

**The role of inositol 1,4,5-trisphosphate
receptors in mammalian oocytes and
preimplantation embryos**

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ABSTRACT

A fertilisation-induced increase in intracellular Ca^{2+} is responsible for initiating all of the events of egg activation. In mammals, the Ca^{2+} increase takes the form of a series of Ca^{2+} oscillations that continue for 3-4 hours. In most cells, intracellular Ca^{2+} is regulated by Ca^{2+} channels in the endoplasmic reticulum known as inositol 1,4,5-trisphosphate receptors (InsP_3Rs). The aim of the experiments presented in this thesis is to investigate the role of three known isoforms of the InsP_3Rs in oocyte maturation, fertilisation and early mammalian development.

Western analysis revealed that the type I isoform accounts for all of the detectable InsP_3R protein in unfertilised mouse oocytes. Furthermore, type I protein levels were dramatically decreased within 4 hours of fertilisation. During development to the blastocyst the level of type I did not return to prefertilisation levels and types II and III remained below our detection limit.

One mechanism that leads to the inhibition of Ca^{2+} transients after fertilisation may be the downregulation of InsP_3Rs . I examined the mechanism of this InsP_3R downregulation and found that neither egg activation nor Ca^{2+} transients are necessary or sufficient for its stimulation. The only stimulus, besides fertilisation, that downregulated InsP_3Rs was microinjection of the potent InsP_3R agonist adenophostin A, suggesting that InsP_3 binding is sufficient for downregulation. InsP_3R downregulation was inhibited by the cysteine protease and proteasome inhibitor ALLN but not by specific proteasome inhibitors MG132 or lactacystin.

To examine the role of InsP_3 -induced Ca^{2+} release during oocyte maturation, at fertilisation and in the preimplantation embryo, I used adenophostin A to produce immature oocytes, MII eggs and fertilised 1-cell embryos which were depleted of InsP_3Rs . I found that sperm-induced Ca^{2+} signaling was inhibited in InsP_3R -depleted MII eggs, indicating that Ca^{2+} signaling at fertilisation is mediated via the InsP_3R . In contrast, I found that endogenous levels of InsP_3Rs are not necessary for GVBD and meiosis I of oocyte maturation or for NEBD of the first cell cycle or for subsequent cell division cycles up to the morula stage.

I conclude that the type I InsP_3R is essential for fertilisation but that less than 5% of the endogenous receptors are necessary for the first meiotic division and the early embryonic cell division cycles. These studies suggest that the previously reported role for Ca^{2+} in mitosis in mammalian cells requires re-evaluation.

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CHAPTER 1:

GENERAL INTRODUCTION

The general introduction will review literature covering the topics relevant to the work of this thesis. Intracellular calcium (Ca^{2+}) release is known to play an important part in the regulation of all cell cycles. In particular, in all species Ca^{2+} triggers the transition of egg to embryo at fertilisation. This thesis investigates the role of one family of intracellular Ca^{2+} release channels, the inositol 1,4,5-trisphosphate receptors, in the stimulation of the events of fertilisation and in subsequent early development. I will begin the review by discussing the meiotic and mitotic cell cycles. I will then review the development of the oocyte up to the time of fertilisation and the preimplantation development of the resulting embryo. This will be followed by a discussion of the mechanisms of Ca^{2+} signaling with specific emphasis on the role of the inositol 1,4,5-trisphosphate receptor. Finally, I will describe what is known about how Ca^{2+} signaling can influence the cell cycle.

1.1 The cell cycle

Early development involves a series of steps progressing through the cell cycle. In order to explain what is known about the role of Ca^{2+} in the cell cycle, and thus in early development, it is first necessary to give an overview of the cell cycle itself. There are two types of cell division: mitosis and meiosis.

1.1.1 Mitosis

Mitotic divisions are responsible for all somatic cell divisions in all eukaryotic cells. At mitosis, the chromatin of the nucleus condenses to form visible chromosomes, which, through an elaborate series of movements, are pulled apart into two identical sets. This is followed by cytokinesis, in which the cell splits into two daughter cells, each receiving one of the two sets of chromosomes. These two events, termed M phase, last only 1-2 hours and generally occur once every 16-24 hours in rapidly proliferating cells. The time in-between M phases is known as interphase, during which preparations for division, such as DNA synthesis occur. Interphase is made up of three stages, named G1, S and G2.

There are six stages of M phase (**Fig. 1.1**). These are prophase, prometaphase, metaphase, anaphase and telophase (which constitute mitosis itself) and cytokinesis, which, in fact, commences during anaphase. At the onset of prophase, the chromatin, which is diffuse in interphase, condenses into well-defined chromosomes (46 in human). Each chromosome has duplicated during the preceding S phase and consists of two sister chromatids. Near the end of prophase, the microtubules of the interphase cytoskeleton disassemble and the mitotic spindle begins to form outside the nucleus. The spindle is a bipolar structure composed of microtubules and associated proteins. Prometaphase begins

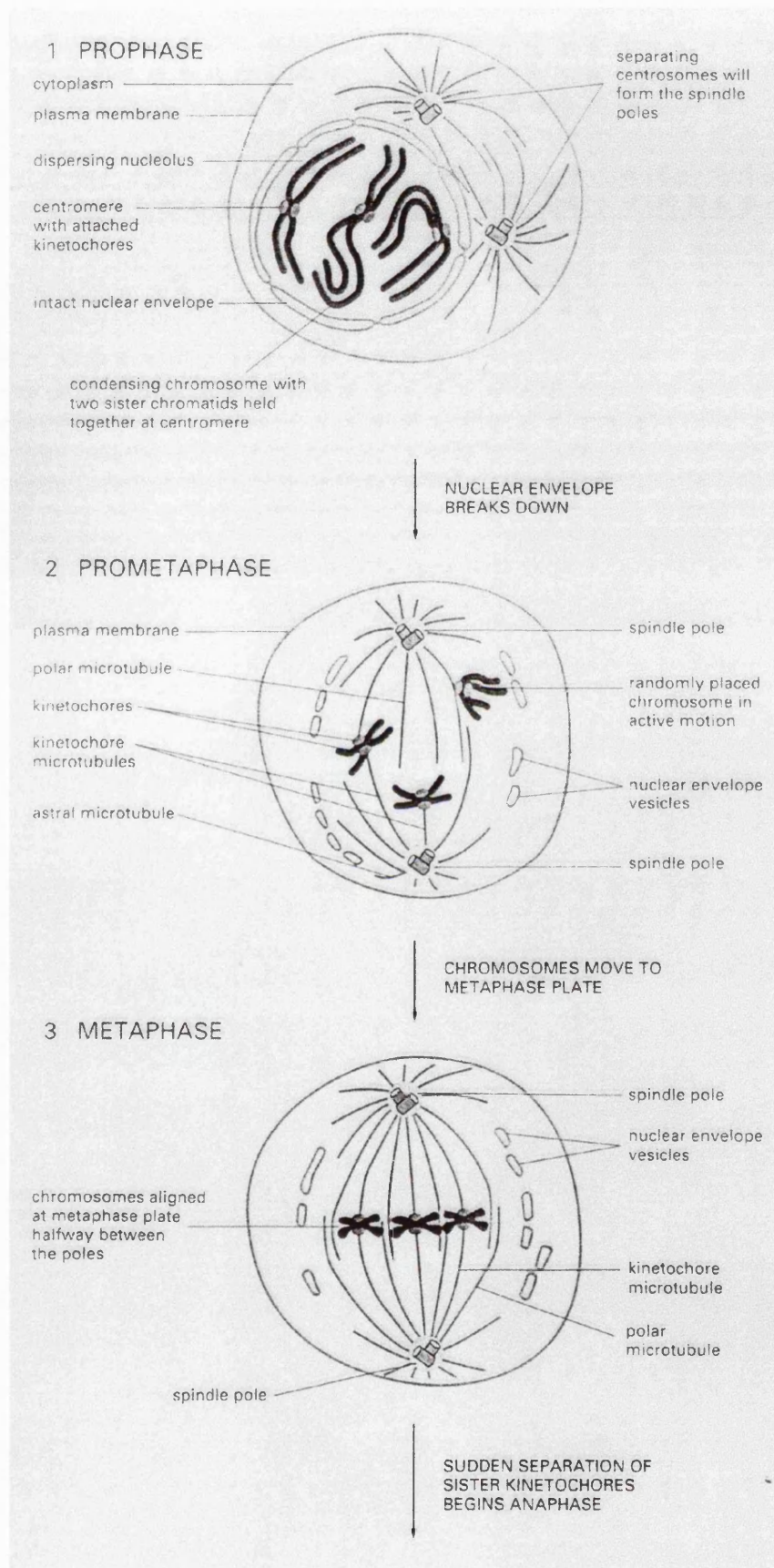


Figure 1.1 The six stages of mitotic cell division. (from Molecular Biology of the Cell, Alberts *et al.*).

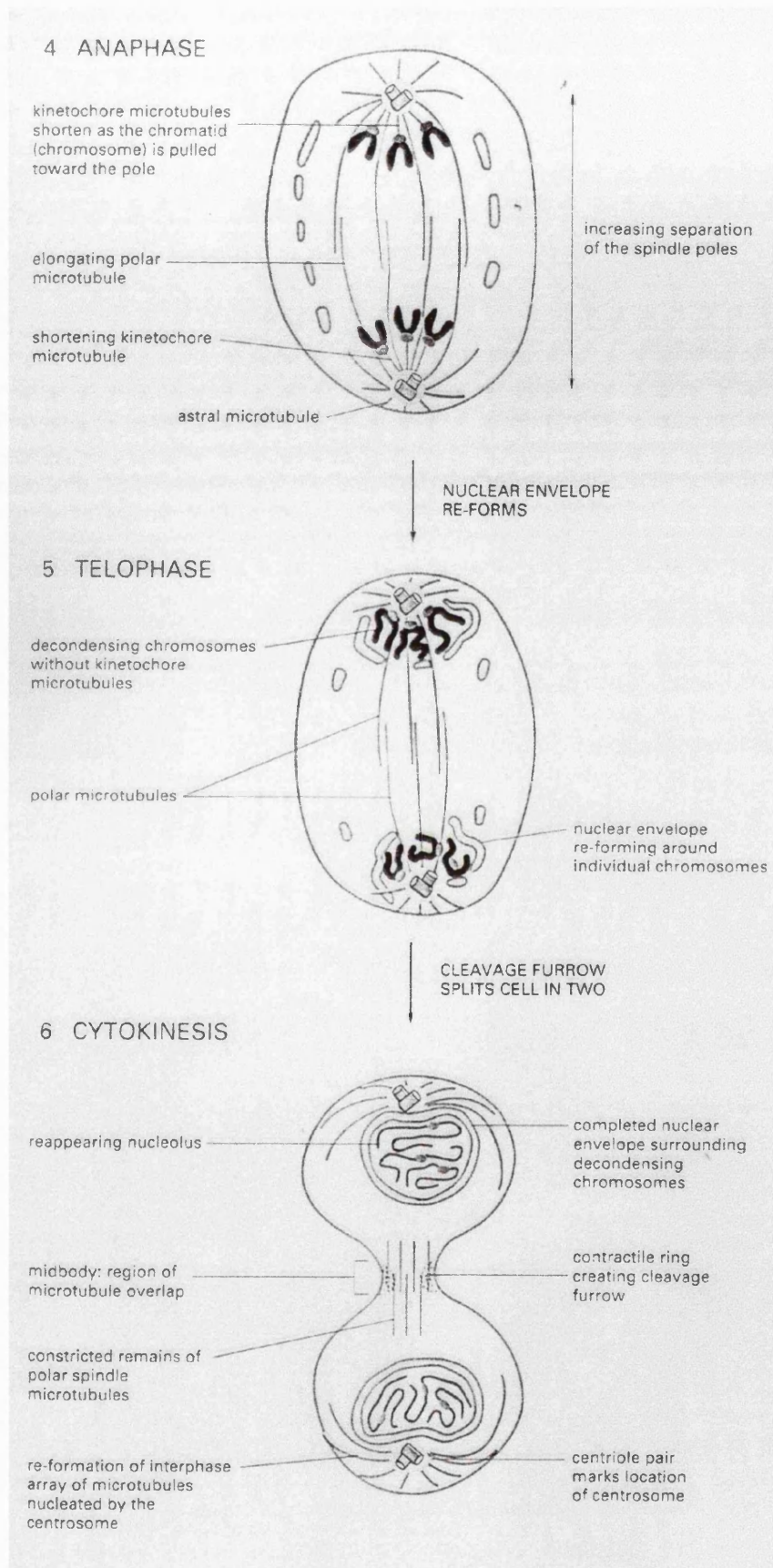


Figure 1.1 (continued) The six stages of mitotic cell division. (from Molecular Biology of the Cell, Alberts *et al.*).

