-nuclear site, and its prolongued nature is probably due to the low buffering capacity within the nucleus (Fox *et al.*, 1997) and the absence of Ca^{2+}-ATPases on the inner nuclear envelope membrane (Humbert *et al.*, 1996). It is unlikely that a role for local cytoplasmic Ca^{2+} puffs in influencing nuclear function will prove universal, however, since localised Ca^{2+} changes in the pancreatic acinar cell usually occur far from the nucleus, and fail to affect $[Ca^{2+}]_{Nu}$ (Gerasimenko *et al.*, 1996;Lipp *et al.*, 1997).

1.3.5 The role of mitochondria in Ca^{2+} signalling

Recently it has become clear that uptake of Ca^{2+} into mitochondria may play an important role in determining the spatiotemporal dynamics of Ca^{2+} release events. Demonstration that the concentration of Ca^{2+} within the mitochondria ($[Ca^{2+}]_m$) changes during cell stimulation (in individual cells) was provided using a mitochondrially-targetted aeqouorin to measure $[Ca^{2+}]_m$ in HeLa cells; stimulation by histamine triggered a rapid rise in $[Ca^{2+}]_m$ (Rizzuto *et al.*, 1992). Similar increases in $[Ca^{2+}]_m$ are now known to occur following cell stimulation in several cell types, and it is widely believed that such increases reflect uptake of cytosolic Ca^{2+} by the mitochondrial Ca^{2+}-uniporter, as a result of Ca^{2+} release from the ER (see (Duchen, 1999;Duchen, 2000;Rizzuto *et al.*, 2000 for review). Since the Ca^{2+} uniporter is believed to have a low affinity for Ca^{2+}, mitochondrial Ca^{2+} uptake may be facilitated by close apposition of mitochondria and ER Ca^{2+} release sites, such that the Ca^{2+} uniporter is exposed to local microdomains of high $[Ca^{2+}]_i$ (Rizzuto *et al.*, 1998;Marchant *et al.*, 2002).

The physiological effects of mitochondrial Ca^{2+} uptake are essentially twofold. Firstly, since several dehydrogenase enzymes involved in the Kreb's cycle are Ca^{2+} sensitive, mitochondrial Ca^{2+} uptake influences the rate of oxidative phosphorylation (Duchen, 1999 for review). Accordingly, cytosolic Ca^{2+} oscillations cause levels of the metabolite NADH to oscillate in several cell types (Pralong *et al.*, 1992;Duchen, 1992;Hajnoczky *et al.*, 1995), and Ca^{2+}-releasing agonists have been shown to trigger increased ATP production in HeLa cells, as monitored by cytosolic and mitochondrially-targeted ATP-sensitive bioluminescent probes (Jouaville *et al.*, 1999). Thus mitochondrial Ca^{2+} uptake following cell stimulation may provide a mechanism whereby ATP production is upregulated to meet the increased metabolic demand of the cell. Secondly, mitochondrial Ca^{2+} uptake can influence the spatiotemporal dynamics of $[Ca^{2+}]_i$ release events. In pancreatic acinar cells $InsP_3$ stimulates local Ca^{2+} oscillations which are restricted to the granular apical region by a barrier of

mitochondria. Inhibition of mitochondrial function subsequently permits Ca^{2+}-release to propagate into the basal region and generation of a global Ca^{2+} wave (Tinel *et al.*, 1999). Thus mitochondria can act as spatial Ca^{2+} buffers. In addition it is thought that mitochondria closely associated with the ER may influence Ca^{2+} signalling by locally buffering Ca^{2+} in the vicinity of the release site. For example, in *Xenopus* oocytes increased mitochondrial function potentiates $InsP_3R$-mediated Ca^{2+} release by buffering $[Ca^{2+}]_i$ locally and thereby relieving the inhibitory effect of high $[Ca^{2+}]_i$ (Jouaville *et al.*, 1995). Conversely, in hepatocytes mitochondrial Ca^{2+} uptake is thought to provide a negative feedback upon the $InsP_3R$ by restraining CICR (Hajnoczky *et al.*, 1999). Thus whilst the effect of mitochondrial Ca^{2+} uptake may vary between cells, it is clear that mitochondria influence Ca^{2+} release from the ER. The role of mitochondria in Ca^{2+} signalling in mammalian eggs will be addressed in a later section.

1.4 Ca^{2+} signalling at fertilisation

As already discussed, the mouse egg was amongst the first cells in which Ca^{2+} oscillations were reported (Cuthbertson and Cobbold, 1985). At that time it had already been suspected for a number of years that Ca^{2+} may play a role at fertilisation, since microinjection of Ca^{2+} was known to cause egg activation (Steinhardt *et al.*, 1974;Fulton and Whittingham, 1978). Since these early studies it has become clear that the generation of repetitive Ca^{2+} transients at fertilisation is the necessary trigger for the resumption of meiosis, and the mechanisms underpinning their generation have been well studied. Since the varying nature of sperm-induced Ca^{2+} signals in different organisms has recently been reviewed (Stricker, 1999), the following account will focus primarily upon mammals.

1.4.1 Spatiotemporal dynamics of the fertilisation Ca^{2+} response in mouse

In mouse, the sperm-induced Ca^{2+} oscillations persist until around the time of pronucleus formation, some 3-4 hours after fertilisation (fig 1.3a; Jones *et al.*, 1995a;Day *et al.*, 2000;Deguchi *et al.*, 2000;Marangos *et al.*, 2003). The rising phase of the first Ca^{2+} transient consists of two steps. Initially a regenerative Ca^{2+} wave originating at the site of sperm-egg fusion propagates across the cytoplasm within 4-5 seconds (at a velocity of ~20μm/sec). Following a brief pause at a so-called shoulder point, maximal $[Ca^{2+}]_i$ is achieved by a spatially homogeneous increase in $[Ca^{2+}]_i$ throughout the cytoplasm (Deguchi *et al.*, 2000). The first Ca^{2+} transient is prolonged, lasting 3-5 minutes, with several small spikes detectable on top of the plateau (fig 1.3b). Subsequent Ca^{2+} transients are shorter in duration and of lesser amplitude than the initial fertilisation transient. The frequency and amplitude of oscillation remain relatively constant within any given egg (though may vary between eggs), the amplitude only becoming dampened shortly prior to pronucleus formation (Deguchi *et al.*, 2000).

1.4.2 The role of Ca^{2+} in egg activation

The crucial importance of the fertilisation Ca^{2+} response is illustrated by two principal lines of evidence. The first is that treatments which stimulate oscillatory, or even monotonic changes in $[Ca^{2+}]_i$, are capable of triggering cortical granule exocytosis and meiotic resumption (Steinhardt *et al.*, 1974;Fulton and Whittingham, 1978;Kline and Kline, 1992b;Swann and Ozil, 1994). Ca^{2+} can therefore be considered sufficient for egg activation. Conversely, activation is inhibited by treatments which prevent Ca^{2+} signals (Kline and Kline, 1992b;Lawrence *et al.*, 1998). This is most simply demonstrated by pre-incubating MII eggs with the membrane permeable Ca^{2+} chelator BAPTA-AM prior to insemination; a treatment which prevents both cortical granule exocytosis and meiotic resumption (Kline and

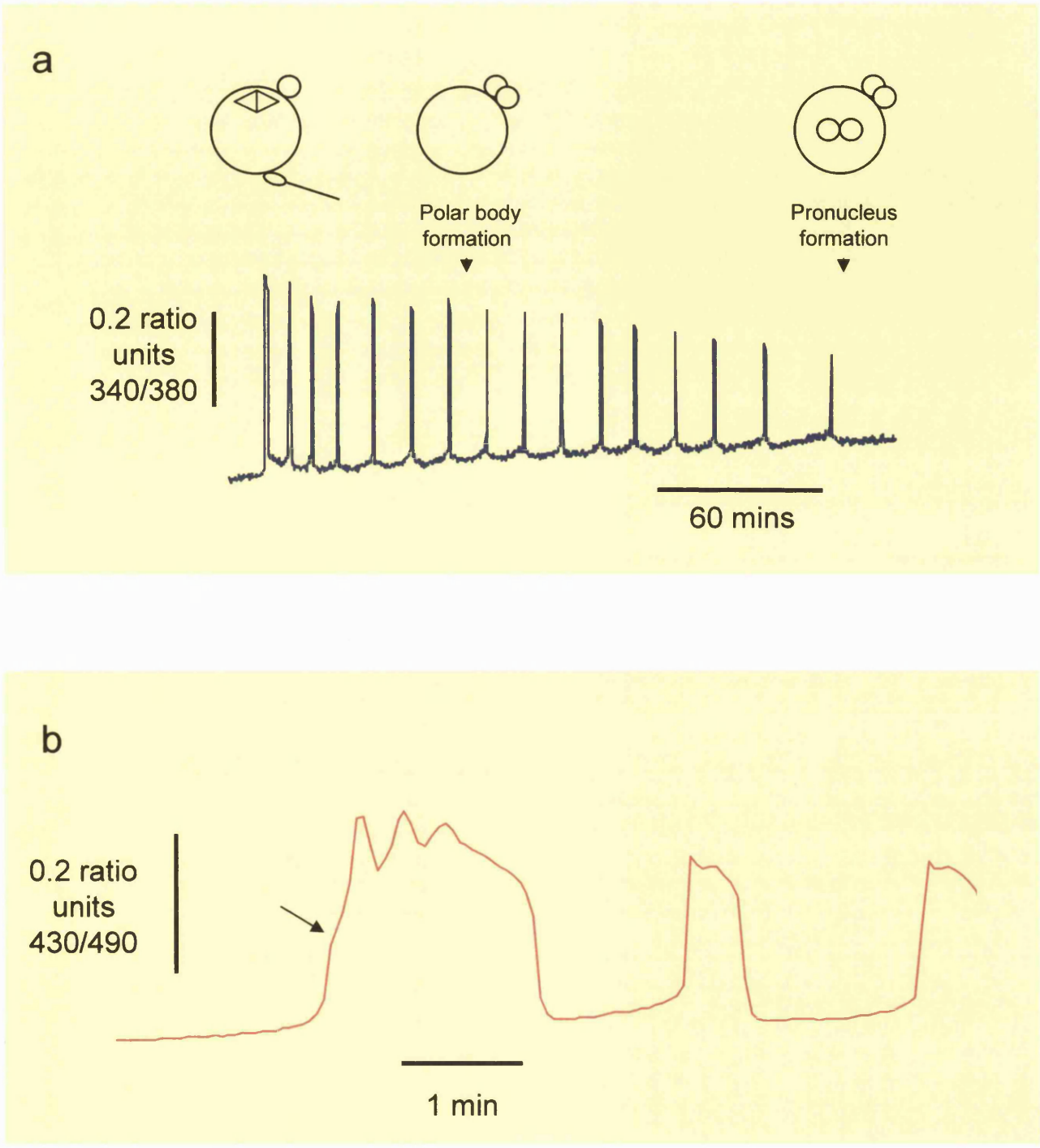


Figure 1.3. The fertilisation Ca^{2+} response in mouse. (A) Typical example of fertilisation-induced Ca^{2+} transients. Notice that the oscillations persist beyond the time of second polar body formation and end at approximately the time that the pronuclei form. $[Ca^{2+}]_i$ was monitored using Fura-2 (courtesy of Petros Marangos). (B) Comparison of the first two sperm-induced Ca^{2+} transients. Note that the initial fertilisation Ca^{2+} transient is longer than subsequent oscillations, and exhibits a characteristic shoulder point (arrow), and small Ca^{2+} spikes on the crest of the transient. Imaging performed using Fura-red (see Chapter 2).

Kline, 1992b;Xu *et al.*, 1996). Moreover, the importance of the oscillatory nature of the fertilisation Ca^{2+} signal is clear, since the rate of cell cycle progression and developmental competence have been shown to be affected by oscillation frequency (Bos-Mikich *et al.*, 1997;Ozil and Huneau, 2001;Ducibella *et al.*, 2002), and oocyte activation is inhibited if only the initial Ca^{2+} transients are permitted (Lawrence *et al.*, 1998). Thus Ca^{2+} oscillations are both necessary and sufficient for egg activation.

1.4.3 The mechanism of generation of sperm-induced Ca^{2+} oscillations

Support for a strategic role of $InsP_3$ in the fertilisation Ca^{2+} response is strong since oscillations are inhibited by $InsP_3$R-specific antibodies (Miyazaki *et al.*,1993;Goud *et al.*, 2002), by $InsP_3$ receptor downregulation (Brind *et al.*, 2000a;Xu *et al.*, 2003), or by the $InsP_3$ receptor antagonist heparin (Fissore and Robl, 1994;Fissore *et al.*, 1995). Futhermore, introduction of $InsP_3$ by microinjection (Miyazaki *et al.*,1993) stimulates Ca^{2+} release in mammalian oocytes, and the oscillatory pattern of fertilisation Ca^{2+} transients can be faithfully reproduced by continual photorelease of small amounts of caged $InsP_3$ (Jones and Nixon, 2000;Halet *et al.*, 2002). The latter result strongly implies that sperm-induced Ca^{2+} oscillations are driven by a stable rather than an oscillating level of $InsP_3$. That PIP_2 serves as the precursor of $InsP_3$ at fertilisation is suggested by the report that the PLC inhibitor U73122 inhibits oscillations (Dupont *et al.*, 1996). The source of PIP_2 is unclear however, since a recent report using a green fluorescent protein (GFP) -tagged PH-domain to measure plasmalemmal PIP_2 failed to uncover hydrolysis at fertilisation; rather a Ca^{2+}-dependent PIP_2-increase was reported (Halet *et al.*, 2002). Such an increase may mask any hydrolysis, especially if phosphoinositide (PI) turnover is small (Jones and Nixon, 2000;Halet *et al.*, 2002). An alternative possibility is provided by experiments which suggest that vesicles can act as a source of PIP_2 in the sea urchin egg homogenate system (Rice *et al.*, 2000). Further

work will be required to ascertain whether intracellular (rather than plasmalemmal) PIP_2 plays a role at fertilisation in the more physiological setting of the intact egg.

Recent work points towards a role for mitochondria in the generation of repetitive Ca^{2+} oscillations at fertilisation in mouse eggs. Inhibition of mitochondrial function is sufficient to terminate sperm induced Ca^{2+} oscillations, and measurement of $[Ca^{2+}]_m$ using Rhod-2 reveals $[Ca^{2+}]_m$ oscillations which reflect $[Ca^{2+}]_i$ at fertilisation (Liu *et al.*, 2001). Moreover, oocyte fragments which contain ER and mitochondria exhibit repetitive Ca^{2+} oscillations in response to Sr^{2+} similar to intact eggs, though fragments rich in ER but devoid of mitochondria exhibit only a monotonic rise in $[Ca^{2+}]_i$. Thus it has been suggested that mitochondrial Ca^{2+} uptake may be necessary for forming proper Ca^{2+} oscillations at fertilisation (Liu *et al.*, 2001). As yet there are no published reports as to whether mitochondrial Ca^{2+} uptake influences mitochondrial metabolism at fertilisation in mammals.

1.4.4 How does the sperm initiate Ca^{2+} oscillations?

The means by which sperm is able to trigger Ca^{2+} release in oocytes is highly controversial, and has been thoroughly reviewed (Swann and Ozil, 1994;Schultz and Kopf, 1995;Swann and Parrington, 1999;Runft *et al.*, 2002). Two main hypotheses predominate. The first is that the sperm interacts with a molecule on the egg surface, thus acting as an extracellular ligand, triggering hydrolysis of PIP_2 by PLC (Schultz and Kopf, 1995; see figure 1.4). Referred to as the 'receptor theory', this hypothesis came about following the discovery that phosphoinisitide turnover is increased at fertilisation in the sea urchin egg (Ciapa and Whitaker, 1986), and that the activation of G-proteins by GTPγS causes egg activation, as gauged by elevation of the fertilisation envelope (Turner *et al.*, 1986). In mammalian eggs however, though inhibition of G-proteins with GTPβS can inhibit the fertilisation Ca^{2+} response (Miyazaki, 1988), a similar result is not reproduced using functional inhibitory anti-

G protein antibodies (Williams *et al.*, 1998). More recent evidence for the receptor hypothesis was provided by reports that mammalian Src homology 2 (SH2) domain constructs, thought to bind the cytosolic domains of receptor tyrosine kinases and prevent PLCγ activation, inhibit sperm-induced Ca^{2+}-release and oocyte activation in sea urchin and starfish (Carroll *et al.*,1997;Carroll *et al.*, 1999). A role for PLCγ at egg activation in vertebrates seems unlikely, however, since SH2 domains fail to inhibit Ca^{2+} release in mouse and *Xenopus* oocytes (Runft *et al.*, 1999;Mehlmann *et al.*, 1998). Finally, the lack of a putative membrane receptor by which sperm can elicit Ca^{2+} release sheds considerable doubt upon the receptor hypothesis (Swann and Parrington, 1999).

The second major hypothesis is that a soluble factor (or sperm factor) diffuses from the sperm to initiate Ca^{2+} release in the egg (see figure 1.4). This hypothesis has been championed primarily by reports that homogenised sperm extracts (SE) trigger Ca^{2+} oscillations similar to those generated at fertilisation, and subsequently trigger egg activation when microinjected (Swann, 1990; for review see Swann and Parrington, 1999;Stricker, 1999;Fissore *et al.*, 1999a). That this phenomenon is explained by a sperm-specific soluble factor is corroborated by the finding that Ca^{2+} oscillations are also stimulated by injection of intact sperm (termed intra-cytoplasmic sperm injection, ICSI; Tesarik and Sousa, 1994;Sato *et al.*, 1999) or by spermatogenic (but not somatic) mRNA (Parrington *et al.*, 2000). The sperm-factor hypothesis is further supported by the occurrence of a latent period between sperm-egg binding and the initiation of Ca^{2+} release (Whitaker and Swann, 1993), and reports that cytoplasmic continuity occurs prior to the initiation of Ca^{2+} signals, allowing the passage of large molecular weight molecules between egg and sperm to precede Ca^{2+} release (Lawrence *et al.*, 1997;Jones *et al.*, 1998a).

A major barrier preventing widespread acceptance of the sperm factor theory has been the requirement for large amounts of sperm extract (more than one sperm equivalent) to

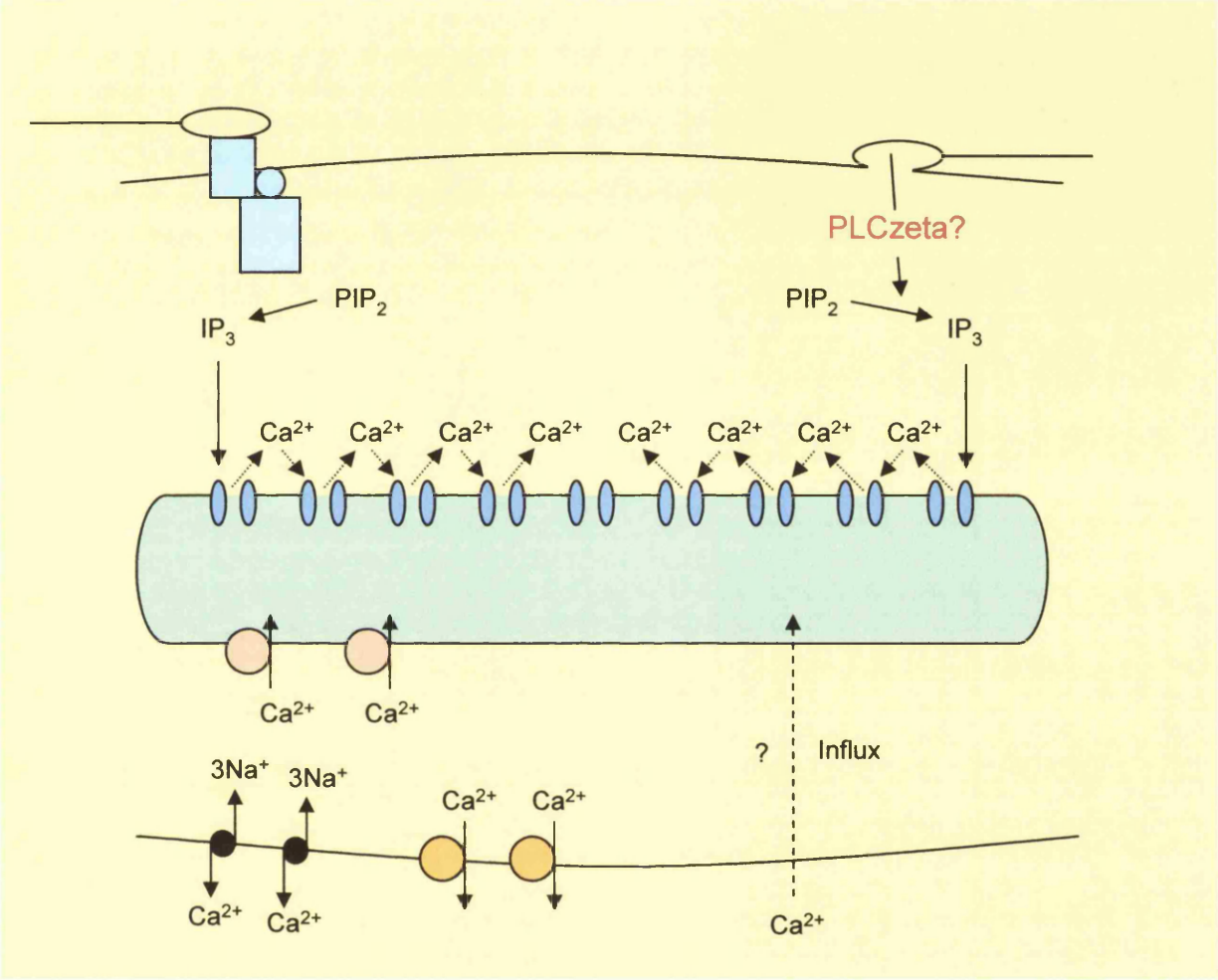


Figure 1.4. The mechanism of sperm-induced Ca^{2+} oscillations in mouse. Diagram illustrating the two major hypotheses for the generation of Ca^{2+} signals at fertilisation. On the left, sperm-interaction with a membrane receptor causes PLC activation and $InsP_3$ production (blue). Alternatively, introduction of a soluble factor, possibly the recently described PLCzeta, leads to $InsP_3$ production (right). $InsP_3$ triggers Ca^{2+} release from the ER (green) through $InsP_3$Rs and RyRs (dark blue). Ca^{2+} ions are removed from the cytosol by SERCA pumps (pink), plasma-membrane ATPases (orange), and Na^+/Ca^{2+} exchangers. A Ca^{2+} influx pathway has been reported in mouse eggs, the mechanism of which is unclear.

cause egg activation (Swann, 1990;Stricker, 1997;Kyozuka *et al.*, 1998). However, this phenomenon is most likely explained by a loss of protein/activity during purification (discussed by Swann *et al.*, 2001), and the sufficiency of a single sperm is clear, since Ca^{2+} oscillations are generated following ICSI (Tesarik and Sousa, 1994;Sato *et al.*, 1999).

1.4.5 The identity of the sperm factor

The past ten years have witnessed several attempts at identifying the sperm factor (reviewed by (Runft *et al.*, 2002). Early suggestions that Ca^{2+} (Jaffe, 1983) or $InsP_3$ (Iwasa *et al.*, 1990;Tosti *et al.*, 1993) stored within the sperm might be the responsible factor have since been discarded since microinjection of a single bolus of either is insufficient to trigger sustained oscillations (Igusa and Miyazaki, 1983;Miyazaki, 1988;Mehlmann and Kline, 1994).

One promising sperm factor candidate was a 33 kDa glucosamine-6-phosphate isomerase termed 'oscillin', which was purified to apparent homogeneity from hamster sperm. The fraction containing oscillin was found to posses Ca^{2+}-releasing ability when microinjected into unfertilised mouse eggs (Parrington *et al.*, 1996). However, microinjection of a recombinant oscillin protein fails to trigger Ca^{2+} oscillations (Wolosker *et al.*, 1998), and subsequent experiments demonstrated that the active fractions of sperm extracts do not always contain oscillin (Wu *et al.*, 1998;Parrington *et al.,* 1999). Later, a sperm specific truncated kit receptor (tr-kit) was reported to activate mouse eggs, presumably by activating phospholipase C (Sette *et al.*, 1997). Further work suggested that tr-kit might activate a PLCγ via a src-like kinase (Sette *et al.*,1998, 2002). These results seem unlikely to reflect physiological mechanisms, however, since Src homology 3 (SH3) domain constructs capable of inhibiting tr-kit mediated activation have no effect upon sperm-induced Ca^{2+}-release (Mehlmann *et al.*, 1998). Crucially, it is not known whether tr-kit stimulates Ca^{2+}-oscillations

when microinjected into oocytes, the key criterion by which a sperm-factor candidate should be judged (discussed by Swann and Parrington, 1999).

More recently, nitric oxide (NO) production was proposed to play a role in egg activation, as high levels of NO synthase activity are present in sperm, and since the NO-scavenger oxyhaemoglobin prevents activation of sea urchin eggs (Kuo *et al.*, 2000). However, fertilisation is not associated with an increase in [NO] in mouse or ascidian eggs, nor do NO-scavengers inhibit fertilisation Ca^{2+} release (Hyslop *et al.*, 2001). Further work in the sea urchin egg revealed that NO modulates rather than initiates Ca^{2+} release (Leckie *et al.*, 2003). Depolymerisation of the actin cytoskeleton was also recently forwarded as a potential mechanism of Ca^{2+} release at fertilisation, since the potent actin depolymerising agent latrunculin A was reported to trigger fertilisation-like Ca^{2+} release in starfish oocytes (Lim *et al.*, 2002). A universal role for such a mechanism seems unlikely however, since latrunculin A does not activate mouse eggs (GF and JC unpublished data).

Most recently, a novel sperm-specific phospholipase C isoform, termed PLCzeta, has been identified and proposed as the sperm factor (Cox *et al.*, 2002;Saunders *et al.*, 2002). PLCzeta appears to stimulate Ca^{2+} transients remarkably similar to those generated at fertilisation at levels approximately equivalent to one sperm. Moreover, removal of PLCzeta from sperm extracts by immunodepletion eliminates the Ca^{2+} releasing ability (Saunders *et al.*, 2002). The claim of PLCzeta is enhanced by its compliance with several prior clues as to the molecular nature of the sperm factor. Firstly, the active factor must be a protein since the Ca^{2+} releasing properties of sperm extracts are sensitive both to heat and to trypsin proteases (Wu *et al.*,1998;Swann, 1990;Kyozuka *et al.*, 1998). Secondly, PLCzeta has a molecular weight of 74kDa, which is roughly in the order predicted by fractionation experiments; sperm factor activity having been found in the 30-70kDa fraction (Saunders *et al.*, 2002;Parrington *et al.*, 2002). Thirdly, and perhaps most compellingly, a study using boar sperm extract and

sea urchin egg homogenate reported that pre-incubation of SE with PIP_2 results in significantly amplified Ca^{2+} release when extract is subsequently added to the homogenate (Jones *et al.*, 1998b), suggesting that a PLC activity resides within the sperm itself. Since conventional PLC isoforms fail to stimulate Ca^{2+} release when microinjected into mammalian eggs (Jones *et al.*, 2000), this report suggests that sperm contain a novel PLC activity.

As such, PLCzeta represents the most promising sperm factor candidate. Conclusive evidence would be provided by demonstration that PLCzeta transfers from sperm to egg prior to the initiation of Ca^{2+} release, and by production of a PLCzeta-null mouse which would, if PLCzeta is indeed the sperm-factor, be infertile.

1.4.6 Ca^{2+} oscillations trigger the inactivation of cdk1-cyclin B activity and MAPK

Though the mechanisms underpinning Ca^{2+} release at fertilisation are well studied, the means by which they trigger meiotic resumption are poorly understood. As discussed above, the metaphase II arrest is maintained by elevated cdk1-cyclin B activity, courtesy of high MAPK activity. Exit from metaphase II is permitted by cyclin B destruction and and thus a decrease in cdk1-cyclin B activity (Kubiak *et al.*, 1993). Cyclin B destruction is triggered by ubiquitination of its destruction-box sequence by the anaphase promoting complex (APC), ubiquitinated cyclin subsequently being subjected to proteolysis by the 26S proteosome (Glotzer *et al.*, 1991;Townsley and Ruderman, 1998).

Several lines of evidence link Ca^{2+} release at fertilisation to the cyclin destruction pathway. Ca^{2+} is sufficient to cause cyclin B destruction and metaphase exit (Lorca *et al.*,1991;Lorca *et al.*, 1993;Winston *et al.*, 1995;Collas *et al.*, 1995), provided there is an intact spindle (Winston *et al.*, 1995). Moreover, recent evidence suggests that individual Ca^{2+} transients trigger incremental bursts of cyclin destruction in mouse eggs (Nixon *et al.*, 2002).

Further, the activity of the 26S proteosome responsible for cyclin proteolysis has been shown to be sensitive to $[Ca^{2+}]_i$ (Kawahara and Yokosawa, 1994;Aizawa *et al.*, 1996).

The ability of Ca^{2+} release to cause cyclin destruction may be mediated by CaMKII. Studies in *Xenopus* oocytes demonstrated that $[Ca^{2+}]_i$ stimulates cyclin destruction at fertilisation by activating CaMKII (Lorca *et al.*, 1993). A constitutively active CaMKII mutant inactivates cdk1-cyclin B and causes parthenogenetic activation, while a peptide sequence which binds the autoinhibitory site and thus inactivates CaMKII prohibits Ca^{2+}-activated cdk1-cyclin B inactivation (Lorca *et al.*, 1993). A similar mechanism appears likely in mouse, since egg activation is inhibited by the calmodulin inhibitor W7 (Xu *et al.*, 1996), and exposure to ethanol (which produces a single monotonic Ca^{2+} rise and activates eggs) causes transient CaMKII activation (Winston and Maro, 1995).

In mouse, destruction of sufficient cyclin B to permit activation is dependent upon the oscillatory nature of Ca^{2+}-release at fertilisation, since meiotic resumption is inhibited if Ca^{2+} transients are infrequent or precociously curtailed (Lawrence *et al.*, 1998;Ducibella *et al.*, 2002). This is most likely because sustained CamKII activity requires repetitive Ca^{2+} oscillations; individual monotonic $[Ca^{2+}]_i$ rises cause only a transient period of CaMKII activation (Winston and Maro,1995;Tatone *et al.*, 2002). Thus, to summarise, whilst it is not known whereabouts in the APC/proteosome pathway Ca^{2+}-CaM-CamKII acts (Nixon *et al.*, 2002), it seems likely that sperm-induced Ca^{2+} oscillations serve to stimulate meiotic resumption by degradation of cyclin B through CamKII-dependent activation of the APC/proteosome pathway.

Following fertilisation, the level of cdk1 activity decreases at about the time of Pb2 extrusion. MAPK activity subsequently declines at the time of Pn formation, 2-3 hours later (Verlhac *et al.*, 1994;Moos *et al.*, 1995;Moos *et al.*, 1996). This timecourse appears paradoxical, since CSF activity was first described as an activity capable of preventing MPF

destruction (Masui and Markert, 1971; discussed above). Moreover, MAPK activity can decrease in the presence of high cdk1-cyclin B activity if oocytes are treated with the microtubule depolymerising agent nocodazole, demonstrating that neither kinase activity is alone sufficient to maintain the activity of the other (Moos *et al.*, 1995). Though these phenomena remain poorly explained, it appears that fertilisaton Ca^{2+} signals might override the stabilising effect of MAPK upon cyclin B, permitting cyclin destruction in the face of high MAPK activity.

1.5 Regulation of Ca^{2+} release during oocyte maturation and following fertilisation

As outlined above, sperm-egg fusion triggers a series of repetitive Ca^{2+} oscillations, the fidelity of which is a determinant both of egg activation and of further developmental potential. It is therefore imperative that the mature oocyte respond to the fertilising sperm by generating Ca^{2+} transients of the correct magnitude and frequency over the appropriate timecourse. The following sections will therefore consider the mechnisms controlling the sensitivity of Ca^{2+} release in the mouse oocyte in the prelude to, and following fertilisation.

1.5.1 Development of Ca^{2+}-release mechanisms during oocyte maturation

Given the dramatic nature and crucial importance of Ca^{2+} release at fertilisation (Ca^{2+} transients being both necessary and sufficient for egg activation), it seems intuitive that the mature, fertilisation competent egg should posses finely tuned Ca^{2+} signalling machinery capable of supporting such a signal. Thus it is perhaps not surprising that in all species studied so far an increase in the sensitivity of Ca^{2+} release mechanisms occurs during oocyte maturation (for review see Carroll *et al.*, 1996). This notion is most clearly demonstrated by comparison of the fertilisation Ca^{2+} response in immature and mature oocytes. This

experiment was first carried out in starfish oocytes, in which ten sperm entries are required to produce a similar Ca^{2+} response in immature oocytes as one sperm in the mature oocyte (Chiba *et al.*, 1990). In mouse, insemination of GV stage oocytes yields an initial fertilisation transient which is both of lower amplitude and shorter duration than that triggered in mature MII oocytes (Mehlmann and Kline, 1994;Jones *et al.*, 1995b;Cheung *et al.*, 2000). Moreover, one group reported that the number of transients subsequently generated is significantly reduced in GV oocytes (Jones *et al.*, 1995b;Cheung *et al.*, 2000). Similar comparisons using sperm extract injection revealed that the initial Ca^{2+} transient in GV oocytes is not only smaller and shorter, but also spatially homeogeneous, whereas Ca^{2+} release in the MII egg takes the form of a regenerative wave (Carroll *et al.*, 1994); a similar disparity is also observed following fertilisation of hamster oocytes (Shiraishi *et al.*, 1995).

Several clues exist as to the mechanisms underpinning these changes. Oocyte maturation is associated with a 1.5-2 fold increase in $InsP_3R$ expression in mammals (Mehlmann *et al.*, 1996;Parrington *et al.*, 1998; Fissore *et al.*, 1999b), a change that correlates with increased Ca^{2+} release in response to $InsP_3$ microinjection (Fujiwara *et al.*, 1993;Mehlmann and Kline, 1994). A causal role for $InsP_3R$ upregulation in the generation of competent Ca^{2+} release machinery was recently shown in a study which demonstrated that oocytes in which $InsP_3$ receptor upregulation is prohibited during maturation by $InsP_3R$-1 double stranded RNA (dsRNA) exhibit fewer fertilisation Ca^{2+} transients, and secrete less cortical granules in response to $InsP_3$ microinjection than control oocytes (Xu *et al.*, 2003). Whilst the timecourse of this enhancement of $InsP_3$-mediated Ca^{2+} release sensitivity has not been studied in mouse, in hamster oocytes the increase occurs in two distinct phases; the magnitude of response to large doses of $InsP_3$ increases around the time of GVBD, while the magnitude of Ca^{2+} release in response to smaller doses of $InsP_3$ increases later, around the time of Pb1 extrusion (Fujiwara *et al.*, 1993).

Since Ca^{2+} release through $InsP_3$ receptors is sensitive not only to $InsP_3$ but also to cytosolic and luminal (ER) $[Ca^{2+}]$ (see above), alterations to other components of the Ca^{2+} signalling machinery may contribute to the increased sensitivity of $InsP_3$ gated Ca^{2+} release during maturation. The magnitude of Ca^{2+} release in response to thapsigargin and ionomycin (thought to reflect the Ca^{2+}-content of the ER and all internal stores respectively) is greatly increased in MII compared to GV oocytes (Tombes *et al.*, 1992;Jones *et al.*, 1995b), and modifications of plasma-membrane Na^{+}/Ca^{2+} exchange also occur during maturation (Carroll, 2000).

Less is known about the effect of oocyte maturation upon the sensitivity of Ca^{2+} induced Ca^{2+} release (CICR). Though ryanodine receptors are not detected in hamster eggs (Miyazaki *et al.*, 1992), mouse oocytes do contain RyRs, the spatial distribution of which changes during maturation (Ayabe *et al.*, 1995). Moreover, an increase in the sensitivity of CICR is alluded to by an increase in the size of ryanodine-releasable Ca^{2+}-stores during maturation (Jones *et al.*, 1995b). Since CICR is considered responsible for regenerative Ca^{2+} release (discussed above), changes in the number, distribution and properties of RyRs (as well as $InsP_3Rs$) may contribute to the switch from homogeneous release (GV oocyte) to propagating waves (MII egg; Carroll *et al.*, 1994;Shiraishi *et al.*, 1995).

An alternative explanation for the exclusivity of propagating waves to mature eggs involves changes in the structure of the endoplasmic reticulum. In mammals the ER undergoes dramatic spatial reorganisation during oocyte maturation, MII oocytes exhibiting cortical accumulations (or clusters) of ER not present at GV stage (Mehlmann *et al.*, 1995;Shiraishi *et al.*, 1995). These ER clusters may house corresponding clusters of IP_3Rs (Mehlmann *et al.*, 1996;Fissore *et al.*, 1999b). Since in mouse eggs Ca^{2+} waves emanate from the ER-rich vegetal cortex regardless of the site of sperm entry, SE introduction, or $InsP_3$ injection (Kline *et al.*, 1999;Oda *et al.*, 1999;Deguchi *et al.*, 2000), cortical clusters of ER

have been proposed to form Ca^{2+} pacemaker sites (Kline *et al.*, 1999;Kline, 2000;Dumollard *et al.*, 2002). In addition, ER reorganisation during oocyte maturation has been implicated in the overall increase in the sensitivity of Ca^{2+} release (Mehlmann *et al.*, 1995;Stricker *et al.*, 1998;Kline, 2000), though precisely why clustering of ER should provide a favourable environment for Ca^{2+} release remains unclear (discussed further in Chapter 3). As yet the mechanism and timecourse of ER reorganisation relative to Ca^{2+} release have not been studied.

The importance of maturation-associated increases in Ca^{2+}-release sensitivity is alluded to by the impaired ability of GV oocytes to undergo CGE, though modifications to Ca^{2+}-independent factors may also contribute (Ducibella and Buetow, 1994). Moreover, since the ability to generate proper repetitive Ca^{2+} transients at fertilisation is a determinant of developmental competence (Ozil and Huneau, 2001;Ducibella *et al.*, 2002), acquisition of Ca^{2+} releasing ability during oocyte maturation undoubtedly represents a key step in the production of a competent oocyte.