with the breakdown of the nuclear envelope (NEBD) into ER-like membrane vesicles. Protein complexes called kinetochores attach the chromatids to some of the spindle microtubules. By metaphase, the microtubules have aligned the chromosomes in a single plane halfway between the spindle poles (the metaphase plate) and they are held in this plane by tension from opposing microtubules. It is at this stage that the mammalian oocyte arrests prior to fertilisation; anaphase is triggered by a specific signal at fertilisation (see 1.2.2). The paired kinetochores on each chromosome separate, allowing each chromatid to be pulled slowly towards a spindle pole. Anaphase typically lasts only a few minutes. In telophase, the separated chromatids arrive at the poles and the microtubules attached to kinetochores disappear. A new nuclear envelope reforms around each group of daughter chromosomes. The chromatin decondenses again, the nucleoli begin to reappear and mitosis ends. At cytokinesis, the membrane around the middle of the cell between the daughter nuclei is drawn inward to form a cleavage furrow, which gradually deepens and then cleaves to form two separated daughter cells (Alberts *et al.*).

1.1.2 Meiosis

Whereas mitosis duplicates diploid cells, meiosis is a special type of cell division designed to produce haploid germ cells (**Fig. 1.2**). In order to ensure that each progeny cell contains only one member of each homologous pair of chromosomes (either maternal or paternal homologue), there are two nuclear divisions rather than one. Meiosis occurs in male and female germ cells. For the purposes of this review, I will describe the events of meiosis as they occur in the female germ-line. In the primary oocyte awaiting the first meiotic division the DNA replicates so that each chromosome consists of two chromatids. At meiosis, the homologous chromosomes pair along their long axes, and chromosomal crossing-over occurs between the chromatids (to introduce genetic variation between the resulting progeny). The cell now remains arrested in prophase of division I of meiosis until the onset of sexual maturity (oocyte maturation, see below). Under hormonal influences the chromosomes condense, dissolution of the germinal vesicle membrane (germinal vesicle breakdown, GVBD) occurs and the replicated homologous chromosomes segregate at anaphase I into two daughter nuclei, each with half the original number of chromosomes. The cytoplasm divides asymmetrically to produce a small first polar body cell and a large secondary oocyte, each containing a daughter nucleus. After a brief interphase, prophase of the second meiotic division begins. Meiosis II resembles a normal mitosis. The sister chromatids of each chromosome are partitioned into separate cells by a process that is identical to mitosis. After anaphase II the cytoplasm of the large secondary oocyte again divides asymmetrically to produce the mature egg and a second small polar body, each with a haploid number of single chromosomes. Both polar bodies eventually degenerate. Note that in most vertebrates, oocyte maturation proceeds to metaphase of meiosis II (MII) and then arrests. At ovulation the MII-arrested secondary oocyte is released from the ovary, and if fertilisation occurs, the oocyte is stimulated to complete meiosis (see 1.2.2) (Alberts *et al.*). The next section refers specifically to the production (by meiosis) of mature germ

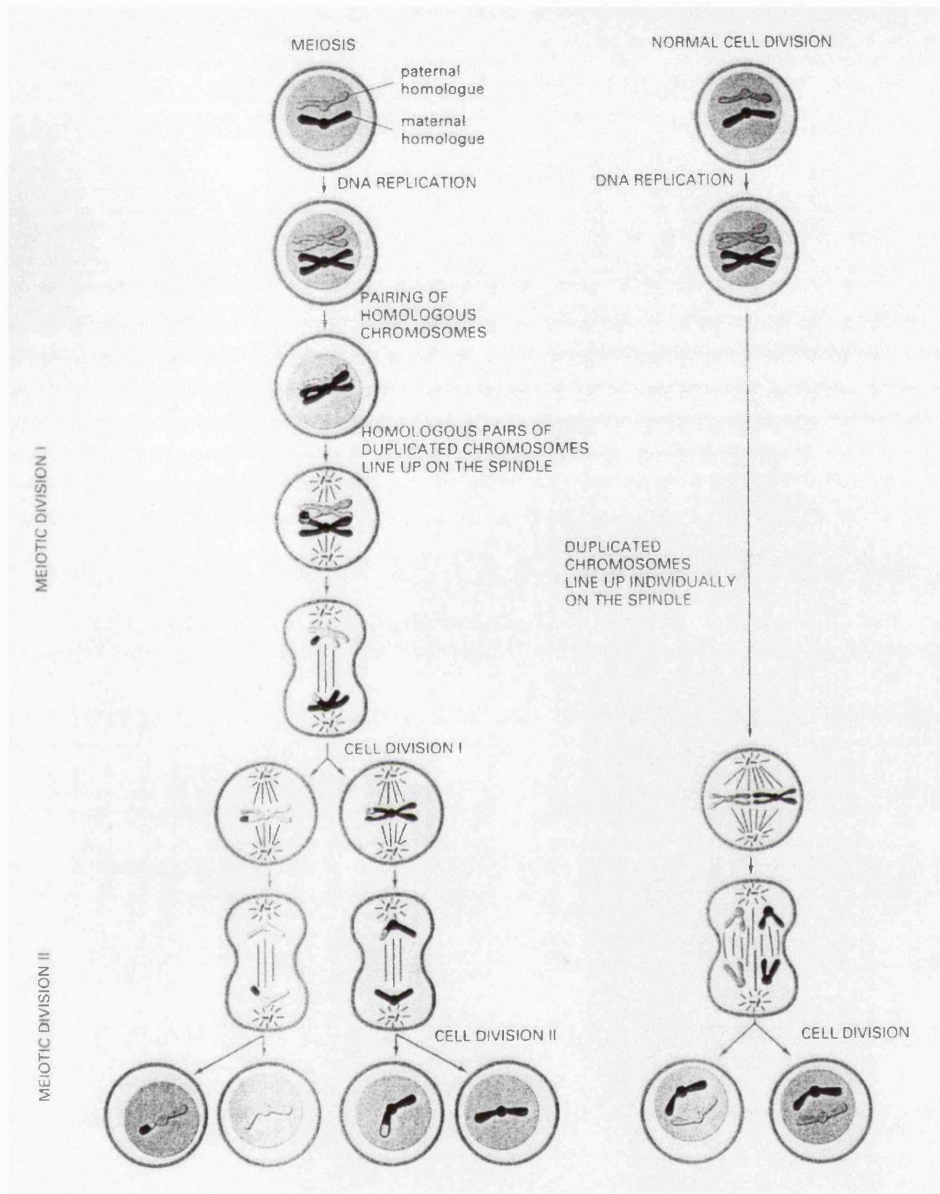


Figure 1.2 Comparison of meiosis and mitotic cell division. Note that only one pair of homologous chromosomes is shown. In meiosis, following DNA replication, two nuclear (and cytoplasmic) divisions are required to produce the haploid gametes. Each diploid cell that enters meiosis therefore produces four haploid cells, whereas each diploid cell that divides by mitosis produces two diploid cells. (from *Molecular Biology of the Cell*, Alberts *et al.*).

cells/oocytes in the female mammal and leads on to a review of mammalian fertilisation and the mitotic divisions of early embryonic development.

1.2 Preimplantation mammalian development

1.2.1 Oocyte growth and maturation

By 5 days after birth, all mammalian oocytes are arrested in prophase of the first meiotic division and are termed primary oocytes (**Fig. 1.3**). Each oocyte is initially contained within a primordial follicle composed of a layer of pregranulosa cells. Beginning sometime before birth, and periodically throughout the reproductive lifespan, a small proportion of follicles are stimulated to grow and leave the primordial follicle pool. Although it is not known what causes certain primordial follicles to begin growing to become developing follicles, the continuing development of such follicles may depend on follicle-stimulating hormone (FSH) secreted by the pituitary gland and on oestrogens secreted by the follicle cells themselves. The granulosa or follicle cells of the developing follicles, which have various roles in oocyte growth and differentiation (Richards *et al.*, 1987), become larger and cuboidal and proliferate to surround the oocyte in a multilayered envelope. A basement membrane separates the follicle cells from the ovarian stroma. The primary oocyte remains in prophase I but grows from approximately 15 to 85µm in diameter over the course of 10-17 days. The growing oocyte gradually synthesises and deposits a layer of extracellular material, called the zona pellucida (Bleil and Wassarman, 1980a; Bleil and Wassarman, 1980b; Greve and Wassarman, 1985), which is about 7µm thick in the fully grown oocyte. A major component of the zona, ZP3 (a glycoprotein), functions as a sperm receptor and initiates the acrosome reaction, which must occur if the sperm is to fertilise the egg (see 1.2.2).

As the oocyte grows larger, it gradually acquires the competence to enter the final stages of meiosis in response to either the correct hormonal stimulus *in vivo* or to release from the follicle *in vitro*. In a natural cycle in the mouse, 6-15 developing follicles respond to an increase in levels of FSH. The follicles accumulate fluid secreted by the follicle cells and move towards the periphery of the ovary, ready for the final maturation and release of the oocyte. These follicles are now termed antral or Graafian follicles. Once a follicle has initiated growth it continues to grow until it either degenerates or releases its oocyte by ovulation. However, oocyte maturation (progression beyond prophase I) and ovulation do not begin until sexual maturity.