1.5.2 Regulation of sperm-induced Ca^{2+} oscillations

As discussed earlier, fertilisation-induced Ca^{2+}-transients continue for around four hours in mouse, ending around the time of pronucleus formation (Jones *et al.*, 1995a;Day *et al.*, 2000;Deguchi *et al.*, 2000;Marangos *et al.*, 2003). The importance of such long lasting repetitive oscillations has also been noted. However, the mechanisms which underpin the continued generation and (perhaps more pertinently) the subsequent cessation of sperm-induced Ca^{2+} oscillations are unclear. Several candidate mechanisms have been proposed:

A role for cdk1-cyclin B activity in control of sperm-induced Ca^{2+} oscillations is suggested by the observation that oscillations continue if fertilised eggs are arrested in M-phase (Jones *et al.*, 1995a;Marangos *et al.*, 2003). Furthermore, the cdk1 inhibitor roscovitine

is sufficient to prevent or curtail Ca^{2+} release (Deng and Shen, 2000). The relationship between cdk1-cyclin B activity and Ca^{2+} release is most clear in ascidian oocytes. In this species fertilisation triggers two distinct series of oscillations, the generation of which is closely coupled to cdk1-cyclin B activity; exogenous cyclin causes prolonged oscillations and abolition of the gap-period between the two oscillation groups, the oscillations subsequently ending when cyclin is eventually destroyed (McDougall and Levasseur, 1998;Levasseur and McDougall, 2000). However, the correlation is not as tight in mouse oocytes, since cdk1-cyclin B activity declines around the time of Pb2 extrusion (Verlhac *et al.*, 1994;Moos *et al.*, 1995;Moos *et al.*, 1996), some 2 hours prior to cessation of the oscillations (Jones *et al.*, 1995a;Day *et al.*, 2000;Deguchi *et al.*, 2000;Marangos *et al.*, 2003). In light of this discrepancy it seems unlikely that cdk1-cyclin B activity should prove responsible for controlling the timecourse of fertilisation oscillations.

In mouse, a better correlation exists between cessation of fertilisation Ca^{2+} oscillations and inactivation of MAPK, since MAPK activity decreases around the time of Pn formation (Moos *et al.*, 1995;Moos *et al.*, 1996). It is unlikely that this decrease is responsible for the pattern of Ca^{2+} transients generated at fertilisation, however, since the MAPK inhibitor UO126 does not prevent sperm-induced Ca^{2+} oscillations (Marangos *et al.*, 2003).

Modifications to the Ca^{2+} signalling machinery may play a role in the temporal organisation of the fertilisation Ca^{2+} response. Fertilisation prompts $InsP_3$ receptor downregulation (He *et al.*, 1999;Jellerette *et al.*, 2000;Brind *et al.*, 2000) which may explain the reduced sensitivity of $InsP_3$-mediated Ca^{2+} release reported in pronucleate embryos (Jones *et al.*, 1995a;Jones and Whittingham, 1996). However, it is unlikely that this downregulation is responsible for the cessation of Ca^{2+} oscillations since the vast majority of $InsP_3R$ downregulation occurs within two hours of fertilisation (Brind *et al.*, 2000), and since

oscillations persist for many hours in eggs arrested in MII using colemid (Jones *et al.*, 1995a). A detailed study of the relationship between the meiotic and mitotic cell cycle and the sensitivity of $InsP_3$ mediated Ca^{2+} release in mouse is yet to be carried out. Another component of the Ca^{2+} homeostatic machinery which may be involved in terminating sperm-induced Ca^{2+} oscillations is the endoplasmic reticulum. One study noted a dramatic ER reorganisation coincident with cessation of fertilisation Ca^{2+} oscillations in oocytes of the Nemertean worm *Cerebratulus lacteus* (Stricker *et al.*, 1998), prompting the suggestion that changes in ER structure may control the timecourse of Ca^{2+} oscillations (Stricker *et al.*, 1998;Kline, 2000). Whilst it has been noted in mouse that no changes occur during the first 7 oscillations following fertilisation (Kline *et al.*, 1999), the spatial organisation of the ER during the later events of activation, including Pb2 extrusion and Pn formation has not been examined in mammals.

Most recently it has been suggested that the duration of the fertilisation Ca^{2+} response in mouse might be regulated by the formation of the pronuclei (Pn), since treatments which prevent pronucleus formation without interfering with cdk1-cyclin B or MAPK activity lead to persistent oscillations (Marangos *et al.*, 2003). Drawing upon a prior report in which pronuclei from fertilised (but not parthenogenetically activated) embryos were shown to have the ability to trigger Ca^{2+} release when transferred to recipient eggs (Kono *et al.*, 1995), and given the close temporal relationship between cessation of Ca^{2+} oscillations and Pn formation, this study proposed that sequestration of a cytoplasmic factor into the developing pronuclei may be the critical step which draws sperm-induced Ca^{2+} oscillations to a close. The evidence both for and against this model will be discussed at length in Chapters 5 and 6.

1.6 Ca^{2+} release in mitosis

1.6.1 Role of Ca^{2+} in mitosis in sea urchin embryos and somatic cells

Whilst exit from meiosis II (at fertilisation) remains by far the best studied example of a role for Ca^{2+} in cell cycle progression, there is much evidence that Ca^{2+} serves to trigger cell cycle transitions during mitosis also. Though a role for Ca^{2+} in mitosis had previously been speculated (Clothier and Timourian, 1972;Timourian *et al.*, 1972), the advent of the Ca^{2+} indicator Fura-2 provided the first report of mitotic Ca^{2+} transients in the sea urchin embryo (Poenie *et al.*, 1985;Grynkiewicz *et al.*, 1985). In this cell type nuclear envelope breakdown (NEBD), the metaphase-anaphase transition and cytokinesis are all associated with transient rises in $[Ca^{2+}]_i$ (Poenie *et al.*, 1985), and their respective roles in cell cycle progression have been studied. Microinjection of Ca^{2+} chelator prior to NEBD is sufficient to prohibit mitosis entry, an effect reversible by microinjection of Ca^{2+} or $InsP_3$ (Twigg *et al.*, 1988;Steinhardt and Alderton, 1988), or by photolysis of the chelator (Wilding *et al.*, 1996). Thus Ca^{2+} release is necessary and sufficient for NEBD in sea urchin embryos. There is strong evidence that Ca^{2+} exerts its effect through CamKII at NEBD, since mitosis entry is inhibited by calmodulin inhibitory peptides (Torok *et al.*, 1998) or by antibodies raised against CamKII (Baitinger *et al.*, 1990). A similarly causal role for Ca^{2+} has been established at the metaphase-anaphase transition. In this case, the onset of anaphase is accompanied by a global Ca^{2+} transient and is reversibly inhibited by Ca^{2+} chelators or heparin. Spindle forces continue to be generated under these conditions, however, suggesting that the role of Ca^{2+} is to permit disjunction of the sister chromosomes (Groigno and Whitaker, 1998). The role of Ca^{2+} at cytokinesis is less well studied in sea urchin, though in *Xenopus* embryos cytokinesis is associated with a local $[Ca^{2+}]_i$ rise which precedes the cleavage furrow; inhibition of which impedes cell division (Fluck *et al.*, 1991;Snow and Nuccitelli, 1993;Chang and Meng, 1995;Muto *et al.*, 1996).

Cell cycle Ca^{2+} transients in sea urchin are apparently $InsP_3$ driven. Measurement of $InsP_3$ reveals cyclic increases corresponding to specific cell cycle events (Ciapa *et al.*, 1994). Moreover, the $InsP_3R$ antagonist heparin, and lithium (which inhibits phosphoinositide turnover), are both capable of blocking NEBD and anaphase onset (Wilding *et al.*, 1996;Becchetti and Whitaker, 1997;Groigno and Whitaker, 1998). In addition, removal of extracellular Ca^{2+} has no effect upon cell cycle progression, demonstrating a reliance upon intracellular (as opposed to extracellular) stores (Wilding *et al.*, 1996).

The role of Ca^{2+} in mitosis in somatic cells is less clear. Ca^{2+} transients have been reported in mitosis in several cells (Poenie *et al.*, 1986;Ratan *et al.*, 1988;Kao *et al.*, 1990), blockade of which by Ca^{2+} chelators reversibly inhibits mitosis entry (Kao *et al.*, 1990). However, several studies have also reported that mitosis can occur in the absence of $[Ca^{2+}]_i$ changes (Tombes and Borisy, 1989;Kao *et al.*, 1990;Whitaker and Larman, 2001). As such, the explanation for this variation is unclear, though localised $[Ca^{2+}]$ changes have been observed during mitosis (Keith *et al.*, 1985;Ratan *et al.*, 1986;Wilding *et al.*, 1996), and it has been argued that such events may be sufficient for cell-cycle progression, and may in some cases go undetected (Whitaker and Patel, 1990;Hepler, 1994). This notion is discussed further below.

1.6.2 Role of Ca^{2+} in the first mitotic division in mouse

A role for Ca^{2+} in mitotic progression in the early mouse embryo is suggested by the finding that nuclear envelope breakdown of the first mitotic division (pronuclear breakdown) is accompanied by a Ca^{2+}-transient, inhibition of which using the Ca^{2+} chelator BAPTA-AM causes cell cycle arrest (Tombes *et al.*, 1992;Kono *et al.*, 1996). Perhaps paradoxically, BAPTA-AM also prohibits NEBD in parthenogenetically activated embryos, in which

nuclear breakdown occurs in the absence of any detectable $[Ca^{2+}]i$ change (Kono *et al.*, 1996).

The reason for this disparity is not yet clear, though two (not necessarily exclusive) hypotheses have been considered. The first comes by way of analogy with NEBD in the sea urchin, in which global Ca^{2+} transients only occur in about half of all cases. In the other half, high magnification confocal microscopy reveals local areas of elevated $[Ca^{2+}]$ around the nucleus, not distinguishable by conventional microscopy (Wilding *et al.*, 1996). Thus it has been argued that NEBD in mouse might be triggered by a similar localised Ca^{2+} release event, which somehow becomes amplified in the presence of a fertilising sperm (Kono *et al.*, 1996).

A second possibility draws from observations that pronuclei transferred from fertilised embryos stimulate Ca^{2+} release and activate recipient eggs when their nuclear membranes subsequently break down. A similar Ca^{2+} releasing ability is not present within nuclei of parthenogenetically activated embryos (Zernicka-Goetz *et al.*, 1995;Kono *et al.*, 1995;Ogonuki *et al.*, 2001). Thus it has been argued that the global Ca^{2+} transient associated with NEBD in the fertilised embryo occurs as a consequence of NEBD, as opposed to serving as its trigger (Kono *et al.*, 1996). These hypotheses will be discussed at length in Chapters Five and Six.

It is not yet known whether detectable Ca^{2+} transients accompany the second or subsequent embryonic divisions in mouse.

1.7 Synopsis

The aim of this thesis is to investigate the relationship between the cell cycle, ER structure, and Ca^{2+} release in the mouse oocyte and early embryo. Experiments presented in Chapter 3 examine the timecourse and mechanisms of ER reorganisation during oocyte maturation and

compare the upregulation in the responsiveness of Ca^{2+} release duing meiosis I with the appearance of ER cortical clusters. Continuing with this theme, Chapter 4 investigates ER structure and the responsiveness of $InsP_3$-induced Ca^{2+} release following fertilisation and during the first mitotic division, in order to test the hypothesis that structural changes of the ER may determine the pattern of Ca^{2+}-release at fertilisation. The aim of Chapter 5 is to establish the relationship between Ca^{2+} release and cell cycle progression during the first mitotic divisions in mouse. Finally, Chapter 6 summarises the data presented and proposes a model of the mechanisms governing Ca^{2+} release in the mammalian oocyte.

2. Materials and methods

2.1 Mice

Oocytes, eggs and one cell embryos were recovered from 21-24 day old female MF1 mice (Harlan, UK), F1 (C57B1xCBA; JAX, ME, USA), LTXBO (Hirao and Eppig, 1997a), or $mos^{-/-}$ mice (Colledge et al., 1994; Hirao and Eppig, 1997b). MF1 mice were used unless otherwise stated. MF1 male mice of proven fertility were used for in vitro fertilisation and mating (Harlan, UK).

2.2 Oocyte and embryo collection

Immature (germinal vesicle stage) oocytes were retrieved from the ovaries of 21-24 day old mice that had been administered a 7 IU intraperitoneal injection of pregnant male serum gonadotrophin (PMSG; Intervet) 48 hours earlier. Mice were culled by cervical dislocation. Ovaries were released into warmed hepes-buffered KSOM medium (Lawitts and Biggers, 1983;Summers *et al.*, 2000) containing 1mg/ml fraction V BSA (Sigma Chemicals, Poole, Dorset, UK) with 200μM IBMX (Sigma UK) to prevent germinal vesicle breakdown, and maintained at 37°C. Oocytes were recovered by puncturing the surface of the ovary with a 27-gauge needle, collected using a mouth-operated pipette, and placed in drops of media under oil to prevent evaporation (Mineral oil; embryo tested, Sigma UK). Only oocytes with an intact layer of cumulus cells were recovered, and cumulus cells were subsequently removed by repeated pipetting with a narrow pipette. Oocytes 3, 6, and 9h after administration of human Chorionic Gonadotrophin (hCG; see below) were recovered by the same method.

To recover mature (MII) oocytes, human Chorionic Gonadotrophin (hCG; Intervet, Milton Keynes, UK) was administered 48 hours after PMSG. Mice were culled and

oviducts removed 14-16 hours post-hCG. Cumulus masses were released into hepes-buffered KSOM by tearing the oviduct using forceps. Cumulus cells were removed by addition of hyalauronidase (300μg/ml; embryo tested grade, Sigma UK) to the media. Following recovery, oocytes were washed through at least three drops of hyalauronidase-free media under oil.

For recovery of pronucleate embryos, female mice were mated with males at the time of hCG administration. Cells were recovered from the oviduct by the same method as for mature oocytes, 27-28h after hCG and mating. Embryos at the two-cell stage were recovered 48 hours after hCG and mating.

2.3 *In vitro* maturation (IVM) of oocytes

In vitro maturation experiments were performed in the lab of Dr John Eppig, Jackson Laboratory, Bar Harbour, Maine. For *in vitro* maturation, cumulus-intact oocytes were placed in 1.5ml drops of either Waymouths media with 10% fetal bovine serum (Sigma Chemicals, St Louis, MO) with 100 IU/l human recombinant FSH (hFSH; National Hormone and Pituitary Program, NIDDK; O'Brien *et al.*, 2003), or in minimum essential media (MEM; all components from Sigma MO) with BSA (3mg/ml; ICN Immunochemicals, Lisle, IL; Hirao and Eppig, 1999). Culture was performed at 37°C in an atmosphere of 5%CO_2, 5%O_2 and 90%N_2. Oocytes were cultured for 16 hours prior to transfer into M2 media (Fulton and Whittingham, 1978) containing 4mg/ml BSA.

2.4 *In vitro* fertilisation (IVF) and parthenogenetic activation

For zona-free IVF, sperm was released from the epididymi of an F1 male into T6 media (Quinn *et al.*, 1982) containing 10mg/ml BSA (Fraction V, Sigma UK) which had been pre-equilibrated to pH7.6 at 37°C, 5%CO_2 in air. Following a 20-30 minute swim-out period,

100µl of sperm solution was added to 100µl T6 under oil, and the resulting 1 in 2 dilution was placed in the incubator for 2 hours to allow capacitation. The zona pellucida was removed from cumulus-free mature oocytes by a brief exposure to an acidified Tyrodes solution (Sigma UK) followed by repeated washing in KSOM-hepes. For IVF on the microscope stage, zona-free oocytes were transferred to 0.5ml BSA-free H-KSOM on the microscope stage to allow the oocytes to adhere to the coverslip. After 5-10 minutes 0.5ml of BSA-containing media was added to the chamber, followed by 10-15µl of the capacitated sperm.

For IVF of zona-intact oocytes, sperm was released into a 1ml drop of fertilisation media (Cook UK, Herts, United Kingdom) under oil, pre-equilibrated at 6%CO_2, 37°C. After 20 minutes, 10 µl of the sperm suspension was added to 90ul drops of equilibrated fertilisation media under oil to produce a one in 10 dilution. Following a 90-minute capacitation period, insemination was performed by transfer of cumulus masses to the dilute sperm suspension. An insemination period of 2 hours was allowed, after which cumulus-free oocytes were collected and washed in further drops of pre-equilibrated fertilisation media (sperm-free). IVF was performed 17-18 hours after administration of hCG both for zona-intact and zona-free IVF.

Parthenogenetic embryos were produced by exposure of cumulus-free metaphase-II arrested oocytes (18 hours after hCG) to a 7% solution of ethanol in hepes-KSOM for 7 minutes at 25°C. Cells were subsequently washed at least three times in warmed ethanol-free media. Activated oocytes were identified by the extrusion of the second polar body and formation of a single pronucleus.

2.5 Microinjection

Oocytes and embryos were pressure injected using a micropipette and Narishige manipulators mounted on a Leica DM IRB inverted microscope (Leica, Wetzlar, Germany). Oocytes were placed in a drop of hepes-KSOM covered with mineral oil to prevent evaporation. Cells were immobilised with a holding pipette while the injection pipette was pushed through the zona pellucida until making contact with the oocyte plasma membrane. A brief over-compensation of negative capacitance caused the pipette tip to penetrate the cell. Microinjection was performed using a fixed pressure pulse through a pico-pump (WPI, Sarasota, FL). Injection volumes were estimated at 5% of total cell volume by cytoplasmic displacement. For cyclin injections, an estimated 8-10pg of cyclin-GFP was delivered. Cyclin injections were performed by Petros Marangos.

2.6 Measurment of intracellular [Ca^{2+}] and photorelease of 'caged-InsP3'

$[Ca^{2+}]_i$ was monitored either with Fura-red or Fura-2-dextran (Molecular Probes, Eugene, OR). Background subtraction was performed online using the Metafluor software.

Fura-red-AM was prepared as a 1 millimolar stock solution in dimethyl sulphoxide (DMSO) with 5% pluronic. For use, 1μl of stock solution was added to 250μl of H-KSOM, to produce a final concentration of 4μM Fura-red. The final solution of Fura-red AM therefore contained 0.4% DMSO and 0.02% pluronic. Oocytes were loaded with 4μM Fura-red for 10 minutes at 37°C. Fura-red-loaded oocytes were placed in a drop of hepes-KSOM under oil in a chamber and placed on an Axiovert microscope (Zeiss, Welwyn Garden City, UK). Fura-red was excited at 427 and 490nm using a monochromator, with emitted light monitored with a 600nm long pass filter placed in front of a cooled CCD camera (MicroMax, Princeton Scientific Instruments, Monmouth Junction, NJ). Imaging was performed using a 20x (air) objective lens with a numerical aperture of 0.75. Changes in

intracellular Ca^{2+} concentration are expressed as the ratio between the emission in response to 430nm and 490nm excitation (430/490).

'Caged $InsP_3$' ($cInsP_3$) was microinjected as described above to an estimated final concentration of 50μM. Photo-release was performed 30-60 minutes after microinjection by brief timed exposures of microinjected oocytes to ultraviolet light. UV light (360nm) was selected using the monochromator and introduced via the epifluoresence port. The duration of exposure to UV was controlled using the metafluor software. In experiments using $cInsP_3$ to compare Ca^{2+}-release in different groups of oocytes and embryos, both experimental groups were placed on the stage and exposed to UV light at the same time. Thus, comparisons of the responsiveness of $InsP_3$-induced Ca^{2+} release were made between groups that were injected with the same pipette of $cInsP_3$, loaded with Fura-red, and exposed to UV light at the same time and under identical conditions.

Fura-2-dextran (10,000Mw) was microinjected into pronucleate-stage embryos as described above to a final concentration of 2-4μM. Fura-2 was excited at 340 and 380nm, and emitted light collected using a 520nm band-pass filter. Ca^{2+} measurements are presented as the ratio of emission at 340nm and 380nm wavelengths.

2.7 Labelling and imaging the endoplasmic reticulum (ER)

To label the endoplasmic reticulum, DiI_{18} (Molecular Probes) was microinjected as a saturated solution in soyabean oil (Sigma UK), 30 minutes before imaging. Imaging was performed using a Bio-Rad μ-radiance confocal scan head (Bio-Rad, Hemmel Hempstead, UK) mounted on a Zeiss Axiovert microscope using a 40x oil-immersion objective lens with a numerical aperture of 1.3. The dye was excited with 514nm light and emitted fluorescence collected with a 600nm longpass filter. Examination of oocytes was performed on a heated microscope stage maintained at 37°C, 30 minutes after microinjection of DiI.

Imaging was performed at a resolution of 512x512 pixels. With the exception of the experiment presented in figure 3.4C, all experiments were performed at the same degree of 'zoom', wherein each pixel is a square with length of approximately 0.16μm. This value was calculated by the *Lasersharp* software, and confirmed empirically on the basis of the known diameter of an egg (70μm).

Optical slice thickness

Imaging was performed using a 4mm (macro) pinhole. This pinhole size was used because it was found to provide the best definition of the reticular nature of DiI-labelled ER over a range of 1-8mm. From the pinhole diameter it is possible to obtain an estimate of the optical slice thickness. The optical slice thickness of a confocal section is influenced by several factors, including the wavelength of excitation used, the magnification and numerical aperture of the objective lens, the refractive index of the sample, and the size of the pinhole (Wilson, 1995). A formula that provides an estimate of the depth of the confocal slice for our confocal microscope is (formula provided by Graham Brown, BioRad, UK):

$$Z = \sqrt{\left(\frac{0.88 \times \lambda \times 10^{-3}}{n - n^2 - NA^2}\right)^2 + \left(\frac{1.4 \times n \times [(PH \times 10^3)/(mag \times 73.2)]}{NA}\right)^2}$$

Where, z= optical thickness of the slice in microns

λ = wavelength of excitation

n= the refractive index of the sample

NA= the numerical aperture of the objective lens

PH= pinhole aperture

Mag= magnification of the objective lens

Based on a configuration that includes wavelength of 514nm, an estimated refractive index of 1.51, a pinhole aperture of 4mm, a numerical aperture of 1.3, and an objective magnification of 40x, this formula provides a z value (optical slice thickness) of 2.23μm. This should be treated as an estimate rather than an absolute since small variations in refractive index and objective lens properties will influence the z value provided for the formula.

All DiI-labelled ER images were obtained using the same microscope settings in order to allow fair comparison between images.

Co-labelling ER and chromatin

To co-label the ER and chromatin, oocytes were microinjected with DiI as described above prior to brief (5 minute) incubation in 1μg/ml Hoechst 33342. Imaging was performed with a Zeis LSM-510 laser-scanning microscope (Carl Zeiss Inc) with a 40x oil immersion objective using the 'multitracking' mode. DiI was excited with a 543nm laser and emitted light collected with a 600nm longpass filter. Hoechst 33342 was excited with a UV laser (360nm) and emitted light collected using a 385/470 bandpass filter.

2.8 Analysis of DiI-labelled endoplasmic reticulum

In Chapter 4 we have attempted to quantify different structural morphologies of ER at different cell cycle stages. The aim is to provide an objective standardized method for counting the number of cortical clusters of ER after different treatments. As can be seen from the images (for example, see Fig 3.1) the ER in the cortex of MII eggs takes the form of a loose reticular network that has 'clusters' of ER that are seen by the eye as small round regions of bright fluorescence. In contrast, the ER network in pronucleate stage eggs is more dense, but with many fewer obvious clusters (see Figure 4.1 for example). Similar clusters of ER to those described in this thesis have been seen in oocytes from mice, *Xenopus* and the

nemertean worm (Mehlmann *et al.*, 1995;Stricker *et al.*, 1998;Terasaki *et al.*, 2001). These studies report the size and number of 'ER clusters', but no systematic method of quantifying the number of ER clusters has been used other than that provided by the eye of the experimenter. Here we have developed a technique that provides a standardized method of counting ER clusters in a slice of the cortex.

Firstly we have been consistent in determining the location of the cortical slice. The focus motor function within the *Lasersharp* software was used to place the confocal slice 6μm from the plasma membrane. We have confirmed that the focus motor is correctly calibrated by stepping through the egg at a number of step sizes, and find that the number of steps required is consistent with the known diameter of a mouse egg (70μm). A step of 6μm was chosen since it is the depth at which we found cortical clusters of ER characteristic of metaphase II oocytes are most evident. In addition, this is similar to the depth previously reported for ER clusters in mouse eggs (5μm; Mehlmann *et al.,* 1995). To confirm that slight changes in the confocal slice depth are not responsible for experimental results obtained using our analysis protocol, we have used *metamorph* software to measure the diameter of cortical slice images of five MII eggs and five pronucleate embryos selected at random (from the experiment described in section 4.2.2). There was no difference in the mean diameter of the cortical slice between these two groups ($P>0.9$). This suggests that differences in the number of ER clusters between cell cycle stages are not attributable to differences in Z-depth.

To count ER clusters we have attempted to provide a standardized method that is consistent with that seen when comparing different treatments by eye. To achieve this, a Region Of Interest (ROI) of 1.5μm (9 pixels) in diameter was used. This diameter was used as it was the size that had been previously reported for ER clusters in MII eggs (Mehlmann *et al.*, 1995) and because it was consistent with a blind analysis where an individual that had no

role in the experiments was asked to draw an ROI around any regions of obvious bright fluorescence in a sample image of an MII cortex. The 1.5μm ROI was then placed over any regions determined by the experimenter to be above the level of fluorescence provided by the reticular staining of the ER network (see below for discussion of controlling for experimenter bias). If the fluorescence filled the ROI, the mean pixel intensity was recorded. In order to normalise the data to account for variability in staining intensity, the mean pixel intensity of the ROI was divided by the mean pixel intensity of the entire cortical slice. This ratio value will subsequently be referred to as a cluster-ratio. Typically, analysis of one cortical confocal slice image would generate 30-60 cluster-ratio values ranging between and 1.0 and 2.5. This range reflects the approach taken to sampling, in that all regions above background, even marginally so, were logged. To define an ER cluster it was then necessary to set a threshold in the range of cluster ratios above which it could be considered that ER within the ROI could be defined as an ER cluster. By determining cluster ratios for a small number of ER clusters in a sample MII egg a cluster ratio of 1.5 was found to select areas of fluorescence consistent with ER clusters previously described (Mehlmann *et al.,* 1995;Kline, 2000). Thus, an ER cluster was then defined as any ROI where the cluster ratio is ≥ 1.5. This threshold yields an average cluster number within a similar range as that previously reported (Mehlmann *et al.,* 1995). To confirm that results obtained using this threshold provides a robust means of analysis and that experimental results are not dramatically altered by small changes in the threshold, one experiment (the experiment presented in Figure 4.2) has been analysed using thresholds of 1.4 and 1.6 (figure 2.1). The results confirm that small changes in the threshold have no significant effects on the nature of the experimental result obtained by this method of analysis.

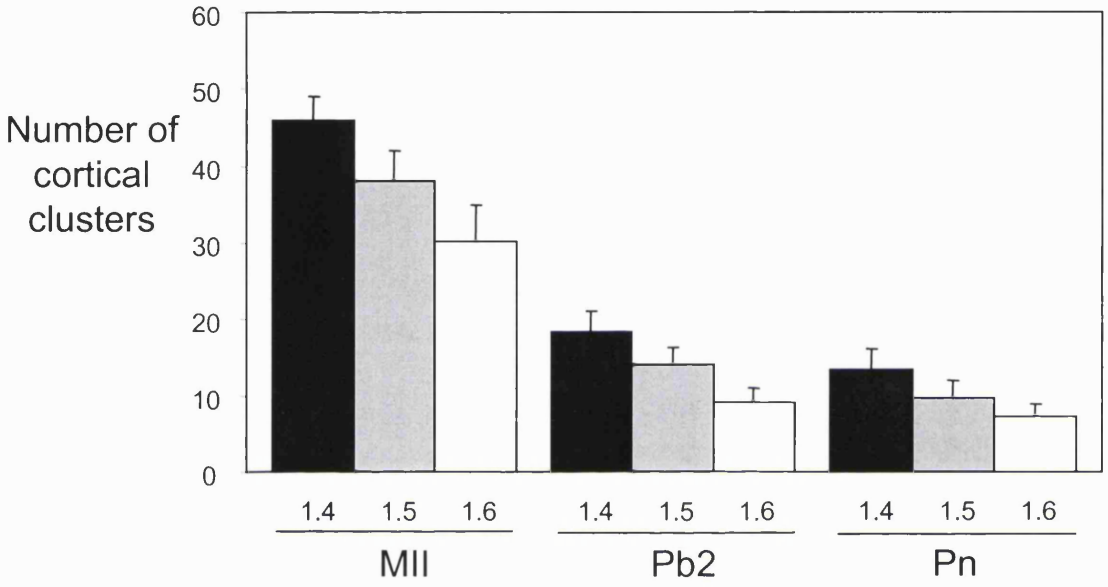


Figure 2.1. The effect of changing region of interest size upon ER cluster analysis results. The cluster-ratios generated by analysis of the experiment presented in figure 4.2.2 were analysed using threshold values of 1.4 (black), 1.5 (grey) and 1.6 (white; see text for detailed description of the analysis protocol). This analysis method reveals that MII oocytes have more cortical ER clusters than Pb2 or Pn stage parthenogenetic embryos ($P<0.01$), regardless of the threshold applied. There is no significant diference between Pb2 and Pn at any threshold ($P>0.1$ in all cases). Thus, small changes in the value of the threshold applied do not alter the nature of the results obtained by this analysis method.

The technique described above is a standardized approach that will allow comparisons between treatments, but it is not absolutely objective and may be subject to operator bias since the experimenter decides where to place the ROI on the optical slice. All efforts have been made by the experimenter to place the ROI over any region of fluorescence that is above that of the background ER network, independent of experimental conditions. Nevertheless, these experiments were not done blind since the experimenter was able to determine the conditions of the experiment based on the ER morphology obvious to the trained eye. To check that the results obtained by the experimenter were not subject to bias an individual untrained in assessing ER morphologies and with no role in the experiments was provided with 10 coded images. Unknown to the tester, the images consisted of 5 MII stage eggs and 5 fertilized pronucletae stage eggs. The 'blind' tester was shown a typical cortical slice of the ER in an MII egg, and was asked to count the number of clusters in each image. This 'blind' analysis also provides an independent means of verifying whether the analysis by ROI and cluster-ratio accurately reports the same as the number of clusters as the untrained eye. After the exercise, the images were decoded and the mean number of clusters counted for MII eggs was 34.8+/-11.2 and 8.8+/-3.0 for pronucletae stage eggs. These values obtained by the blind analysis compare favourably to those obtained by the experimenter 38.1+/-3.9 and 9.8+/-2.3, for MII eggs and pronucleate embryos, respectively. This simple blind test suggests that the method of analysis described above is not influenced by operator bias and that the ROI and threshold values assigned do indeed discriminate between clustered and non-clustered ER. Thus, the technique has been used to provide a standardized means of counting cortical clusters of ER in different experimental conditions.

At the suggestion of my PhD examiners, I have subsequently further confirmed the validity of the results obtained using this method by re-analysing an entire experiment using an unrelated and entirely objective method; this second method produced results

similar to the first. Details of this method and the results obtained are presented in Appendix 2.

2.9 Monitoring formation and breakdown of nuclei

In order to monitor nuclear membrane permeability, fluorescein dextran (77kDa; Sigma) was microinjected into one pronucleus. The injected embryos were subsequently loaded with Fura-red (see above), placed on the microscope stage in a drop of KSOM-hepes under oil, and monitored through NEBD. Fluorescein was excited with 490nm light and emission collected with a 520mn band-pass filter. Fura-red was excited with 430nm and 490nm light, and emission collected with a 665nm longpass filter.

To monitor nuclear envelope reformation, embryos were microinjected with fluorescein isothiocyanate-labelled BSA tagged with a nuclear targeting signal (FITC-NLS-BSA; provided by Mark Jackman, Cambridge University). In these experiments embryos were co-injected with Fura-2-dextran (described above). Imaging was performed as described for Fura-2. FITC-NLS-BSA was monitored using the same excitation and emission filters as for Fura-2. Experiments examining the second mitotic division were carried out using an excitation filter wheel. Fura-2 was excited using 340 and 380nm light, fluorescein was excited with 490nm light. A 510nm dichroic mirror and a 520nm longpass filter were used to collect emitted light.

2.10 Manipulation of cdk1-cyclin B activity

In order to maintain high cdk1-cyclin B activity activity following fertilisation or parthenogenetic activation, the proteosome inhibitor MG132 (50μM) was used. MG132 was prepared as a 50mM stock solution in DMSO. For use, 1μl of stock solution was diluted in 1ml of H-KSOM to provide a final concentration of 50μM MG132, 0.1% DMSO. Oocytes

were preincubated in MG132 for 30 minutes prior to insemination or activation by ethanol, and maintained in MG132 for the duration of the experiment.

To inhibit cdk1-cyclin B activity, oocytes were incubated in media containing the cdk1 inhibitor roscovitine. Roscovitine was prepared as a 25mM solution stock in DMSO. 1µl of stock solution was diluted in 330µl H-KSOM to provide a final solution of 75µM roscovitine, 0.3% DMSO. MII oocytes were transfered to media containing roscovitine for either 1 or 2 hours prior to examination of the ER. Oocytes remained in roscovitine during imaging.

2.11 Downregulation of $InsP_3$ receptors

To provoke $InsP_3$ receptor downregulation, oocytes and embryos were microinjected with Adenophostin A (Calbiochem, San Diego, CA) to an estimated final concentration of 2.5µM (Brind *et al.*, 2000).

2.12 Statistical analysis

All t-tests are two-tailed and based upon two samples (unpaired) with similar variance. Where shown on figures, error bars indicate the standard error (sem). Analysis of variance

3. Endoplasmic reticulum structure and $InsP_3$ sensitive Ca^{2+} release in oocyte maturation

3.1 Introduction

Fertilisation of the mouse egg stimulates a remarkable sequence of Ca^{2+} signalling events; an initial explosive release of Ca^{2+} is followed by repetitive transients which last until the time of pronucleus formation, some 4 hours after fertilisation (Cuthbertson and Cobbold, 1985;Jones *et al.*, 1995a;Day *et al.*, 2000;Deguchi *et al.*, 2000;Marangos *et al.*, 2003). Since the integrity of these Ca^{2+} oscillations is a determinant not only of successful activation, but also of pre- and post-implantation development (Lawrence *et al.*, 1998;Ozil and Huneau, 2001), the development of Ca^{2+}-release mechanisms capable of sustaining this signal is a key step in oogenesis (as discussed by Carroll *et al.*, 1996).

It is now well established that an essential component of this development occurs during oocyte maturation. Fertilisation stimulates Ca^{2+} transients in GV oocytes which are markedly smaller and with a slower initial rate of rise than MII oocytes (Mehlmann and Kline, 1994;Jones *et al.*, 1995b;Cheung *et al.*, 2000). Additionally, it has been noted that the number of oscillations generated following insemination is significantly less in GV stage oocytes (Jones *et al.*, 1995b;Cheung *et al.*, 2000). This increased responsiveness of the MII egg to sperm is reflected by increased ability to release Ca^{2+} in response to $InsP_3$, the physiological Ca^{2+}-releasing agonist at fertilisation (Mehlmann and Kline, 1994). Several modifications of the Ca^{2+} signalling machinery occur during oocyte maturation which may contribute to this upregulation in sensitivity. These include an increase in the amount of $InsP_3R$ protein (Parrington *et al.*, 1998;Fissore *et al.*, 1999b), thapsigargin-sensitive Ca^{2+}-

stores (Jones *et al.*, 1995b), Na^+/Ca^{2+} exchange (Carroll, 2000), and changes in the three-dimensional structure of the ER (Mehlmann *et al.*, 1995; see Chapter 1).

ER structural reorganisations have been shown to accompany oocyte maturation in all species studied so far including *Xenopus* (Terasaki *et al.*, 2001), sea urchin (Henson *et al.*, 1990), the nemertean worm (Stricker *et al.*, 1998), starfish (Jaffe and Terasaki, 1994), hamster (Shiraishi *et al.*, 1995), mouse (Mehlmann *et al.*, 1995) and human (GF and JC, unpublished observations). In mouse this reorganisation involves the generation of small microdomains, or clusters of ER 1-2μm in diameter in the oocyte cortex (Mehlmann *et al.*, 1995). The mechanism and timecourse of this reorganisation are yet to be addressed.

Previous studies of mammalian oocytes have used the lipophilic dicarbocyanine dye DiI_{18} to label the ER (Mehlmann *et al.*, 1995;Shiraishi *et al.*, 1995;Kline *et al.*, 1999;Kline, 2000). In this protocol DiI is dissolved in soybean oil to produce a saturated solution, which can then be microinjected into the oocyte cytoplasm. DiI subsequently transfers to any adjacent structures and its lipophilic nature causes it to diffuse throughout any continuous lipophilic membranes (Terasaki and Jaffe, 1993;Kline, 2000). The validity of using DiI as an ER marker has been confirmed by a study in which the distribution of DiI in the starfish oocyte was shown to be identical to that of a green fluorescent protein (GFP) targetted to the ER by means of a KDEL retention sequence (Terasaki *et al.*, 1996).

In this chapter we use DiI to investigate the timecourse of reorganisation during oocyte maturation in mouse. The experiments presented suggest that ER first accumulates around the MI spindle following GVBD by a microtubule-dependent mechanism, prior to relocating to the cortex concomitant with spindle migration. ER cortical clusters form close to the time of, but independently of first polar body extrusion. Finally, using 'caged' $InsP_3$ to compare the responsiveness of Ca^{2+} release between groups of oocytes, we demonstrate that

a significant proportion of the upregulation in responsiveness occurs in the final hours of maturation, over which timecourse cortical clusters of ER are generated.

3.2 Results

3.2.1 The timecourse of ER reorganisation during oocyte maturation

Whilst it has been established that the ER undergoes dramatic structural reorganisation during meiotic maturation in mouse, the MII egg being characterised by a network of cortical accumulations not present in the GV oocyte (Mehlmann *et al.*, 1995;Kline, 2000), the developmental stage at which these ER clusters are formed is unknown. Therefore, in order to investigate the timecourse of ER reorganisation, we have examined the structure of ER at various times during maturation using the lipophilic marker DiI_{18}.

To first verify that the pattern of DiI labelling was the same here as previously reported, we microinjected DiI into GV and MII stage oocytes and examined the ER using confocal microscopy. We found the ER structure to be similar to previous studies (see figure 3.1). In GV oocytes the ER was characterised by small accumulations of ER in the cytoplasm. The germinal vesicle membrane was prominently stained, confirming continuity between ER and nuclear membranes. There was no labelling within the germinal vesicle. In MII oocytes, the ER extended throughout the cytoplasm in a reticular nature. No labelling was seen in the area presumed to surround the MII spindle. Clusters of ER similar to those previously described were observed in the cortical confocal slice.

Initial experiments revealed that *in vitro* maturation was unable to support the generation of cortical ER clusters in our laboratory (see Chapter 6 for further discussion). Thus, to investigate the timecourse of ER reorganisation during oocyte maturation, oocytes were microinjected with DiI and ER structure investigated with confocal microscopy at various times following administration of hCG. At each timepoint, oocytes were collected from the ovary and the ER examined within one hour of retrieval. The ER was examined in GV stage oocytes, and in MI oocytes 3, 6 and 9 hours after hCG (fig 3.2A). Hyalauronidase

was used to remove cumulus cells from oocytes in the 3, 6 and 9h groups as cumulus expansion had commenced (see Chapter 2).