The female mouse reaches sexual maturity at approximately 6 weeks of age, depending on the strain and environmental conditions. At this point, each ovary contains approximately 10⁴ oocytes at different stages of maturity. There are established techniques for isolating and culturing immature oocytes from preantral follicles and from antral follicles (Eppig and Telfer, 1993). Germinal vesicle (GV) stage oocytes from antral follicles mature

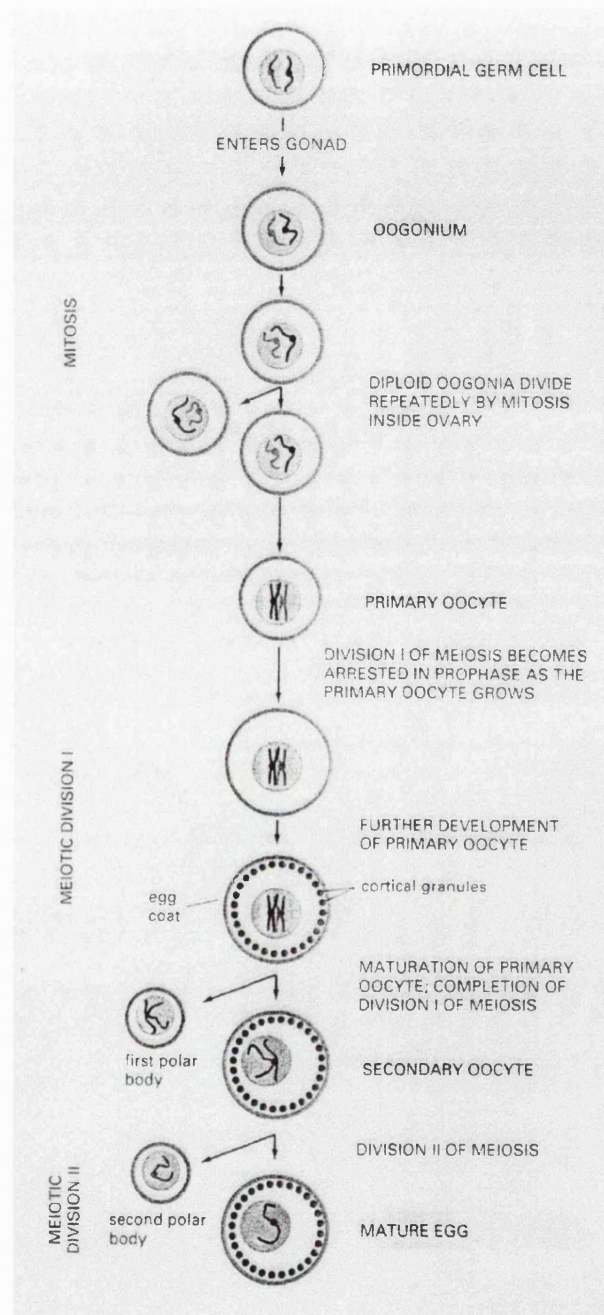


Figure 1.3 The stages of oogenesis. Oogonia develop from primordial germ cells that migrate into the developing gonad early in embryogenesis. After a number of mitotic divisions, oogonia begin meiotic division I, after which they are called primary oocytes. In mammals, primary oocytes are formed very early (between 3 and 8 months of gestation in the human embryo) and remain arrested in prophase of meiotic division I until the female becomes sexually mature. Then a small number periodically mature under the influence of hormones, completing meiotic division I to become secondary oocytes, which eventually undergo meiotic division II to become mature eggs. The stage at which the egg or oocyte is released from the ovary and is fertilised varies from species to species. In most vertebrates oocyte maturation is arrested at metaphase of meiosis II and the secondary oocyte completes meiosis II only after fertilisation. All polar bodies eventually degenerate. In most animals the developing oocyte is surrounded by specialised accessory cells that help isolate and nourish it (not shown). (from *Molecular Biology of the Cell*, Alberts *et al.*).

spontaneously under these culture conditions and can be fertilised *in vitro* and develop normally afterwards (Schroeder and Eppig, 1984).

Ovulation requires coordinated signaling from both the follicle cells and the oocyte and occurs once every 4 days in the mouse, under optimal conditions. However, cycle length can be influenced by environmental factors and can be induced artificially by hormone injection (superovulation, see below). Ovulation occurs in response to a surge in the level of luteinising hormone (LH), which is produced by the pituitary at each cycle, once sexual maturity has been reached. LH first stimulates the meiotic maturation of primary oocytes enclosed in Graafian follicles. The first sign of meiotic maturation is GVBD. After GVBD the chromosomes assemble on the spindle and move towards the cell periphery where the first meiotic division takes place. One set of homologous chromosomes, surrounded by a small amount of cytoplasm, is extruded as the first polar body. The other set progresses to and arrests at metaphase of the second meiotic division and the oocyte is finally released from the follicle.

The ovulated oocyte is surrounded by the zona and a mass of follicle cells (cumulus cells). The egg is swept into the infundibulum of the oviduct by the action of the numerous cilia on the surface of the oviduct epithelium. At the time of ovulation, the end section of the oviduct enlarges to form an ampulla where fertilisation takes place. In a natural ovulation in the mouse, 8-12 eggs are released but the process is not synchronous and occurs over a period of 2-3 hours (Hogan *et al.*, 1994). Superovulation of mice may be used in order to obtain sufficiently large numbers of eggs or preimplantation embryos, which are synchronised in their development. This involves administering gonadotrophins to females prior to mating or egg collection to increase the number of eggs that are ovulated. Pregnant mare's serum gonadotrophin (PMSG) is used to mimic follicle-stimulating hormone (FSH), and human chorionic gonadotrophin (hCG) is used to mimic luteinising hormone (LH).

1.2.2 Fertilisation: *in vivo* and *in vitro*

Approximately 58×10^6 sperm are released into the female reproductive tract per ejaculation. Before the sperm become competent for fertilisation, they must undergo a process of maturation. This process is known as capacitation and lasts for about one hour, but its mechanism is unknown. To reach the surface of the egg, the sperm must penetrate both the cumulus mass and the zona pellucida. The glycoprotein ZP3 binds the sperm to the zona and triggers the acrosomal reaction, a process in which the acrosome (a structure in the sperm head similar to a secretory vacuole) fuses with the plasma membrane of the sperm head, and releases various hydrolytic enzymes. This reaction allows the sperm to pass through the zona and must take place for the sperm to be able to fertilise the egg. Fertilisation is triggered by fusion of the posterior part of the sperm head with the egg membrane. Early events of fertilisation include a change in the egg surface, which inhibits the fusion of additional sperm, and the Ca^{2+} -dependent exocytosis of the cortical granules

beneath the plasma membrane. The latter event initiates the zona reaction, which involves modifications of the zona so that it no longer binds sperm or triggers the acrosomal reaction. These events both help to prevent polyspermy (and thus removal of the zona must be accompanied by experimental precautions designed to limit polyspermy).

The ovulated mammalian oocyte is arrested at metaphase of the second meiotic division. Fertilisation stimulates completion of the second meiotic division, extrusion of the second polar body and entry into the first mitotic cell division (**Fig. 1.4**). This process is known as egg activation. The second polar body is extruded at 2-3 hours post insemination (IVF figures). At 4-6 hours (IVF figures), nuclear membranes form around the maternal and paternal chromosomes, forming separate haploid male and female pronuclei that move towards the centre of the egg. The embryo is now in interphase. During this migration DNA replication takes place. The membranes of the pronuclei break down and the chromosomes assemble on the spindle. This is followed by the first mitotic cleavage at 17-20 hours (IVF figures). Due to the asynchrony inherent in ovulation and fertilisation, the first cleavage occurs over a number of hours in a population of naturally fertilised zygotes. Therefore, *in vitro* fertilisation (IVF) is frequently used to obtain more synchronous development.

IVF is a technique used to obtain large numbers of early cleavage stage embryos that are developing more synchronously than those collected after natural matings. IVF involves superovulation of female mice and fertilisation of the eggs obtained with sperm taken from the epididymis of the male. Sperm from the epididymis have been found to give a more synchronous development than sperm from the *vas deferens* (Pratt, unpublished).

Oocytes of different species arrest and await fertilisation at different stages of maturation. To give a few examples, oocytes of mammals, frog (*Xenopus*) and fish are all arrested at MII whereas oocytes of ascidians arrest at MI, sea urchin oocytes arrest at G1 of the first mitosis and starfish oocytes lack a natural arrest point once GVBD has been triggered (Stricker, 1999). Thus the stage of the cell cycle stimulated by the sperm at fertilisation will differ between species.

1.2.3 Preimplantation development of the fertilised egg

As described above, the first mitotic cleavage occurs at 17-20 hours post insemination (IVF figures) (Howlett and Bolton, 1985). *In vivo*, the second cleavage (3-4 cells) occurs between 46 and 54 hours post fertilisation, the third (5-8 cells) between 50 and 60 hours, formation of the compacted morula between 60 and 74 hours and finally, formation of the blastocyst between 84 and 96 hours (3.5-4 days) (Hogan *et al.*) (**Figs. 1.5, 1.6 and 1.7**). During these divisions the embryo is transported along the oviduct and into the uterus. During the fifth day of development, the blastocyst “hatches” from the zona and

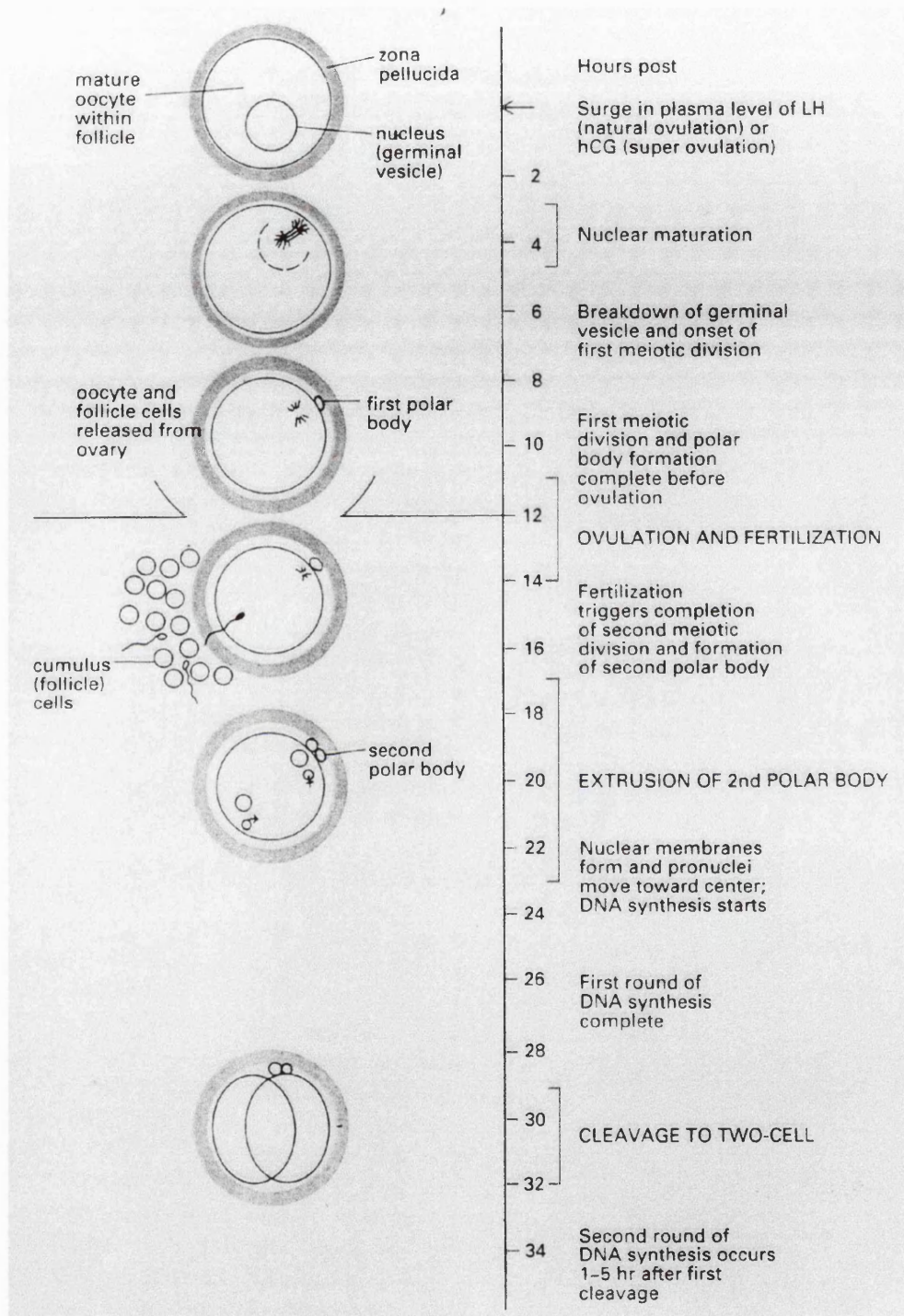


Figure 1.4 Ovulation and fertilisation in the mouse. (from *Manipulating the Mouse Embryo*, Hogan *et al.*).