As before, no cortical clusters were observed in GV stage oocytes, rather, the ER was characterised by small accumulations of fluorescence deeper in the cytoplasm. Oocytes retrieved 3 hours after hCG had undergone germinal vesicle breakdown. In these oocytes the ER was characterised by a bright ring of fluorescence around a central ER-free region presumed to contain the developing meiotic spindle. Similar rings of ER were seen in oocytes 6 and 9 hours after hCG. This ER ring was positioned in the cortex in oocytes of the 9-hour group (fig 3.2A, top row). Cortical clusters were not seen in oocytes 3, 6 or 9 hours after hCG (fig 3.2A, bottom row). Oocytes examined 14h after administration of hCG had been ovulated, and were therefore retrieved from the oviduct (see Chapter 2). All ovulated oocytes had extruded Pb1. Cortical clusters of ER were present in these oocytes as described previously. These data suggest that cortical clusters of ER are formed late in the maturation process, close to the time of Pb1 extrusion.

3.2.2 ER accumulation at GVBD is initially bipolar

In order to examine the process by which the ER accumulates as a ring in the oocyte interior at GVBD, the ER was investigated in oocytes in which the germinal vesicle was still visible 90 minutes after release from the ovary. These oocytes were therefore presumed close to the time of GVBD. Large accumulations of ER around the MI spindle characteristic of oocytes which have undergone GVBD were not seen. Rather, smaller dense accumulations of ER were observed at opposing poles of the germinal vesicle. In addition, fine protrusions of DiI-labelled ER were seen to invaginate the GV. A representative example of such an oocyte is displayed in figure 3.2B. This result suggests that ER accumulation at GVBD is initially bipolar.

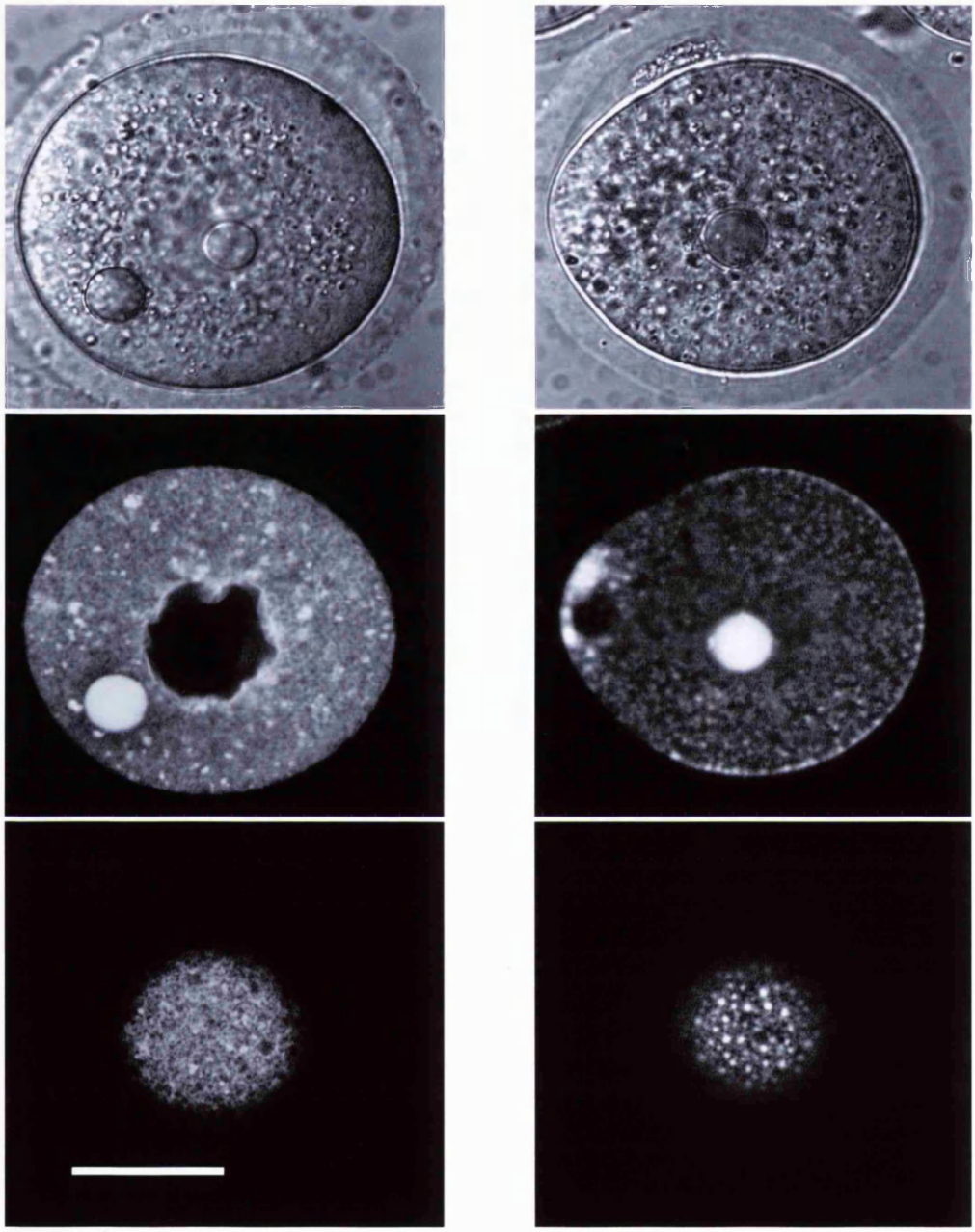


Figure 3.1. The appearance of DiI-labelled ER in GV (left) and MII (right) stage oocytes. Oocytes were microinjected with DiI and the ER examined with confocal microscopy 30 minutes after injection. Equatorial (middle) and cortical (bottom) confocal slices, and brightfield images (top) are shown. The ER in MII oocytes is characterised by prominent clusters in the cortex, not observed in GV stage oocytes. A dark ER-free region surrounds the cortical spindle in the equatorial slice at MII. The germinal vesicle membrane is clearly visible in the GV-stage oocyte, confirming continuity between ER and nuclear envelope. Note also the fluorescent DiI-containing oil droplet in both oocytes. This pattern of labelling is similar to that previously reported (Mehlmann et al, 1995). Scale Bar 30μm.

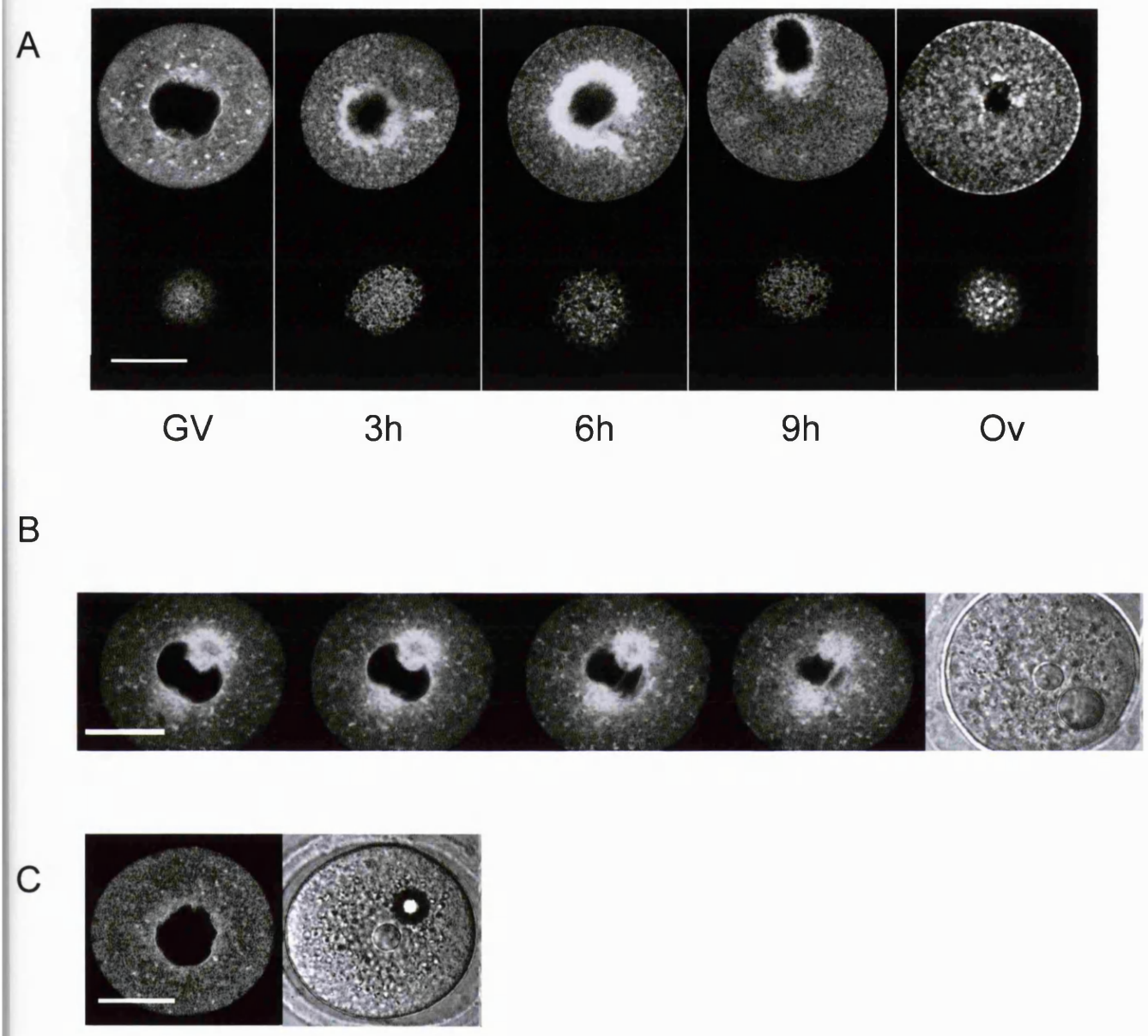


Figure 3.2. Endoplasmic reticulum reorganisation during oocyte maturation. (A) Oocytes were collected and ER examined in GV stage oocytes, 3, 6, and 9 hours after administration of hCG, and in ovulated oocytes (14 hours after hCG; Ov). Equatorial (top row) and cortical confocal slices (bottom row) are shown at each timepoint. A minimum of 7 oocytes were examined over the course of at least two separate experiments for each time point. Notice that cortical clusters of ER are only seen following extrusion of Pb1. (B) The ER was examined 1.5 hours after release from the ovary, close to the time of GVBD (n=4). The figure shows a representative z-stack through the GV (3μM steps), and a brightfield image of one such oocyte. Note that ER accumulation at this stage is bipolar and that DiI-Labelled ER invaginates the germinal vesicle. (C) The ER was examined in oocytes arrested in IBMX for 6 hours. No ER accumulation occurs in these oocytes (n=11), thus ER accumulation at GVBD is cell-cycle dependent, and not attributable to manipulations in vitro. Scale bar 30μm.

3.2.3 ER reorganisation at GVBD is cell-cycle dependent

To confirm that the GVBD-associated ER accumulation in the oocyte interior is dependent upon germinal vesicle breakdown, the ER was examined in oocytes arrested at GV stage for 6h in the phosphodiesterase inhibitor IBMX (fig 3.2C). The ER of IBMX-treated oocytes was similar to that of freshly collected GV oocytes, demonstrating that ER accumulations observed after GVBD are dependent upon cell cycle progression, and not a result of manipulations *in vitro*.

3.2.4 The ER envelops and invades the MI spindle following GVBD

In order to clarify the spatial relationship between the meiotic spindle and the dense ring of ER, we co-labelled MI stage mouse oocytes (6 hours after hCG) with DiI to label the ER, and with Hoechst 33342 to label the chromosomes. Confocal images of these oocytes confirm that the ER ring surrounds the chromosomes (fig 3.3A). High magnification images reveal that the ER invades the spindle, forming close associations with the chromosomes (fig 3.3B). These data suggest that the ER envelopes and invades the MI spindle following GVBD, and remains around the chromosomes during centro-cortical migration.

3.2.5 Endoplasmic reticulum reorganisation following GVBD is inhibited by nocodazole but not by latrunculin A

ER restructuring is known to be controlled by components of the cytoskeleton in a variety of cell types (Voeltz *et al.*, 2002). However, the mechanism of ER reorganisation during mammalian oocyte maturation is not known. Latrunculin A is a toxin known to cause depolymerisation of the actin cytoskeleton by forming a complex with actin monomers (Spestor *et al.*, 1989;Wakatsuki *et al.*, 2001;Lim *et al.*, 2002). Nocozazole causes microtubule depolymerisation by binding tubulin monomers and has been widely used to

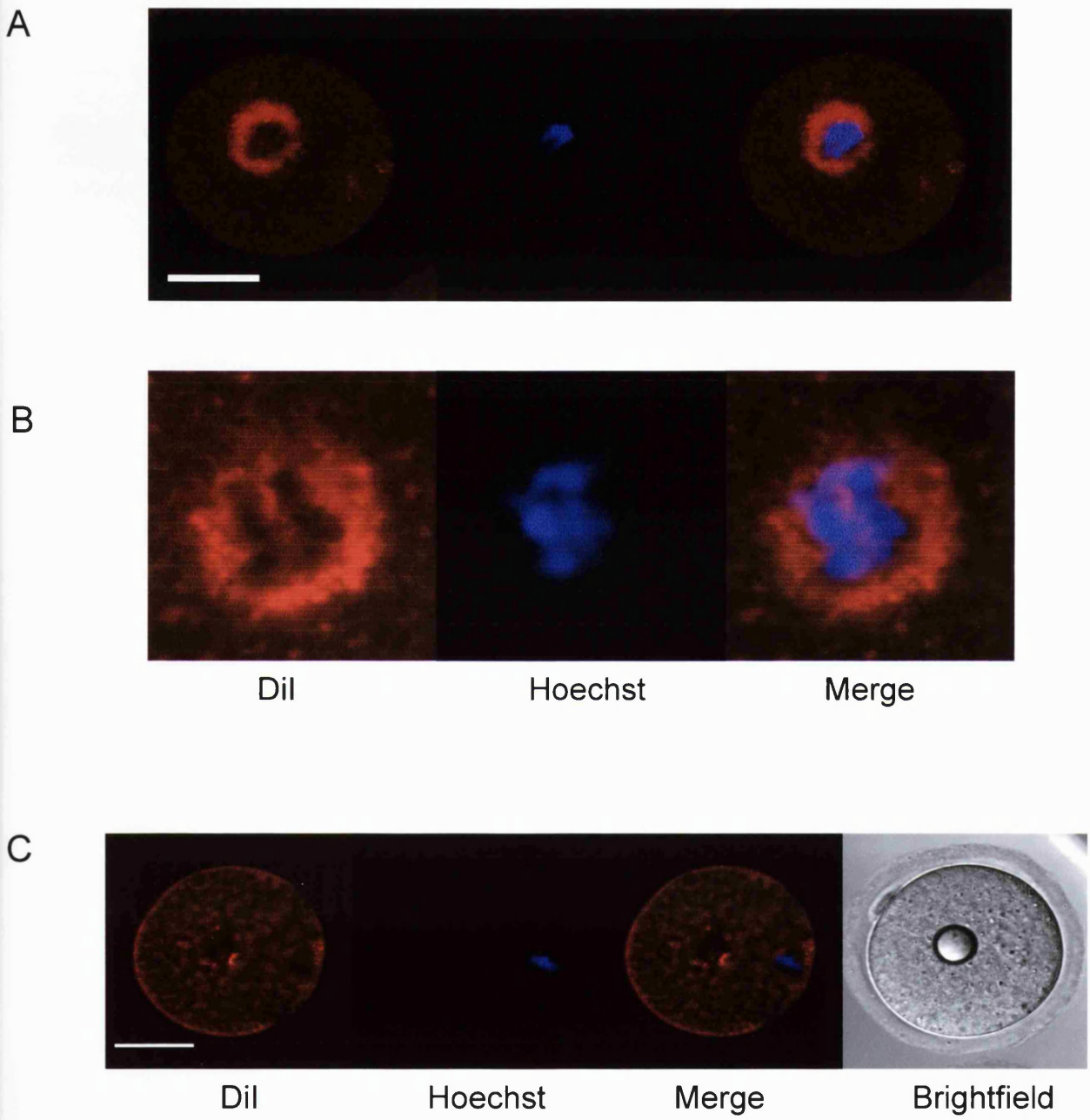


Figure 3.3. Endoplasmic reticulum closely envelops the chromosomes during metaphase I. (A) Oocytes 6 hours after release from the ovary (which had undergone GVBD) were co-labelled with DiI to label the ER, and Hoechst 33324 to label chromatin (n=8). Note that the chromosomes lie within the dense ring of ER. (B) High magnification of the ER-ring reveals close associations between ER and the chromosomes. (C) MII egg for comparison (n=4). Scale Bar 30μm.

investigate the effect of microtubule depolymerisation in mouse oocytes (Wickramasinghe and Albertini, 1992; Kubiak *et al.,* 1993;Winston *et al.,* 1995). Here we have used latrunculin A and nocodazole to investigate the respective roles of microfilaments and microtubules in ER reorganisation at GVBD.

To first confirm the effectiveness of each drug, MII oocytes were activated by exposure to ethanol prior to transfer to media containing latrunculin A or nocodazole. Pb2 extrusion was inhibited in both cases, indicating that both drugs were effective in disrupting the cytoskeleton (Maro *et al.*, 1984;Winston *et al.*, 1995; fig 3.4A).

To investigate the effect of latrunculin A and nocodazole upon ER reorganisation at GVBD, GV stage oocytes were transferred to media containing each drug one hour after release from the ovary, at which time GVBD had not occurred. Neither drug exerted any effect upon the proportion of oocytes which underwent GVBD (data not shown). Oocytes were microinjected with DiI and examined 3-4 hours later, following GVBD. Untreated oocytes displayed the typical MI appearance, characterised by a ring of fluorescence surrounding the MI spindle. Latrunculin A had no obvious effects upon the development of the ER ring around the spindle. In contrast, ER accumulation did not occur following GVBD in nocodazole treated oocytes (fig 3.4B). Rather, nocodazole treated oocytes displayed a small area of bright fluorescence in the centre of the oocyte, presumed to be remnants of the GV membranes (fig 3.4B,C). Thus, reorganisation of ER associated with GVBD is inhibited by nocodazole, but not by latrunculin A.

3.2.6 Cortical clusters of ER are not generated by oocytes of the mos$^{-/-}$ mouse

The results above demonstrate that around the time of GVBD the MI spindle becomes enveloped in ER, and that this ER remains around the spindle during migration to the cortex. One possible interpretation of this data is that the peri-spindle accumulation of ER might

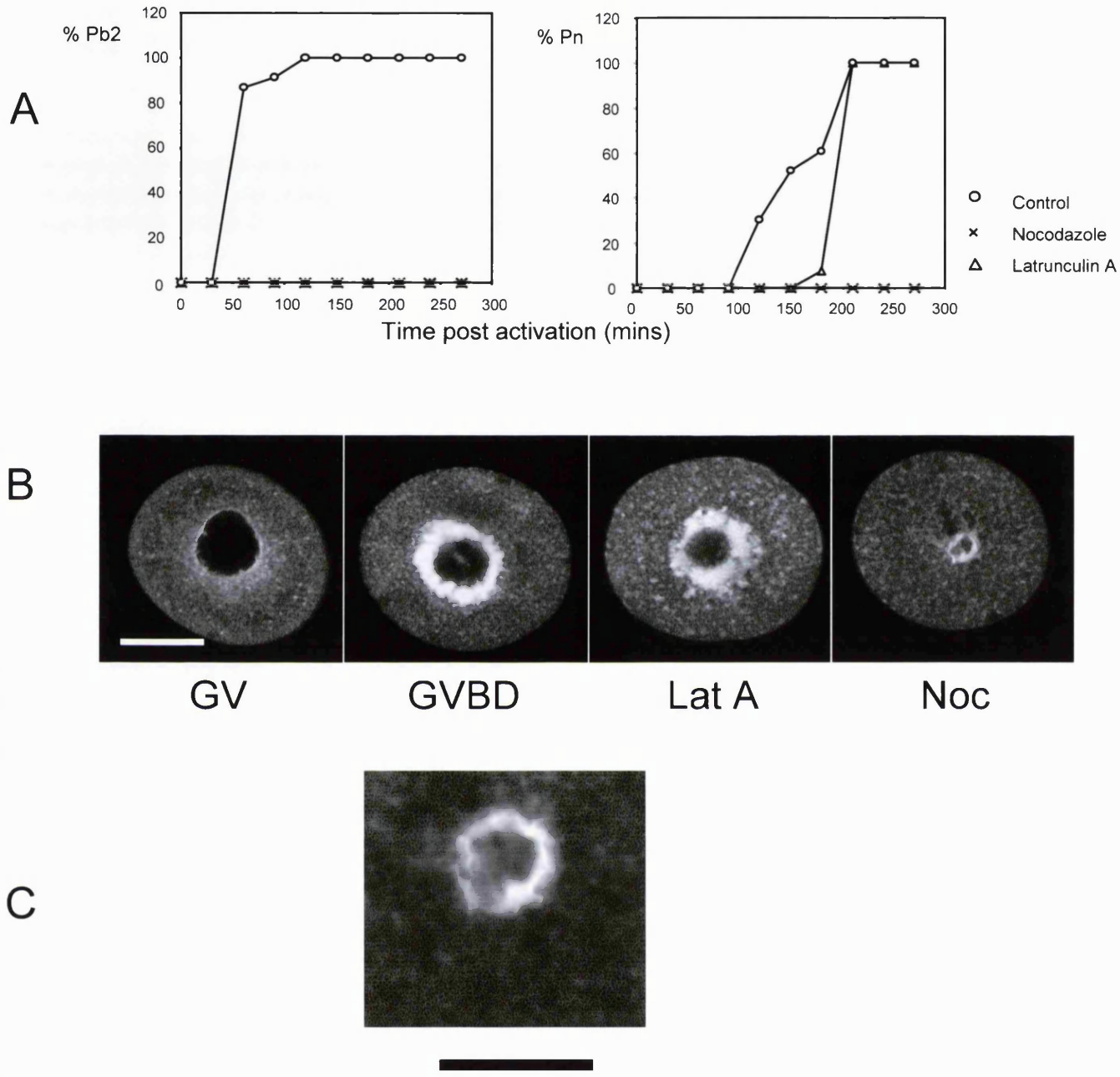


Figure 3.4. Endoplasmic retuculum reorganisation following germinal vesicle breakdown is inhibited by nocodazole but not by latrunculin-A. (A) Latrunculin-A and nocodazole both inhibit Pb2 extrusion. MII eggs were activated by exposure to ethanol then transferred to media containing nocodazole (10μM) or latrunculin A (6μM). Oocytes were examined for second polar bodies and pronuclei at half hour intervals. Each group comprises between 10 and 25 oocytes; data is representative of two independent trials and experiments A and B were performed on the same day. (B) GV stage oocytes were transferred into media containing latrunculin A (Lat A) or nocodazole (Noc) one hour after release from the ovary. Oocytes were labelled with DiI and the ER examined 3 hours later, following GVBD. Untreated oocytes (GVBD; n=5) exhibit the peri-spindle ER accumulation characteristic of MI. Similar ER accumulation around the spindle is exhibited by latrunculin-A treated oocytes (n=10). Nocodazole-treated oocytes do not display the ring of fluorescence at metaphase I, instead a small area of bright fluorescence is seen in the centre of the oocyte (n=8). Scale bar 30μm. (C) High magnification image of nocodazole-treated oocyte. Scale bar 15μm.

provide a mechanism of redistributing the ER from the interior of the oocyte to the cortex during oocyte maturation. Within such a model one might predict that inhibition of spindle migration may prevent the formation of cortical clusters at MII.

Spindle migration does not occur in oocytes of the $mos^{-/-}$ mouse. In the first meiotic division in these oocytes, one spindle pole remains stationary during spindle elongation, while the other moves nearer to the cortex (Verlhac *et al.*, 2000). Thus an asymmetric cell division takes place, and a first polar body is extruded which is larger than usual (Choi *et al.*, 1996;Verlhac *et al.*, 2000). Hence oocytes of the $mos^{-/-}$ mouse provide a model with which to test the role of spindle migration in ER reorganisation during oocyte maturation.

Cumulus-enclosed GV stage $mos^{-/-}$ and F1 oocytes were cultured in Waymouth's media with serum. Cumulus cells were removed and the ER examined in oocytes which had extruded Pb1 after 14 hours of *in vitro* maturation (IVM), the time at which the highest proportion of oocytes would be expected to be at MII in this protocol (John Eppig, personal communication). Oocytes that showed signs of pronucleus formation, either by means of brightfield optics or DiI staining, were not examined. ER clusters developed normally in control in vitro matured oocytes. In contrast, cortical clusters of ER were not visible in $mos^{-/-}$ oocytes (fig 3.5). Nevertheless, examination of the equatorial slices revealed the cortex to be rich in ER. These data suggest that redistribution of ER to the cortex is not inhibited in $mos^{-/-}$ oocytes, though cortical clusters of ER characteristic of MII oocytes appear not to be generated.

3.2.7 Cortical clusters of ER are generated independently of the MI-MII transition

The finding that cortical clusters of ER characteristic of MII oocytes are formed close to the time of Pb1 extrusion raises the question of whether polar body formation is a necessary

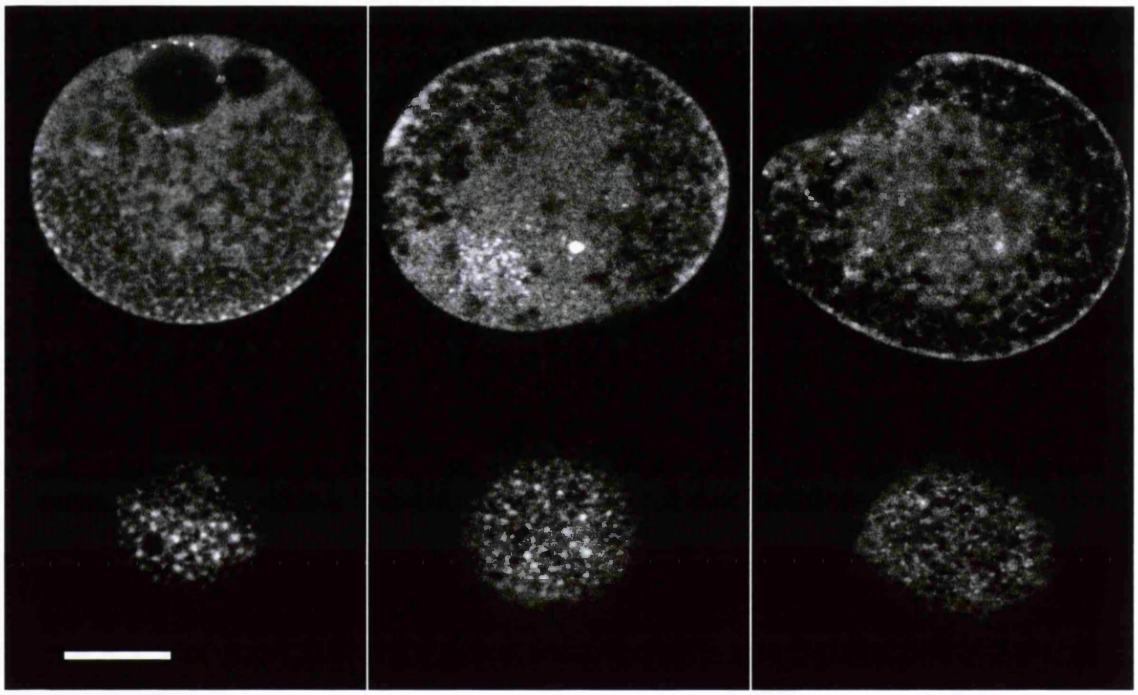


Figure 3.5. Cortical clusters of ER are not generated in oocytes of the mos$^{-/-}$ mouse. GV stage oocytes from F1 and mos$^{-/-}$ mice were collected and cultured for 14 hours prior to examination of the ER. Equatorial (top row) and cortical (bottom) confocal slices are shown of ovulated F1 oocytes, and of in vitro matured oocytes of both F1 and c-mos$^{-/-}$ oocytes. All oocytes examined had extruded Pb1 and were without pronuclei. Cortical clusters of ER present in MII ovulated (n=4) and in vitro matured (n=11) oocytes were not seen in mos$^{-/-}$ oocytes (n=14). Scale bar 30 μM.

requirement for cluster generation. In order to address this we have investigated the ER in oocytes of mouse strain LTXBO. An abnormally high proportion of oocytes of mouse strain LTXBO arrest at metaphase I, thus failing to extrude the first polar body (O'Neill and Kaufman, 1987;Hirao and Eppig, 1997b). LTXBO oocytes therefore provide an ideal model with which to address this question.

Oocytes from mouse strains LTXBO and F1 (control) were isolated and matured *in vitro*. Oocytes were labelled with DiI and the ER examined after 18 hours IVM (fig 3.6). *In vitro* matured and ovulated oocytes from F1 mice both displayed clusters of ER in the cortical slice characteristic of MII eggs. Moreover, MI-arrested LTXBO and F1 oocytes which spontaneously arrested at MI displayed cortical ER clusters similar to those observed in ovulated eggs. Similar results were observed when in vitro maturation was performed in either Waymouth's media with serum, or in MEM-BSA. Cortical clusters of ER were not seen in GV stage oocytes from either F1 or LTXBO mice. Thus the restructuring of ER into cortical clusters is not critically dependent upon the transition from MI to MII.

3.2.8 $InsP_3$-mediated Ca^{2+} release in GV and MII stage oocytes

An increase in the responsiveness of $InsP_3$-mediated Ca^{2+} release has previously been reported to occur during meiotic maturation in mouse (Mehlmann and Kline, 1994). In this study MII oocytes were shown to exhibit $[Ca^{2+}]_i$ changes of greater amplitude than GV stage oocytes in response to microinjection of a known concentration of $InsP_3$. It has been argued that this protocol may result in co-injection of Ca^{2+} from the media (Jones and Nixon, 2000). Given that $InsP_3$-mediated Ca^{2+} release is exquisitely sensitive to changes in $[Ca^{2+}]_i$ (Bezprozvanny *et al.*, 1991), Ca^{2+} introduced from the extracellular media may modify $InsP_3$-induced changes in $[Ca^{2+}]_i$. One solution to this problem is to use photolysis of caged-$InsP_3$ ($cInsP_3$) as the source of $InsP_3$. $cInsP_3$ is microinjected in its inactive (caged) form.

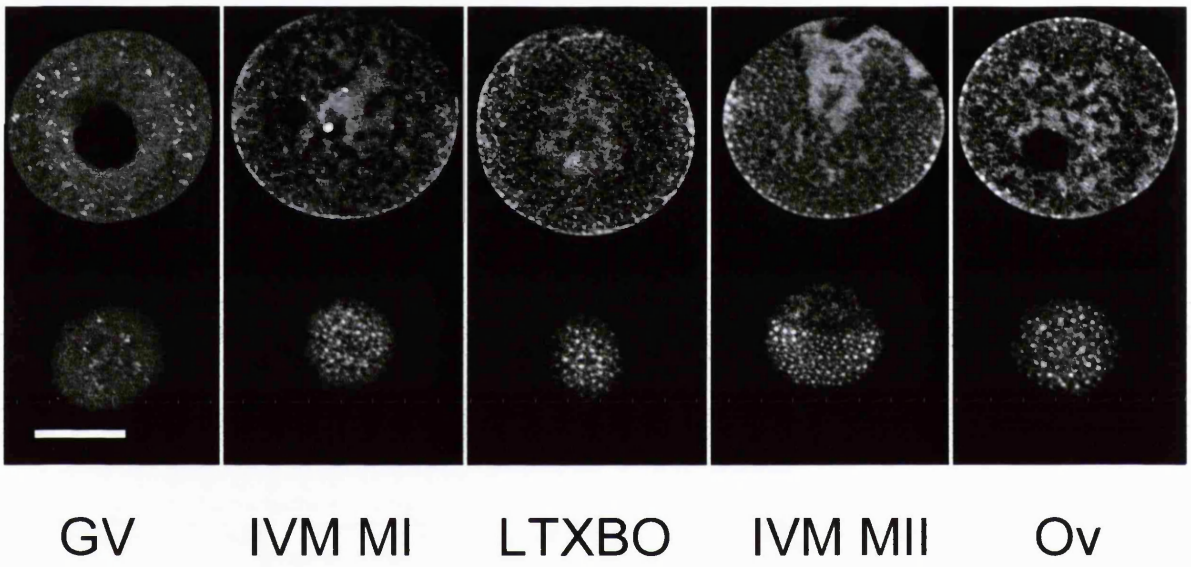


Figure 3.6. Generation of cortical ER clusters occurs independently of the MI-MII transition. GV oocytes from F1 and LTXBO mice were cultured for 18 hours prior to examination of the ER. Representative equatorial (top row) and cortical confocal slices are shown. Metaphase II-stage *in vitro* matured F1 oocytes (IVM MII) displayed cortical clusters of ER similar to those seen in ovulated (Ov) oocytes. MI-arrested LTXBO oocytes and spontaneously arrested MI oocytes from F1 mice (IVM MI) both displayed cortical clusters of ER 18 hours after collection despite failing to extrude Pb1. GV stage oocytes were without cortical clusters. The ER was similar in F1 and LTXBO GV stage oocytes (data not shown). 10-15 oocytes were examined in each group, and results were similar after maturation in two different culture conditions (see Chapter 2). Scale bar 30μm.

Subsequent exposure to UV light causes photolysis of $cInsP_3$ and liberation of $InsP_3$. Photolysis may be performed many minutes after injection, thus eliminating artefacts associated with microinjection.

Microinjection of high concentrations of caged $InsP_3$ is thought to provide a reservoir within the cell which is not readily exhausted by repeated short photolysis events (Callamaras and Parker, 1998;Jones and Nixon, 2000); thus the magnitude of Ca^{2+} response is not reduced by repeated exposures to UV light (Jones and Nixon, 2000). Therefore a further advantage of this method is that each oocyte may be sequentially exposed to a variety of durations of UV light, thereby providing the opportunity of investigating the effect of different concentrations of $InsP_3$ upon $[Ca^{2+}]_i$.

To use such a protocol, it was first necessary to establish conditions in which repeated photolysis of $cInsP_3$ results in a consistent level of Ca^{2+} release. When photorelease was performed 30-60 minutes after microinjection, a sequence of six 1000ms exposures at 2 minute intervals triggered six similar Ca^{2+} transients (figure 3.7Ai). No significant difference in peak change in Fura-red ratio was observed between the first and sixth transient ($P>0.7$; fig 3.7Aii). Thus repeated photo-release does not limit the availability of $cInsP_3$ for subsequent photolysis under these conditions. This validates the use of multiple photolysis events to establish stimulus-response relationships between the amount of $cInsP_3$ photo-released and peak change in Fura-red ratio.

To compare the responsiveness of $InsP_3$-mediated Ca^{2+} release in GV and MII oocytes, we simultaneously exposed $cInsP_3$-injected oocytes to a series of different durations of UV light exposure, whilst recording $[Ca^{2+}]_i$ using Fura-red. To ensure that both groups had been treated similarly, GV and MII stage oocytes were microinjected using the same pipette of $cInsP_3$, and placed on the stage at the same time, thus allowing simultaneous exposure to UV light and Ca^{2+} measurement. MII and GV stage oocytes were exposed to 10, 100, 300,

600, 1200, 3000 and 6000 ms durations of UV light at 2 minute intervals (fig 3.7B). Peak change in Fura-red ratio was significantly greater in MII oocytes than in GV oocytes in response to all UV exposure durations ($P<0.05$ for 10 and 100 ms exposures, and $P<0.002$ for all other exposures).

To confirm that the amplitudes of the Ca^{2+} transients elicited by photolysis of $cInsP_3$ under these conditions are physiologically relevent, Ca^{2+} was monitored following fertilisation of MII eggs using the same imaging protocol as for the photolysis experiment described above. The mean peak change in Fura-red ratio stimulated in MII oocytes by maximal photolysis under these conditions (6000ms exposure to UV light) was of greater magnitude than that of the second fertilisation Ca^{2+} transient, but smaller than the first fertilisation Ca^{2+} transient (fig 3.7B). Thus the amplitudes of $[Ca^{2+}]_i$ transients caused by photolysis of $cInsP_3$ under these conditions are within the physiological range.

These results demonstrate an increase in the magnitude of $InsP_3$-induced Ca^{2+} release during oocyte maturation which is independent of Ca^{2+} introduced from the extracellular media, and confirm that the protocol of uncaging varying amounts of $cInsP_3$ to establish a stimulus-response relationship is efficient in distinguishing differences in the responsiveness of $InsP_3$-induced Ca^{2+} release between two populations of oocytes.