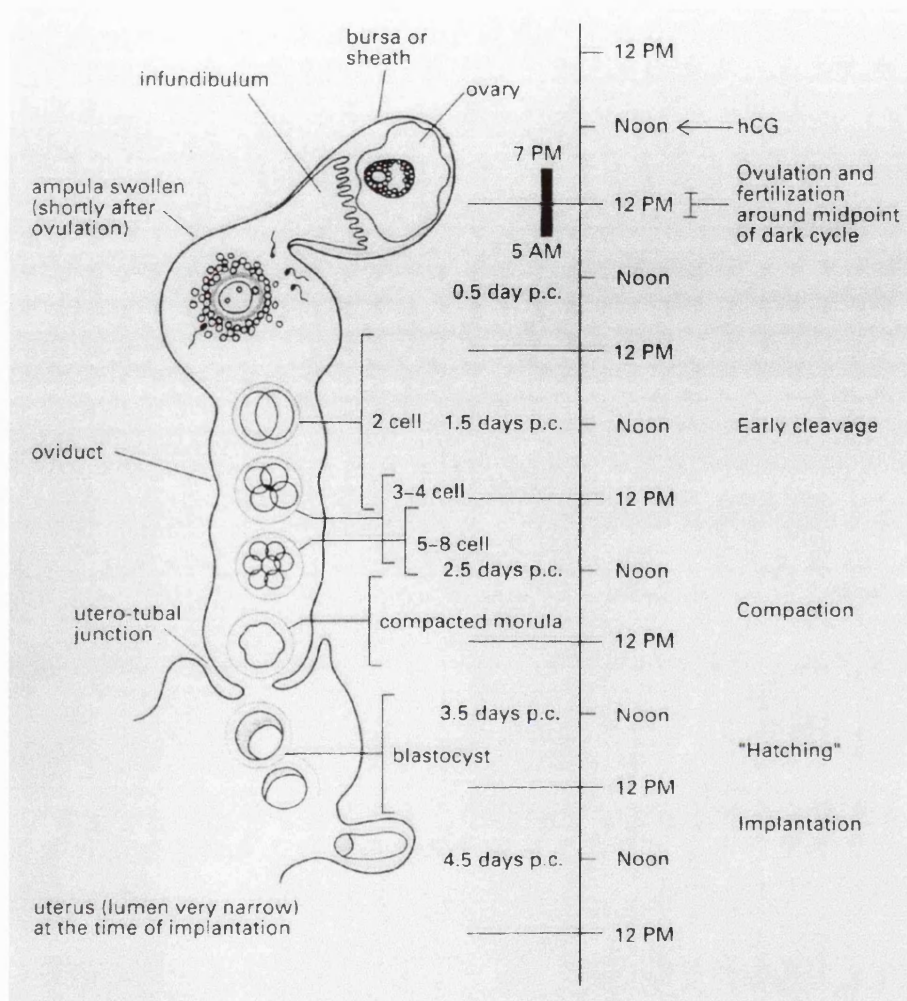


Figure 1.5 Summary of preimplantation development in the mouse.
(from *Manipulating the Mouse Embryo*, Hogan *et al.*).

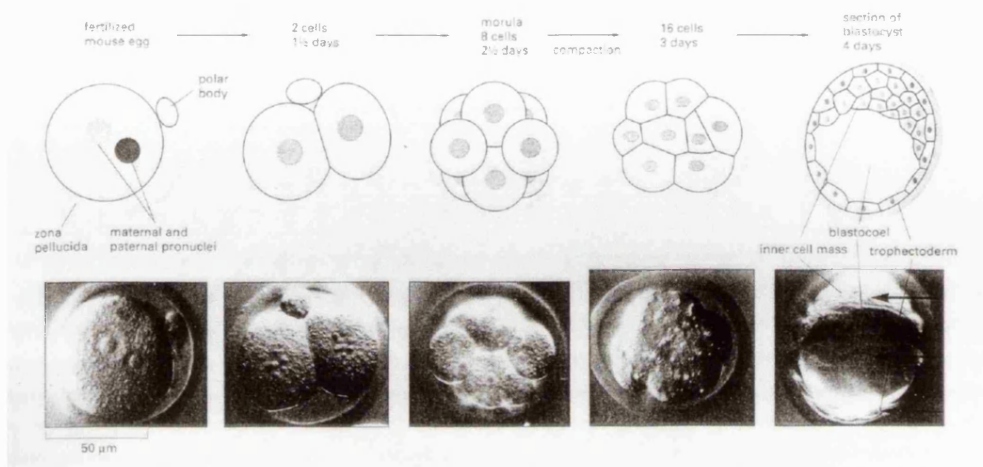


Figure 1.6 The early stages of mouse development. (from Molecular Biology of the Cell, Alberts *et al.*).

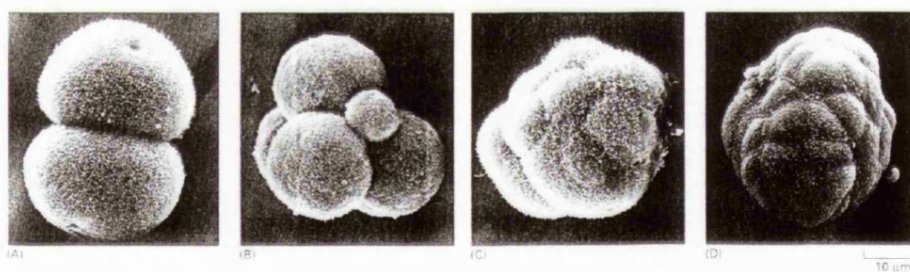


Figure 1.7 Scanning electron micrographs of the early mouse embryo. The zona pellucida has been removed. (A) 2-cell stage. (B) 4-cell stage (a polar body is visible in addition to the 4 blastomeres). (C) 8-16-cell morula, compaction occurring. (D) Blastocyst (from Molecular Biology of the Cell, Alberts *et al.*).

implants in the uterine wall. The blastocyst consists of an outer cell layer (trophectoderm) with an accumulation of cells inside at one pole (inner cell mass).

1.3 Induction of egg activation

1.3.1 Sperm-induced Ca^{2+} oscillations at fertilisation

The role of Ca^{2+} in the cell cycle is addressed later in this review. However, having described mammalian preimplantation development above, it seems appropriate at this point to discuss the Ca^{2+} signal induced by the sperm at mammalian fertilisation. It has been demonstrated that the signal responsible for egg activation is a sperm-induced increase in intracellular Ca^{2+} (Kline and Kline, 1992a; Whitaker and Swann, 1993). In mammals, the Ca^{2+} signal at fertilisation takes the form of a series of repetitive Ca^{2+} oscillations that continue for 3-4 hours and stop around the time of pronucleus formation (Jones ~~*et al.*~~, 1995a; Kline and Kline, 1992a). ^{Jones *et al.*, 1995a} In any one oocyte the amplitude and frequency of these oscillations remains fairly constant and only decreases shortly prior to pronuclear formation (Jones *et al.*, 1995a).

The repetitive nature of the sperm-induced Ca^{2+} signal is necessary for complete mammalian oocyte activation. Inhibition of Ca^{2+} transients with the Ca^{2+} chelator, BAPTA-AM, completely blocks mouse egg activation (Kline and Kline, 1992a). The necessity for oscillations is demonstrated by the finding that at least nine Ca^{2+} spikes are required at fertilisation for the formation of two pronuclei (Lawrence *et al.*, 1998). This same study used Ca^{2+} chelators to stop oscillations prematurely and showed that the number or frequency of Ca^{2+} spikes is correlated with the timing of pronuclear formation and mitosis in mouse eggs (Lawrence *et al.*, 1998). The timing of cessation of transients has in fact been shown to be related to pronucleus formation and entry into interphase (Jones *et al.*, 1995a) as the sperm is able to induce transients during metaphase but not during interphase. One hypothesis is that sperm-induced Ca^{2+} oscillations stop at pronuclear formation because the Ca^{2+} -releasing activity introduced by the sperm becomes localised within the pronucleus itself or associated with perinuclear material such as ER. An alternative explanation is that the mechanism of sperm-induced Ca^{2+} release is inactivated during interphase (Jones *et al.*, 1995a) (see section 1.7.4). However, more recent evidence has found that the cessation of spiking does not require the formation of a pronucleus and that sequestration of some factor into the pronucleus cannot be an essential step in spiking cessation (Day *et al.*, 2000). The regulation of sperm-induced Ca^{2+} oscillations by cell cycle proteins is discussed later in this review.

As well as being necessary, Ca^{2+} transients are also sufficient as stimuli for egg activation. Single Ca^{2+} transients can be induced by stimuli such as ethanol, ionomycin or Ca^{2+} injection and can be sufficient to activate mouse eggs (Swann and Ozil, 1994; Xu *et al.*, 1997) (see 1.3.2). However, the occurrence of activation using these agents is low in

freshly ovulated oocytes compared to that in oocytes 2-3 hours post ovulation; stimuli that generate multiple Ca^{2+} transients, such as repetitive pulsatile electrical stimulation, achieve a higher activation rate in freshly ovulated oocytes (Collas *et al.*, 1995; Ozil, 1990; Xu *et al.*, 1997).