3.2.9 The upregulation of $InsP_3$-mediated Ca^{2+} release during oocyte maturation is incomplete 9h after hCG

Whilst it has been established that the sensitivity of $InsP_3$-mediated Ca^{2+} release is significantly greater in MII than in GV stage oocytes, the time course of this upregulation has not been studied in mouse. Several studies have implied that the generation of cortical clusters of ER should play a crucial role in providing the increased sensitivity of Ca^{2+} release of the MII egg (Mehlmann *et al.*, 1995;Stricker *et al.*, 1998;Kline *et al.*, 1999). Therefore, to

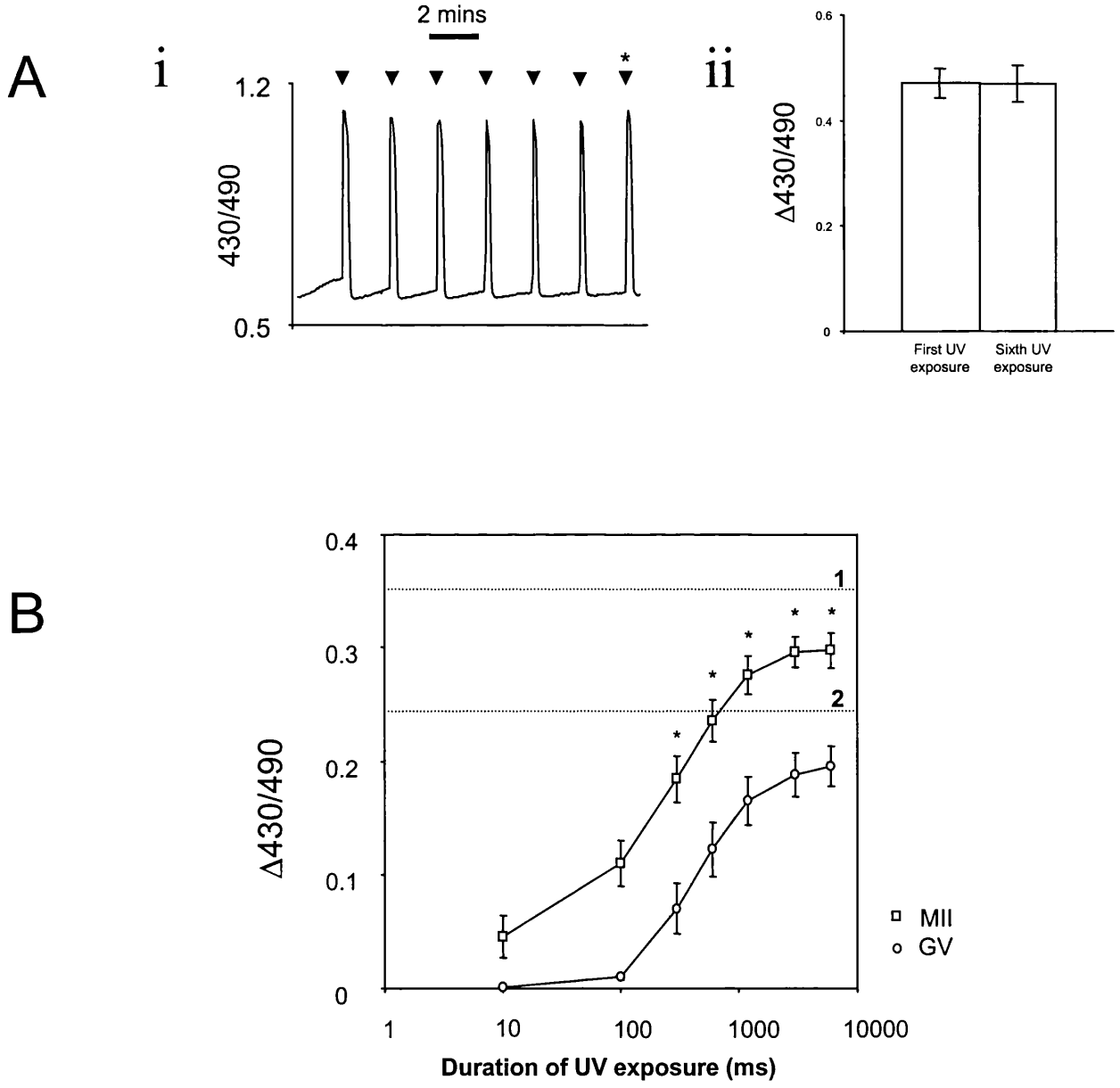


Figure 3.7. $InsP_3$-releasable Ca^{2+} stores are upregulated during oocyte maturation. (A) Injection of $cInsP_3$ into MII oocytes provides a reservoir of $InsP_3$ that is not significantly depleted by repeated photolysis events. Oocytes were microinjected with 'caged'-$InsP_3$ to a final concentration of 50μM and exposed to 1000ms durations of UV light (arrowhead). $[Ca^{2+}]_i$ was monitored using Fura-red (Ai). *The final UV exposure was 3000ms in duration. There was no significant difference in Δ430/490 between first (0.48+/-0.025 ratio units) and sixth (0.47+/-0.026r.u.) uncaging events ($P>0.7$, n=10) (Aii). (B) cInsP3-injected GV and MII stage oocytes were exposed to 10, 100, 300, 600, 1200, 3000 and 6000ms exposures of UV light. Each point represents the mean change in Fura-red ratio of several cells (n=17 for MII, n=12 for GV). Δ430/490 was significantly greater in MII eggs than in GV stage oocytes in response to all UV exposures ($P<0.05$, $^*P<0.002$). The mean change in Fura-red ratio exhibited by MII oocytes by the first (1) and second (2) Ca^{2+} transients at fertilisation are shown by the dotted lines for comparison (Δ430/490 first transient, 0.36+/-0.02; second transient, 0.25+/-0.02; n=8).

address the question of whether the timing of this upregulation tracks the generation of cortical clusters, we have used $cInsP_3$ to test the relative extent to which upregulation occurs during the first 9 hours of maturation, and during the final stages of meiotic maturation when the clusters are formed.

In the first series of experiments, GV stage and MI oocytes 9h after hCG were microinjected with $cInsP_3$ and simultaneously exposed to 10, 100, 1000 and 3000ms exposures of UV light (fig 3.8A). Peak change in Fura-red ratio was significantly greater in MI oocytes than in GV stage oocytes in response to all UV exposures ($P<0.05$ for 10ms exposure, $P<0.01$ for 100, 1000 and 3000ms exposures). In the second series of experiments, the change in Fura-red ratio in response to photolysis of $cInsP_3$ was compared in maturing oocytes 9 hours after hCG oocytes and ovulated MII oocytes 14 hours after hCG (fig 3.8B). MII oocytes exhibited a significantly greater change in Fura-red ratio than oocytes 9 hours after hCG for all UV exposures ($P<0.05$ for 10ms exposure, $P<0.01$ for 100, 1000 and 3000ms exposures). Thus there is an increase in the responsiveness of $InsP_3$ mediated Ca^{2+} release mechanism associated with the first 9 hours of oocyte maturation, and also with the latter stages of maturation.

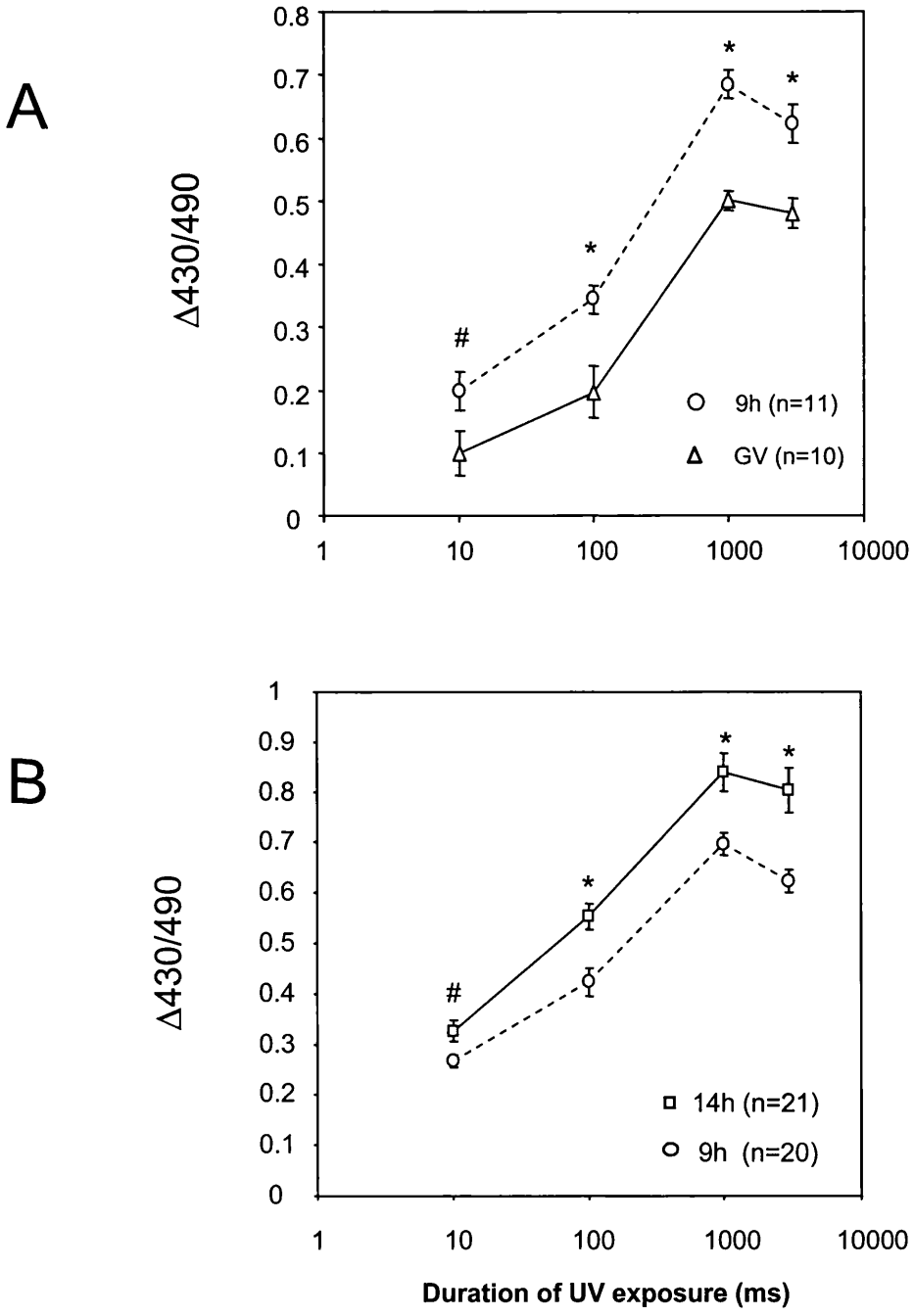


Figure 3.8. The maturation-associated upregulation of $InsP_3$ mediated Ca^{2+} release is incomplete 9 hours after hCG. (A) Caged $InsP_3$ was simultaneously photoreleased in GV stage oocytes and oocytes 9h after administration of hCG, as described in Chapter 2. The mean change in Fura-ratio (Δ430/490) is significantly greater in 9h oocytes in response to all exposure durations (* $P<0.01$, #$P<0.05$). (B) Caged $InsP_3$ was photoreleased in oocytes 9 hours and 14 hours (ovulated) after hCG. The mean change in Fura-ratio (Δ430/490) is significantly greater in ovulated oocytes in response to all exposure durations.

3.3 Discussion

The experiments presented in this chapter reveal that ER reorganisation during oocyte maturation is a multi-step process. Initially, GVBD is accompanied by a microtubule-dependent accumulation of ER firstly at the spindle poles, then as a dense ring around the MI spindle. The subsequent relocation of this ER ring to the oocyte cortex takes place concomitantly with spindle migration. Cortical clusters of ER are formed close to the time of, but in a manner independent of Pb1 extrusion. Finally, we confirm that oocyte maturation is associated with an increase in the responsiveness of $InsP_3$-mediated Ca^{2+} release, and demonstrate that a significant proportion of this increase occurs in the final hours of maturation, during which time ER clusters are formed. These results raise two main issues for discussion; the mechanisms underpinning ER reorganisation during oocyte maturation, and the role of ER cortical clusters in Ca^{2+} release.

The mechanism of ER reorganisation in meiosis

The first maturation-associated changes in ER structure become evident 90 minutes after the oocyte is released from the ovary, close to the expected time of GVBD. These changes have two noteworthy features. First, accumulations of ER are seen on the GV membrane at two opposing poles. These accumulation sites are most likely the spindle poles, since remarkably similar ER accumulations occur at the spindle poles at mitosis entry in sea urchin embryos (Terasaki, 2000;Whitaker and Larman, 2001). Secondly, DiI-labelled projections are visible in the formerly ER-free germinal vesicle. These protrusions most likely represent invaginations of the nuclear envelope, since the ER and nuclear membranes are continuous, and similar 'fingerlike projections' of ER invade the nucleus prior to permeablisation of the nuclear envelope (NE) in sea urchin embryos (Terasaki, 2000). In somatic cells these

invaginations of nuclear membranes are microtubule dependent and have been implicated in 'tearing' the NE at NEBD (Georgatos *et al.*, 1997;Beaudouin *et al.*, 2002).

The mechanisms underpinning ER reorganisations in the mouse oocyte had not previously been studied. Here we show that GVBD is associated with an accumulation of ER as a ring enclosing the MI spindle. This reorganisation is cell cycle dependent, as ER accumulation does not occur in germinal vesicle stage-arrested oocytes. Further, ER accumulation around the spindle at GVBD is inhibited by the microtubule depolymerisng drug nocodazole. This is consistent with previous studies where it has been show that microtubules often co-localise with ER tubules (Terasaki *et al.*, 1986), and microtubule depolymerisation is known to cause the collapse of the ER network in cultured eukaryotic cells (Terasaki *et al.*, 1986;Lee *et al.*, 1989). Further, similar changes in ER structure to those reported here occur at NEBD in the early sea urchin embryo and are also microtubule dependent (Terasaki, 2000). Finally, it is known that GVBD in mouse is associated with significant movement of tubulin from the cytoplasm towards the centre of the cell as a prelude to spindle formation (Combelles and Albertini, 2001).

Microtubule networks of cultured cells are organised with their growing ends (plus ends) in the cell periphery, and with their minus ends at the microtubule organising centre (MTOC), adjacent to the nucleus. Motor proteins responsible for the transport of vesicles and organelles along microtubules fall into two categories; the kinesin family mediate transport towards the plus-end, and dyneins facilitate transport towards minus-end (see Allan and Schroer, 1999 for review). Since ER reorganisation at GVBD involves redistribution to the centrally placed GV and spindle, it seems likely that this reorganisation might be dynein-mediated. Whilst dynein-mediated organelle motility has previously been reported to be regulated by the cell cycle, early indications from *Xenopus* egg extracts studies were that M-phase entry serves to reduce dynein-mediated ER transport in metaphase by

uncoupling dynein from its membranous cargo (Allan and Vale, 1991;Niclas *et al.*, 1996). However, one recent report using GFP-α-tubulin-expressing cells demonstrated a dramatic inward movment of microtubule bundles at mitotic prophase/pro-metaphase which is blocked by antibodies raised against the intermediate chain of dynein (Rusan *et al.*, 2002). A similar dynein-mediated inward movement of microtubule bundles in prophase/pro-metaphase may prove responsible for ER accumulation at GVBD. Moreover, a role for dynein has been described in the microtubule-depenent invagination of the nuclear envelope in mitosis described above (Salina *et al.*, 2002). Thus, while further work will be required to confirm whether similar mechanisms operate in meiosis and mitosis, it seems plausible that dynein may play a role in controlling ER dynamics at GVBD. Finally, though we have shown that ER does not accumulate around the chromosomes in nocodazole-treated oocytes, small areas of fluorescence remain in the oocyte interior. This may reflect GV membrane remnants which persist in the absence of the microtubule contribution to the membrane-tearing mechanism which has been demonstrated in somatic cells (Georgatos *et al.*, 1997;Beaudouin *et al.*, 2002).

Our data suggest that the MI spindle remains tightly enclosed within a ring of ER during migration to the cortex. It has been argued that movement of ER may in turn provide motility to other cell compartments (Kachar and Reese, 1988;Allan V, 1996). Thus one possible explanation considered for this close association was that enveloping the chromosomes within ER may serve to ensure faithful transport of the spindle to the oocyte cortex. This seems unlikely to be the case, however, since migration of the chromosomes at MI is actin-dependent (inhibited by cytochalasin B) and unaffected by nocodazole (Longo and Chen, 1985;Van Blerkom and Bell, 1986), which we have demonstrated prevents formation of the ER ring. An alternative explanation is that the ER-spindle association functions as a mechanism of transporting ER to the cortex. As already discussed, a key

feature of meiotic maturation in mammals is the generation of clusters of ER in the oocyte cortex (Mehlmann *et al.*, 1995;Shiraishi *et al.*, 1995). ER accumulation around the MI spindle following GVBD might provide a mechanism whereby ER from the interior of the cell is relocated to the cell periphery in preparation for ER cortical cluster generation. Within this model one might predict that ER would not redistribute to the cortex if spindle migration were inhibited. An explanation for the structure of the ER in the $mos^{-/-}$ oocyte is therefore not immediately clear. Spindle migration does not occur in oocytes of the $mos^{-/-}$ mouse, rather a larger than usual first polar body is generated by an asymmetric spindle elongation at anaphase (Choi *et al.*, 1996;Verlhac *et al.*, 2000). Thus the apparent abundance of ER in the cortex of the $mos^{-/-}$ oocyte suggests that ER redistribution is not critically dependent upon spindle migration. Interestingly, despite the presence of ER in the mos-/- oocyte periphery, cortical clusters were not observed. A separate mechanism may therefore exist which serves to organise cortical ER into discrete clusters. It has previously been speculated that the architecture of the oocyte cortex might be regulated by the activity of cell cycle associated kinases (Sardet *et al.*, 2002). This may prove accurate, since the activities of cdk1-cyclin B and MAPK, the major meiotic kinases, are altered in the $mos^{-/-}$ oocyte (Verlhac *et al.*, 1996). The relationship between meiotic kinases and ER organisation is addressed in Chapter 4.

Our initial observation that cortical clusters of ER are not formed until close to the time of Pb1 formation provoked the question whether polar body extrusion is a prerequisite of cluster formation. We have tested this possibility by investigating the ER in oocytes of mouse strain LTXBO, a considerable proportion of which exhibit precocious MI arrest (O'Neill and Kaufman, 1987;Hirao and Eppig, 1997b). Cortical clusters similar to those described in MII eggs were clearly visible in MI-arrested oocytes, demonstrating unequivocally that formation of ER clusters occurs independently of the MI-MII transition. Previous studies have shown that the ability to generate Ca^{2+} transients and competence to

undergo activation can be attained by MI-arrested oocytes, demonstrating that nuclear and cytoplasmic maturation can be uncoupled (Eppig *et al.*, 1994;Jones *et al.*, 1995b;Carroll *et al.*, 1996). Our data support this view, revealing that the maturation of ER structure does not depend upon nuclear maturation.

The role of cortical clusters of ER at MII

The precise role of ER clusters at MII is unknown. Several reports have argued that the organisation of the ER into discrete clusters might lead to an increase in the sensitivity of Ca^{2+} release mechanisms (Carroll *et al.*, 1996;Stricker *et al.*, 1998;Kline, 2000;Dumollard *et al.*, 2002). Such a model is supported by two principal lines of evidence; the first of which is a temporal correlation between the presence of cortical clusters and the sensitivity of Ca^{2+} release. Clusters of ER are generated during oocyte maturation in mouse (Mehlmann *et al.*, 1995), hamster (Shiraishi *et al.*, 1995), *Xenopus* (Terasaki *et al.*, 2001), nemertean (Stricker *et al.*, 1998) and human (GF and JC, unpublished observations). Thus ER clusters are present at fertilisation when demands upon Ca^{2+} signalling machinery are greatest. Further, in the Nemertean worm, ER clusters subsequently disperse at the same time that sperm-induced Ca^{2+} oscillations terminate (Stricker *et al.*, 1998). Additionally, the sensitivity of $InsP_3$-mediated Ca^{2+} release has been shown to increase following oocyte maturation (and ER cluster formation) in mouse (Mehlmann and Kline, 1994), hamster (Fujiwara *et al.*, 1993) and *Xenopus* (Terasaki *et al.*, 2001). Our data here corroborate this model, demonstrating that in mouse the maturation-associated increase in responsiveness is incomplete after 9 hours of maturation. Thus whilst an initial increase in responsiveness occurs independently of cortical ER-cluster formation, a significant component of the upregulation occurs during the period that the clusters form.

The second line of evidence is that Ca^{2+} release mechanisms are most sensitive in the oocyte cortex, the region in which the ER clusters reside. In mouse, whilst the initial fertilisation Ca^{2+} wave originates from the site of sperm egg fusion, the wave initiation site subsequently moves to the vegetal cortex (Deguchi *et al.*, 2000). This region therefore serves as the pacemaker site for the later Ca^{2+} waves regardless of the site of sperm-egg fusion (Kline *et al.*, 1999). Further, injection of sperm extracts or $InsP_3$ results in a Ca^{2+} wave with origin in the ER-rich cortex, regardless of injection depth (Carroll *et al.*, 1994;Kyozuka *et al.*, 1998;Oda *et al.*, 1999). Finally, and perhaps most compellingly, in *Xenopus* oocytes photorelease of small amounts of $InsP_3$ throughout the entire cell results in local Ca^{2+} release events (Ca^{2+} puffs) predominantly in a 6μm thick layer in the cortex (Callamaras and Parker, 1999), the same region in which $InsP_3$R-rich ER clusters are found (Kume *et al.*, 1997;Terasaki *et al.*, 2001).

Why a clustered arrangement of ER should augment the responsiveness of Ca^{2+} release is not clear. One possibility is that clusters of ER house clusters of $InsP_3$. A clustered distribution of $InsP_3$ receptors in the cortex of mouse eggs has been reported in two studies (Mehlmann *et al.*, 1996;Fissore *et al.*, 1999b), though not in a third (Parrington *et al.*, 1998). Nevertheless it seems likely that clustering of ER produces a similarly clustered arrangement of $InsP_3$ receptors. Given that both Ca^{2+} and $InsP_3$ are necessary for $InsP_3$ receptor opening (Bezprozvanny *et al.*, 1991), clustering of $InsP_3$ receptors might result in locally elevated $[Ca^{2+}]$ in the vicinity of the clusters, and thus increased receptor sensitivity (Kline, 2000). This hypothesis is supported by recent mathematical models which suggest that the arrangement of $InsP_3$Rs into small clusters may optimise the sensitivity of Ca^{2+} release, permitting Ca^{2+} responses when a similar number of homogeneously distributed channels might not respond (Shuai and Jung, 2002, 2003).

While the organisation of the ER into a network of clusters may serve to increase the sensitivity of Ca^{2+} release, the positioning of the clusters close to the plasma membrane may also be important (Dumollard *et al.*, 2002). Though it is widely believed that sperm-induced Ca^{2+} transients are $InsP_3$-driven (Miyazaki *et al.*, 1993;Jones and Nixon, 2000;Brind *et al.*, 2000;Xu *et al.*, 2003), the source of $InsP_3$ is unclear. Two models have been proposed. In the first model the sperm factor introduced in to the egg at fertilisation triggers $InsP_3$ production by hydrolysing plasma membrane PIP_2 (Halet *et al.*, 2002); in the second the sperm factor acts upon PIP_2 located on intracellular membranes (Rice *et al.*, 2000). In either case, the positioning of ER clusters close to the plasma membrane would result in their exposure to the highest possible concentrations of $InsP_3$. This highly specialised arrangement may be of key importance in signal amplification at fertilisation as relatively low levels of PIP_2 hydrolysis (Halet *et al.*, 2002), result in low levels of $InsP_3$ production (Jones and Nixon, 2000;Halet *et al.*, 2002) resulting in dramatic and long-lasting changes in $[Ca^{2+}]_i$.

Whilst our results show a correlation between cortical ER-cluster formation and increased responsiveness of $InsP_3$-mediated Ca^{2+} release in the latter stages of oocyte maturation, these changes may be attributable to other factors thought to influence the responsiveness of Ca^{2+} release during the cell cycle, such as $InsP_3R$ expression, the degree of Ca^{2+}-store loading, and activities of Ca^{2+} pumps and exchangers (discussed in Chapter 1). An alternative possibility is that the intrinsic buffering capacity of the oocyte is modified during oocyte maturation. The role of cytosolic Ca^{2+} buffers in mouse oocytes is yet to be investigated. Other components of the Ca^{2+} signalling machinery which may contribute to maturation-associated upregulation of Ca^{2+} release responsiveness that are yet to be examined include the contribution of mitochondria, and plasmalemmal Ca^{2+} influx. Demonstration of a functional link between ER structure and the responsiveness of Ca^{2+} release will require manipulation of ER structure independently of other factors.

Summary

ER is reorganised to the MI spindle at the time of GVBD in a microtubule-dependent mechanisam. However, neither migration of the meiotic spindle, nor the MI-MII transition are critical for the subsequent redistribution of this ER to the oocyte cortex. Consistent with these results, we propose a model whereby the delivery of the ER to the cortex depends upon a close association between the plasma membrane and the ER ring, rather than being strictly dependent upon spindle migration or polar body extrusion. Confirmation of this model will require experiments in which the position of the ER ring can be manipulated independently of the cell cycle. The mechanisms underpinning the subsequent distribution of ER into the vegetal hemisphere for formation of cortical clusters remain uninvestigated, though one might speculate a role for plus-end directed transport of ER from MII spindle centromeres along microtubules in the oocyte periphery.

4. ER structure and $InsP_3$-mediated Ca^{2+} release following fertilisation and in mitosis

4.1 Introduction

The previous Chapter presented experiments which examined ER reorganisation and the responsiveness of $InsP_3$-mediated Ca^{2+} release during oocyte maturation. Changes in the organisation of the ER are also known to occur following fertilisation in several species. In starfish and sea urchin oocytes fertilisation is associated with a transient loss of ER membrane continuity immediately following fertilisation (Jaffe and Terasaki, 1993;Terasaki *et al.*, 1996). Since in these species fertilisation triggers only a single monotonic Ca^{2+} rise, and since no change in ER structure is seen in the first minutes after fertilisation in species which exhibit repetitive Ca^{2+} transients (Stricker *et al.*, 1998;Kline *et al.*, 1999), it has been argued that ER fragmentation following fertilisation may prohibit the generation of multiple Ca^{2+} oscillations (Kline, 2000).

Further evidence that ER reorganisations may determine the timecourse of sperm-induced Ca^{2+} oscillations arises from a study of Nemertean worm oocytes. Oocytes of this species contain cortical ER clusters which disappear 40-60 minutes after fertilisation, at approximately the same time that sperm-induced oscillations stop (Stricker *et al.*, 1998). Since clusters of ER are believed to contribute to the sensitivity of Ca^{2+}-release in eggs (discussed in Chapter 3), it was suggested that the disappearance of ER clusters may terminate fertilisation Ca^{2+} transients in the nemertean (Stricker *et al.*, 1998;Kline, 2000).

In mammals, though it has been reported that no dramatic changes in ER structure take place during the first seven Ca^{2+} oscillations, the structure of the ER during the later events of activation, including Pb2 extrusion and formation of the pronuclei is yet to be

examined (Kline *et al.*, 1999). On the basis of the studies discussed above, it has been predicted that ER clusters may disappear when Ca^{2+} oscillations conclude, at the time of pronucleus formation (Kline, 2000). The initial aim of this chapter is to test this hypothesis and to investigate the relationship between the cell cycle and ER structure during the completion of meiosis and the first mitotic division.

Whilst the relationship between sperm induced Ca^{2+} release and ER structure is unknown, there is growing evidence to suggest that Ca^{2+} release at fertilisation is influenced by the cell cycle (Nixon *et al.*, 2000;Carroll, 2001). Fertilisation of ascidian eggs triggers two separate series of oscillations which accompany progression through MI and MII. It is clear that this pattern of Ca^{2+} release is governed by cdk1-cyclin B activity since microinjection of exogenous cyclin causes prolongued oscillations and abolition of the gap-period between the two oscillation groups, the oscillations subsequently ending when cyclin is eventually destroyed (McDougall and Levasseur, 1998;Levasseur and McDougall, 2000).

The relationship between cdk1-cyclin B activity and Ca^{2+} release is not as obvious in mouse oocytes since sperm-induced Ca^{2+} oscillations continue until the pronuclei form (Jones *et al.*, 1995a;Marangos *et al.*, 2003), though cyclin B is largely destroyed by the time of Pb2 extrusion (Moos *et al.*, 1996;Nixon *et al.*, 2002). Nevertheless, maintenance of M-phase leads to persistent oscillations both in meiosis and mitosis (Jones *et al.*, 1995a;Kono *et al.*, 1996). That modulation of the sensitivity of Ca^{2+}-release may contribute to these phenomena can be inferred from reports that strontium is able to induce Ca^{2+} oscillations in mitosis, but not interphase (Kono *et al.*, 1996), and less Ca^{2+} is released by pronucleate embryos than unfertilised eggs in response to $InsP_3$ microinjection (Jones *et al.*, 1995a;Jones and Whittingham, 1996). However, a detailed study of the relationship between cell cycle stage and the responsiveness of $InsP_3$-releasable Ca^{2+}-stores in mammalian oocytes and embryos is yet to be presented.

The aim of this chapter is therefore to investigate the relationship between the cell cycle, $InsP_3$ mediated Ca^{2+} release, and the spatial organisation of the ER following activation and during the first mitotic division. The results of the experiments presented demonstrate that ER cortical clusters do not persist throughout sperm-induced Ca^{2+} oscillations, rather they disperse around the time of Pb2 extrusion as a result of decreased cdk1-cyclin B activity. A downregulation in the responsiveness of $InsP_3$-induced Ca^{2+} release occurs by the time of Pb2 extrusion which is independent of the fertilising sperm. Cortical clusters of ER do not reappear when cdk1-cyclin B activity returns in mitosis, instead the ER accumulates around the mitotic spindle. Finally, mitosis entry is associated with an increase in the responsiveness of $InsP_3$ induced Ca^{2+} release both in fertilised and parthenogenetic embryos. These results demonstrate a close relationship between ER structure, $InsP_3$-mediated Ca^{2+} release, and the cell cycle.

4.2 Results

4.2.1 Reorganisation of ER following fertilisation of MII oocytes

To observe the structure of the endoplasmic reticulum of the fertilised oocyte at different stages of development, metaphase II arrested (MII) oocytes were fertilised *in vitro*, microinjected with DiI, and the ER examined at different times after fertilisation. Fertilised oocytes were selected on the basis of incorporation cones. Oocytes which had been fertilised but had not yet extruded the second polar body (Pb2) were examined on the confocal microscope 2-2.5 hours after insemination. Oocytes which had extruded Pb2 but in which no pronuclei were visible were examined 3-3.5 hours after insemination. Pronucleate stage embryos were examined at least five hours after addition of sperm. As in Chapter 3, examination of DiI-injected MII oocytes revealed the ER to be reticular in nature throughout the cytoplasm, with a dark ER-free area where the metaphase spindle resides, and ER clusters in the cortical slice. The ER of the fertilised oocyte prior to formation of Pb2 was similar to that of the MII oocyte, exhibiting cortical accumulations of ER similar to those seen in the MII oocyte. Cortical accumulations of ER were not as evident in oocytes which had extruded Pb2. Instead, the ER of Pb2 oocytes and pronucleate embryos were characterised by larger areas of bright fluorescence deeper in the cytoplasm not seen prior to Pb2 formation. The Pn membrane was stained demonstrating continuity between ER and pronuclear membranes.

These results suggest that an ER reorganisation takes place around the time of Pb2 extrusion. In order to quantify these changes, we have counted the number of ER clusters in the cortical slice of each oocyte and embryo (see figure 4.1B). The criteria for this quantification are set out in Chapter 2. There was no significant difference between the number of ER clusters found in the cortex of the MII oocyte (35.8+/-2.6; n=13) and following fertilisation (36.3+/-3.9). The number of cortical clusters was significantly reduced following extrusion of Pb2 (Pb2, 7.8+/-1.7; Pn, 7.7+/-1.5; $P<0.001$; fig 4.1b). Thus a

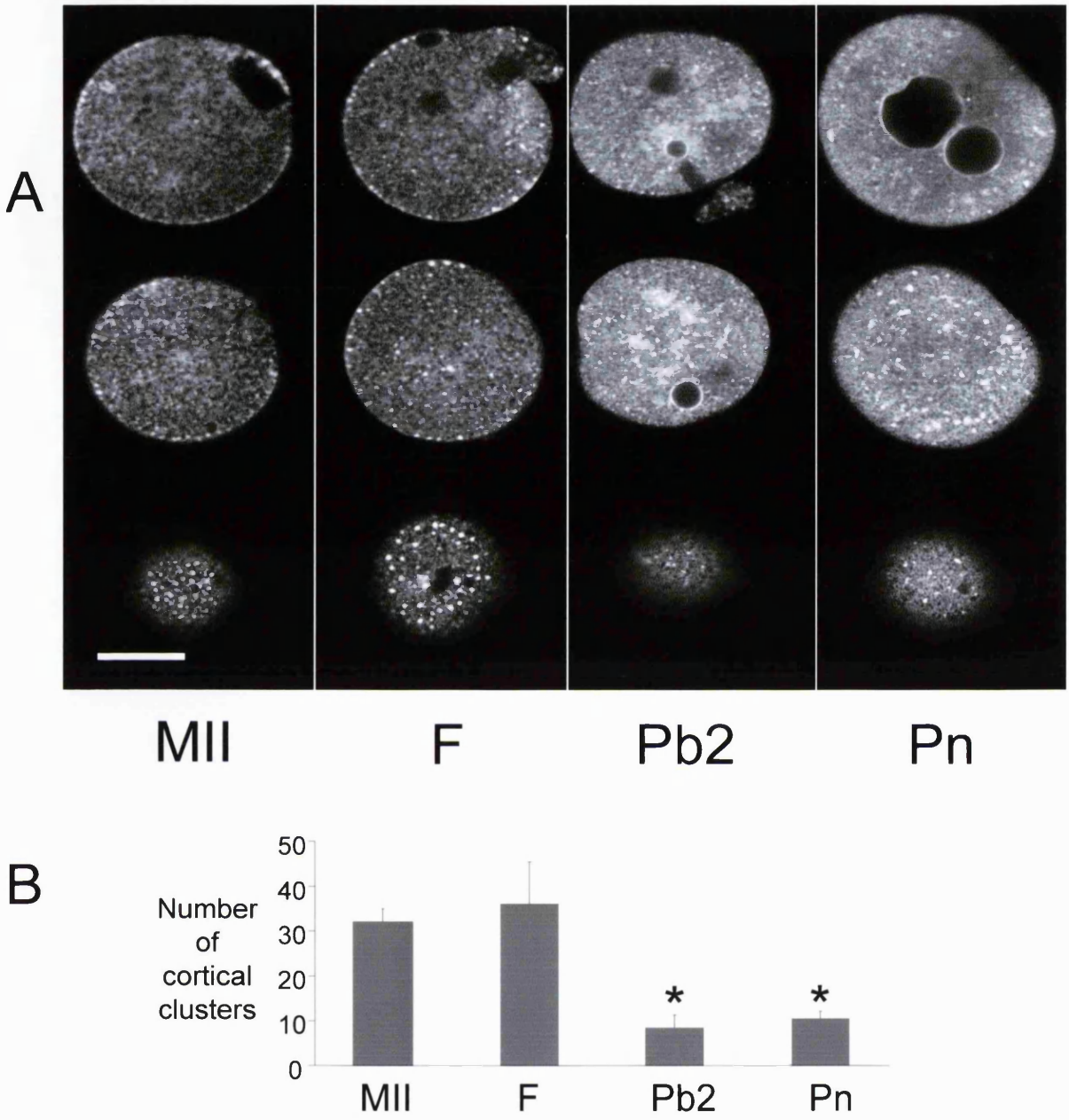


Figure 4.1. ER reorganisation following fertilisation. (A) Unfertilized metaphase II arrested oocytes (MII), fertilized oocytes prior to polar body formation (F), fertilized oocytes after polar body formation (Pb2) and pronucleate stage (Pn) embryos were injected with DiI and examined using confocal microscopy. Equatorial sections (top row) and sections estimated to be 18-20μm (middle row) and 5-7μm (bottom row) from the coverslip are displayed. Note the clear presence of ER clusters in the cortex of MII and F stages and their absence in Pb2 and Pn stages. Pronuclei at various stages of development are evident in the Pb2 and Pn stages and the ER can be seen to be continuous with the pronuclear membranes. Scale bar is 30μM. (B) The numbers of cortical clusters were quantified at the different stages of development (as described in Chapter 2). There were significantly less cortical clusters in Pb2 and Pn stages than in oocytes prior to Pb2 extrusion (*P<0.01). Data are from 13 MII, 7 fertilised, 9 Pb2 and 10 Pn stage eggs examined over the course of two separate experiments. Scale bar 30μm. .

statistically significant reorganisation of the cortical ER takes place at around the time of Pb2 extrusion.

4.2.2 Reorganisation of the ER following parthenogenetic activation

To investigate whether ER reorganisation is specific to fertilisation, ER structure was investigated following parthenogenetic activation by ethanol. The ER was examined in MII oocytes, following Pb2 extrusion, and after pronucleus formation (fig 4.2). MII oocytes displayed ER in the pattern previously described, exhibiting 38.1+/-3.9 cortical clusters (quantification criteria as above). After Pb2 extrusion the ER had significantly fewer cortical clusters (8.0+/-2.4; $P<0.01$), and displayed spatial organisation similar to that of fertilised embryos at the same developmental stage. Pronucleus stage parthenogenetic embryos also exhibited significantly fewer cortical clusters of ER than MII oocytes (9.8+/-2.3; $P<0.01$; fig 4.2). Thus the timecourse of ER reorganisation following parthenogenetic activation is similar to fertilisation. These data therefore demonstrate that reorganisation of the ER and loss of cortical clusters occurs at around the time of Pb2 extrusion in a manner not dependent upon a fertilising sperm. In addition, given that parthenogenetic activation with ethanol produces a monotonic rise in $[Ca^{2+}]_i$ (Steinhardt *et al.*, 1974), these data demonstrate that this ER reorganisation is not dependent upon repetitive Ca^{2+} transients.

4.2.3 Mechanism of ER reorganisation at fertilisation: effect of MG132

The previous experiments demonstrate that ER reorganisation takes place around the time of Pb2 extrusion both in fertilised and in parthenogenetic embryos. Since it is known that a decrease in cdk1-cyclin B activity is temporally associated with, and necessary for Pb2 formation (Verlhac *et al.*, 1994; Moos *et al.*, 1996), we have investigated a possible role for cdk1-cyclin B in the control of ER re-organisation. MG132 is an inhibitor of the proteosome,

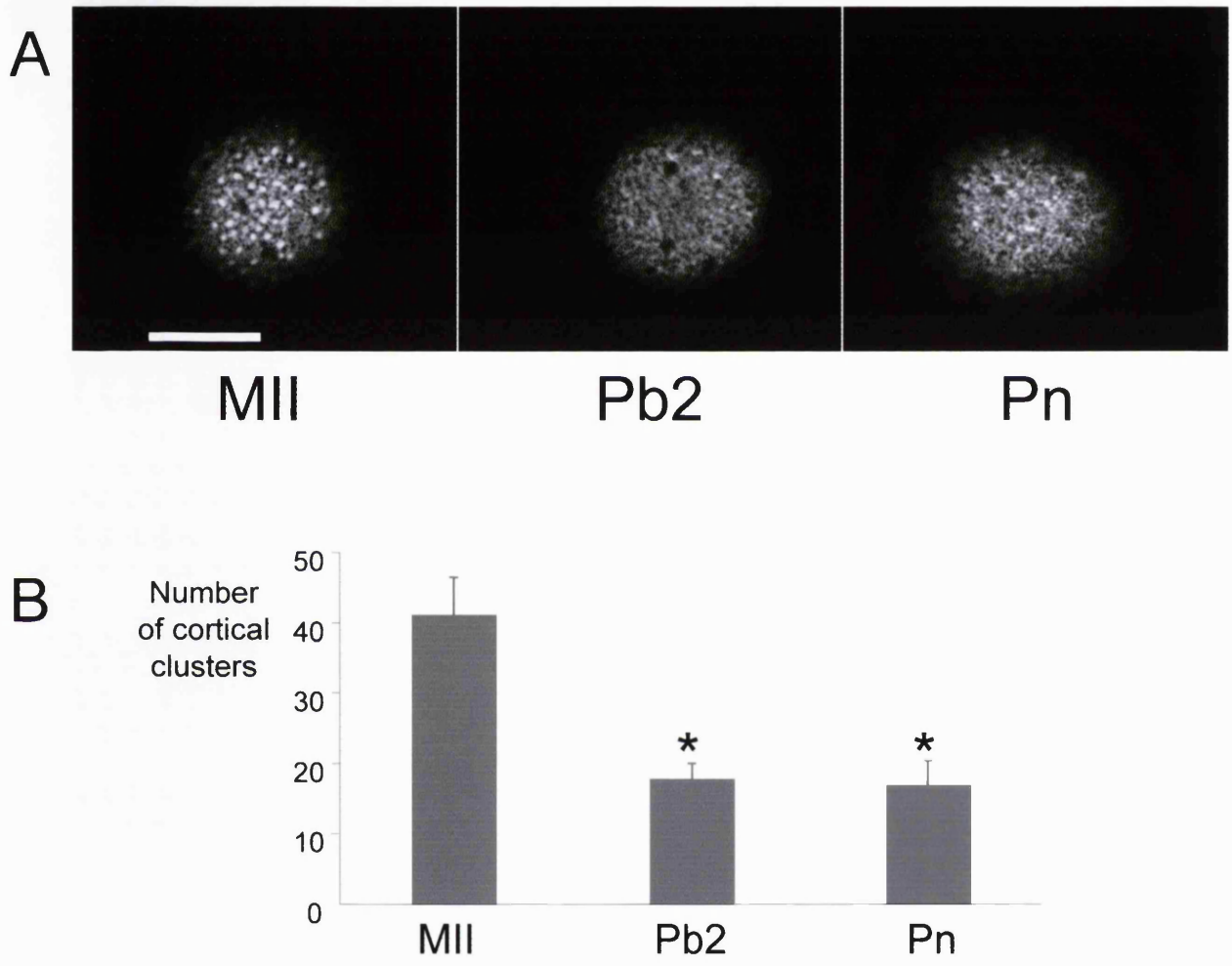


Figure 4.2. ER reorganization following parthenogenetic activation. (A) Parthenogenetic embryos were produced by incubating MII oocytes in 7% ethanol for 7 minutes. Representative cortical slices of oocytes at MII, Pb2 and Pn stages are shown. (B) Cortical clusters of ER were quantified (see Chapter 2). The number of cortical clusters was significantly reduced after egg activation (*P< 0.01). Thus ER reorganisation does not require fertilisation and the associated Ca^{2+} oscillations. Data are from two experiments on 8, 12 and 13 MII, Pb2 and Pn stage oocytes, respectively. Scale bar 30μm.

which prevents cell cycle progression at fertilisation by inhibiting cyclin destruction (Brind *et al.*, 2000; FitzHarris *et al.*, 2003). Here we have used MG132 to establish the effect of inhibiting cyclin B destruction upon ER redistribution following fertilisation.