1.3.2 Parthenogenetic activation

As introduced above, parthenogenetic activation of unfertilised eggs can be stimulated by exposing them to a variety of agents including ethanol, strontium (Sr^{2+}) and Ca^{2+} ionophores such as A23187, all of which induce an increase in the intracellular Ca^{2+} concentration. Parthenogenesis is also stimulated by protein synthesis inhibitors such as cycloheximide and by hyaluronidase, heat/cold shock and anaesthetics. In addition, a small percentage of oocytes, depending on the strain, undergo spontaneous activation, either in the oviduct or the ovary or in culture media. The genotype of the parthenogenetic embryo varies depending on the experimental conditions and the postovulatory age of the activated oocyte. Parthenogenetic activation in this study almost always resulted in the successful extrusion of the second polar body. Therefore the genotype of all cells of the embryo should have been haploid. Most parthenogenotes, particularly these uniform haploids, die before the blastocyst stage *in vivo*, although Sr^{2+} -activated diploid parthenogenotes have been successfully cultured *in vitro* up to the blastocyst stage (O'Neill *et al.*, 1991). The reason for failure of normal development beyond the blastocyst lies in the inactivation of specific genes by imprinting during oogenesis, thus preventing the differentiation of certain tissues which require both male and female genomes.

Ca^{2+} therefore plays an essential role at fertilisation and parthenogenetic activation of the mammalian oocyte. Ca^{2+} also plays an important regulatory role at other stages of the cell cycle during early development and this is discussed later in the review. The next section addresses how Ca^{2+} is involved in cell signaling pathways.

1.4 Ca^{2+} signaling

1.4.1 Overview of intracellular Ca^{2+} signaling

Ca^{2+} functions as a ubiquitous intracellular messenger. The resting concentration of free Ca^{2+} in the cytosol of any cell is extremely low ($\leq 100\text{nM}$), whereas its concentration in the extracellular medium is high ($\approx 1\text{-}2\text{mM}$). Distinct Ca^{2+} stores exist within subcompartments of the endoplasmic reticulum (ER) and nuclear envelope (NE) (Pozzan *et al.*, 1994; Subramanian and Meyer, 1997), in which the Ca^{2+} concentration is in the region of $500\text{-}600\mu\text{M}$. The difference in Ca^{2+} concentration between inside these stores and in the cytosol permits the regulated release of Ca^{2+} down its concentration gradient, from the stores into the cytosol, triggered by an extracellular signal. Increases in cytosolic Ca^{2+} concentration (to the region of $5\mu\text{M}$) resulting from this Ca^{2+} release regulate many aspects of cellular behaviour such as fertilisation and apoptotic death (Berridge, 1993), by stimulating Ca^{2+} -

responsive proteins in the cell. For this signaling mechanism to work, the resting concentration of Ca^{2+} in the cytosol must be kept low. One of the ways in which this is achieved is via ATP-dependent Ca^{2+} -ATPases in the plasma membrane and ER membrane that pump Ca^{2+} out of the cytosol into the extracellular medium or back into the stores, against the concentration gradient. In most animal cells, depletion of the Ca^{2+} stores is able to trigger Ca^{2+} entry across the plasma membrane (Putney, 1990), thus the stores regulate their own refilling (see 1.4.2). Regulated release from Ca^{2+} stores is permitted via Ca^{2+} release channels situated across the ER membrane. The major families of these channels are inositol 1,4,5-trisphosphate receptors (InsP_3Rs) and ryanodine receptors. This review will focus on InsP_3Rs (see 1.5) since these are the channels thought to be involved in Ca^{2+} release at fertilisation. Besides the ER membrane, InsP_3Rs have been detected in the plasma membrane in T-lymphocytes (Kuno and Gardner, 1987), the nuclear envelope (Gerasimenko *et al.*, 1995; Humbert *et al.*, 1996) and the Golgi membrane (Pinton *et al.*, 1998).

In most cells Ca^{2+} stores are mobilised in response to a signaling molecule such as a hormone or growth factor binding to its receptor on the plasma membrane. Many different cell-surface receptors have been shown to utilise the inositol phospholipid signaling pathway. Responses mediated in this way include smooth muscle contraction after acetylcholine stimulation and histamine secretion by mast cells after antigen binding. Receptor stimulation leads to the activation of phospholipase C (PLC) at the plasma membrane, which cleaves plasma membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) by hydrolysis, generating the Ca^{2+} -releasing second messenger, inositol 1,4,5-trisphosphate (InsP_3), and diacylglycerol (DAG). InsP_3 can diffuse rapidly through the cytosol and leads to an increase in intracellular Ca^{2+} by binding to and stimulating the opening of the InsP_3R in the ER membrane (Berridge, 1993).

1.4.2 Ca^{2+} influx

Another important component of the Ca^{2+} -signaling pathway in cells is Ca^{2+} influx through channels in the plasma membrane. Depletion of Ca^{2+} from internal stores triggers a capacitative influx of extracellular Ca^{2+} across the plasma membrane through what have been termed “store-operated” Ca^{2+} channels (SOCCs). In mouse oocytes, there is a stimulation of Ca^{2+} influx associated with onset of the first and subsequent fertilisation Ca^{2+} spikes (McGuinness *et al.*, 1996). It has been proposed that Ca^{2+} influx at fertilisation is controlled by a capacitative mechanism and that it sets the interspike period by determining the time taken to refill stores to a point where they are again sensitive enough to initiate the next spike (McGuinness *et al.*, 1996). Furthermore, the “conformational coupling” model of capacitative Ca^{2+} entry proposes that information on store depletion is transferred to the plasma membrane via the InsP_3R (Berridge, 1990; Berridge, 1995). In support of this, there is good evidence for activated InsP_3Rs functionally interacting with SOCCs and

regulating the Ca^{2+} release-activated current ($I(\text{crac})$) (Kiselyov *et al.*, 1998; Ma *et al.*, 2000).

1.4.3 Ca^{2+} signaling at fertilisation: the role of InsP_3Rs

InsP_3 -induced Ca^{2+} release is thought to be the main mechanism by which Ca^{2+} transients are generated and propagated at fertilisation. For example, an increase in InsP_3 mass has been demonstrated at fertilisation in sea urchin (Ciapa *et al.*, 1994; Turner *et al.*, 1984) and *Xenopus* oocytes (Stiith *et al.*, 1994). Furthermore, inhibition of the InsP_3R with a functionally inhibitory anti-type I InsP_3R antibody (18A10) inhibits sperm-induced Ca^{2+} release and egg activation in mouse and hamster oocytes (Mehlmann *et al.*, 1996; Miyazaki *et al.*, 1993; Miyazaki *et al.*, 1992; Xu *et al.*, 1994). In sea urchin eggs, a Ca^{2+} wave occurs in the presence of inhibitors of InsP_3Rs or RyRs but not when both inhibitor types are used, which suggests that the two receptor types function redundantly during fertilisation (Galione *et al.*, 1993; Lee *et al.*, 1993). However, since fertilisation requires InsP_3 -mediated Ca^{2+} release (Carroll *et al.*, 1999; Lee and Shen, 1998) and functional $\text{PLC}\gamma$ (Carroll *et al.*, 1999), it is unclear if sea urchin RyRs can fully substitute for InsP_3Rs during normal development. In starfish (Carroll *et al.*, 1997; Stricker, 1995) and ascidians (Russo *et al.*, 1996; Yoshida *et al.*, 1998), Ca^{2+} also seems to be mobilised predominantly through InsP_3Rs . Nevertheless, contributions from non- InsP_3 -mediated Ca^{2+} release cannot be ruled out for some species. For example, in ascidians, other receptors may contribute to highly localised transients required for regulating plasma membrane capacitance and currents (Albrieux *et al.*, 1998; Albrieux *et al.*, 1997; Wilding *et al.*, 1998).

The signaling mechanism that leads to the generation of InsP_3 at fertilisation remains unknown. In mouse oocytes, this mechanism does not appear to involve a (Gq family) G-protein-coupled receptor (Williams *et al.*, 1998). The most likely mechanism involves the introduction of a factor from the sperm that causes InsP_3 production in the oocyte (Fissore *et al.*, 1999b; Swann and Lai, 1997; Williams *et al.*, 1998). The fertilising sperm may increase InsP_3 production by introducing either a PLC or a factor that leads to the activation of egg PLCs. Evidence for a sperm-derived PLC comes from experiments using boar sperm extracts added to sea urchin egg homogenates (Jones *et al.*, 1998). However, it is unclear whether there is a sufficient amount of the putative PLC activity in a single sperm to trigger Ca^{2+} release at fertilisation. The nature of the putative sperm-derived PLC is also unknown. There is strong evidence for activation of egg PLCs in sea urchin, starfish and ascidian but not *Xenopus* or mammals. Microinjection of $\text{PLC}\gamma$ SH2 domains to inhibit activation of $\text{PLC}\gamma$ blocks the fertilisation Ca^{2+} transient in sea urchin (Carroll *et al.*, 1999; Carroll *et al.*, 1997; Giusti *et al.*, 1999; Shearer *et al.*, 1999). In addition, the src-related kinase, fyn, may activate $\text{PLC}\gamma$ at fertilisation since SH2 domains from fyn also inhibit egg activation (Abassi *et al.*, 2000; Giusti *et al.*, 2000; Runft and Jaffe, 2000). However, the same SH2 proteins have no effect in mouse and *Xenopus* eggs

(Mehlmann *et al.*, 1998; Runft and Jaffe, 2000). In these animals, egg PLC isoforms other than PLCgamma may be involved. Tyrosine kinases have been implicated in Ca^{2+} release at fertilisation in the mouse (Sette *et al.*, 1997; Sette *et al.*, 1998) but tyrosine kinase inhibitors do not affect the initiation of transients at fertilisation (Dupont *et al.*, 1996). Invertebrates and vertebrates may have adopted different approaches, during the period between sperm-egg fusion and the calcium increase, to achieve the generation of InsP_3 at fertilisation.

1.4.4 Ryanodine receptors

The other major family of Ca^{2+} release channels, and close relatives of InsP_3Rs , are the ryanodine receptors (RyRs). RyR subtypes are found in skeletal muscle (type 1) and cardiac muscle (type 2) and a widely distributed isoform (type 3) is predominantly found in smooth muscle (Coronado *et al.*, 1994; Meissner, 1994; Sutko and Airey, 1996). In cardiac muscle the RyR-mediated mechanism for excitation-contraction coupling is Ca^{2+} -induced Ca^{2+} release (CICR), whereas in skeletal muscle, although an essential role for RyRs has been clearly demonstrated (Takeshima *et al.*, 1994), the precise role of RyR-mediated CICR is less clear (Sutko and Airey, 1996). The role of RyRs in other tissue types also remains uncertain. Although RyRs are expressed in mouse oocytes (types II and III) (Ayabe *et al.*, 1995) and in mature bovine oocytes (Yue *et al.*, 1995), the role of RyRs in the generation of Ca^{2+} transients at fertilisation in mammals is controversial (Ayabe *et al.*, 1995; Carroll *et al.*, 1996; Jones *et al.*, 1995b). The InsP_3Rs will therefore be the focus of this part of the review. However, the following provides a brief comparison between RyRs and InsP_3Rs . The two families share structural and functional characteristics (Galione *et al.*, 1993; Striggow and Ehrlich, 1996). The predicted structure of the P_{400} protein (see below) resembles that of the ryanodine receptor (Takeshima *et al.*, 1989); both receptors have a large N-terminal region located on the cytoplasmic side and a short C-terminal region spanning the membrane (Furuichi *et al.*, 1989).