MII oocytes were fertilised *in vitro* in the presence of 50μM MG132. Unfertilised MII oocytes, control fertilised oocytes and MG132 fertilised oocytes were microinjected with DiI and the ER examined 7 hours after sperm addition, at which time control fertilised oocytes had formed pronuclei. In all cases MG132 treated oocytes failed to form Pb2. Unfertilised eggs and control pronucleate stage oocytes displayed similar arrangements of ER as described earlier, containing 32.1+/-1.5 and 7.7+/-1.2 ER clusters in their cortical slices respectively (fig 4.3A). The ER in MG132 fertilised oocytes was similar in appearance to that of unfertilised eggs, exhibiting 32.5+/-1.2 cortical clusters. The mean number of clusters seen in the cortical slice of MG132 fertilised oocytes was not significantly different to that of unfertilised eggs ($P>0.8$), but significantly greater than that of fertilised control oocytes ($P<0.01$; fig 4.3B).

4.2.4 Effect of MG132 and exogenous cyclin upon ER following parthenogenetic activation

To investigate whether maintaining a high level of cdk1-cyclin B activity is sufficient to inhibit ER reorganisation following parthenogenetic activation, we examined the ER following activation by ethanol in eggs first microinjected with excess cyclin B in the form of a cyclin B1-GFP fusion protein (Clute and Pines, 1999), and in eggs treated with MG132. Oocytes were injected with DiI and the ER examined four hours after exposure to ethanol, at which point control activated oocytes had extruded Pb2. There was no significant difference in the number of cortical clusters exhibited by non-activated MII (34.8+/-1.9 cortical clusters), cyclin-injected (40.9+/-5.7) and MG132-treated oocytes (30.7+/-2.7). Significantly

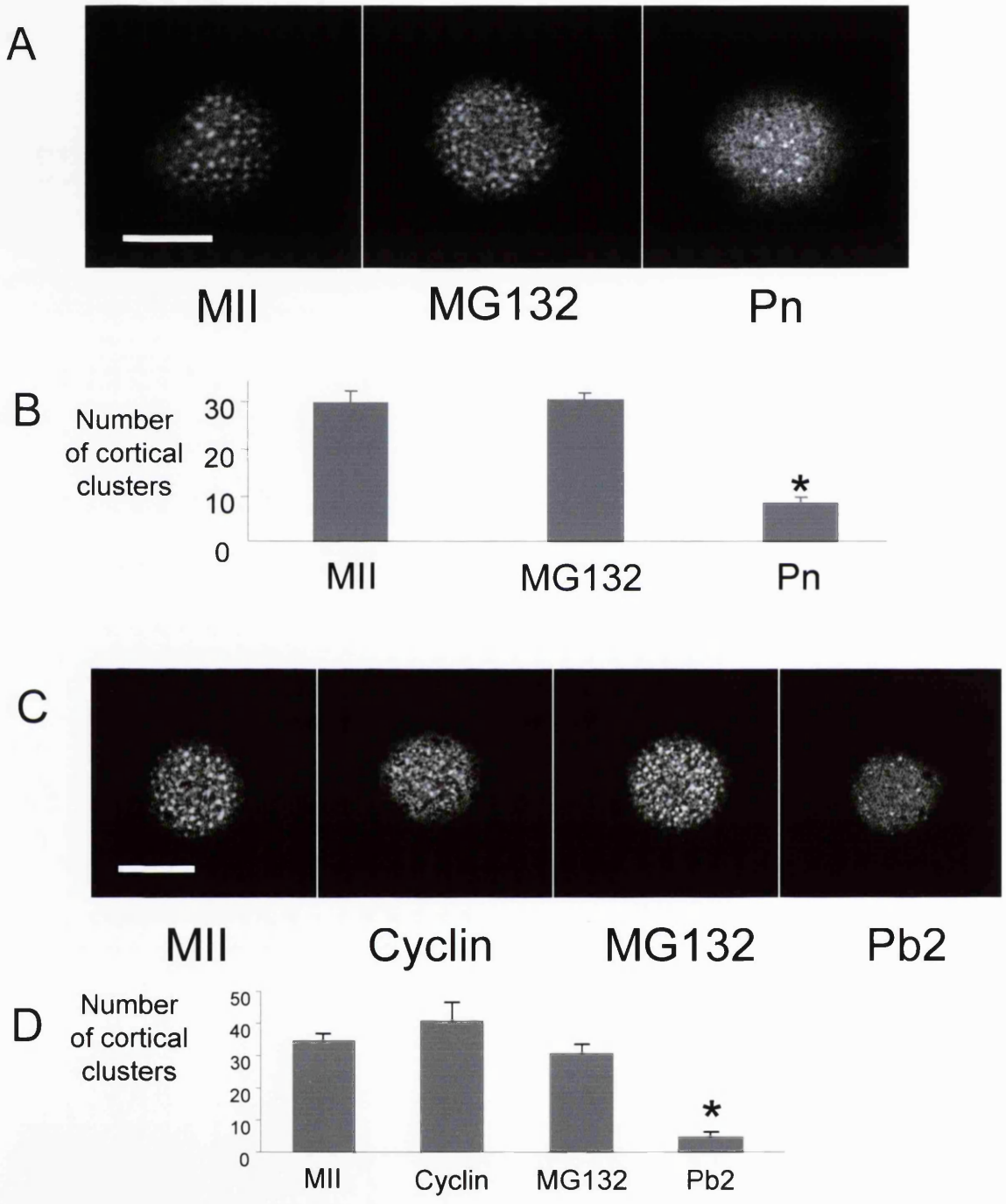


Figure 4.3. ER reorganisation is inhibited in M-phase arrested oocytes. (A) The ER was compared in unfertilised (MII), fertilised MG132 treated, and control fertilised (Pn) oocytes. Representative confocal sections through the cortex are shown. (B) A significant ER reorganisation occurs following fertilisation, but not in the presence of MG132 (*P<0.01; n=10 for MII, n=9 for MG132 and n=12 for Pn). The presence of a fertilising sperm in MG132-treated oocytes was confirmed using Hoechst 33342 (not shown). (C) ER reorganisation following activation by ethanol exposure is inhibited by MG132 or exogenous cyclin. ER was examined three hours after exposure to ethanol. Representative cortical slices are shown. (D) Pb2-stage oocytes (n=12) exhibit significantly fewer cortical clusters of ER than unactivated (MII; n=6), Cyclin-GFP injected (n=7) and MG132-treated oocytes (n=10; *P<0.01). Scale bar 30μm.

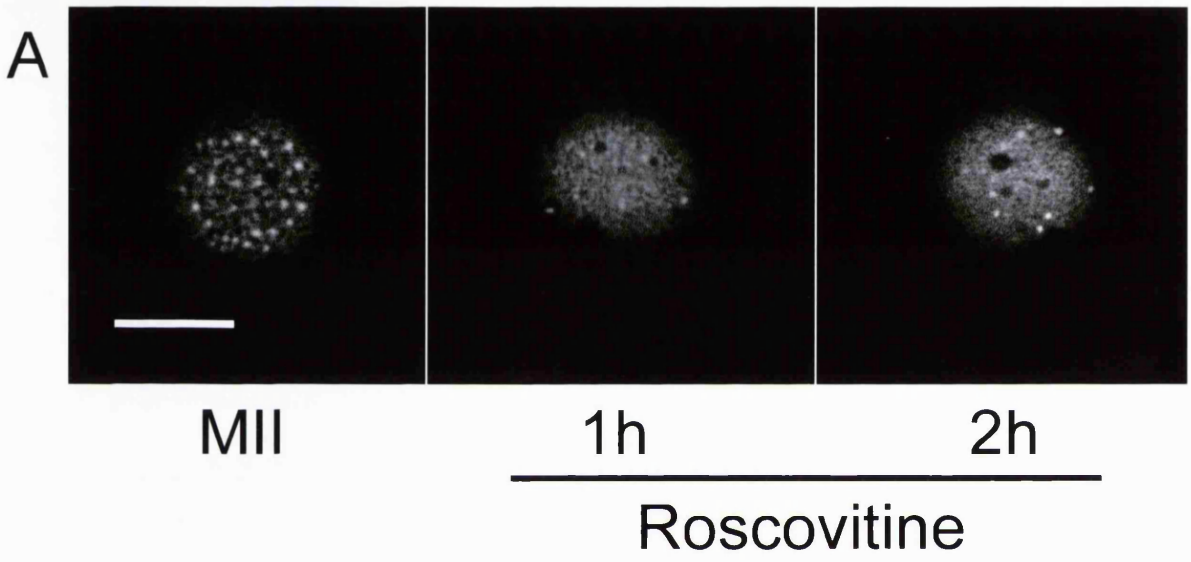
fewer cortical ER clusters were observed following Pb2 extrusion (4.8+/-1.3; $P<0.01$). Thus these data demonstrate using two independent methods that the reorganisation of ER seen following activation is dependent upon the decrease in cdk1-cyclin B activity which occurs at the time of Pb2 formation.

4.2.5 Effect of roscovitine upon ER structure

To further investigate the relationship between cdk1-cyclin B activity and ER reorganisation, we have examined the effect of roscovitine, a specific inhibitor of the cdk1-cyclin B complex (Meijer *et al.*, 1997;Deng and Shen, 2000), upon the arrangement of DiI-labelled ER in mouse oocytes. Unfertilised eggs were incubated in 75μM roscovitine for 1 or 2 hours prior to ER examination. After 1 hour none of the oocytes had extruded Pb2, whereas 7 of 11 oocytes had extruded Pb2 by 2 hours. Untreated eggs displayed ER structure typical of MII arrested oocytes, their cortices containing 27.1+/-2.1 clusters (fig 4.4A). The ER of oocytes treated with roscovitine for either one or two hours exhibited significantly fewer clusters in the cortical slice (8.7+/-1.9 and 4.3+/-0.9 respectively) than that of MII oocytes ($P<0.01$; fig 4.4B). Thus, inhibition of cdk1 activity in MII oocytes is sufficient to cause ER reorganisation and disappearance of cortical clusters of ER.

4.2.6 $InsP_3$-mediated Ca^{2+} release following parthenogenetic activation

Our results show that cortical clusters of ER disperse around the time of Pb2 extrusion. To investigate the relationship between this ER reorganisation and the responsiveness of $InsP_3$-releasable Ca^{2+} stores we compared Ca^{2+} release in MII eggs, Pb2 and Pn stage parthenogenetic embryos using the method of photoreleasing caged $InsP_3$ ($cInsP_3$), whilst monitoring $[Ca^{2+}]_i$ with Fura-red (see Chapter 3). $[Ca^{2+}]_i$ was monitored in $cInsP_3$-injected oocytes during exposure to 10, 100, 1000 and 3000ms of UV light. Peak $[Ca^{2+}]_i$ change was



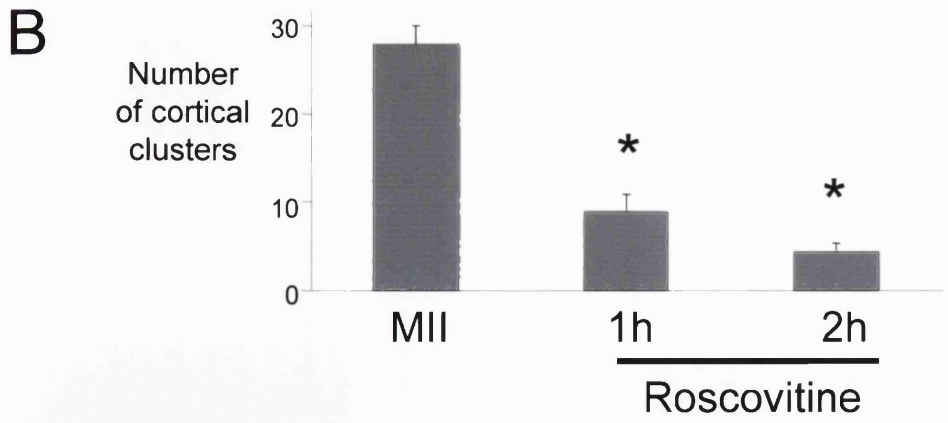


Figure 4.4. Roscovitine causes ER-reorganisation. (A) MII oocytes were incubated in roscovitine (75μM) for 1 or 2 hours prior to examination of the ER. Representative cortical confocal slices are shown. (B) Significantly fewer ER cortical clusters are exhibited by roscovitine-treated oocytes (MII, n=10; 1h, n=9; 2h, n=15; *P<0.01). Scale bar 30μm.

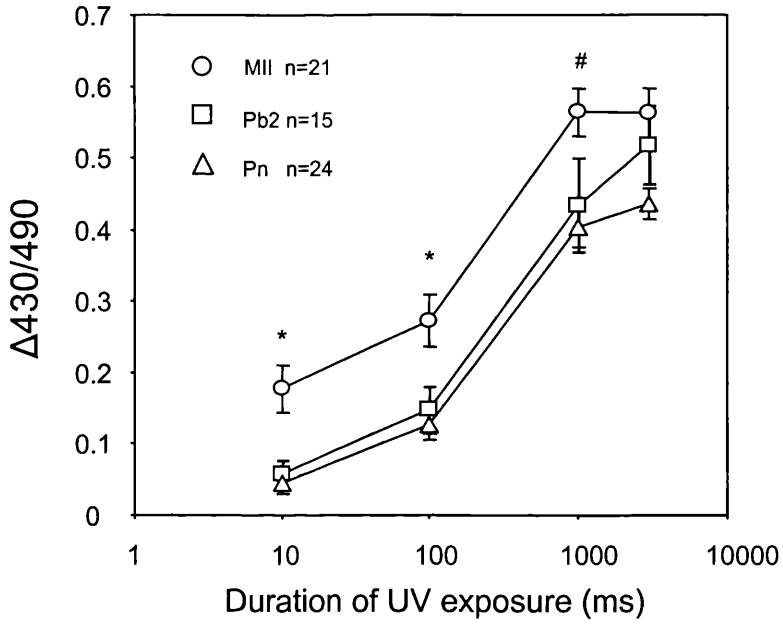
recorded in MII oocytes, 2 hours after activation by exposure to ethanol (following second polar body formation), and 6 hours after activation (following pronucleus formation). The timings of PMSG and hCG administration, and of oocyte activation, were staggered in order to simultaneously produce oocytes at each of these stages. Oocytes at each cell-cycle stage were microinjected with the same pipette of $cInsP_3$ and placed on the microscope stage at the same time for exposure to UV light and Ca^{2+} measurement. The mean change in Fura-red ratio was significantly greater in MII oocytes than Pn stage parthenotes in response to all four exposure durations ($P<0.01$), and than Pb2 parthenotes in response to 10 and 100ms exposures ($P<0.01$ and $P<0.05$ for 10ms and 100ms exposures respectively, see fig 4.5A). There was no significant difference in mean Fura-red ratio change between Pb2 and Pn stage embryos in response to any length of UV exposure.

In order to verify that the observed changes in Ca^{2+} release were due to activation of the oocyte, and not attributable to oocyte aging, we investigated the effect of photoreleasing $cInsP_3$ in oocytes 18 hours post hCG (fresh oocytes), and 24 hours after hCG (aged oocytes). No significant difference in Ca^{2+} release was seen between fresh and aged oocytes in response to any duration time tried (fig 4.5B). Thus a significant downregulation in the responsiveness of $InsP_3$ medaited Ca^{2+} release occurs by the time of Pb2 extrusion which is not attributable to oocyte aging.

4.2.7 Reorganisation of the endoplasmic reticulum during mitosis

The dependence of the disappearance of cortical clusters of ER following fertilisation and parthenogenetic activation upon the decrease in cdk1 activity which occurs following fertilisation raises the question of whether cortical clusters of ER reappear when cdk1 activity increases again in mitosis. To address this question we have examined the structure of the ER during mitosis in *in vitro* fertilised (fig 4.6A) and parthenogenetic (fig 4.6B)

A



B

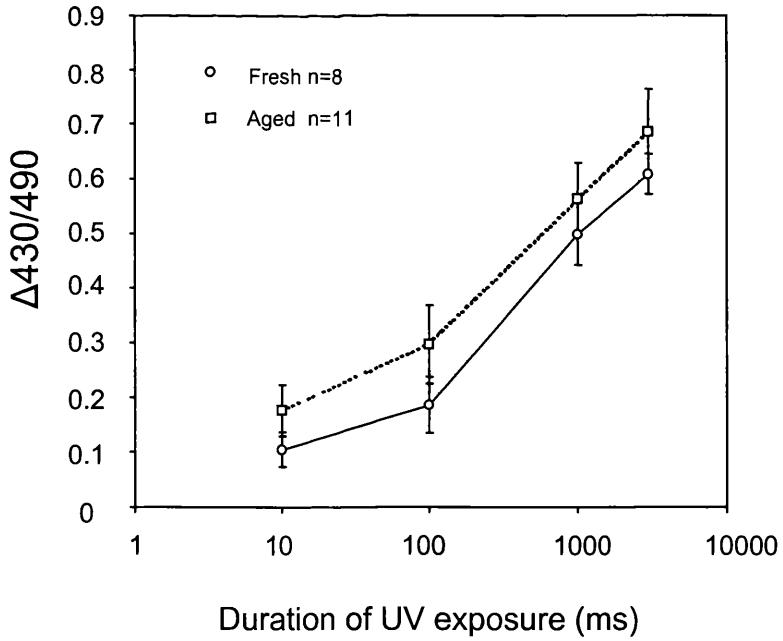


Figure 4.5. The sensitivity of $InsP_3$-induced Ca^{2+} release is reduced in oocytes that have extruded the second polar body. (A) $InsP_3$ was photo-released in MII oocytes and ethanol-activated parthenogenetic embryos at the Pb2 and Pn stages (see Results and Chapter 2). The peak change in Fura-red ratio was greater in MII oocytes for a given dose of $InsP_3$ than those that have been activated (*P<0.01; #P<0.03). (B) The peak change in Fura-red ratio exhibited by aged (24h post hCG) and fresh (18h post hCG) oocytes is similar suggesting time from ovulation does not affect the sensitivity of $InsP_3$ mediated Ca^{2+} release over this timecourse.

embryos. Embryos were injected with DiI, and the ER examined with confocal microscopy at three developmental stages: 2-3 hours before the predicted time of NEBD, shortly after NEBD, and following cytokinesis (fig 4.6). Embryos prior to NEBD displayed a similar arrangment of ER as described for pronucleate embryos above. In embryos which had undergone NEBD, a bright ring of fluorescence was observed in the centre of the cell. There was no evidence of cortical clusters of ER such as those seen in metaphase II eggs. Analysis of 5-7 cells from each cell cycle stage using *integrated morphometry analysis* (see Appendix 2) reveals no significant difference in the number of cortical ER clusters between pronucleate and mitotic embryos ($P>0.1$ both for fertilised and parthenogenetic embryos). Following cytokinesis, the ER of individual blastomeres closely resembled that of pronucleate stage embryos. There was no discernable difference in ER structure between fertilised and parthenogenetic embryos at any stage examined.

4.2.8 $InsP_3$-mediated Ca^{2+} release during the first mitotic cell cycle

The results of the previous experiments demonstrate that the cortical clusters do not re-appear during mitosis, rather the ER accumulates around the mitotic spindle following NEBD. To investigate the responsiveness of Ca^{2+} releasing mechanisms over this timecourse, we have compared $InsP_3$-mediated Ca^{2+} release in interphase and mitosis.

Experiments were designed so as to allow uncaging of $cInsP_3$ in interphase and mitotic embryos simultaneously. In order to achieve this, two sets of IVF or activations were performed so as to produce pronucleate and mitotic embryos at the same time. The timings of hCG administrations were staggered so that activations and inseminations took place in similarly aged oocytes. For parthenogenetic embryos, $cInsP_3$ was microinjected 12-13 and 17-18 hours after exposure to ethanol. For fertilised embryos, $cInsP_3$ was photoreleased 17-18 and 21-22 hours after fertilisation. In both cases, these timings provided one group in

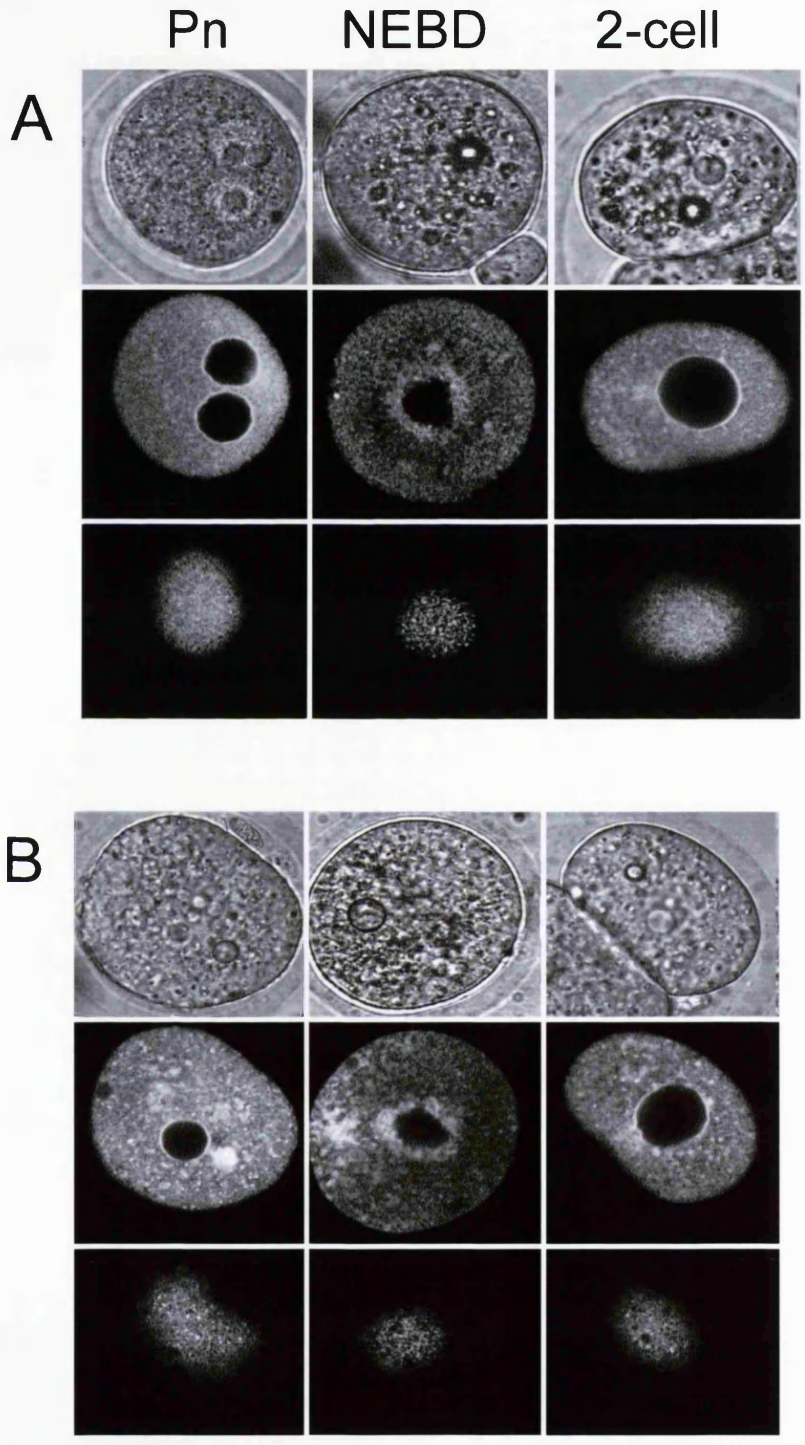


Figure 4.6. Cortical clusters of ER do not re-appear in mitosis. Fertilized (A) and parthenogenetic (B) one-cell embryos were injected with DiI at different times of the first mitotic division; at Pn stage 2-4 hours prior to the expected time of NEBD, in mitosis after NEBD and after cleavage to the 2-cell stage. Bright field (top row), equatorial (middle row) and cortical (bottom row) confocal sections are illustrated. Note that in the cortical sections there is no evidence of ER clusters. The ER in the 2-cell embryo is similar to that of the one cell embryo in interphase. Note also an accumulation of ER around the mitotic spindle. A minimum of 10 cells were examined at each stage over the course of two days.

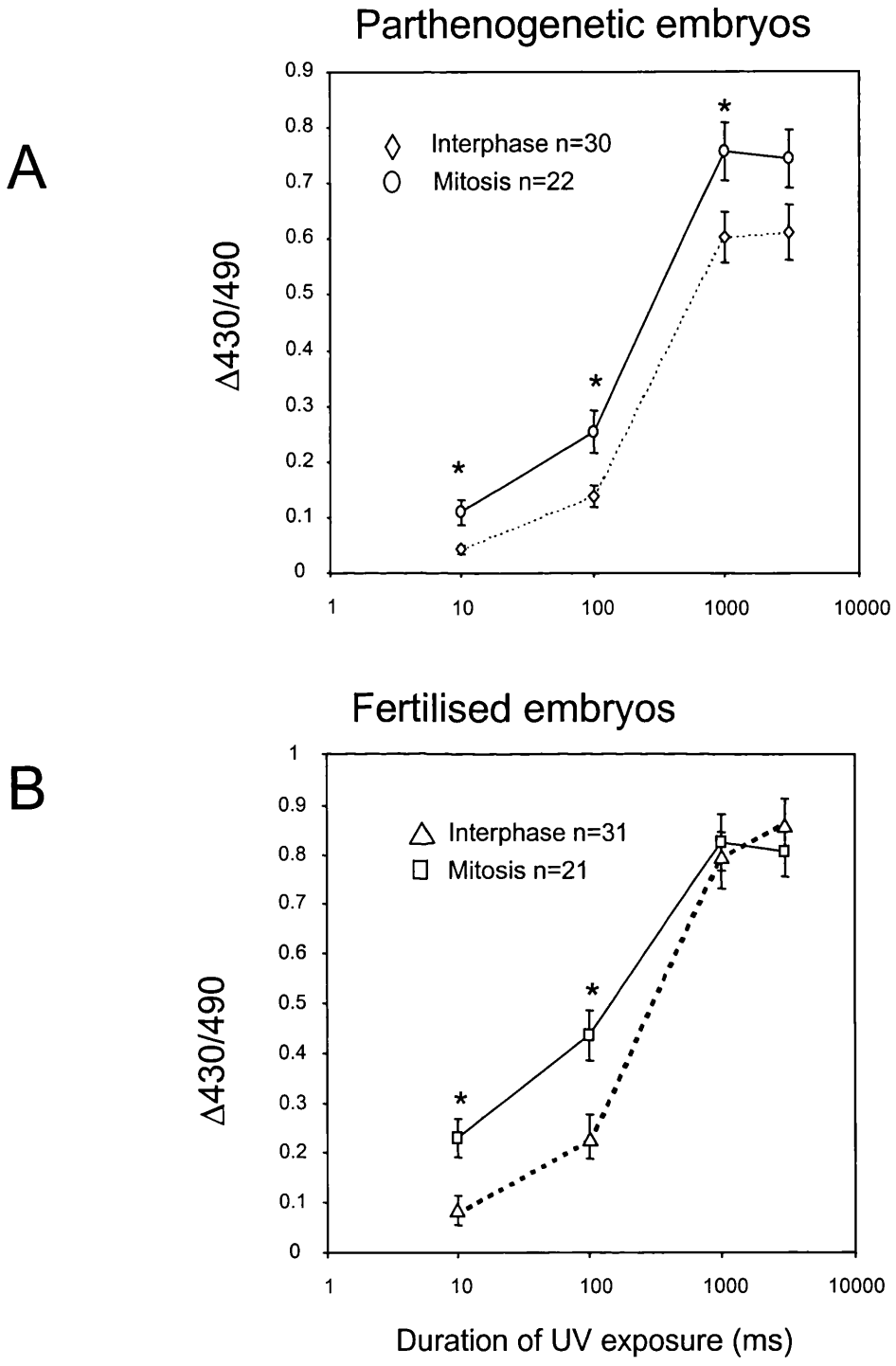


Figure 4.7. The responsivness of $InsP_3$-induced Ca^{2+} release increases during mitosis. Interphase and mitotic parthenogenetic (A) and fertilized (B) embryos were injected with $cInsP_3$ and loaded with Fura-red. The peak change in Fura-red ratio is significantly greater in mitosis than in interphase (*P<0.01).

which all embryos were in interphase and one group in which at least 50% were mitotic. Embryos were microinjected with the same pipette of $cInsP_3$ and together exposed to UV light whilst $[Ca^{2+}]_i$ was monitored as previously described.

The results of these experiments are shown in figure 4.7. Mitotic parthenotes released significantly more Ca^{2+} in response to 10ms, 100ms and 1000ms exposures to UV light than interphase parthenotes ($P<0.01$; fig 4.7A). Similar changes in Fura-red ratio were seen in interphase and mitotic parthenotes in response to 3000ms exposures. Similarly, mitotic fertilised embryos exhibited a greater change in Fura-red ratio than interphase embryos in response to 10ms and 100ms exposures of UV light (fig 4.7B). There was no significant difference between interphase and mitotic embryos in response to 1000ms and 3000ms exposures. These experiments demonstrate that the responsiveness of $InsP_3$-mediated Ca^{2+} release is upregulated in mitosis, both in fertilised and parthenogenetic embryos.

4.3 Discussion

In this Chapter we have demonstrated a dramatic cell-cycle associated reorganisation of the endoplasmic reticulum which occurs at the time of second polar body extrusion. This reorganisation is characterised most notably by the disappearance of ER cortical clusters. An accompanying decrease in the responsiveness of $InsP_3$-mediated Ca^{2+} release is reported which is not attributable to oocyte aging. Cortical clusters of ER do not reappear during the first mitotic division, rather the ER accumulates in the region surrounding the mitotic spindle. Finally, entry into the first mitotic division is accompanied by a maternally driven increase in the responsiveness of $InsP_3$-releasable Ca^{2+} stores.

Reorganisation of endoplasmic reticulum during the second meiosis and first mitosis is cell cycle dependent

The experiments presented demonstrate that in the mouse oocyte ER cortical clusters disappear following exit from metaphase II. Given the cell cycle-dependency of this restructuring, the principal candidates for its control are provided by the two main kinase activities which control cell cycle progression in meiosis; MAP kinase and cdk1-cyclin B (see Chapter 1).

Prior to fertilisation the oocyte is arrested at metaphase of the second meiotic division virtue of an elevated level of cdk1-cyclin B activity (Kubiak *et al.*, 1993). Successful fertilisation stimulates proteolytic destruction of cyclin B and a decrease in cdk1 activity (Kubiak *et al.*, 1993;Moos *et al.*, 1996;Nixon *et al.*, 2002), which in turn serves as the trigger for Pb2 extrusion. Thus there is a close temporal correlation between ER reorganisation and cyclin B destruction. We used two independent methods of maintaining cdk1-cyclin B activity to test the hypothesis that the decrease in cdk1-cyclin B activity might be the trigger for ER reorganisation; MG132 and microinjection of excess cyclin B. In both cases,

maintenence of elevated levels of cdk1-cyclin B activity prevented the disappearance of ER cortical clusters. In addition, we demonstrated that inhibition of cdk1-cyclin B activity with roscovitine is sufficient for the disappearance of ER cortical clusters. Indeed, the ability of roscovitine to bring about ER reorganisation within one hour of treatment, prior to Pb2 extrusion, suggests that this ER reorganisation is dependent upon a decrease in cdk1-cyclin B activity but independent of Pb2 extrusion itself.

Data presented in Chapter 3 suggested that cortical clusters of ER are not formed in oocytes of the $mos^{-/-}$ mouse. Given that oocytes from $mos^{-/-}$ mice do not have MAPK activity (Verlhac *et al.*, 1996), this result raises the possibility that MAPK might play a role in cluster generation. The data presented in this chapter do not support a role for MAPK activity in ER reorganisation following fertilisation for two main reasons. Firstly, the timing of ER reorganisation described here does not coincide with a change in MAPK activity, since MAPK activity decreases around the time of Pn formation (Moos *et al.*, 1995;Moos *et al.*, 1996), some two hours after cortical cluster dispersal. Secondly, roscovitine treatment sufficient for ER reorganisation (and polar body extrusion) causes a significant decrease in cdk1 activity, but has no effect upon the level of MAPK activity (FitzHarris *et al.*, 2003; PM, GF and JC unpublished result). Thus a role for MAPK in the control of ER reorganisation following fertilisation seems unlikely. In light of data presented here, a more appealing explanation for the apparent lack of ER clusters in the $mos^{-/-}$ mouse is that cdk1-cyclin B activity is ultimately responsible for the maintenance of ER clusters, and that their absence in $mos^{-/-}$ oocytes may be attributed to the failure of cdk1-cyclin B activity to remain elevated at MII in these oocytes (Verlhac *et al.*, 1996).

Temporal correlations between ER clustering in oocytes and cdk1-cyclin B activity have previously been noted (Terasaki *et al.*, 2001;Sardet *et al.*, 2002). Mature nemertean oocytes display microdomains of ER analogous to the cortical clusters seen in

mouse. These microdomains form prior to fertilisation, and disappear some 40 minutes after insemination (Stricker *et al.*, 1998), which coincides with cyclin B destruction and completion of meiosis II. A similar temporal relationship is apparent in the *Xenopus* oocyte, in which ER clusters first appear following GVBD. Clusters are not detected in the MI-MII transition, but then reappear in the MII oocyte and ultimately disappear again when cdk1-cyclin B activity decreases following egg activation (Terasaki *et al.*, 2001). Thus in *Xenopus* and the nemertean, the presence of ER clusters closely follows the activity of cdk1-cyclin B, implying that the mechanism of ER reorganisation in meiosis may be conserved. Direct studies will be required to establish a causative link between cdk1-cyclin B activity and the control of ER reorganisation in these species.

Several studies have suggested a role for fertilisation-induced ER reorganisation in controlling the duration of Ca^{2+} signalling at fertilisation. In species which generate a single, monotonic rise in $[Ca^{2+}]_i$, the ER undergoes dramatic reorganisation following fertilisation (Jaffe and Terasaki, 1993;Terasaki *et al.*, 1996). In contrast, no changes in ER organisation are seen in the first minutes after fertilisation in species which generate repetitive transients (Stricker *et al.*, 1998;Kline *et al.*, 1999). Further, in the nemertean worm, Ca^{2+} oscillations end around the same time that cortical microdomains of ER disperse (Stricker *et al.*, 1998). The data presented in this chapter demonstrate that in mouse, cortical clusters of ER disperse at the time of Pb2 extrusion, some 2 hours prior to the cessation of Ca^{2+} signalling (Jones *et al.*,1995a ;Day *et al.*, 2000;Deguchi *et al.*, 2000;Marangos *et al.*, 2003). Thus in mouse, reorganisation of the ER is not sufficient for the cessation of fertilisation Ca^{2+} transients, though a more subtle effect upon Ca^{2+} signals cannot be ruled out (discussed below).

The dependence of ER cortical clusters upon high cdk1-cyclin B activity in meiosis raised the question of whether the clusters reappear in mitosis when cdk1-cyclin B

activity returns. To address this question we studied the structure of the ER during the first mitotic division in fertilised and parthenogenetic embryos. Cortical clusters of ER do not reform during mitosis, rather the ER becomes clustered around the spindle. The ER in blastomeres of the two-cell embryo is similar to that of the pronucleate embryo, without significant clustering in the cortex.

The functional significance of the ring of ER around the mitotic spindle is not known. A role for Ca^{2+} has been established in several mitotic cell cycle events both in somatic and embryonic cells (Whitaker and Larman, 2001), and Ca^{2+} transients are known to occur during the first mitotic division in fertilised mouse embryos (Tombes *et al.*, 1992;Kono *et al.*, 1996). The spatiotemporal dynamics of mitotic Ca^{2+} transients in mouse is not known. By analogy with fertilisation Ca^{2+} transients, one might speculate that clustering of ER around the spindle might act as pacemaker sites for Ca^{2+} release, such that mitotic Ca^{2+} waves emanate from the centre of the embryo. Conversely, mitotic Ca^{2+} transients are not detected at NEBD in parthenogenetic embryos (Kono *et al.*, 1996). It has been suggested that in the absence of global $[Ca^{2+}]_i$ transients NEBD may be driven by local changes in $[Ca^{2+}]_i$ in the peri-nuclear region, not detectable by conventional fluorescence microscopy (Wilding *et al.*, 1996;Kono *et al.*, 1996). Concentrating the ER around the spindle may provide the embryo with the means of producing such localised Ca^{2+} changes. Additionally, the organisation of ER around the mitotic spindle may have an impact upon organelle partitioning; enveloping the mitotic spindle with ER potentially provides a mechanism of ensuring equal distribution into daughter cells. This is in stark contrast with the second meiotic division, where the ER is concentrated in the vegetal hemisphere such that the majority of the ER is retained in the developing embryo.

Cell cycle mediated changes in the responsiveness of $InsP_3$-mediated Ca^{2+}-release in meiosis and mitosis

The results of our experiments using $cInsP_3$ in meiosis demonstrate a decrease in the responsiveness of $InsP_3$ mediated Ca^{2+} release which occurs by the time of Pb2 extrusion. A reduced sensitivity of $InsP_3$-mediated Ca^{2+}-releasing mechanisms in pronucleate stage fertilised (Jones *et al.*, 1995a;Parrington *et al.*, 1998) and parthenogenetic (Jones and Whittingham, 1996) embryos has previously been noted. Two separate studies have attributed this downregulation to oocyte aging rather than progression of the cell cycle. In the first, a slower rate of rise of the first sperm-induced Ca^{2+} transient, and a lesser response to $InsP_3$ injection was noted in aged MII oocytes (Jones and Whittingham, 1996), though oocyte age was variable and vastly exceeded that of activated counterparts. In the second, the investigators reported an age-dependent down-regulation in the sensitivity of Ca^{2+} release over a time-course similar to that which we investigated, though the study relied upon the use of a single-wavelength indicator to compare the magnitude of $[Ca^{2+}]_i$ changes in different oocytes (Takahashi *et al.*, 2000). Here we have used age-matched oocytes and ratiometric Ca^{2+}-imaging, using a technique in which oocytes of different ages are simultaneously challenged with $InsP_3$, and have failed to uncover aging-related changes in $InsP_3$-sensitive Ca^{2+} release in non-activated oocytes. Thus we report a maternally-driven decrease in the responsiveness of $InsP_3$-mediated Ca^{2+} release which is complete by the time of Pb2 formation (2 hours after activation), and which occurs in a manner not attributable to oocyte aging. In addition, we have revealed a subsequent increase in the responsiveness of $InsP_3$-mediated Ca^{2+} release upon entry into the first mitosis. This increase is maternally regulated also, since a similar upregulation is seen during mitosis in both fertilised and parthenogenetic embryos.

The mechanisms underpinning these changes in responsiveness to $InsP_3$. A downregulation of the type I $InsP_3$ receptor is known to occur following fertilisation in bovine (He *et al.*, 1999) and mouse eggs (Jellerette *et al.*, 2000;Brind *et al.*, 2000). This effect cannot be held responsible for the reduction in sensitivity reported here following activation, however, as $InsP_3R$ downregulation does not occur following activation with ethanol (Brind *et al.*, 2000).

Several correlations have previously been noted between the sensitivity of Ca^{2+} release mechanisms and the level of cdk1-cyclin B activity. Ca^{2+} oscillations are detected during mitosis (but not interphase) in several somatic cell types (See Chapter 1, or Whitaker and Larman, 2001 for review). In mouse, invoking a metaphase arrest is sufficient to extend the duration of Ca^{2+} oscillations both following fertilisation (Jones *et al.*, 1995a;Marangos *et al.*, 2003) and during the first mitotic division (Kono *et al.*, 1996). Further, fertilisation oscillations can be prevented or drawn to a close by the cdk1 inhibitor roscovitine, apparently independently of pronucleus formation (Deng and Shen, 2000). Moreover, a cell-cycle related increase in sensitivity in mitosis is suggested by the observation that Sr^{2+} (Kono *et al.*, 1996) and sperm extracts (Tang *et al.*, 2000) are capable of producing oscillations in mitotic but not interphase parthenotes. In ascidians Ca^{2+} oscillations pause in between MI and MII, during which time cdk1-cyclin B activity transiently decreases (McDougall and Levasseur, 1998), and the duration of Ca^{2+} oscillations can be controlled by modulation of cdk1-cyclin B activity (Levasseur and McDougall, 2000). However, the sensitivity of $InsP_3$-mediated Ca^{2+} release does not decrease during interkinesis (Yoshida *et al.*, 1998;McDougall and Levasseur, 1998), suggesting a role for cdk1-cyclin B activity in production of, rather than sensitivity to $InsP_3$. The data presented here demonstrate that in mouse, the responsiveness of $InsP_3$-mediated Ca^{2+} release mechanisms decreases at around the time of Pb2 extrusion, and increases again at the time of mitosis entry. Thus, in mouse, the responsiveness of $InsP_3$-

mediated Ca^{2+} release mirrors changes in cdk1-cyclin B activity both in parthenogenetic and fertilised embryos.

A causal link between cdk1-cyclin B activity and the responsiveness of $InsP_3$-induced Ca^{2+} release is yet to be established. Nevertheless, one appealing possibility is that changes in cdk1-cyclin B activity might contribute to the sensitivity of Ca^{2+} release by orchestrating the structure of the ER. It has been argued that the organisation of ER into a network of clusters should serve to optimise the sensitivity of $InsP_3$ mediated Ca^{2+} release (discussed at length in Chapter 3). Thus whilst dispersal of cortical clusters of ER is insufficient to cause cessation of Ca^{2+} signals at fertilisation, it remains possible that this reorganisation may be responsible for the more subtle decrease in responsiveness of Ca^{2+} release seen at the time of Pb2 formation. Thus cdk1-cyclin B might be (indirectly) responsible for the decrease in responsiveness to $InsP_3$ which we have described by triggering dispersal of ER clusters. The subsequent increase in sensitivity which occurs in mitosis may in turn be as a result of the reformation of ER clusters, this time around the spindle. Confirmation of these hypotheses will be dependent upon the demonstration of a causative link between clustering of ER and the responsiveness of Ca^{2+} release. Such direct evidence will require manipulation of ER structure independently of the cell cycle. A protocol which will achieve this is not immediately obvious.

Given that sperm-induced Ca^{2+} oscillations last until pronucleus formation (Jones *et al.*, 1995a;Marangos *et al.*, 2003), the role of the decrease in Ca^{2+}-releasing responsiveness at Pb2 extrusion is unclear. Close analysis has revealed that the dynamics of individual transients are essentially similar before and after Pb2 formation, the rising phase of a given transient remaining wavelike even after Pb2 extrusion (Deguchi *et al.*, 2000). Thus neither cortical ER clusters nor the associated increased responsiveness to $InsP_3$ are required for wavelike propogation of fertilisation Ca^{2+} transients, the vegetal cortex retaining its Ca^{2+}

pacemaker properties even after the ER clusters have dispersed. A potential role for the subsequent increase in responsiveness to $InsP_3$ in mitosis is more obvious. Ca^{2+} transients similar to those seen at fertilisation accompany entry into the first mitosis in mouse (Tombes *et al.*, 1992;Kono *et al.*, 1996). Thus it seems plausible that this upregulation in $InsP_3$-sensitive Ca^{2+} release should serve to potentiate mitotic Ca^{2+} transients. This increase in Ca^{2+} releasing ability is not sufficient for mitotic Ca^{2+} transients, however, since a similar increase occurs in both fertilised and parthenogenetic embryos, and Ca^{2+} transients are not detected in parthenogenetic embryos (Kono *et al.*, 1996). Thus mitotic transients Ca^{2+} are sperm specific, but may be aided by a maternally regulated increase in the responsiveness of $InsP_3$ mediated Ca^{2+} release mechanisms (discussed further in Chapters 5 and 6).

Summary

The main finding here is that ER cortical clusters disperse at the time of Pb2 extrusion, some two hours before fertilisation Ca^{2+} oscillations stop, thus discounting the hypothesis that the presence of ER cortical clusters may dictate the pattern of Ca^{2+} oscillations at fertilisation. Moreover our data demonstrate that ER restructuring is triggered by the decrease in cdk1-cyclin B activity which occurs following fertilisation. Though the mechanism by which cdk1-cyclin B activity regulates ER structure is unknown, it has recently been shown in somatic cells that cyclin B2 colocalises with the golgi, from where it exerts an influence upon intracellular membrane structure (Jackman *et al.*, 1995;Lowe *et al.*, 1998;Draviam *et al.*, 2001). Further work will be required to determine whether a member of the cyclin family is located at the ER in oocytes, allowing cdk1 to specifically phosphorylate ER proteins and cause structural changes in meiosis and mitosis.

5. The role of Ca^{2+} release in mitosis in early mouse embryos

5.1 Introduction

A large proportion of our current understanding of the role of Ca^{2+} in the mitotic cell cycle comes from study of the sea urchin embryo. The reasons for this pertain to the ease of use of sea urchin embryos; the initial cell divisions occur rapidly in the sea urchin, and the large translucent cell lends itself to investigation by fluorescent probes. Consequently, a causal relationship between Ca^{2+}-release and cell-cycle progression is now well established at nuclear envelope breakdown and the metaphase-anaphase transition in sea urchin (see Chapter 1, or Whitaker and Larman, 2001 for review).

The case for Ca^{2+} in mitosis is not as tight in other cells. Ca^{2+} transients have been reported during mitosis in several somatic cell types (Poenie *et al.*, 1986;Ratan *et al.*, 1988;Kao *et al.*, 1990). A causal link between mitotic Ca^{2+} transients and mitosis entry has been demonstrated in Swiss 3T3 fibroblasts. In this cell type the Ca^{2+} buffer BAPTA blocks NEBD, and NEBD can be prematurely induced by photorelease of 'caged' Ca^{2+} (Kao *et al.*, 1990). However, the same study reported that removal of serum from the culture media resulted in abolition of the Ca^{2+} transients, without preventing mitosis. Moreover, other studies have reported mitosis to occur without detectable change in $[Ca^{2+}]_i$ (Tombes and Borisy, 1989;Whitaker and Larman, 2001).

As such, the explanation for this variation is unresolved, though one hypothesis has predominated; that in the absence of global Ca^{2+} transients localised $[Ca^{2+}]_i$ changes occur which would be spatially restricted and/or too low in magnitude to be uncovered by

conventional epi-fluorescence microscopy (Kao *et al.*, 1990; discussed by Hepler, 1994). Such a hypothesis is corroborated by recent reports that elementary Ca^{2+} signals alter cell function locally (see Chapter 1, Bootman *et al.*, 2001b). More direct support, however, comes from a study of sea urchin embryos which showed that the Ca^{2+} transient which triggers NEBD can, in some cases, remain localised to the area around the nucleus (Wilding *et al.*, 1996).

The situation appears somewhat paradoxical in mouse. NEBD of the first mitotic division in fertilised embryos is accompanied by a Ca^{2+} transient that is not detected in parthenogenetically activated embryos (Tombes *et al.*, 1992;Kono *et al.*, 1996). Nevertheless, BAPTA-AM efficiently inhibits mitosis entry in both cases (Kono *et al.*, 1996). Similar to cultured somatic cells, the proposed explanation for these results is that a putative local Ca^{2+} transient triggers NEBD in parthenogenetic embryos, and that this local transient becomes amplified to produce a global transient in the presence of a fertilising sperm (Kono *et al.*, 1996c).

The aim of the experiments presented in this Chapter is to further investigate the relationship between Ca^{2+} release and NEBD. The experiments presented adopt two broad strategies. Firstly we investigate the effect of inhibiting Ca^{2+} signals using (a) Ca^{2+}-buffers and (b) $InsP_3$ receptor downregulation upon the ability of pronucleate embryos to enter mitosis. Secondly we use a novel technique to closely examine the temporal relationship between NEBD and the accompanying Ca^{2+} transient. Finally, we investigate whether global Ca^{2+} transients accompany the second mitotic division in mouse.

5.2 Results

5.2.1 The effect of Ca^{2+} chelators upon mitosis entry and the metaphase-anaphase transition

Two previous studies have reported that entry into the first mitotic division in mouse can be inhibited by the membrane-permeable Ca^{2+} chelator BAPTA-AM (Tombes *et al.*, 1992;Kono *et al.*, 1996), suggesting that NEBD may be Ca^{2+}-dependent. It is known that Ca^{2+} chelators are differentially potent in blocking Ca^{2+}-dependent cell-cycle events in vivo according to their affinities for Ca^{2+} (Speksnijder *et al.*, 1989;Snow and Nuccitelli, 1993;Hepler, 1994). Thus, to further explore the Ca^{2+}-dependency of NEBD, we investigated the ability of Ca^{2+} chelators with different affinities for Ca^{2+} to block NEBD. In these experiments we have microinjected free-acid forms of EGTA (Kd=80nM), BAPTA (Kd=160nM) and dibromoBAPTA (Kd=1.6μM), each to an estimated final concentration of 10mM.

To first demonstrate the ability of free-acid Ca^{2+} chelators to buffer changes in $[Ca^{2+}]_i$ in mouse eggs, we examined the effect of BAPTA-injection upon Ca^{2+} release in *in vitro* fertilised and parthenogenetic embryos using the method of repeated photolysis of $cInsP_3$ (see Chapters 3 and 4). The results of these experiments are shown in figure 5.1. The change in Fura-red ratio was all but inhibited in BAPTA injected embryos compared to controls in response to 100, 1000 and 3000ms UV exposures ($P<0.05$ for 100 ms exposure, $P<0.01$ for 1000 and 3000ms exposures) both in fertilised embryos and parthenotes, demonstrating that free-acid BAPTA is effective in buffering $[Ca^{2+}]_i$ changes in pronucleate embryos.

To study the effect of free-acid Ca^{2+} chelators upon mitosis entry, fertilised embryos and parthenotes were microinjected with Ca^{2+} buffer 14-15 hours after IVF or exposure to ethanol; 1-2 hours prior to the predicted time of NEBD. Embryos were subsequently examined for the presence of pronuclei using light microscopy. The results of

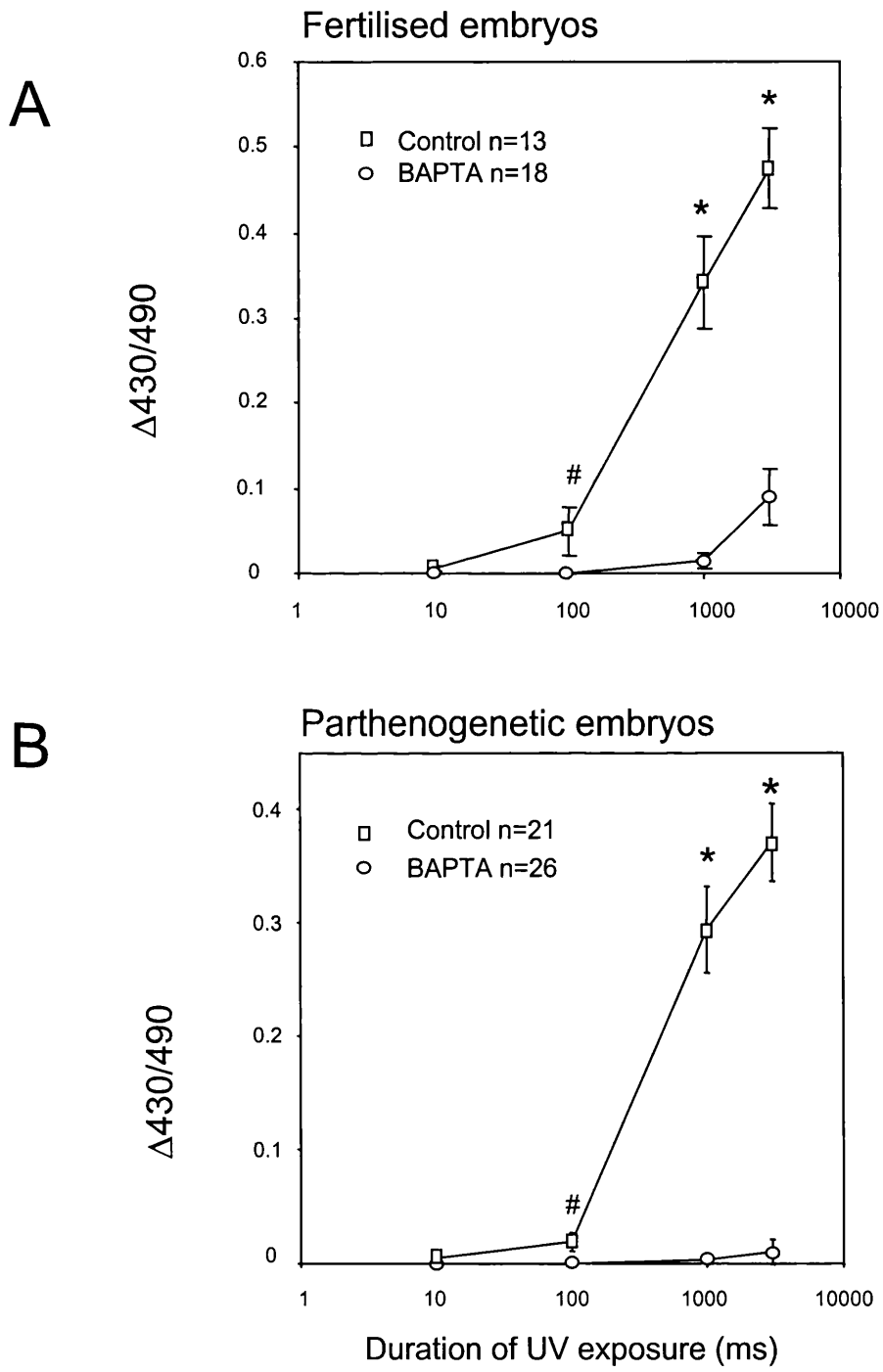


Figure 5.1. Ca^{2+} release in response to $InsP_3$ is inhibited by BAPTA in fertilised and parthenogenetic embryos. Pronucleus stage *in vitro* fertilised (A) and parthenogenetically activated (B) embryos were injected with $cInsP_3$ or BAPTA (10mM final concentration) and $cInsP_3$, and loaded with Fura-red. Embryos were exposed to 10, 100, 1000 and 3000 ms durations of UV light as described in Chapter 2. Peak change in Fura-red ratio is significantly reduced in BAPTA-injected embryos (#P<0.05, *P<0.01).

these experiments are displayed in figure 5.2A. NEBD was efficiently inhibited by a 30-minute incubation in 10μM BAPTA-AM as previously described. However mitosis entry was not inhibited by microinjection of salt form EGTA, BAPTA or Br_2BAPTA. Thus NEBD is prevented by BAPTA-AM, but not by free-acid Ca^{2+} chelators.

To investigate whether the metaphase-anaphase transition can take place in the absence of mitotic Ca^{2+} transients, fertilised embryos were microinjected with BAPTA 1-2 hours prior to the predicted time of NEBD, and subsequently loaded with Hoechst 33342 at the two-cell stage to observe the distribution of chromatin (fig 5.2B). Chromatin was observed within the nuclear envelope in both blastomeres in 9 of 10 control embryos, and in all 12 BAPTA-injected embryos, suggesting that 10mM BAPTA was not sufficient to prevent chromosome disjunction at anaphase.

Finally, in order to verify the ability of BAPTA to inhibit endogenous mitotic Ca^{2+} transients, $[Ca^{2+}]_i$ was monitored during the first mitotic division in BAPTA-injected fertilised embryos (fig 5.2C). Untreated embryos exhibited between 2 and 12 mitotic transients during mitosis (6.3+/-1.1 transients per embryo). In contrast, Ca^{2+} transients were not seen during mitosis in BAPTA-injected embryos. Nevertheless, small fluctuations in Fura-red ratio (more than 10-fold smaller in amplitude than the NEBD Ca^{2+} transient) were seen at NEBD in BAPTA-injected embryos in all cases (amplitude of NEBD Ca^{2+} transient in control embryos 2.0+/-0.1 r.u., BAPTA embryos 0.15+/-0.02 r.u.). Therefore, perhaps surprisingly, these results demonstrate that 10mM BAPTA is insufficient to prevent all changes in $[Ca^{2+}]_i$ at NEBD. It is unlikely that these changes are a result of Ca^{2+}-independent effects of NEBD on the indicator since parthenogenetic embryos do not exhibit even the small Ca^{2+} change detected here (Kono *et al.*, 1996; GF and JC, unpublished results).

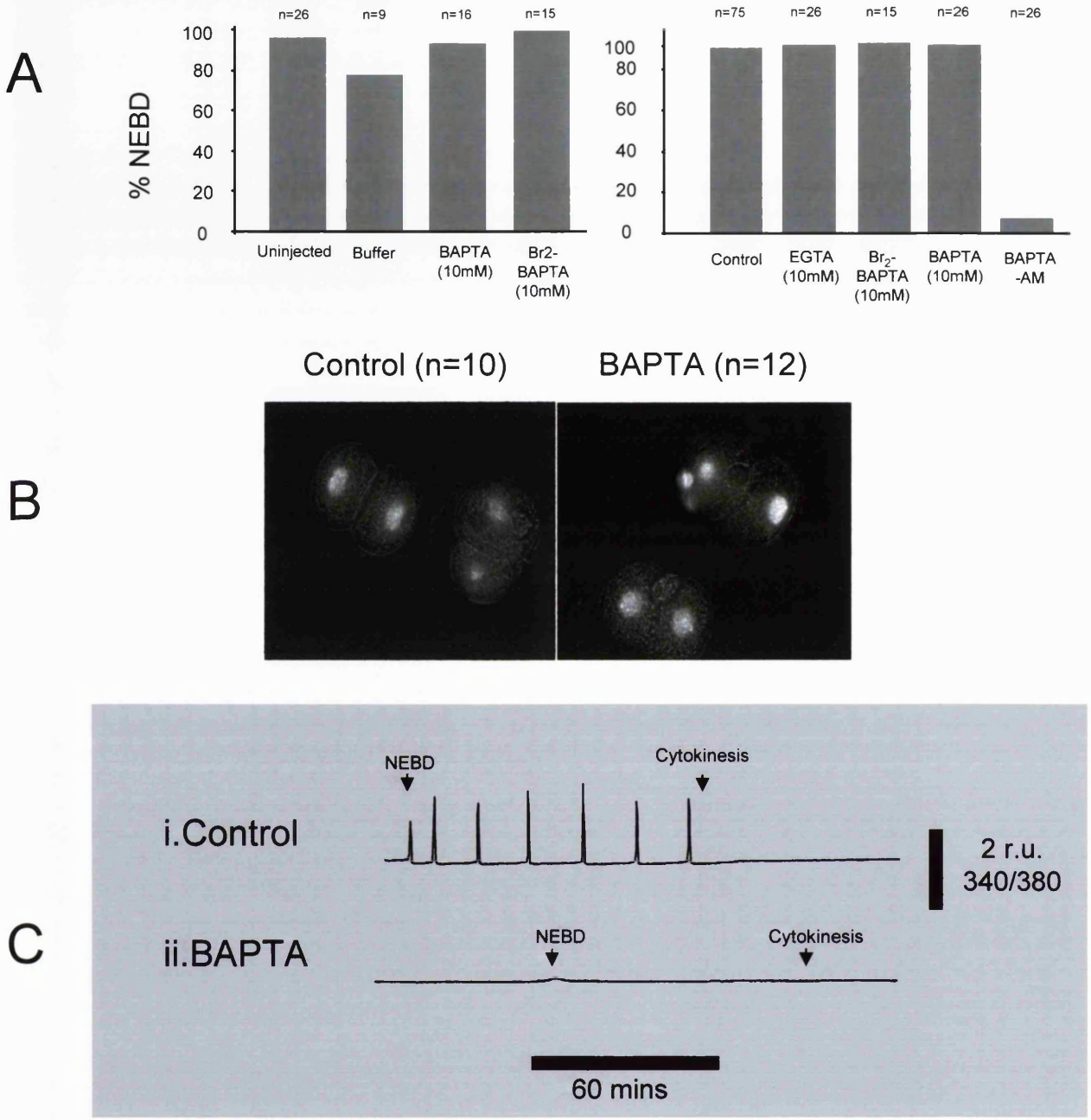


Figure 5.2. Inhibition of mitotic Ca^{2+} transients by BAPTA does not affect the ability of pronucleus stage embryos to undergo NEBD. (A) *In vitro* fertilised and parthenogenetic embryos were injected with BAPTA, Br_2BAPTA, EGTA, injection-buffer or loaded with BAPTA-AM 1-2 hours prior to the predicted time of NEBD. Embryos were subsequently examined for the presence of pronuclei at 1 hour intervals. Data are expressed as the percentage of embryos which had undergone NEBD within 20 hours of IVF or parthenogenetic activation. Results shown are data from at least two different days. (B) BAPTA does not prevent chromosome separation at the first mitotic division. Fertilised pronuclear stage embryos were injected with BAPTA or injection buffer 1-2 hours prior to NEBD. Embryos were loaded with Hoechst at the two cell stage and examined. Bright staining indicating the presence of chromatin was observed in both blastomeres in 9/10 control embryos, and 12/12 BAPTA-injected embryos. (C) BAPTA inhibits Ca^{2+} release at NEBD in fertilised embryos. Embryos were injected with Fura-2-dextran, or co-injected with BAPTA (10mM) and Fura-2-dextran prior to NEBD. Mitotic Ca^{2+} transients were observed in all control embryos (n=14). NEBD occurred without mitotic Ca^{2+} transients in BAPTA-injected embryos (n=19). Note however, a small fluctuation in $[Ca^{2+}]_i$ was seen in all cases at NEBD in BAPTA-injected embryos.

5.2.2 The effect of $InsP_3$-receptor downreglation upon mitosis

Given the inability of BAPTA to completely inhibit changes in $[Ca^{2+}]_I$ at NEBD, we have adopted an alternative strategy to investigate the effect of blocking Ca^{2+} signalling upon mitosis entry. Adenophostin A is a potent $InsP_3$ receptor agonist (Takahashi *et al.*, 1993) demonstrated to induce a dramatic downregulation of $InsP_3$ receptors (to below detectable levels) when microinjected into mammalian oocytes (He *et al.*, 1999;Brind *et al.*, 2000) and early embryos (Brind, 2001). Adenophostin A therefore provides a tool with which to analyse the effect of $InsP_3$ receptor downregulation upon mitotic Ca^{2+} signals.

First we used photolysis of $cInsP_3$ to confrm the ability of Adenophostin A to inhibit $InsP_3$-triggered Ca^{2+} responses in pronucleate embryos. $cInsP_3$ was photoreleased and $[Ca^{2+}]$ monitored in pronucleate fertilised embryos four hours after microinjection of adenophostin A or injection buffer (fig 5.3A). Peak change in Fura-red ratio was significantly greater in untreated and buffer-injected embryos than in adenophostin A-injected embryos in response to 300, 1200 and 3000ms exposures of UV light ($P<0.01$), confirming the ability of Adenophostin A to downregulate the responsiveness of $InsP_3$ mediated Ca^{2+} release in pronucleate embryos.

To investigate the effect of $InsP_3R$ downregulation upon mitotic Ca^{2+} release, $[Ca^{2+}]_i$ was monitored during mitosis in adenophostin A-treated embryos (fig 5.3B). Fertilised embryos were microinjected with adenophostin A and Fura-2-dextran 28 hours after hCG. Embryos were transferred to the microscope stage 1-2 hours before the predicted time of NEBD, at least four hours after Adenophostin A treatment. NEBD was accompanied by a Ca^{2+} transient in all control embryos, 7 of 12 subsequently exhibiting further mitotic transients (2.3+/-0.5 transients per embryo). No changes in $[Ca^{2+}]_i$ were observed at NEBD or during mitosis in adenophostin A-treated oocytes, indicating that $InsP_3R$ downregulation with adenophostin A is sufficient to inhibit all detectable $[Ca^{2+}]_i$ changes at NEBD.

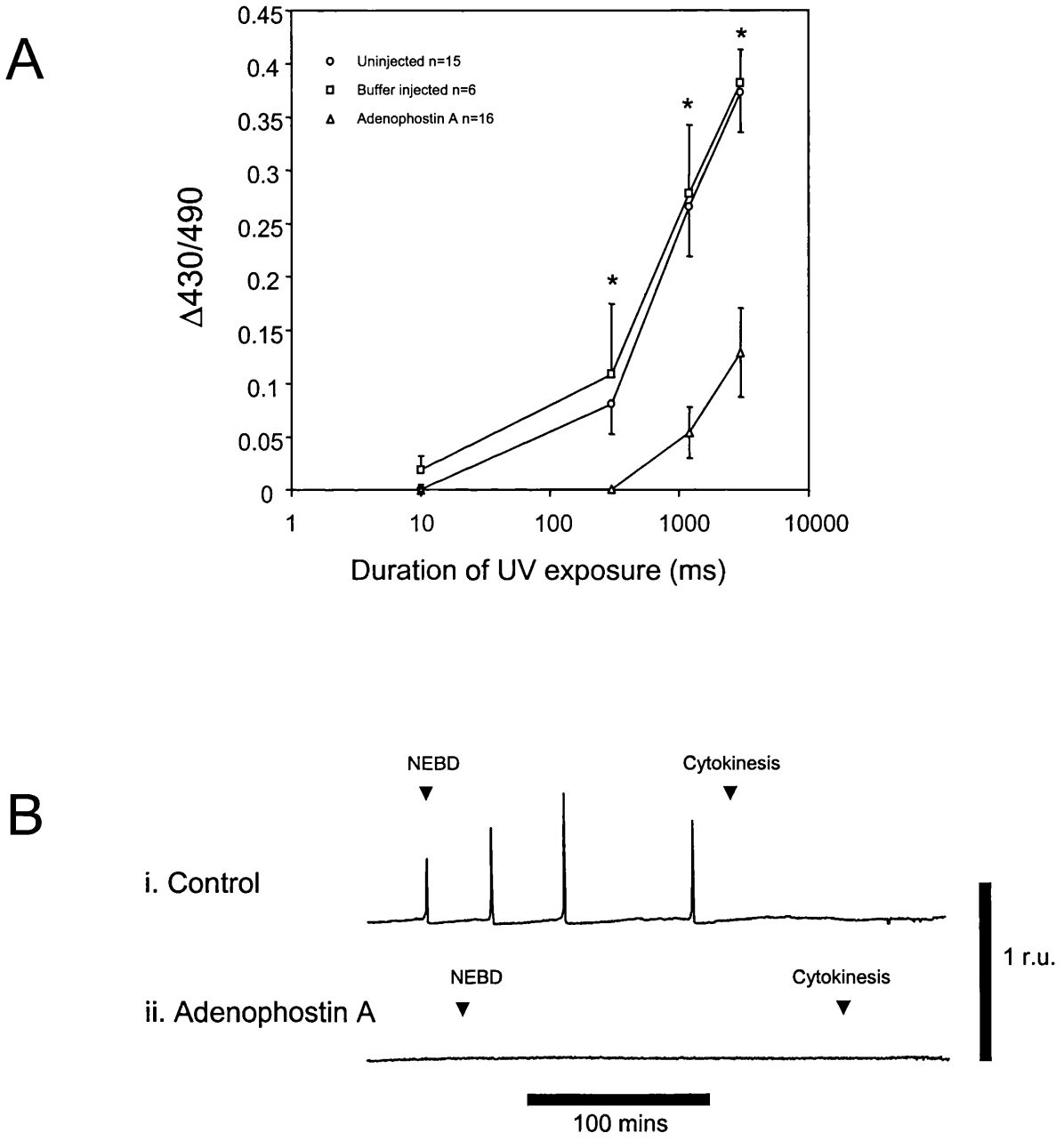
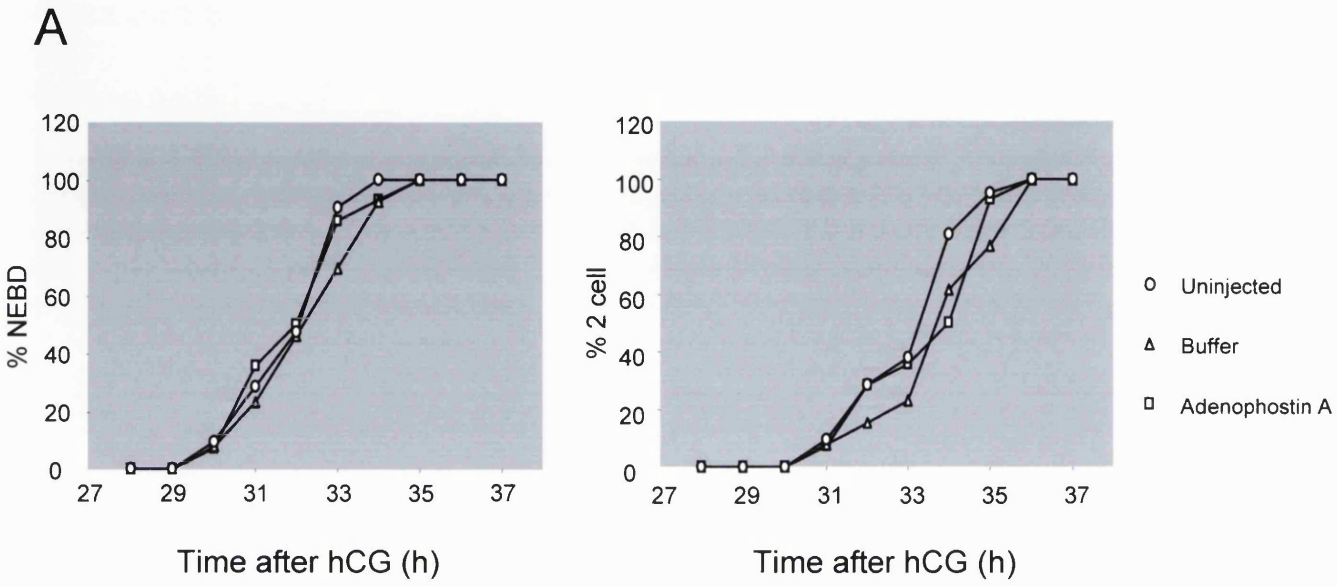


Figure 5.3. Mitotic Ca^{2+} transients are inhibted by $InsP_3$ receptor downregulation. (A) Pronucleus stage embryos were injected with adenophostin A to induce $InsP_3$ receptor downregulation, or injected with buffer 24 hours after hCG and mating. $InsP_3$-mediated Ca^{2+} release was subsequently compared in adenophostin-injected, buffer-injected and uninjected control oocytes using $cInsP_3$ and Fura red 28 hours after hCG. Ca^{2+} release was significantly inhibited in adenophostin-injected embryos compared to controls (*P<0.01). (B) $[Ca^{2+}]_i$ was monitored throughout mitosis in $InsP_3$R-downregulated and control embryos using Fura-2-dextran. Typical Fura-2 traces are shown. At least one Ca^{2+} transient was seen at NEBD in all controls (n=12). No changes in $[Ca^{2+}]_i$ were observed during mitosis in adenophostin-injected oocytes (n=9).



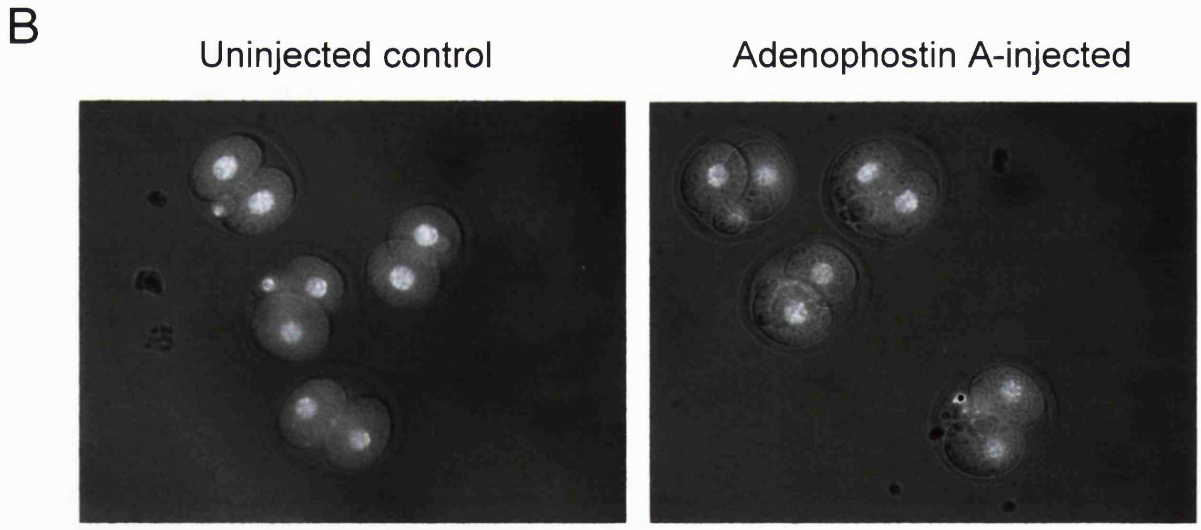


Figure 5.4. Adenophostin A has no effect upon the first embryonic division. (A) *In vivo* produced one-cell embryos were microinjected with adenophostin A (2.5μM) or injection buffer 26 hours after hCG. Embryos were subsequently examined at 1 hour intervals. Data is representative of two independent trials. Each group comprises between 13 and 20 embryos. On each day the ability of adenophostin A to activate unfertilised MII eggs was confirmed (data not shown). (B) Adenophostin A-treated and control embryos were loaded with Hoechst to examine the distribution of chromatin following cytokinesis. Bright areas of fluorescence were observed within the nucleus in all embryos examined, indicating that adenophostin A treatment does not prohibit chromosome separation at the metaphase-anaphase transition (n=12 for adenophostin A; n=19 for controls).

Finally, to investigate the effect of this $InsP_3R$ downregulation upon the first mitotic division, the timing of NEBD and cytokinesis was compared in adenophostin A-treated and control fertilised embryos. Embryos were microinjected with adenophostin A or injection buffer 26h after hCG, and subsequently examined for the presence of pronuclei and cytokinesis at one hour intervals. Adenophostin A had no appreciable effect upon the timing of NEBD or cytokinesis (fig 5.4A). The resultant 2-cell embryos were subsequently loaded with Hoechst in order to examine distribution of chromatin. Bright areas of fluorescence were visible within the nuclei of both blastomeres in all cases (fig 5.4B), indicating that the metaphase-anaphase transition had successfully occurred. Thus $InsP_3R$ downregulation has no effect upon the capability of fertilised embryos to undergo mitosis, nor upon the timing of NEBD or duration of the first embryonic cell division.

Together these data suggest that mitotic Ca^{2+} transients are mediated by the $InsP_3$ receptor, and demonstrate that $InsP_3R$ downregulation completely abolishes the global Ca^{2+} transients associated with mitosis without any effect upon the first mitotic division itself.

5.2.3 The relationship between nuclei and mitotic Ca^{2+} release

A role for Ca^{2+} in nuclear envelope breakdown would predict that the initial NEBD-associated transient detected in fertilised embryos would precede the first signs of pronucleus breakdown. The timing of the Ca^{2+} transient relative to NEBD itself has previously only been addressed using brightfield observations, and reports have differed as to which occurs first (Tombes *et al.*, 1992;Kono *et al.*, 1996;Day *et al.*, 2000). Thus the precise relationship between NEBD and the accompanying Ca^{2+} transient is yet to be determined. To address this question we micro-injected a 77KDa fluorescein dextran into one of the pronuclei of fertilised embryos. By monitoring the loss of fluorescence from the pronucleus we were able to precisely establish the time at which the nuclear membranes become permeable to

molecules of this size. Embryos were loaded with Fura-red so as to simultaneously monitor NEBD and $[Ca^{2+}]_i$. A representative example of this experiment is illustrated in figure 5.5. The initial loss of nuclear fluorescence preceded the peak of the NEBD Ca^{2+} transient by 9.1+/-1.4 minutes. Thus the first indication of NEBD occurs prior to the generation of the NEBD Ca^{2+} transient.