1.5 The InsP_3R

1.5.1 Discovery of the InsP_3R

The InsP_3R was first identified as a membrane glycoprotein known as P_{400} with a relative molecular mass (M_r) of 250kDa. During a study on cerebellar ataxic mutant mice this protein was found to be enriched in Purkinje cells from normal mice but reduced in the cerebellar of Purkinje cell-deficient mutants (Mallet *et al.*, 1976; Mikoshiba *et al.*, 1979). An InsP_3 -binding protein with M_r of 260kDa was later purified from the rat cerebellum (Supattapone *et al.*, 1988b). P_{400} was then shown to be a receptor for InsP_3 and to be identical to the InsP_3R protein (Furuichi *et al.*, 1989; Maeda *et al.*, 1990). Different subtypes of InsP_3R , transcribed from different genes, have since been identified in mammals (Blondel *et al.*, 1993; Furuichi *et al.*, 1989; Ross *et al.*, 1992; Sudhof *et al.*, 1991).

The importance of the InsP₃R *in vivo* has been examined by using knockout mice. Most mice lacking type I InsP₃R, which is the predominant InsP₃R subtype in the central nervous system (Furuichi *et al.*, 1994; Yamamoto-Hino *et al.*, 1995), generated by gene-targeting, die *in utero* and others have severe ataxia and epileptic seizures (Matsumoto *et al.*, 1996). Thus type I InsP₃R is essential for proper brain function.

1.5.2 Structure of the InsP₃R

The cloned cDNAs of mouse and rat type I InsP₃Rs code for a protein with seven or eight membrane-spanning domains near the C-terminal end (Furuichi *et al.*, 1989; Mignery *et al.*, 1990; Mignery and Sudhof, 1990), and two PKA consensus phosphorylation sites in the midportion of the sequence. InsP₃R monomers assemble to form tetramers in the membrane of the endoplasmic reticulum. Both homo- (Hirota *et al.*, 1995) and hetero-tetramers (Monkawa *et al.*, 1995; Wojcikiewicz and He, 1995) can form using the three mammalian InsP₃R subtypes (see below). The assembled protein consists of a large cytoplasmic domain containing the InsP₃-binding site, a membrane-spanning region containing the Ca²⁺ channel, and a small cytoplasmic C-terminus (Berridge, 1993; Mikoshiba, 1993) (**Fig. 1.8**). Early mutational analysis of the expressed rat receptor suggested that the InsP₃-binding site was at the N-terminal end and that binding of InsP₃ causes a conformational change that presumably activates channel opening (Mignery and Sudhof, 1990; Miyawaki *et al.*, 1991). The negatively charged InsP₃ molecule interacts with positively charged amino acids, which are found throughout the binding domain, but more recent experiments with amino acid substitution have revealed that there are only 10 crucial residues for InsP₃ binding (Yoshikawa *et al.*, 1996). Each InsP₃R subunit binds one InsP₃ molecule (Miyawaki *et al.*, 1991).

1.5.3 InsP₃R isoforms and splice variants

There are three distinct isoforms of the InsP₃R in mammals that are expressed at different levels in different cell types (De Smedt *et al.*, 1994; De Smedt *et al.*, 1997; Nakagawa *et al.*, 1991; Wojcikiewicz, 1995). Besides mammals, InsP₃Rs have also been cloned in *Xenopus* eggs (Kume *et al.*, 1993) and in *Drosophila* (Yoshikawa *et al.*, 1992). They have also been detected in sea urchin eggs using a type I InsP₃R antibody (Parys *et al.*, 1994) and in starfish eggs (Chiba *et al.*, 1990; Picard *et al.*, 1985) and ascidians (Dale, 1988; McDougall and Sardet, 1995), by the ability of InsP₃ to cause a rise in intracellular Ca²⁺ concentration.

In mammals, the full sequence of mouse type I (Furuichi *et al.*, 1989) and rat type I (Mignery *et al.*, 1990), rat type II (Sudhof *et al.*, 1991), rat type III (Blondel *et al.*, 1993) and sequences of partial clones of mouse types II and III (De Smedt *et al.*, 1997), have been determined. These isoforms are structurally similar and are encoded by homologous genes, isoform pairs showing 60-75% identity within compared nucleotide sequences in

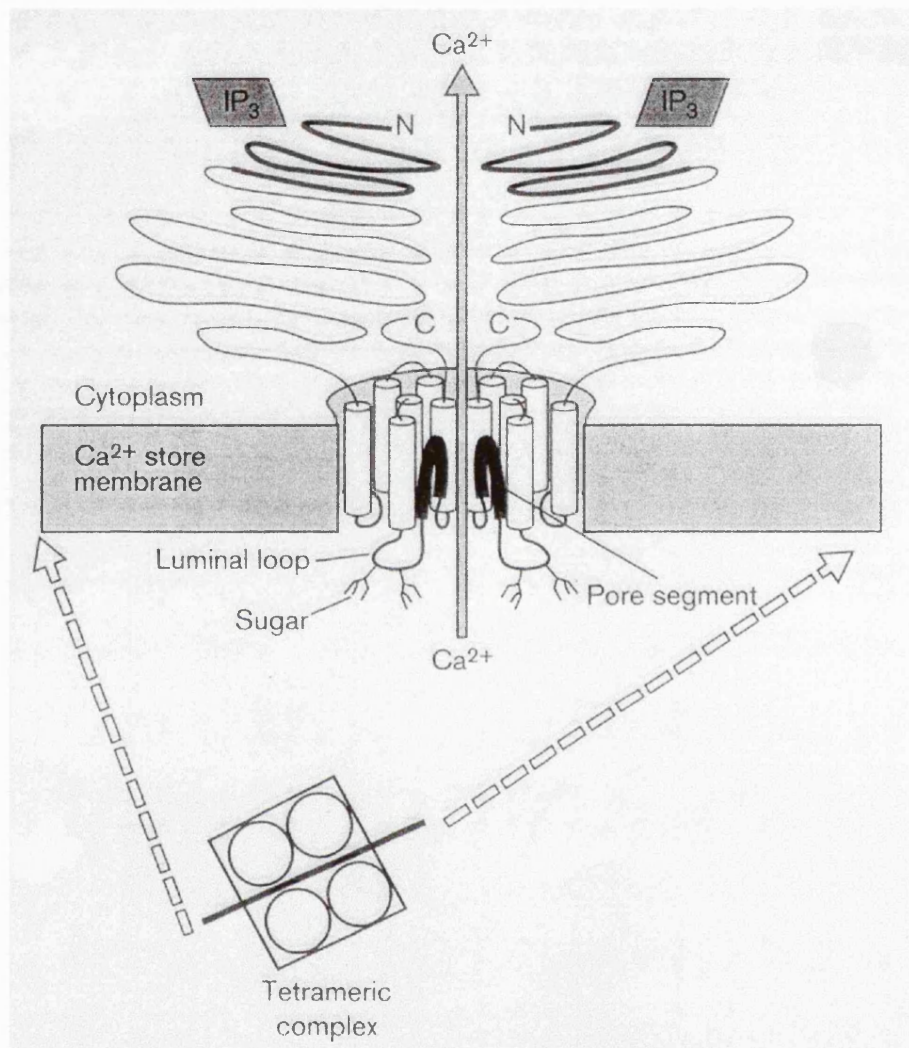


Figure 1.8 Topological model of the InsP₃R in the Ca²⁺ store membrane. (from Mikoshiba, 1997).

the mouse (De Smedt *et al.*, 1997). Furthermore, nucleotide sequence comparison has revealed 94.5% identity between mouse and rat InsP₃Rs for all three isoforms (De Smedt *et al.*, 1997). The amino acid sequence of mouse type I InsP₃R shares 90% identity with that of *Xenopus* InsP₃R (Kume *et al.*, 1993) and also shares an extensive homology with that of *Drosophila* InsP₃R (Yoshikawa *et al.*, 1992). Additional subtypes have previously been reported but most likely reflect minor species differences (De Smedt *et al.*, 1997).

In addition, multiple splice variants of type I, due to the existence of two small variably spliced segments, have been identified in the mouse (Nakagawa *et al.*, 1991). One segment (SI) is located within the InsP₃ binding domain and another (SII) is located near putative sites for phosphorylation and ATP binding (for modulation of the effect of InsP₃ on Ca²⁺ release) (Nakagawa *et al.*, 1991). Moreover, evidence suggests the existence of intermediately spliced forms containing partial versions of the SII splice. It has recently been demonstrated that the SI region, although within the InsP₃ binding domain, is not part of the binding site itself and may be involved in a function other than InsP₃-induced Ca²⁺ release (Lievremont *et al.*, 1996), such as the interaction of the InsP₃R with other proteins in the cell and the organisation of the intracellular Ca²⁺ stores. These splice variants and intermediately spliced forms of type I are also expressed in a tissue-specific and a developmentally-specific manner (Nakagawa *et al.*, 1991). In the same year, two distinct transcripts of InsP₃R were independently identified in the rat, which were derived by alternative splicing of a 120-nucleotide insert between the two cAMP-dependent protein kinase phosphorylation consensus sequences (Danoff *et al.*, 1991). This insert was found to be exactly the same length as SII. Furthermore, type I transcripts which included the insert were found in adult brain and appeared to be exclusively neuronal, whereas splice variants of type I lacking the insert appeared to represent a non-neuronal receptor (Danoff *et al.*, 1991). However, in contrast to the mouse SII splice variant, intermediately spliced forms are not found in rat (Schell *et al.*, 1993). The SI, at least, is found only for the type I receptor (Lievremont *et al.*, 1996) and both SI and SII domains are absent in the sequenced *Xenopus* InsP₃R (Kume *et al.*, 1993).

1.5.4 InsP₃R isoform expression

In *Xenopus* (Kume *et al.*, 1993; Parys and Bezprozvanny, 1995), mouse (Parrington *et al.*, 1998) and bovine (He *et al.*, 1999) oocytes, the type I InsP₃R is by far the most abundant isoform. However, at least in the mouse oocyte, mRNAs for all three isoforms are present (Parrington *et al.*, 1998). In terms of mRNA expression in other mouse tissues, the three subtypes are co-expressed in almost all tissues and cell lines so far investigated (De Smedt *et al.*, 1997; Newton *et al.*, 1994; Wojcikiewicz, 1995). Cell types studied thus almost invariably express some type I receptors. However, type I InsP₃R mRNA is particularly prominent in brain and cerebellum and type II mRNA is prominent in epithelia such as kidney as well as in both cardiac and skeletal muscle (De Smedt *et al.*, 1997). Type III mRNA has been found to be highly expressed in all cultured cell types and in tissues

with high cell turnover, such as testis (De Smedt *et al.*, 1997), suggesting that it may have a role in cell proliferation.