Previously, brightfield observations have suggested that Ca^{2+} oscillations which follow NEBD are subsequently confined to mitosis (Kono *et al.*, 1996). To more accurately examine the relationship between cessation of Ca^{2+} oscillations and exit from mitosis, we have carefully recorded the timing of nucleus formation at the two-cell stage using a FITC-tagged nuclear localisation sequence (FITC-NLS-BSA; Jackman *et al.*, 2002). Fertilised embryos were microinjected with FITC-NLS-BSA and Fura-2-dextran at pronucleus stage and transferred to the microscope stage following NEBD. A representative example of this experiment is shown in figure 5.6. Mitotic Ca^{2+} transients were observed in 13 of 14 embryos. 10 of the 13 exhibited a Ca^{2+} transient after the appearance of the mitotic cleavage furrow, within 20 minutes of the first sign of nucleus formation. The final Ca^{2+} transient occurred shortly prior to cytokinesis in the other three. Ca^{2+} was monitored for at least two hours after nucleus formation in all embryos. Ca^{2+} transients were not seen after first sign of nucleus formation in 13 of 14 embryos, the first sign of nuclear formation preceding the final Ca^{2+} transient by one minute in the other embryo. Thus there is a tight temporal correlation between formation of nuclei in the two-cell stage blastomeres, and cessation of mitotic Ca^{2+} transients.

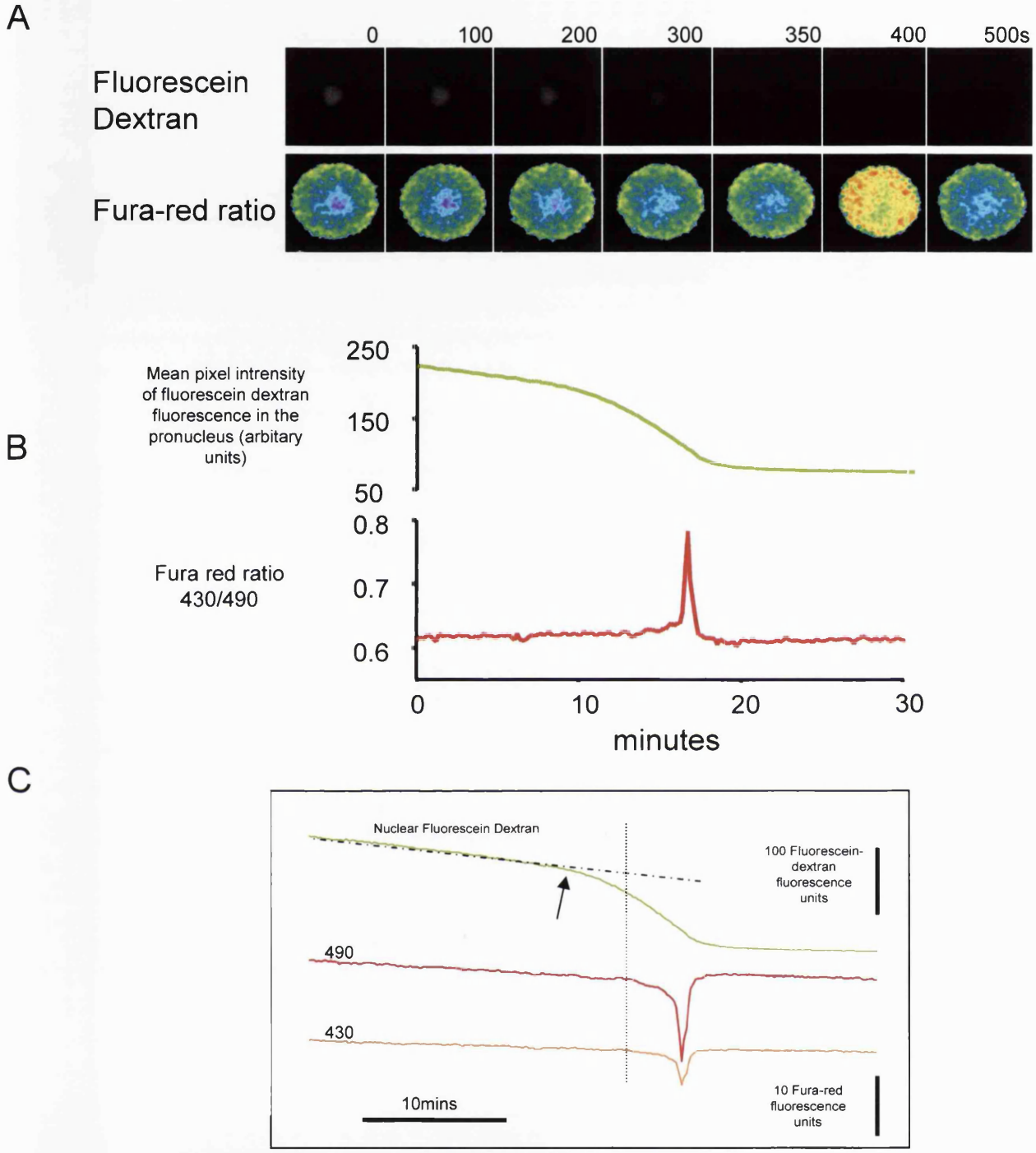


Figure 5.5. The nuclear membranes become permeable to large molecular weight molecules prior to generation of the first mitotic Ca^{2+} transient. (A) Fluorescein dextran (77kDa) was injected into one of the pronuclei of *in vivo* fertilised embryos to monitor the permeability of the pronuclear membrane, and Ca^{2+} was monitored simultaneously using Fura-red. Nuclear fluorescein dextran is shown in the top panel of images and the Fura-red ratio images are shown in the bottom row. The Fura-red ratio is presented as pseudocoloured images, wherein warmer colours correspond to higher ratio values. The ratio values are indicated in (B). The lower ratio value in the centre of the embryo is caused by bleed through of fluorescein signal into the 490nm Fura-red image. (B) Fluorescence traces of the fluorescein dextran and Fura-red ratio are shown. Note the Ca^{2+} transient takes place after the nuclear fluorescence has started to decrease; the initial loss of fluorescence preceeded the Ca^{2+} transient by 9.1+/-1.4 minutes (n=24). (C) The same experiment as in A and B, showing the individual 430nm and 490nm Fura-red traces, over a slightly extended time-scale. Note that the initial decrease in nuclear fluorescein signal (indicated by an arrow) occurs many minutes before any change in Fura-red signal (highlighted by the vertical dotted line). This demonstrates that the initial decrease in nuclear fluorescein signal is not attributable to cross-talk from Fura-red signal.

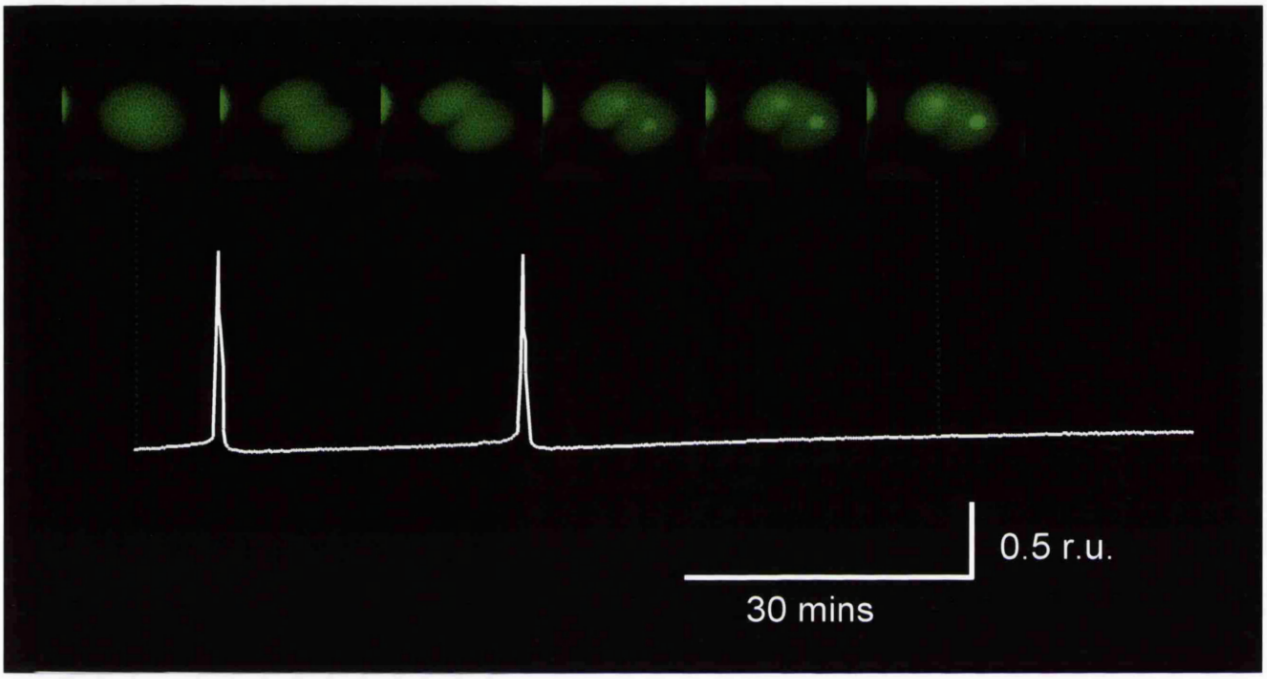


Figure 5.6. Mitotic Ca^{2+} transients cease shortly prior to formation of two-cell stage nuclei. *In vivo* fertilised embryos were microinjected with Fura-2-dextran and FITC-NLS-BSA at pronucleus stage. Embryos were subsequently transferred to the microscope stage following nuclear envelope breakdown. 13 of 14 embryos exhibited at least one Ca^{2+} transient under observation. In 10 of these 13, the final Ca^{2+} transient occurred in the 20 minutes immediately preceeding the first sign of nucleus formation. No Ca^{2+} transients occurred after nuclear envelope reformation (NER) in 12 of the 13, the final transient occurring within 1 minute of NER in the other embryo. On average, the final Ca^{2+} transient preceeded the first indication of NER by 12.8+/-3.6 minutes (n=13).

5.2.4 The effect of fertilisation upon the responsiveness of $InsP_3$ mediated Ca^{2+} release in mitosis

In the previous chapter we used $cInsP_3$ to investigate the responsiveness of Ca^{2+} release before and after nuclear envelope breakdown. The results of these experiments demonstrated an upregulation in responsiveness of Ca^{2+} release in mitosis both in fertilised and parthenogenetic embryos. The design of these experiments did not allow comparisons between fertilised and parthenogenetic embryos. Therefore, to investigate whether the fertilising sperm makes a contribution to the upregulation in responsiveness of Ca^{2+}-release in mitosis, $InsP_3$ was photoreleased in fertilised and parthenogenetically activated embryos simultaneously. Oocytes were fertilised in vitro or activated with ethanol so as to produce embryos and parthenotes of the same cell cycle stage. To compare interphase embryos, fertilised eggs and parthenotes 17-18 hours after IVF and 12-13 hours after activation respectively were injected with $cInsP_3$ and Ca^{2+} monitored during photolysis. To compare mitotic embryos, photolysis was performed 21-22 and 17-18 hours after IVF and ethanol respectivly, at which time at least 50% of the cells each group had undergone NEBD. No difference in peak fura ratio change was seen between interphase fertilised and parthenogenetic embryos in response to any UV duration exposure (fig 5.7A). In contrast, fertilised mitotic embryos released significantly more Ca^{2+} than parthenotes in response to 10ms durations of UV exposure (fig 5.7B). Ca^{2+} release was similar in fertilised and parthenogenetic embryos in response to longer UV exposures. Thus, whilst the responsiveness of Ca^{2+}-release is similar in interphase, fertilised mitotic embryos are more sensitive to photorelease of small amounts of $cInsP_3$ than parthenogenetic counterparts. Together with the data presented in Chapter 4, these experiments demonstrate that an upregulation in the responsiveness of $InsP_3$-mediated Ca^{2+}-release occurs at mitosis entry which is partly attributable to the maternal cell cycle, and partly provided by the sperm.

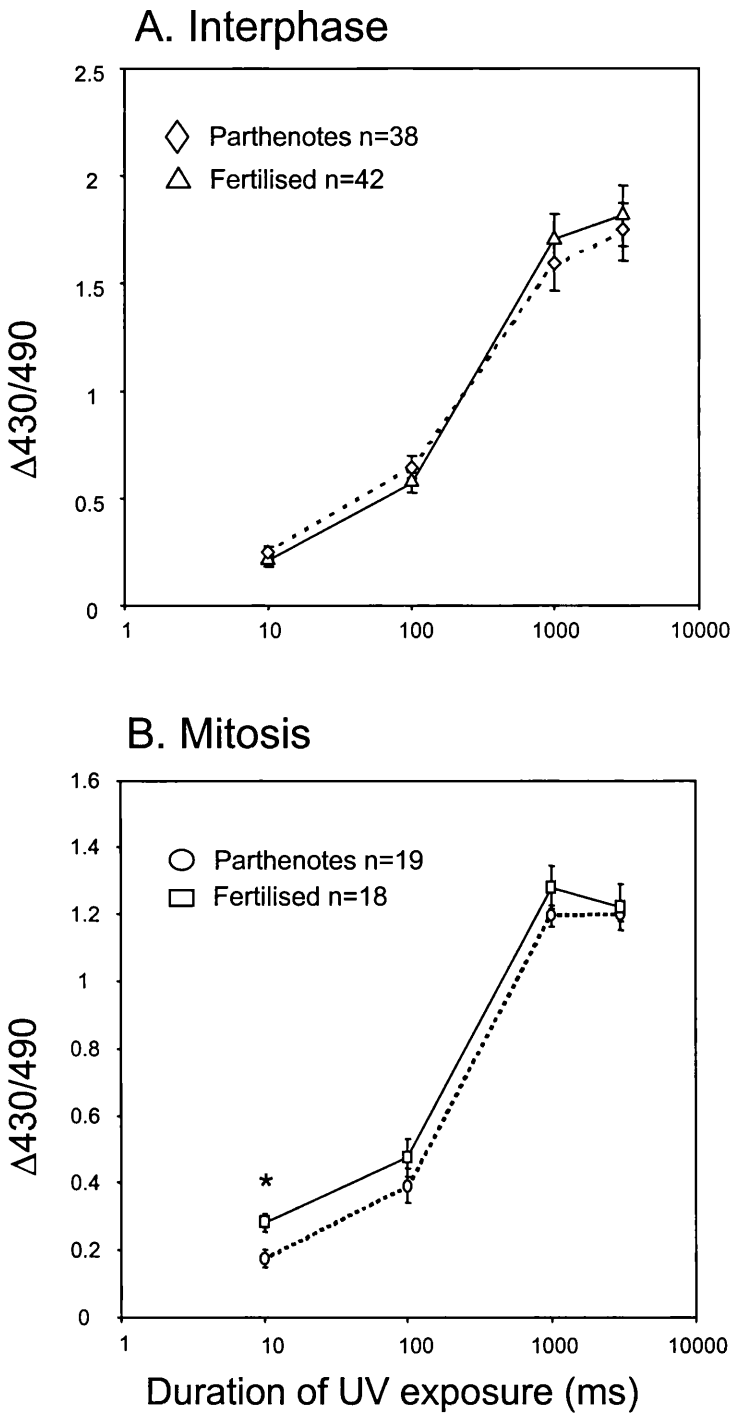


Figure 5.7. Mitotic fertilised embryos are more sensitive than mitotic parthenogenetic embryos to a low dose of $InsP_3$. The ability of parthenogenetic (P) and fertilised (F) embryos to release Ca^{2+} was compared using $cInsP_3$ and Fura-red (as previously). $InsP_3$ was photo-released in interphase (A) and mitotic (B) embryos under similar conditions. (A) No difference in Ca^{2+} release was detected between fertilised and parthenogenetic embryos in interphase (ANOVA; $P>0.7$). (B) In contrast, fertilised mitotic embryos are significantly more responsive to $InsP_3$ than parthenogenetic counterparts (ANOVA; $P<0.03$). In response to a 10ms pulse of UV light mitotic fertilised embryos generated a larger Ca^{2+} transient than mitotic parthenogenetic embryos (* students T-test, $P<0.01$). No significant difference was detected in response to 100ms or longer exposures. Data are from 3-4 experiments with a total of 19 mitotic parthenotes, 18 mitotic fertilised embryos, 38 interphase parthenotes and 42 interphase fertilised embryos.

5.2.5 Monitoring $[Ca^{2+}]_i$ during the second mitotic division

It is not known whether changes in $[Ca^{2+}]_i$ accompany the second mitotic division in mammals. To address this question blastomeres of embryos at the 2-cell stage were microinjected with Fura-2-dextran to record $[Ca^{2+}]_i$, and FITC-NLS-BSA to visualise nuclear envelope breakdown and reformation. Embryos were placed on the microscope stage shortly prior to the predicted time of mitosis entry (approximately 55 hours after hCG) and monitored thoroughout mitosis (fig 5.8). Images were collected at 10 second intervals. No changes in 340/380 ratio were detected during the second mitotic division. To verify the ability to detect changes in $[Ca^{2+}]_i$, embryos which had undergone the 2-4 cell transition on the microscope stage were subjected to the Ca^{2+} ionophore ionomycin (5μM). A substantial changes in 340/380 ratio was detected following ionomycin addition (fig 5.8). This result demonstrates clearly that the second mitotic division in mouse proceeds without global changes in $[Ca^{2+}]_i$.

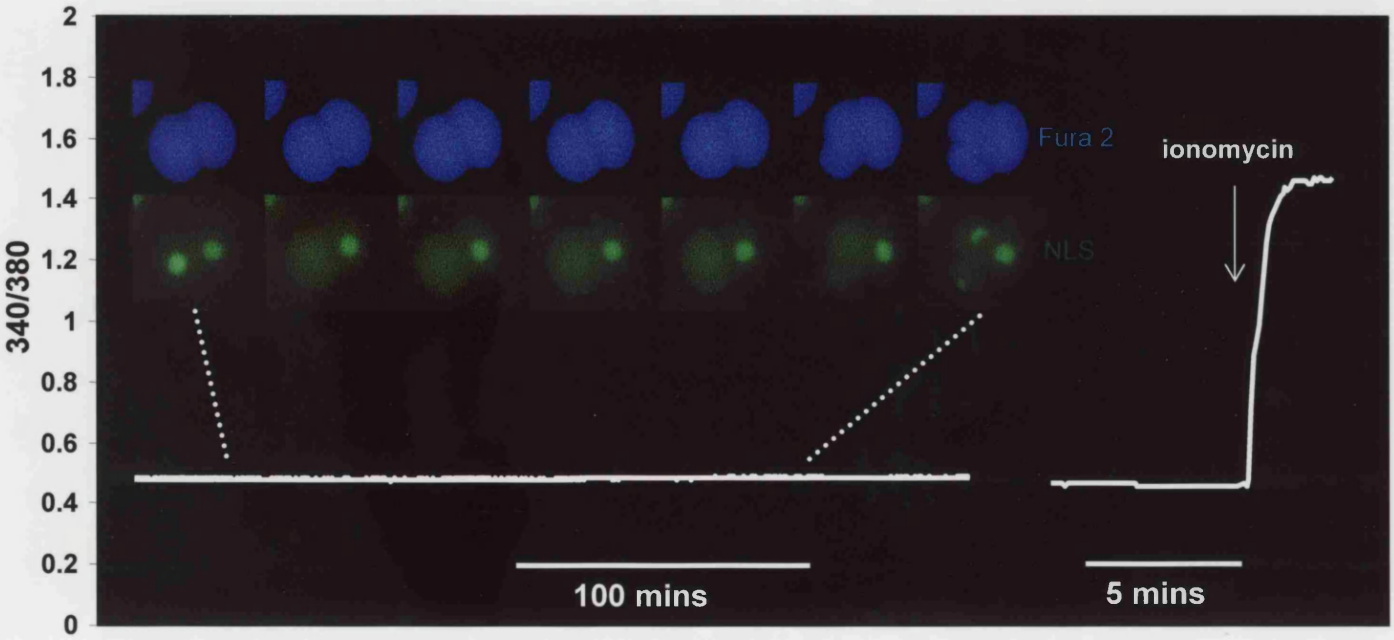


Figure 5.8. Mitotic Ca^{2+} transients are not generated during the second embryonic division. 2-cell embryos were microinjected with Fura-2-dextran and FITC-NLS-BSA to monitor $[Ca^{2+}]_i$ and the presence of nuclei respectively, and placed on the microscope stage prior to the second mitotic division. Images were acquired every 10 seonds. Note the disappearance of nuclear staining at NEBD, and the formation of two new nuclei following cytokinesis. No Ca^{2+} transients were seen during the second mitotic division (n=9). Blastomeres were subsequently challenged with ionomycin (5μM) to confirm the ability to measure changes in $[Ca^{2+}]_i$. Images were taken at 10 second intervals.

5.3 Discussion

The experiments presented in this chapter were designed to determine the role of Ca^{2+} in the first mitotic division in mouse. The main finding is that the abolition of mitotic Ca^{2+} transients has no effect upon the first embryonic division; thus NEBD can occur in the absence of detectable $[Ca^{2+}]_i$ changes not only in parthenotes, but also in fertilised embryos. Moreover the finding that the NEBD precedes the initial Ca^{2+} transient in fertilised embryos suggests that the generation of a global Ca^{2+} transient at mitosis entry may be a consequence of NEBD, rather than its cause. Subsequent Ca^{2+} transients are confined to mitosis, the final transient being generated shortly prior to nucleus formation in the two-cell embryo. Finally, we have shown that global Ca^{2+} transients do not accompany the second embryonic division. The following discussion of these data will primarily address two issues: the role of Ca^{2+} in mitosis, and the mechanisms governing the generation of Ca^{2+} oscillations in mitosis.

The role of Ca^{2+} in mitosis

Reported detection rates of mitotic Ca^{2+} transients have varied. Transients have been reported to occur at NEBD occasionally (Tombes *et al.*, 1992;Day *et al.*, 2000), in all cases (Tang *et al.*, 2000;Gordo *et al.*, 2002), and then repetitively throughout mitosis (Kono *et al.*, 1996). Attempts to explain this apparent variation have included strain differences between mice, and the 'inactivation state of (an unidentified) maternal machinery' (Tang *et al.*, 2000). Though strain differences cannot be ruled out, a more plausible explanation can be found in terms of the Ca^{2+}-indicator used. Previous studies have all employed membrane permeable Ca^{2+}-chelators, which progressively compartmentalise following loading, limiting the available cytosolic indicator (al Mohanna *et al.*, 1994;Carroll *et al.*, 1994). This may explain the variability, since the timing of NEBD is unpredictable. Using a dextran-conjugated Ca^{2+}

indicator (Fura-2-dextran) we report NEBD Ca^{2+} transients in all cases, the vast majority of embryos (~90%) exhibiting subsequent transients during mitosis.

Whilst Ca^{2+} transients accompany pronuclear breakdown in fertilised embryos, no change in $[Ca^{2+}]_i$ is detected at NEBD in parthenotes (Kono *et al.*, 1996). Nevertheless BAPTA-AM, a widely used membrane permeable Ca^{2+} chelator, is effective in preventing NEBD in both cells. A potential explanation for this paradox has been suggested by analogy with NEBD in the sea urchin embryo. The Ca^{2+}-dependency of mitosis entry in the sea urchin is established, since photo-release of Ca^{2+} is sufficient to trigger NEBD in interphase-arrested cells (Wilding *et al.*, 1996). Global Ca^{2+} transients are detected at NEBD in sea urchin embryos approximately half of the time. In their absence confocal microscopy uncovers localised areas of elevated $[Ca^{2+}]_i$ around the nucleus (Wilding *et al.*, 1996). Thus it has been argued that NEBD in mouse might be triggered by local release of Ca^{2+}, undetected by conventional epifluorescence microscopy. It has been suggested that this putative local $[Ca^{2+}]$ change may provide the stimulus for global transients when amplified in the presence of a fertilising sperm (Kono *et al.*, 1996). Intriguingly, the experiments in Chapter 4 demonstrate that the ER accumulates around the spindle at around the time of NEBD. Though the precise timing of this redistribution is not known, clustering of the ER may provide an environment in which locally high Ca^{2+} concentrations may be generated. In this Chapter we have demonstrated that injection of free-acid Ca^{2+} chelators and downregulation of $InsP_3$ receptors, whilst capable of thwarting the generation of mitotic Ca^{2+} transients, are insufficient to prevent mitosis entry in either embryos or parthenotes. Thus, if localised Ca^{2+} signals are to be held responsible for triggering NEBD in mouse, they must be sufficiently robust to not be inhibited by cytosolic Ca^{2+} buffer or by extensive $InsP_3$ receptor downregulation. Such a functional reserve seems unlikely, but may be provided by ER (and therefore presumably $InsP_3R$) clustering around the spindle. Use of high-resolution confocal

microscopy to monitor Ca^{2+} at NEBD in parthenogenetic embryos will prove crucial in determining whether localised Ca^{2+} transients play a similar role at NEBD in mouse as in sea urchin.

Why NEBD is inhibited by membrane permeable and not by free-acid BAPTA is unclear. A non-specific effect of -AM loading seems unlikely since a variety of -AM Ca^{2+} indicators have been shown not to block NEBD (Tombes *et al.*, 1992;Kono *et al.*, 1996; present study). A key difference between the two treatments is that whilst free-acid BAPTA is confined to the cytosol, BAPTA-AM is able to penetrate organelles. Thus the effectiveness of BAPTA-AM in preventing NEBD may relate to its ability to disturb luminal Ca^{2+} homeostasis. Such a hypothesis is supported by a study reporting that the ability of BAPTA-AM to inhibit NEBD is enhanced in Ca^{2+}-free media, conditions which would be expected to cause Ca^{2+} store depletion (Tombes *et al.*, 1992). An alternative explanation stems from a report that BAPTA-AM treatment can lead to protein synthesis inhibition in mouse eggs (Lawrence *et al.*, 1998). Since mitosis entry is critically dependent upon synthesis of cyclin B, and protein synthesis inhibitors can prevent NEBD (Howlett, 1986;Day *et al.*, 2000), it seems plausible that BAPTA-AM may prevent NEBD by disrupting cyclin synthesis rather than by inhibiting Ca^{2+} signals.

Our experiments using adenophostin A to downregulate $InsP_3$ receptors prior to mitosis reveal that the complete inhibition of global mitotic Ca^{2+} transients (as determined by whole-cell imaging) has no effect upon the timing or duration of mitosis, nor upon the metaphase-anaphase transition. Though these results demonstrate that mitotic Ca^{2+} transients are dispensable in terms of progression through the first mitotic division, a role in further development cannot be excluded. Embryos treated with low concentrations of ethanol to elevate $[Ca^{2+}]_i$ for 24h at the one- or two-cell stage exhibit an increased rate of blastocyst formation (Leach *et al.*, 1993). Perhaps more pertinently, incubation of parthenotes in Sr^{2+}-

containing media during the first mitosis both stimulates Ca^{2+} transients and increases the inner cell mass of resulting blastocysts (Bos-Mikich *et al.*, 1997). Thus developmental potential can be influenced by the generation of Ca^{2+} transients not only following fertilisation (Ozil and Huneau, 2001; discussed in Chapter 1), but also in mitosis. The mechanism underpinning this corollary is unclear, though it is known that Ca^{2+} oscillation number can govern gene expression in somatic cells by determining the genes expressed and the efficiency of their manufacture (Li *et al.*, 1998;Dolmetsch *et al.*, 1998). Modification of gene expression may explain how mitotic Ca^{2+} transients influence the constitution of resultant blastocysts without altering the duration of the first mitotic division itself.

The control of Ca^{2+} release in mitosis

The results of our experiments using nuclear-injected fluorescein dextran to assess the temporal relationship between NEBD and the accompanying Ca^{2+} transient reveal that the nuclear membranes become permeable prior to generation of the transient. Whilst these data do not preclude the possibility of an earlier localised Ca^{2+} signal (discussed above), they further demonstrate that the global transient cannot be held responsible for dissolution of the nuclear envelope. Indeed, given the consistent nature of this relationship, these data are more compatible with a model in which breakdown of the pronuclei is the cause of the Ca^{2+} transient rather than its consequence. Such a proposal obviously raises the question as to why NEBD should initiate Ca^{2+} release. One possible explanation can be drawn from the recent report that pronucleus formation is the critical step in the cessation of fertilisation Ca^{2+} transients (Marangos *et al.*, 2003). This study showed that oscillation cessation is closely coupled to pronucleus formation, and that oscillations continue if pronuclear reformation is inhibited. Incorporating the observations that nuclei from fertilised but not parthenogenetic embryos are able to stimulate Ca^{2+} release in surrogate oocytes (Kono *et al.*, 1995;Ogonuki *et*

al., 2001), and that mitotic Ca^{2+} transients are specific to fertilised embryos (Kono *et al.*, 1996), this study proposed a model to explain the control of fertilisation Ca^{2+} oscillations; that the sequestration of a Ca^{2+}-releasing activity into the developing pronuclei might terminate sperm-induced Ca^{2+} oscillations. Within this model, the subsequent dissolution of the pronuclear membranes at NEBD would be expected to cause the liberation of the activity back into the cytosol and thus the re-initiation of Ca^{2+} oscillations. Our data support this hypothesis by demonstrating that NEBD Ca^{2+} transients occur after the nuclear membrane becomes porous to large molecules. Additionally, the results of our experiments using FITC-NLS-BSA to monitor the formation of nuclei at the exit of mitosis demonstrate a tight temporal relationship between the first signs of nucleus formation, and the cessation of mitotic Ca^{2+} spiking, suggesting a role for nuclei in drawing mitotic oscillations to a close. Therefore, whilst other factors such a cell-cycle kinases, ER reorganisations and modulation of Ca^{2+} homeostasis machinery may serve to modulate the responsiveness of Ca^{2+} release in the cell cycle independently of the pronuclei (discussed at length in Chapters 3 and 4), compartmentalisation of a soluble factor within the forming pronuclei appears to be the predominant mechanism responsible for controlling the duration of Ca^{2+} transients in meiosis and mitosis (Marangos *et al.*, 2003).

Data which do not support this hypothesis do exist. Firstly, one group reported that Ca^{2+} oscillations stop at approximately the same time in nucleate and anucleate halves of oocytes bisected prior to pronucleus formation, suggesting that cessation of oscillations can occur in the absence of pronuclei (Day *et al.*, 2000). Such an experimental protocol is unduly invasive, however, and may result in unequal distribution of organelles or sperm-factor to the daughter fragments. Secondly, cytoplasmic transfer experiments reveal that whist cytoplasts from recently fertilised eggs are able to activate recipient oocytes, transfer of the second polar body is without effect (Ogonuki *et al.*, 2001). Though initially surprising, the absence

of the Ca^{2+} releasing ability from the polar body is not unreasonable since it is not known whether the cytoplasmic makeup of the second polar body reflects that of the fertilised egg, and the animal pole (the origin of the Pb2) is deprived of other components of Ca^{2+} signalling machinery also (Mehlmann *et al.*, 1995;Halet *et al.*, 2002).

In contrast, a nuclear compartmentalisation model may help explain several previous findings; (i) that recipient oocytes are activated by cytoplasts from recently fertilised, but not pronucleate embryos (Ogonuki *et al.*, 2001), (ii) that induction of metaphase arrest produces continued oscillations both in meiosis and mitosis (Jones *et al.*, 1995a;Kono *et al.*, 1996), (iii) that sperm-extract injections are incapable of triggering Ca^{2+} oscillations in the presence of a pronucleus (Tang *et al.*, 2000), (iv) that no Ca^{2+} transients are generated in embryos arrested in interphase by inhibition of protein synthesis (Day *et al.*, 2000), and (v) that NEBD Ca^{2+} transients are more pronounced in polyspermic sea-urchin embryos (Whitaker and Patel, 1990;Browne *et al.*, 1996).

A model incorporating the liberation of a putative Ca^{2+} releasing activity from the pronuclei at NEBD is consistent with the results of our experiments in which we used caged-$InsP_3$ to compare the responsiveness of Ca^{2+} release in fertilised and parthenogenetic embryos. These data establish that mitotic fertilised embryos generate larger Ca^{2+} transients in response to low levels of $InsP_3$ than parthenotes. Since similar transients are generated by fertilised and parthenogenetic embryos in interphase; this result suggests that a sperm-specific contribution to the responsiveness of Ca^{2+} release is made available following NEBD. A comparable result has been noted by microinjecting $InsP_3$ (Tang *et al.*, 2000). It seems plausible that the same factors whose sequestration into the pronuclei terminates fertilisation oscillations, might augment the responsiveness of $InsP_3$-mediated Ca^{2+} release when liberated into the cytosol in mitosis. This increase in responsiveness may be responsible (at least in part) for the resumption of Ca^{2+} oscillations at NEBD.

Finally, we have monitored $[Ca^{2+}]_i$ during the second mitotic division. These experiments demonstrate unequivocally that global Ca^{2+} transients such as those generated by fertilised embryos during the first mitotic division do not occur during the transition from the 2-cell to the 4-cell stage. A similar observation has been made in the sea urchin embryo, in which Ca^{2+} transients accompany the first, but not subsequent cell divisions (Browne *et al.*, 1996). These results could imply either of two things: that the nuclear sequestered factor has been degraded or inactivated by the time of second mitosis, or that the cytoplasm is no longer able to support the generation of Ca^{2+} transients by the liberated factor. Strong support for the former is provided by reports that nuclei transferred from pronucleate, but not late 2-cell or 4-cell stage embryos can activate recipient eggs (Kono *et al.*, 1995;Zernicka-Goetz *et al.*, 1995). Thus the nuclear-sequestered factor which is both able to activate recipient eggs or cause Ca^{2+} oscillations upon release from pronuclei, is eliminated following the first embryonic division such that no Ca^{2+} transients accompany the second mitotic division and nuclei no longer activate eggs.

The identity of the sequestered molecule or molecules is unknown. Whilst the sperm factor responsible for triggering Ca^{2+} release at fertilisation provides the most obvious candidate, the sequestration of a substrate or critical cofactor can not be ruled out (Marangos *et al.*, 2003). Intriguingly, a putative nuclear localisation sequence has been identified in PLCzeta, the recently discovered sperm-specific PLC isoform proposed to be the sperm factor (Mark Larman and Karl Swann; personal communication). Demonstration that PLCzeta localises to the pronuclei would provide strong support both for the proposed nuclear compartmentalisation model, and for the identity of PLCzeta as the sperm factor.

6. Conclusion

This thesis has presented the results of work investigating ER structure and Ca^{2+} release in meiosis and mitosis. The work has resulted in several original findings. Firstly, ER reorganisation during oocyte maturation is microtubule-dependent, and can occur independently of the MI-MII transition. Secondly, ER cortical clusters disperse around the time of Pb2 extrusion as a result of the decrease in cdk1-cyclin B activity which occurs at that time. Thus ER cortical clusters are not required for the continued generation of Ca^{2+} oscillations to the time of Pn formation. Third, ER cortical clusters do not re-form during mitosis, instead the ER accumulates around the spindle similarly to meiosis I. Fourthly, mitotic Ca^{2+} transients are dispensable in terms of progression through the first embryonic division, and commence after NEBD has begun. Fifthly, no global Ca^{2+} transients occur during the second mitotic division. Finally, the responsiveness of $InsP_3$-mediated Ca^{2+} release tracks the level of cdk1-cyclin B activity during meiosis and the first mitosis, such that the ability to release Ca^{2+} is greatest in M-phase. Since these findings are of particular relevance to the regulation of Ca^{2+} release at fertilisation, this final section will now summarise the mechanisms controlling Ca^{2+} release at fertilisation in mouse eggs, and draw these together into a model to explain the complex pattern of sperm-induced Ca^{2+} signals.

In mouse, fertilisation triggers two series of Ca^{2+} oscillations. The first series occurs shortly after sperm-egg fusion and ends some four hours later, around the time of pronucleus formation. A second series occurs much later as an accompaniment to the first embryonic division. Since no transients are detected during mitosis in parthenogenetic embryos, the mitotic transients in fertilised embryos can also be considered part of the sperm-induced Ca^{2+} response. Whilst much attention has focused upon the molecular nature of the trigger which

initiates Ca^{2+} release at fertilisation, the mechanisms which govern the subsequent pattern of oscillations have been less well studied. Recently it has become clear that modifications of the Ca^{2+} signalling machinery occur during early development which may dictate this characteristic pattern. Broadly, these pathways can be categorised as those which encourage Ca^{2+} release ('On' pathways), and those which tend to hinder Ca^{2+} release ('Off' pathways).

The increase in responsiveness of Ca^{2+} release during oocyte maturation; 'On' pathways

The upregulation of Ca^{2+} signalling machinery which occurs in the prelude to fertilisation has several key features. First, the temporal pattern of Ca^{2+} release triggered by fertilisation is modified during oocyte maturation; whilst reports have varied as to whether long-lasting Ca^{2+} oscillations can only be generated by MII oocytes, the magnitude of individual Ca^{2+} transients and the rate of Ca^{2+} release is undoubtedly greater in MII than in GV oocytes (Mehlmann and Kline, 1994;Jones *et al.*, 1995b;Cheung *et al.*, 2000). Second, the spatial patterning of Ca^{2+} release is modified during oocyte maturation; the characteristic wave-like propagation of Ca^{2+} release exhibited by mature MII oocytes has not been reported following fertilisation or SE injection in GV oocytes, rather the Ca^{2+} oscillations appear to be spatially homogeneous (Carroll *et al.*, 1994;Shiraishi *et al.*, 1995). Thirdly, the response to Ca^{2+}-releasing agonists such as $InsP_3$ increases during oocyte maturation (Fujiwara *et al.*, 1993;Mehlmann and Kline, 1994; see Carroll *et al.*, 1996 for review).

It is now well established that the upregulation of $InsP_3R$ expression during meiosis I is a key contributor to the elevated sensitivity of Ca^{2+} release at MII (Mehlmann *et al.*, 1996;Parrington *et al.*, 1998;Fissore *et al.*, 1999b). A causal relationship between $InsP_3$ R expression and the generation of fully competent Ca^{2+} release machinery was recently demonstrated using double stranded RNA (dsRNA) to prevent upregulation of $InsP_3RI$ during maturation (Xu *et al.*, 2003). dsRNA-injected eggs exhibited fewer sperm-induced

Ca^{2+} transients and produced an initial fertilisation Ca^{2+} transient which was shorter in duration than controls. However, the amplitude of Ca^{2+} release and the ability to generate repetitive oscillations in response to sperm was not significantly affected by dsRNA. This illustrates both the importance of $InsP_3R$ upregulation in the attainment of MII Ca^{2+} release machinery, and the existence of other 'On' pathways during maturation.

Evidence is accruing that the sensitivity of Ca^{2+} release is greatest in M-phase (reviewed in Chapters 1 and 4). Our method of simultaneously 'uncaging' $InsP_3$ in two or more populations of oocytes has allowed us to draw up a profile of the responsiveness of $InsP_3$-mediated Ca^{2+} release during oocyte maturation, following activation, and in mitosis. The responsiveness of Ca^{2+} release increases gradually during oocyte maturation. Following activation a decrease in the responsiveness of $InsP_3$ mediated Ca^{2+} release occurs which is complete by the time of Pb2, and which occurs independently of the fertilising sperm. Subsequently, the responsiveness increases again in mitosis both in fertilised and parthenogenetic embryos. On the basis of these experiments it appears that the responsiveness of $InsP_3$ mediated Ca^{2+} release may reflect the level of cdk1-cyclin B activity and that, as such, cell cycle progression to metaphase II may in itself represent an 'On' pathway. Further experiments would help expand this picture. Principally, these data only provide a temporal correlation and, in mouse, a functional relationship between the cell cycle and Ca^{2+} release is yet to be established. Comparison of activated and MII-arrested oocytes (eg by MG132 or excess cyclin; see Chapter 4), and examination of the effect of (the cdk1 inhibitor) roscovitine upon $InsP_3$ induced Ca^{2+} release may provide some indication as to whether there is a causal link between cdk1-cyclin B activity and Ca^{2+} release in mouse oocytes. Results of the latter experiment would be difficult to interpret however, since roscovitine has also been reported to affect thapsigargin-sensitive Ca^{2+} release (Deng and

Shen, 2000) and ER structure (present study). Candidate mechanisms by which cdk1-cyclin B activity might influence Ca^{2+} release are discussed below.