1.5.5 Differences between isoforms

Genetic evidence has shown that each subtype is capable of mediating InsP_3 -induced Ca^{2+} release from intracellular Ca^{2+} stores (Sugawara *et al.*, 1997). However, the three subtypes differ in several ways, such as affinity for InsP_3 . For example, InsP_3 interacts with the type II receptor with 3-fold higher affinity than the type I receptor (Newton *et al.*, 1994; Sudhof *et al.*, 1991) and type III has been shown to have an approximately 10-fold lower affinity for InsP_3 than does type I (Newton *et al.*, 1994). However, a more recent study has concluded that the affinity order is type I > type II > type III in both cell lines and rat tissues (Wojcikiewicz and Luo, 1998a). This data appears to be more reliable since it measured binding to whole InsP_3R tetramers rather than to monomeric receptor fragments as in the earlier studies. These latter differences in ligand-binding affinity appear to correlate well with the potency of InsP_3 as a Ca^{2+} -mobilising agent (Wojcikiewicz and Luo, 1998a). The selective expression of a particular receptor type will thus influence the sensitivity of cellular Ca^{2+} stores to InsP_3 . The subtypes and splice variants are also differentially regulated (see below). The differences between the subtypes and splice variants may confer upon them distinct biological roles in the cells in which they are co-expressed.

1.5.6 InsP_3R regulation

The large N-terminal cytoplasmic region provides the main region for cytoplasmic regulators to act. InsP_3Rs are regulated by small modulators such as InsP_3 and Ca^{2+} itself (discussed in more detail in section 1.5.7) (Taylor and Traynor, 1995), adenine nucleotides (Bezprozvanny and Ehrlich, 1993; Missiaen *et al.*, 1997), pH (Joseph *et al.*, 1989), redox state (Bootman *et al.*, 1992; Renard-Rooney *et al.*, 1995) and phosphorylation (Joseph, 1996; Supattapone *et al.*, 1988a; Wojcikiewicz and Luo, 1998b). For example, cAMP-dependent protein kinase (PKA) phosphorylates the InsP_3R in intact cells and enhances InsP_3 -induced Ca^{2+} mobilisation in several cell lines (Wojcikiewicz and Luo, 1998b). However, PKA decreases sensitivity to InsP_3 in cerebellar microsomes (Cameron *et al.*, 1995; Supattapone *et al.*, 1988a), although this result may have been an artefact of the microsome preparation and may not apply to intact cerebellum cells.

InsP_3Rs are also modulated by larger protein-protein interactions with accessory proteins such as calmodulin (Patel *et al.*, 1997; Yamada *et al.*, 1995) and FKBP12 (Cameron *et al.*, 1995). The InsP_3R has also been shown to play a role in the organisation of the intracellular Ca^{2+} stores. For example, in the Purkinje cells of the cerebellum the type I appears to participate in the formation of stacks of ER (Rusakov *et al.*, 1993; Takei *et al.*, 1994) and in other tissues type I interacts with cytoskeletal proteins like ankyrin (Bourguignon and Jin, 1995; Bourguignon *et al.*, 1993b; Joseph and Samanta, 1993) or actin (Rossier *et al.*, 1991). More recently, the actin cytoskeleton has been shown to

mediate an interaction between the Sec6/8 complex (involved in exocytosis) and Ca^{2+} signaling complexes incorporating the type I and III InsP_3Rs , in pancreatic acinar cells (Shin *et al.*, 2000). Furthermore, anti-Sec6 and anti-Sec8 antibodies inhibit Ca^{2+} signaling at a step upstream of Ca^{2+} release by InsP_3 (Shin *et al.*, 2000). The InsP_3R can thus integrate information from many signaling pathways to determine sensitivity to InsP_3 .

Subtypes and splice variants appear to be differentially regulated. For example, type III InsP_3Rs , unlike type I InsP_3Rs , are not stimulated by sulfhydryl oxidation (by thimerosal) and are less sensitive to ATP as well as InsP_3 (Missiaen *et al.*, 1998). Furthermore, type I InsP_3R is much more susceptible to phosphorylation than types II and III (Wojcikiewicz and Luo, 1998b). The neuronal (SII+) and non-neuronal (SII-) splice variants of type I InsP_3R (described above) also have different phosphorylation kinetics and patterns (Danoff *et al.*, 1991). With respect to CICR, Ca^{2+} differentially regulates the InsP_3 -affinity states of type I and type III InsP_3Rs (Cardy *et al.*, 1997; Yoneshima *et al.*, 1997) (see section 1.5.7). The subtypes also exhibit differences in their resistance to downregulation (Wojcikiewicz, 1995) (see section 1.5.9).

1.5.7 Regulation by Ca^{2+} and InsP_3 : regenerative Ca^{2+} transients

The InsP_3Rs of many tissues are biphasically regulated by cytosolic Ca^{2+} . During sustained exposure to Ca^{2+} , responses to submaximal concentrations of InsP_3 are enhanced by modest increases in cytosolic Ca^{2+} concentration ($\leq 300\text{nM}$), while responses to most InsP_3 concentrations are inhibited by more substantial increases in Ca^{2+} concentration ($>300\text{nM}$) (Taylor, 1998). These effects were first described in guinea pig smooth muscle cells in 1990 (Iino, 1990) when the terms CICR (Ca^{2+} -induced Ca^{2+} release) and IICR (InsP_3 -induced Ca^{2+} release) were first used. It was also shown that InsP_3Rs need to be bound to InsP_3 in order to be activated by Ca^{2+} (Bezprozvanny *et al.*, 1991; Iino, 1990). It is thought that InsP_3 binding to a single receptor subunit (Yoshikawa *et al.*, 1996) causes a large conformational change (Mignery and Sudhof, 1990) that exposes a Ca^{2+} -binding stimulatory site (Marchant and Taylor, 1997; Parker *et al.*, 1996). As the concentration of cytosolic Ca^{2+} increases, it can bind to a second inhibitory site which is accessible whether or not the receptor has InsP_3 bound (Taylor, 1998). The stimulation of InsP_3Rs by Ca^{2+} is thought to contribute to the regenerative recruitment of InsP_3Rs in intact cells, whereas the inhibitory effect of Ca^{2+} may provide the negative feedback that limits the duration and amplitude of elementary Ca^{2+} release events (Berridge, 1997). More recently, this idea has been extended into a theory of lateral inhibition. This hypothesises that InsP_3 switches its receptor from a state in which only an inhibitory Ca^{2+} -binding site is accessible to one in which only a stimulatory site is available. This ensures that Ca^{2+} released by an active InsP_3R may rapidly inhibit its unliganded neighbours, but it cannot terminate the activity of a receptor with InsP_3 bound, thus providing increased sensitivity to InsP_3 and allowing rapid graded recruitment of InsP_3Rs (Adkins and Taylor, 1999).

The biphasic effect of cytosolic Ca^{2+} has been observed in tissues expressing predominantly the type I, II or III InsP_3R (Swatton *et al.*, 1999; Taylor, 1998) and is thus widely thought to be a feature of all subtypes. However, another study on single-channel function of receptor subtypes suggests that they may differ in this form of regulation. Whereas single type I channel activity is a biphasic function of Ca^{2+} concentration, single type II channel activity is not inhibited by high Ca^{2+} concentration (Ramos-Franco *et al.*, 1998). Finally, one way in which Ca^{2+} regulates InsP_3 -induced Ca^{2+} release is by regulating InsP_3 binding. For example, Ca^{2+} has different effects on InsP_3 binding to types I and III InsP_3R (Cardy *et al.*, 1997; Yoneshima *et al.*, 1997). One study (on recombinant mammalian InsP_3Rs in insect Sf9 cells) found that Ca^{2+} increases inhibit InsP_3 binding to type I receptors by causing 50% to adopt a conformation with undetectable affinity for InsP_3 . In contrast, the same Ca^{2+} increases unmask InsP_3 -binding sites on type III receptors but, as Ca^{2+} increases further, there is a substantial decrease in their affinity for InsP_3 (Cardy *et al.*, 1997). In other words, it appears under these conditions that Ca^{2+} causes only inhibition of binding to type I whereas it biphasically regulates InsP_3 binding to type III InsP_3Rs . Accessory proteins and the direct effect of Ca^{2+} on channel opening also play a role in determining the biphasic regulation of InsP_3 -induced Ca^{2+} release.

The experiments used in describing biphasic regulation were mostly not done in intact cells but on permeabilised cells or single InsP_3R channels incorporated into planar lipid bilayers (Bezprozvanny *et al.*, 1991; Swatton *et al.*, 1999). In a physiological situation, this form of regulation may be significantly affected by InsP_3R density since it is dependent upon diffusion of Ca^{2+} from the locality of one receptor to that of another. Downregulation of InsP_3R expression (see below) will thus have consequences for CICR through the InsP_3R . The underlying mechanisms of CICR are not completely understood. However, as suggested above, it is generally proposed that the two effects of Ca^{2+} are probably mediated by two distinct Ca^{2+} -binding sites, which may reside on the InsP_3R itself or on accessory proteins (Taylor, 1998).

In addition to regulation by binding to Ca^{2+} , InsP_3Rs are regulated in a complex manner by InsP_3 itself (Marchant and Taylor, 1998). Firstly, InsP_3Rs exhibit cooperative responses to InsP_3 , enabling an all-or-nothing response (Callamaras *et al.*, 1998; Marchant and Taylor, 1997; Meyer *et al.*, 1988) and thus preventing spontaneous activation. Binding of InsP_3 to its receptor unmasks a site to which Ca^{2+} can then bind, and when all four subunits of the receptor have bound both ligands, the intrinsic Ca^{2+} channel opens to its maximal activity (Marchant and Taylor, 1997). Limiting spontaneous opening of channels is important since such positive feedback (CICR) could potentially lead to the generation of inappropriate Ca^{2+} transients or empty all Ca^{2+} stores and raise cytosolic Ca^{2+} to toxic levels (Dowd, 1995).

Secondly, experiments suggest that the InsP_3R may be inactivated by InsP_3 (Hajnóczky and Thomas, 1994; Hajnóczky and Thomas, 1997; Marchant and Taylor, 1998). However, these experiments were carried out in permeabilised hepatocytes and the role of calcium in InsP_3 -induced receptor inactivation remains controversial. Also, hepatocytes have predominantly type II InsP_3Rs (De Smedt *et al.*, 1997) so this type of inactivation may not be a feature of all subtypes. In these experiments, InsP_3 -induced receptor activation was followed by an obligatory time-dependent inactivation, which appeared to be a direct consequence of InsP_3 binding (Hajnóczky and Thomas, 1994; Hajnóczky and Thomas, 1997). As described above, binding of InsP_3 to each of its four receptor subunits unmasks a site to which Ca^{2+} must bind before the channel can open to maximal activity. However, a different study in support of the above experiments suggests that InsP_3 binding also initiates a slower switch of the receptor to a higher-affinity but less active (more closed) conformation which is about 40% less able to mediate Ca^{2+} release (Marchant and Taylor, 1998). Thus it appears that InsP_3Rs may be switched to a less active conformation/state by both Ca^{2+} and InsP_3 . These two pathways must together determine the permeability of the InsP_3R to Ca^{2+} and thus the local rise in cytosolic Ca^{2+} concentration.