Several lines of evidence support the notion that distribution of the ER (and thus presumably also $InsP_3Rs$) into discrete clusters generates highly sensitive foci for Ca^{2+} release (see Chapter 3). Data presented in this thesis demonstrate that ER cortical clusters are formed close to the time of Pb1 extrusion, and disappear shortly after fertilisation. Thus, under normal circumstances, ER clusters are specific to MII, when the demand upon the Ca^{2+} signalling machinery is greatest. The corollary between ER clusters and Ca^{2+} release has been extended by the finding that cortical clusters are generated in MI-arrested oocytes (Chapter 3), and MI-arrested oocytes generate sperm-induced Ca^{2+} oscillations similar to those generated at MII (Jones *et al.*, 1995b). However, a causal relationship between ER/$InsP_3R$ clustering and the sensitivity of Ca^{2+} release remains to be demonstrated (see below).

These 'On' pathways together contribute towards the generation of cytoplasm capable of mounting a proper Ca^{2+} response to the fertilising sperm.

The cessation of sperm-induced Ca^{2+} oscillations; 'Off' pathways

Several of the same mechanisms which contribute to the increased responsiveness of Ca^{2+} release at MII may be involved in defining the duration of sperm-induced Ca^{2+} oscillations. $InsP_3R$ expression is dramatically downregulated following fertilisation, raising the possibility that downregulation of $InsP_3Rs$ below a critical threshold might determine the time at which Ca^{2+} oscillations end (Parrington *et al.*, 1998;Brind *et al.*, 2000;Jellerette *et al.*, 2000). However, it is unlikely that this mechanism should prove ultimately responsible for the cessation of Ca^{2+} oscillations, since the vast majority of $InsP_3R$ downregulation occurs within two hours of fertilisation (Brind *et al.*, 2000), though sperm-induced Ca^{2+} oscillations

continue for around four hours without significant effect upon oscillation rate or amplitude (Brind, 2000). Moreover, Ca^{2+} oscillations persist if fertilised eggs are arrested at MII in the presence of nocodazole (Jones *et al.*, 1995a), conditions which have no effect upon the ability of sperm to cause $InsP_3R$ downrgualtion (Brind, 2001). Thus the purpose of $InsP_3R$ upregulation prior to fertilisation may simply be to provide a significant functional reserve at MII when the need is greatest. The subsequent downregulation which occurs following fertilisation may then be important in preventing aberrant Ca^{2+} release which may be inappropriate in S-phase. Perhaps surprisingly, our experiment comparing Ca^{2+} release in fertilised and ethanol-activated embryos revealed no significant difference in the response of fertilised and parthenogenetic embryos to $InsP_3$ at interphase. Since activation by ethanol fails to cause $InsP_3R$ downregulation (Brind *et al.*, 2000), this result implies that the effect of sperm-induced $InsP_3R$-downregulation upon the sensitivity of Ca^{2+} release may be minimal, though such a comparison does not account for any contribution of the fertilising sperm in interphase. A more direct examination of the contribution of $InsP_3R$ downregulation to the responsiveness of Ca^{2+} release would require a method of downregulating $InsP_3Rs$ to the same extent as fertilisation without altering other factors which influence Ca^{2+} release (such as cdk1-cyclin B activity and ER structure). Nevertheless, though the role of $InsP_3R$ downregulation is not yet resolved, it is clear that it is not alone sufficient to terminate sperm-induced Ca^{2+} oscillations.

A role for ER structural changes in determining the duration of Ca^{2+} oscillations at fertilisation was initially alluded to by reports that the ER becomes discontinuous shortly after generation of the solitary sperm-induced Ca^{2+} transient in sea urchin and starfish oocytes (Jaffe and Terasaki, 1993;Terasaki *et al.*, 1996). More compelling evidence came from the nemertean worm, in which cytoplasmic clusters of ER disperse at the same time that Ca^{2+} oscillations stop (Stricker *et al.*, 1998). Thus it was speculated that, in mouse, cortical

clusters of ER might disperse when the oscillations stop, close to the time of pronucleus formation (Kline, 2000), a plausible suggestion given the correlation between ER clusters and the responsiveness of Ca^{2+} release (see Chapter 3). A principal aim of this thesis was to test this hypothesis. The data presented demonstrate unambiguously that ER reorganisation is not the foremost mechanism by which the timecourse of Ca^{2+} oscillations at fertilisation is determined, since ER clusters disperse at the time of Pb2 extrusion. Thus ER reorganisation is not the 'Off' pathway which ultimately defines the temporal patterning of Ca^{2+} release at fertilisation in mammals. Nevertheless, the possibility that ER cortical clusters present a more subtle contribution to the responsiveness of Ca^{2+} release cannot been ruled out, since the disappearance of cortical clusters coincides with the reduction in responsiveness of Ca^{2+} release which occurs independently of $InsP_3R$ downregulation following fertilisation (see below).

It is now well established that progression form metaphase II to interphase (pronucleate stage) is accompanied by a decrease in the sensitivity of $InsP_3$-mediated Ca^{2+} release (Jones *et al.*, 1995a;Jones and Whittingham, 1996;Parrington *et al.*, 1998; present study). Using caged $InsP_3$ to investigate the responsiveness of Ca^{2+} release following parthenogenetic activation, we have shown that a component of this decrease is maternally regulated, independent of $InsP_3R$ downregulation (since activation by ethanol does not trigger $InsP_3R$-downregulation), and complete by the time of Pb2 extrusion. Thus exit from M-phase decreases the responsiveness of Ca^{2+} release. There are several components of the Ca^{2+} homeostasis machinery which are now thought to be regulated by the cell cycle. Data in this thesis demonstrate a causal link between cdk1-cyclin B activity and ER structure. Given the close correlation between ER clustering and the sensitivity of Ca^{2+} release, we speculate that cdk1-cyclin B activity may influence Ca^{2+} release by dictating ER structure; high cdk1-cyclin B activity causes ER clustering (in the cortex at MII, around the spindle in mitosis),

which in turn increases the responsiveness of the cytoplasm to $InsP_3$. Confirmation of this model would require manipulation of the ER without disturbing other Ca^{2+} signalling machinery. Another aspect of the Ca^{2+} homeostasis which may be regulated by the cell cycle in mouse eggs is store loading, since it has been reported that roscovitine limits Ca^{2+} release in response to (the SERCA pump inhibitor) thapsigargin (Deng and Shen, 2000). It is unclear whether this result is soley attributable to the level of store loading, however, since it is likely that roscovitine would cause ER restructuring under these conditions (see Chapter 4). Cdk1-cyclin B activity has also been implicated in the regulation of Ca^{2+} influx. Store operated Ca^{2+} influx is inactivated following GVBD in *Xenopus* oocytes, and during mitosis in somatic cells, suggesting that high cdk1-cyclin B activity may uncouple Ca^{2+} release from influx (Preston *et al.*, 1991;Machaca and Haun 2000, 2003). However, it is not known whether a similar mechanism exists in mouse, and it is unclear why decreased Ca^{2+} entry in M-phase should encourage Ca^{2+} release. Finally, in ascidian oocytes the cytoplasm remains sensitive to $InsP_3$ during the gap period between cdk1-cyclin B activity-driven Ca^{2+} oscillations, suggesting that cdk1 activity may control the level of $InsP_3$ production rather than the sensitivity of $InsP_3$ mediated Ca^{2+} release in this species (McDougall and Levasseur, 1998;Levasseur and McDougall, 2000). Cell cycle regulation of PI turnover is yet to be examined in mouse oocytes.

Though many of the above 'Off' pathways are likely to contribute to the decrease in responsiveness of Ca^{2+} release following fertilisation, it has recently been shown that the time at which the Ca^{2+} oscillations stop is ultimately defined by the formation of the pronuclei (Marangos *et al.*, 2003). Thus it appears that the sequestration of a sperm-derived Ca^{2+} releasing ability into the pronuclei is the critical permissive step which terminates sperm-induced Ca^{2+} oscillations (discussed at length in Chapter 5). Nuclear sequestration of the sperm-derived factor should not be considered solely responsible for cessation of the

oscillations, however, since it occurs on a background of several other 'Off' pathways, and as such, it is not known whether the putative sequestration event is alone sufficient. Though a paternally-derived PLC presents the most obvious candidate for the compartmentalised factor, sequestration of its substrate (PIP_2) or a cofactor remain a possibility. Some support for the idea of a nuclear-localised PLC is provided by the finding that PLCβ1 has a nuclear localisation signal (NLS) and is located in the nucleus in several cell types (Avazeri *et al.*, 2000;Cocco *et al.*, 2002;Irvine, 2003). PLCβ1 is thought to stimulate a nuclear phosphoinositide turnover leading to the generation of nuclear $InsP_3$ and diacylglycerol (Cocco *et al.*, 2002; Irvine, 2003). PLC zeta has a putative NLS (Mark Larman and Karl Swann, personal communication) but no data has been published with regard to its role in cell signalling. Clearly, if PLCzeta is localised to the nucleus it must be incapable of generating sufficient $InsP_3$ to trigger Ca^{2+} transients during interphase.

'On' and 'Off' pathways in mitosis

Prior to the experiments presented in this thesis, little was known about the regulation of Ca^{2+} release in mitosis in the early mouse embryo. It was known that Ca^{2+} transients were specific to fertilised embryos, and were not detected in interphase, and that strontium triggers Ca^{2+} release in mitosis but not interphase (Kono *et al.*, 1996). Here we have shown that entry into mitotic M-phase is accompanied by an upregulation in the responsiveness of $InsP_3$-mediated Ca^{2+} release both in fertilised and parthenogenetic embryos. In addition we have shown that Ca^{2+} release occurs as a result of NEBD, and that mitotic Ca^{2+} oscillations subsequently end close to the time of nuclear envelope reformation at the 2-cell stage. Thus we confirm that mitotic Ca^{2+} transients are triggered by release of the nuclear sequestered Ca^{2+} releasing factor into an $InsP_3$-sensitive environment, and that the subsequent re-sequestration of the cytoplasmic factor into the developing 2-cell nuclei concludes the oscillations. The

mechanisms driving sperm-induced Ca^{2+} release in meiosis and the first mitotic division are therefore remarkably similar.

In contrast, Ca^{2+} oscillations are not generated in the second, or presumably subsequent mitotic divisions. We can therefore report that the first mitotic division represents the final contribution of the sperm in terms of Ca^{2+} signalling. Since there is no reason to suggest that the cytoplasm of the mitotic 2-cell embryo would not be receptive to the paternally derived Ca^{2+} releasing activity, it seems reasonable to speculate that the sperm-derived factor(s) are degraded or inactivated with time, such that insufficient remain to trigger Ca^{2+} release at in the second division, some two days after fertilisation. A gradual degradation of the sperm factor may also explain the diminished frequency of Ca^{2+} oscillation in the first mitosis; infrequent Ca^{2+} oscillations may reflect a lower level of $InsP_3$ production in mitosis as a result of sperm factor degradation at the pronucleus stage. Destruction of the sperm-factor may therefore constitute the 'Off' pathway which ultimately restricts sperm-induced Ca^{2+} release to meiosis and the first mitosis. It will be interesting to see whether mitotic Ca^{2+} transients accompany later mitotic divisions in polyspermic mouse eggs, or following egg activation by excessive amounts of PLCzeta.

These 'On' and 'Off' pathways which control Ca^{2+} release in mouse eggs are summarised in figure 6.1. In this model, upregulation of $InsP_3R$ expression, ER reorganisation and progression to M-phase generate a responsive cytoplasm at MII, such that introduction of PLCzeta at fertilisation triggers Ca^{2+} oscillations. The oscillations end upon entry to interphase due to sequestration of PLCzeta into the developing pronuclei, aided by an environment of reduced responsiveness to $InsP_3$. Mitotic Ca^{2+} oscillations are subsequently triggered by release of PLCzeta from the pronuclei into M-phase cytoplasm, and are terminated by its resequestration into the nuclei at the 2-cell stage. Insufficient PLCzeta prevents NEBD from triggering Ca^{2+} release in the second embryonic division. As

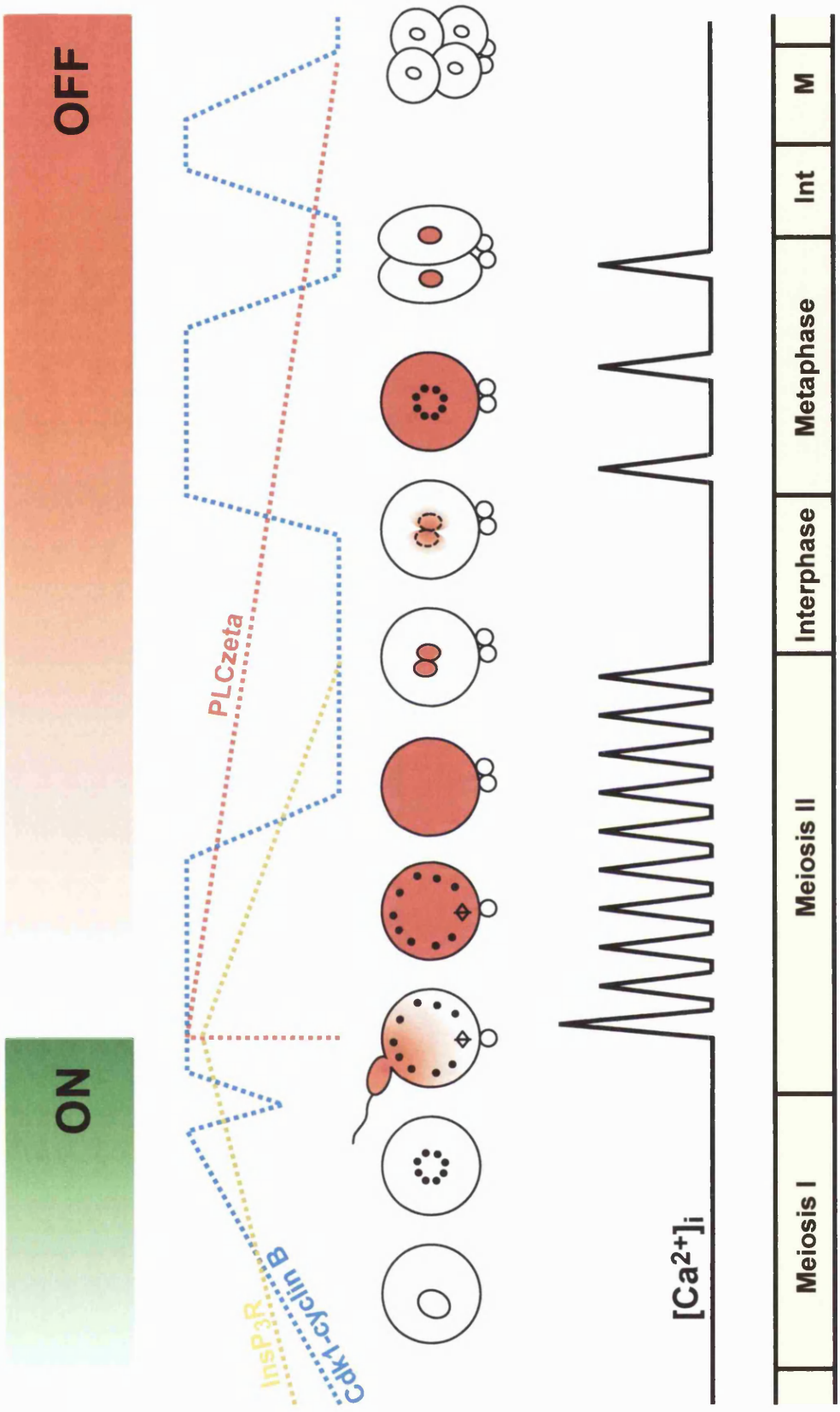


Figure 6.1. 'On' and 'Off' pathways regulating Ca^{2+} release in meiosis and mitosis. Upregulation of $InsP_3Rs$ (yellow dashes), increased cdk1-cyclin B activity (blue dashes) and ER clustering (black dots) during oocyte maturation contribute towards the generation of metaphase II cytoplasm which is highly sensitive to the introduction of sperm-derived Ca^{2+}-releasing factors (red shading). Introduction of these factors at fertilisation triggers Ca^{2+} oscillations which subsequently end as a result of nuclear sequestration of the sperm-factor(s), on a background of decreased sensitivity of Ca^{2+} release (see text). Ca^{2+} oscillations resume in mitosis when the sperm-factor(s) are released back into the M-phase cytoplasm. Degradation of the sperm-factor below a critical threshold (red dashes) prevents Ca^{2+} release in the second mitotic division. Cell cycle stage is shown in yellow.

previously discussed, this model differs significantly from that of the ascidian, in which evidence is strong that cdk1-cyclin B activity is the principal regulator of Ca^{2+} release (discussed in Chapters 1 and 4). Hence, whilst it is not yet clear at what time the pronucleus forms relative to Ca^{2+} oscillation cessation in ascidians, it appears likely that the mechanisms governing the timecourse of Ca^{2+} spiking may differ between species.

'On' and 'Off' pathways and oocyte competence

Although the mechanisms which dictate the pattern of Ca^{2+} release in laboratory animals are now becoming clear, the situation may be somewhat different in the clinical setting, where the oocyte or sperm may be compromised. One illustrative example is the use of *in vitro* matured oocytes for human IVF cycles.

Completion of meiosis I can occur in vitro for all species studied, the vast majority of oocytes isolated from antral follicles subsequently progressing to MII (Trounson *et al.*, 2001). However, with the possible exception of mice (Schroeder and Eppig, 1984), the proportion of in vitro matured oocytes which fertilise and subsequently develop to blastocyst is remarkably low (Trounson *et al.*, 2001). The generation of a developmentally competent egg is dependent both upon the completion of meiosis, and upon an assortment of molecular changes which prepare the egg for fertilisation collectively termed cytoplasmic maturation (Eppig *et al.*, 1994;Moor *et al.*, 1998). Since the percentage of oocytes which complete nuclear maturation in vitro is usually high, poor developmental competence may relate to aberrations in cytoplasmic maturation. Aspects of the cytoplasm known to be modified during oocyte maturation include the redistribution of cytoplasmic organelles, an increase in glutathione (GSH) levels important for formation of the male pronucleus (Perreault *et al.*, 1988;Zuelke *et al.*, 2003), changes in the number and distribution of cortical granules

(Ducibella *et al.*, 1988) and, of course, the 'On' pathways which allow the oocyte to mount a proper Ca^{2+} response at fertilisation.

The failure of *in vitro* maturation systems to support these 'On' pathways is an attractive potential contributor to the developmental failure of IVM oocytes. It is now well established that the fidelity of Ca^{2+} oscillations is a determinant of egg activation and developmental competence. In mouse, egg activation is dependent upon a critical number of Ca^{2+} oscillations (Lawrence *et al.*, 1998;Ducibella *et al.*, 2002), and embryonic development is be influenced by Ca^{2+} signalling regimen both immediately following fertilisation and in mitosis (Bos-Mikich *et al.*, 1997;Ozil and Huneau, 2001). Recently it has been shown that the process of *in vitro* maturation may affect the sperm-induced Ca^{2+} oscillations. Comparing oocytes from 19-day old mice one group noted that *in vitro* matured oocytes generate fewer Ca^{2+} transients than ovulated counterparts, and exhibit impaired development to 2-cell stage (Cheung *et al.*, 2000). Thus, whilst major deficits in the Ca^{2+} signalling machinery can prevent oocyte activation, more subtle shortcomings acquired during maturation may contribute to the high levels of embryo loss reported after insemination of IVM oocytes. It is conceivable that 'Off' pathways such as ER reorganisation, exit from M-phase and $InsP_3R$ downregulation which reduce the responsiveness of Ca^{2+} release without stopping Ca^{2+} oscillations in healthy eggs, may be sufficient to draw oscillations to a close in oocytes in which the Ca^{2+} signalling machinery were initially imperfect at the time of fertilisation. The compromised development of IVM oocytes may therefore relate to premature cessation of sperm-induced Ca^{2+} oscillations.

It is not yet known which aspects(s) of the 'On' pathways are compromised during *in vitro* maturation. In initial experiments designed to examine the timecourse of ER reorganisation during oocyte maturation, we noted that ER cortical clusters fail to form *in vitro* in our laboratory (our unpublished observations), though the vast majority of oocytes

progress to MII. Oocytes matured according to the same protocol release less Ca^{2+} in response to photolysis of caged $InsP_3$ than ovulated eggs. In contrast, cortical clusters of ER similar to those found in ovulated oocytes were generated during IVM in Dr John Eppig's lab (see Chapter 3). ER reorganisation may therefore be an example of an 'On' pathway susceptible to IVM protocols.

To conclude, it is clear that the generation of Ca^{2+} signalling machinery capable of supporting a proper response to fertilisation is a key component of oogenesis, and failure of IVM systems to sustain this generation may well contribute to the impaired developmental potential of *in vitro* matured oocytes. Development of protocols capable of supporting this aspect of cytoplasmic maturation may improve the developmental potential of *in vitro* matured oocytes.

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Appendix 1. Monitoring $[Ca^{2+}]_I$ in mouse oocytes using Fura-red.

Fura-red is a Ca^{2+} indicator suited to ratiometric imaging techniques. The wavelengths of maximum light absorbtion of Fura-red in solution are around 445nm and 470nm in its Ca^{2+}-bound and Ca^{2+}-free forms respectively. The emission maxima for both forms of the dye lie between 650 and 670 nm (*Molecular Probes* website). Since the isosbestic wavelength of Fura-red is found at approximately 440nm in living cells (Bolsover *et al.*, 2001), ratiometric Ca^{2+} imaging is typically performed by exciting the dye at approximately 430 and 490nm, and collecting emitted fluorescence using an emission filter which collects light over 600nm. Using such a protocol, an increase in $[Ca^{2+}]_i$ causes a moderate increase in fluorescence collected as a result of excitation at 430nm, and a more substantial decrease in fluorescence in response to 490nm (Bolsover *et al.*, 2001). Ca^{2+} release events thus cause an increase in the 430/490 ratio.

In order to comply with the changes requested by my PhD examiners, it was necessary to re-examine individual wavelength traces for Fura-red in the experiment investigating mitotic Ca^{2+} release (figure 5.5). In the process of this re-analysis I noted that the amount of fluorescence emitted by Fura-red in response to excitation at 430 and 490nm both decreased when $[Ca^{2+}]_i$ became elevated following mitosis entry. Subsequent reanalysis revealed the same to be true of all experiments presented in this thesis in which Fura-red has been used; increases in $[Ca^{2+}]_i$ have been recorded as decreases in both 430nm and 490nm traces. Since the magnitude of fluorescence decrease is much greater for the 490nm wavelength than 430nm, changes in $[Ca^{2+}]_i$ are still recorded as positive changes in the Fura-red ratio. One such example of this has now been presented in figure 5.5, and a further example is presented in figure A1a. This discovery was initially something of a surprise, since we had originally

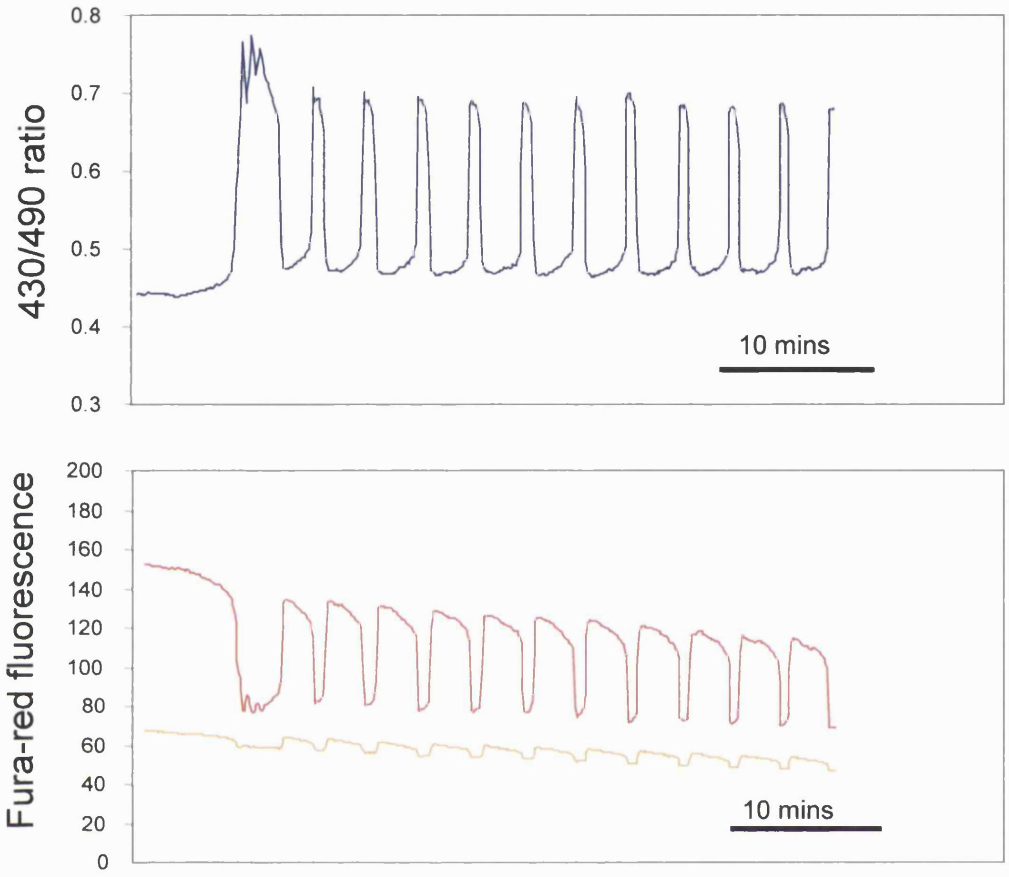

Figure A1a. Ca^{2+} transients stimulate a decrease in fluorescence emission when Fura-red is excited with 430nm light. MII oocytes were inseminated on the microscope stage and $[Ca^{2+}]_i$ monitored using Fura-red as described in Chapter 2. Typical fertilisation-induced Ca^{2+} oscillations are detected by the 430/490 ratio (blue). Examination of the individual wavelength traces reveals that each Ca^{2+} oscillation is associated with a transient decrease in fluorescence emitted both in response to excitation of Fura-red at 490nm (red), and also at 430nm (orange). Data from the experiment originally presented in figure 3.7B.

chosen our imaging protocol based upon the published properties of Fura-red, and upon previous reports in which Fura-red had been used in mouse eggs (Lawrence *et al.*, 1998;Parrington *et al.*, 1999;Jones *et al.*, 2000) .

In order to ascertain why both 430nm and 490nm traces decrease in response to high $[Ca^{2+}]_i$, I have subsequently investigated the spectral properties of Fura-red in mouse eggs by examining the effect of $[Ca^{2+}]_i$ oscillations upon fluorescence emission when Fura-red is excited with light of different wavelengths. Fura-red-loaded eggs were inseminated on the microscope stage. Typical sperm-induced Ca^{2+} transients were monitored using the 490nm trace. At the same time the change in fluorescence emission caused by Ca^{2+} oscillations at different excitation wavelengths (in the range of 405-430nm) was investigated by using the *metafluor* software to control the wavelength of light selected by the monochromator. A typical example of this experiment is shown in figure A1b. Ca^{2+} oscillations (as monitored using the 490nm trace) were accompanied by increases in fluorescence detection when Fura-red was excited with 405, 410, 415 or 420nm light. However, Ca^{2+} oscillations were accompanied by small changes in fluorescence when Fura-red was excited at 425nm. As before, the fluorescence collected in response to 430nm excitation decreased slightly during each Ca^{2+} oscillation. Thus it appears that the spectral properties of Fura-red are shifted in the mouse egg, such that the isosbestic point is around 425nm, rather than 440nm. This phenomenon is not attributable to unexpected properties of our monochromator, since we find the isosbestic point of Fura-2 to be around 358nm, which is similar to that reported in the literature. Moreover, subsequent reanalysis of experiments performed in a different laboratory has revealed a similar result (Karl Swann and Mark Larman, personal communication).

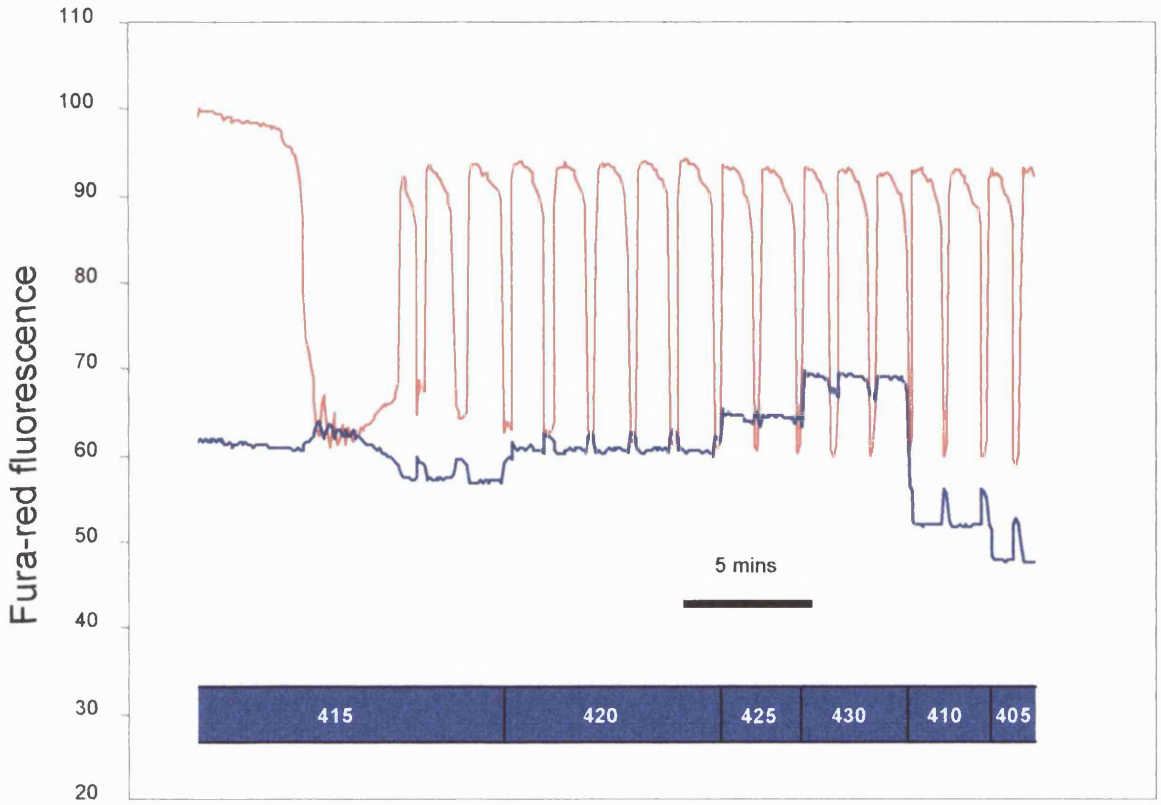

Figure A1b. The isosbestic point of excitation of Fura-red is found at approximately 425nm. Metaphase II oocytes were inseminated on the microscope stage, and typical sperm-induced Ca^{2+} transients observed by exciting the dye with 490nm light, and collecting fluorescence emitted using a 600nm long pass filter (red trace). At the same time, the effect of changing the wavelength of excitation was examined by using the metafluor software to control the monochromator (blue trace). The wavelengths selected are indicated by the blue bar. Ca^{2+} transients cause a transient increase in fluorescence collected in response to excitation at a wavelength less than 425nm. As before, each Ca^{2+} oscillation stimulates a decrease in fluorescence collected if the dye is excited at 430nm. Little change in fluorescence is detected at an excitation wavelength of 425nm. The example shown is representative of 15 oocytes examined over the course of two days.

It is important to note that this discovery does not in any way alter the findings presented in this thesis. Though it appears that we have in fact been monitoring $[Ca^{2+}]_i$ by exciting Fura-red at two different points on the same side of the isosbestic point, our measurements are still ratiometric, and thus still control against differences in dye-loading. Moreover, all oocytes have been treated similarly. Review of the available literature reveals several publications in which Fura-red has been used ratiometrically to monitor $[Ca^{2+}]_i$ in mouse eggs, in which the dye has been excited at 430-440nm and 490nm (Lawrence *et al.*, 1998;Parrington *et al.*, 1999;Jones *et al.*, 2000;Cox *et al.*, 2002;Saunders *et al.*, 2002). In light of the data presented here, it seems likely that fluorescence emission in response to both individual wavelengths will have decreased in response to high $[Ca^{2+}]_i$ in these experiments also. Whilst the unexpected properties of Fura-red do not in any way prejudice the findings of these reports, sampling on the same side of the isosbestic point undoubtedly reduces the dynamic range of the ratio. Therefore future investigations in which Fura-red is used in mouse eggs should be carried out using a wavelength less than 420nm.

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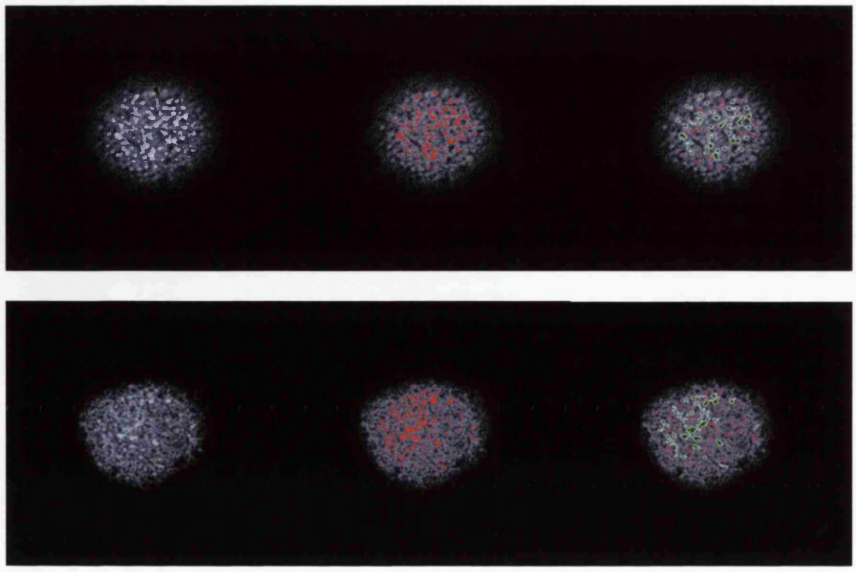
Appendix 2. Analysing cortical endoplasmic reticulum using metamorph.

In Chapters 2 and 4 I present a novel method of analysing cortical ER clusters. At the suggestion of my PhD examiners, I have investigated potential alternative methods of analysing these data. Here I describe a method of using metamorph to quantify cortical clusters of ER which is unrelated to the original method presented, completely objective, and which cannot be biased by the experimenter.

Firstly, the threshold function within the *metamorph* software is used to select pixels above a given intensity. We have chosen to use a threshold which is the mean pixel intensity of the given cortical slice plus one standard deviation. Performing this operation consistently selects pixels in the clustered regions of ER, but also highlights many individual pixels and small groups of pixels that should not be considered to be ER clusters. It is important therefore to define an ER cluster as a group of pixels of a given size. Since ER clusters have been described as 1-2μm in diameter, we have used the *integrated morphometry analysis* function in *metamorph* to automatically count the number of regions in which a minimum of 30 pixels, which corresponds to the area of a circular region of 1μm diameter. This process is demonstrated in figure A2a.

I have subsequently used this method to re-analyse one entire experiment (the experiment investigating the effect of egg activation upon ER structure; section 4.2.2, figure 4.2). The result of this re-analysis is presented in figure A2b. Under the criteria described above, the *integrated morphometry analysis* function counted 35.1+/-2.0 clusters in MII arrested eggs (n=8), 26.4+/-2.5 clusters in Pb2 stage parthenotes (n=11), and 20.8+/-2.1 clusters in pronucleate stage parthenotes (n=14). The number of clusters counted is significantly greater in MII eggs than in either group of parthenotes ($P<0.01$). There is no significant difference in the number of

A2a



A2b

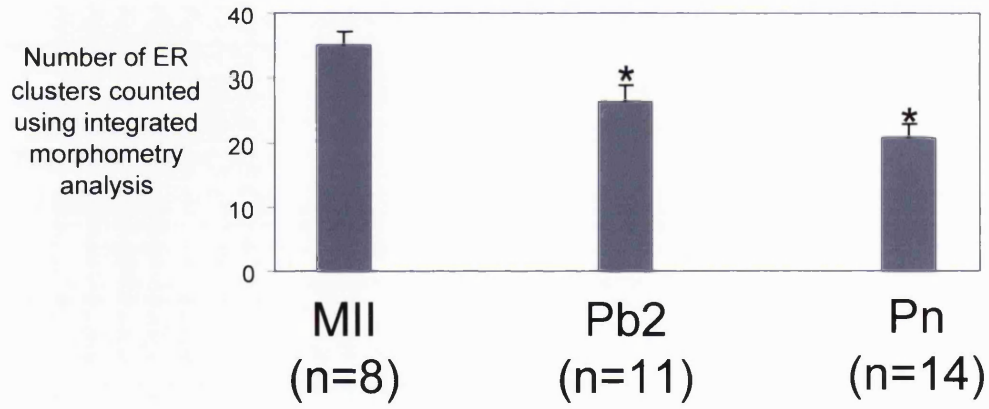


Figure A2. (a) An alternative method of analysing cortical ER. Two examples (one MII egg, and one pronucleate parthenogenetic embryo; Pn) of using the *integrated morphometry analysis* function in the *metamorph* software to objectively count cortical ER clusters. First, the threshold is set as the mean plus one standard deviation of the pixel intensities in the cortical slice. The pixels selected by this threshold are indicated in red (middle column). The integrated morphometry analysis function is used to define ER clusters as thresholded regions with a minimum area of 30 pixels (green). The software automatically counts these regions. In these typical examples, there are 33 clusters in the MII egg, and 23 clusters in the pronucleate embryo. (b) Reanalysis of the experiment presented in section 4.2.2 using *integrated morphometry analysis*. *The number of cortical ER clusters counted is significantly less in Pb2 and Pn stage parthenogenetic embryos than in MII eggs ($P<0.01$). These is no significant difference the number of ER clusters identified by this method between Pb2 and Pn groups ($P>0.1$). This result concurs with the result presented in 4.2.2, confirming a reorganisation of ER which occurs following egg activation.

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