In addition to InsP_3 -binding cooperativity and Ca^{2+} - and InsP_3 -regulated channel activation and partial inactivation, there are other elements which play a part in generating repetitive Ca^{2+} transients (Meyer and Stryer, 1988). InsP_3 is continuously destroyed by a phosphatase and therefore new InsP_3 must be generated. This may involve the stimulation of a Ca^{2+} -dependent PLC by released Ca^{2+} (positive feedback). Furthermore, as well as being pumped out of the cell, Ca^{2+} ions that enter the cytosol are continuously pumped back into the ER by Ca^{2+} -ATPase pumps. When the Ca^{2+} stores have been refilled to a critical level, the next spike may be generated. It has been proposed that the time required for the Ca^{2+} level in the ER to reach a threshold sets the interspike interval (Meyer and Stryer, 1988).

1.5.8 Generation of Ca^{2+} waves and oscillations

Localised, InsP_3 -generated Ca^{2+} “events” have recently been described (mostly in *Xenopus* oocytes), that are thought to represent the elementary building-blocks of larger, global Ca^{2+} increases and waves. In *Xenopus* oocytes InsP_3 -induced transient “puffs” (peak free Ca^{2+} 100-200nM) and “blips” (peak free Ca^{2+} <40nM) have been evoked by photorelease of InsP_3 from a caged precursor (Parker and Yao, 1996; Yao *et al.*, 1995). Blips are thought to arise through the opening of single InsP_3Rs whereas puffs may arise from the concerted opening of several clustered InsP_3Rs (Parker and Yao, 1996). Other examples of elementary events are Ca^{2+} “sparks” in cardiac muscle cells (Cheng *et al.*, 1993; Lipp and Niggli, 1994) and the Ca^{2+} puffs in HeLa cells (Bootman *et al.*, 1997a). These events are short-duration and dissipate rapidly due to diffusion of Ca^{2+} in the cytoplasm and sequestration into the intracellular stores. In terms of location, Ca^{2+} puffs have been observed specifically in the perinuclear region of HeLa cells (Lipp *et al.*, 1997). This is

consistent with the findings that the NE is a functional Ca^{2+} store (Gerasimenko *et al.*, 1995) and that the inner NE membrane expresses both InsP_3Rs and RyRs (Humbert *et al.*, 1996; Santella and Kyojuka, 1997). Nuclei also appear to have the ability to generate InsP_3 which is distinct from generation at the plasma membrane (Divecha *et al.*, 1991).

Ca^{2+} waves, such as the first transient induced at fertilisation, allow efficient delivery of Ca^{2+} signals to cytosolic targets. The mean velocities of fertilisation-induced Ca^{2+} waves fall within a relatively narrow range of about 10-30 $\mu\text{m/s}$, consistent with a fundamentally conserved reaction-diffusion based mechanism of wave propagation (Jaffe, 1991; Jaffe, 1995). Ca^{2+} waves are thought to reflect successive rounds of Ca^{2+} diffusion and CICR from InsP_3Rs (and RyRs). In other words, there appears to be a saltatoric propagation of Ca^{2+} between functionally distinct elementary release sites, which underlies the regenerative spread of Ca^{2+} throughout the cell (Bootman *et al.*, 1997a). This has been detected in both HeLa cells and *Xenopus* oocytes (Bootman *et al.*, 1997a; Callamaras *et al.*, 1998). Recruitment of elementary events for a regenerative global signal appears to involve increasing the frequency, amplitude and number of events. This drives the cytosolic Ca^{2+} concentration towards a threshold where the signal becomes regenerative (Bootman *et al.*, 1997b). Complex repetitive signals such as Ca^{2+} oscillations at fertilisation are thought to be controlled by mechanisms involving all the above forms of regulation.

1.5.9 Downregulation of InsP_3R protein expression

A final mechanism for the regulation of InsP_3 -induced Ca^{2+} signaling is downregulation of the InsP_3R by proteolysis. This form of regulation differs from those discussed above as it takes place over hours instead of seconds. Proteolytic degradation of the type I InsP_3R was first demonstrated in microsomal preparations (Magnusson *et al.*, 1993). InsP_3R downregulation has since been characterised in several cell lines in response to chronic exposure to agonists (Sipma *et al.*, 1998; Wojcikiewicz, 1995; Wojcikiewicz *et al.*, 1994). In these cell lines InsP_3R downregulation appears to be an adaptive response which occurs during activation of cell surface receptors that stimulate PIP_2 hydrolysis, and leads to a reduction in the sensitivity of Ca^{2+} stores to InsP_3 (Wojcikiewicz *et al.*, 1994; Wojcikiewicz and Nahorski, 1991; Wojcikiewicz *et al.*, 1992). Type I, II and III InsP_3Rs can all be downregulated, but with different characteristics. For example, type II receptor downregulation is limited relative to that of type I and III receptors, reflecting resistance of the type II receptor to degradation (Wojcikiewicz, 1995). This could be due to sequence differences between the receptors making the type II less susceptible to the protease(s) responsible for downregulation.

InsP_3R downregulation appears to involve ubiquitination and subsequent degradation by the proteasome (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999) (see 1.6). Activation of PLC-linked receptors causes InsP_3R ubiquitination in a range of cell types and the downregulation induced by activation of these receptors is blocked by proteasome

inhibitors (Oberdorf *et al.*, 1999). With respect to the cause of ubiquitination, which occurs while InsP_3Rs are membrane-bound, ubiquitination and downregulation are stimulated only by agonists that elevate InsP_3 concentration persistently (Oberdorf *et al.*, 1999). The idea that ubiquitination is stimulated by the binding of InsP_3 to its receptor is supported by the finding that a deletion within the ligand-binding domain of the type I InsP_3R renders it unable to bind InsP_3 and blocks its ubiquitination (Zhu and Wojcikiewicz, 2000) and downregulation (Zhu *et al.*, 1999). Also in these studies only the endogenous InsP_3Rs in binding-defective-monomer-containing hetero-tetramers were ubiquitinated (Zhu and Wojcikiewicz, 2000) and degraded (Zhu *et al.*, 1999) in response to agonist stimulation. This indicates that the targeting of InsP_3Rs for ubiquitination is a highly selective process and is inconsistent with any suggestion that mobilised Ca^{2+} might activate ubiquitination, in which case degradation of all subunits should have occurred independently of InsP_3 binding. Finally, the reduced sensitivity of the type II receptor to degradation (Wojcikiewicz, 1995) may be explained by the finding that cellular homomeric type II and type III InsP_3Rs can be resistant to ubiquitination and thus degradation, whereas type II and III receptors in association with type I are readily ubiquitinated (Oberdorf *et al.*, 1999).

It has been proposed that an InsP_3 -binding-induced conformational change in the receptor (Mignery and Sudhof, 1990; Miyawaki *et al.*, 1991) exposes a degradation signal that leads to ubiquitination. The putative degradation signal and the enzymes that mediate InsP_3R ubiquitination have yet to be established. An example of InsP_3R downregulation in a physiological situation is the secretagogue-stimulated ubiquitination and downregulation of InsP_3Rs in rat pancreatic acinar cells (Wojcikiewicz *et al.*, 1999). In these cells, it is believed that secretagogues simultaneously activate an additional proteolytic pathway besides the ubiquitin-proteasome pathway, since proteasome inhibitors do not block InsP_3R downregulation.

Lastly, downregulation of the type I InsP_3R by increased proteolysis may in certain situations be enhanced by reduced InsP_3R -I synthesis. Firstly, one study in SHSY-5Y cells demonstrated that type I InsP_3R mRNA levels fall after 3 hours of muscarinic receptor stimulation but then return to basal levels (Wojcikiewicz *et al.*, 1994). Secondly, inhibited protein synthesis may play an important role in downregulation of type I InsP_3R in a murine mesangial cell line, stimulated by Transforming Growth Factor (TGF)- $\beta 1$. TGF- $\beta 1$ inhibited the expression of the type I InsP_3R mRNA with the reduction in mRNA preceding the reduction in protein (Sharma *et al.*, 1997). It is not known whether mRNA levels are decreased by an effect on transcription of the type I InsP_3R gene or by enhancement of the degradation rate of the mRNA. The former mechanism has been suggested by a third study. Experiments in rat A7r5 smooth muscle cells transiently expressed a luciferase coding sequence, placed under control of the rat InsP_3R -I promoter, and examined the effect of agonists on transcription-initiation from the InsP_3R promoter. It was found that stimulation of cells with agonists which caused downregulation of InsP_3Rs , also caused

reduced luciferase activity, indicating a reduction of type I InsP₃R promoter activity (Sipma *et al.*, 1998). Despite these studies suggesting that reduction of mRNA levels might contribute to the downregulation of InsP₃Rs, the estimated half-life of the InsP₃R, determined in WB rat liver epithelial cells, is about 11 hours (Joseph, 1994). Therefore, since InsP₃R downregulation generally takes place over a few hours, it is expected that in these cell types as well, an enhanced rate of InsP₃R proteolysis is the major determinant causing downregulation of InsP₃Rs.

1.6 Downregulation of protein expression

There are several ways in which proteins can be degraded in cells. The two major routes of degradation are the ubiquitin-proteasome pathway and lysosomal proteolysis. The ubiquitin-proteasome pathway is the principal mechanism for the turnover of short-lived proteins in eukaryotic cells. Degradation of a protein by this pathway involves two distinct and successive steps. The first is a covalent attachment of multiple ubiquitin molecules to the target protein and the second is the degradation of the tagged protein by the 26S proteasome (or, in certain cases, by lysosomes). Conjugation of ubiquitin to the substrate involves three steps. Ubiquitin is first activated by the ubiquitin-activating enzyme, E1. Following activation, one of several E2 enzymes (ubiquitin-carrier proteins or ubiquitin-conjugating enzymes, UBCs) transfers ubiquitin from E1 to a member of the ubiquitin-protein ligase family, E3, to which the substrate protein is specifically bound. E3 catalyses the last step in the conjugation process, covalent attachment of ubiquitin to a lysine residue of the target protein. In successive reactions, a branched polyubiquitin chain is synthesised by transfer of additional ubiquitin subunits to a lysine residue of the previously conjugated subunit. It is thought that the chain acts as a recognition marker for the proteasome. The ATP-dependent 26S proteasome, which is composed of a 20S catalytic subunit and a regulatory subunit complex, rapidly degrades “marked” proteins.

Regulation of cell cycle progression depends on ubiquitin-mediated proteolysis of many cell cycle proteins. The 1500kDa cyclosome (Sudakin *et al.*, 1995) or anaphase promoting complex (APC) (King *et al.*, 1995) is an E3 multienzyme complex with a ubiquitin ligase activity specific for cell cycle regulators, such as the M phase cyclins. Substrate proteins targeted by the APC contain a nine amino acid motif termed the “destruction box” and are degraded during M phase. The APC is activated by MPF (see below) at the end of meiosis and mitosis and is inactive during interphase. E2-C is the specific E2 which acts along with the APC in the degradation of some cell cycle regulators (Aristarkhov *et al.*, 1996; Townsley *et al.*, 1997). One example of ubiquitin-mediated proteolysis of a cell cycle protein is the degradation of cyclin B1 at the metaphase-anaphase transition both at fertilisation and exit from mitosis (Glutzer *et al.*, 1991). Another example is the proteolysis of proteins other than the known cyclins, which is required to dissolve the linkage between sister chromatids at the initiation of anaphase at fertilisation (Holloway *et al.*, 1993). These

proteins are thought to include the Cut2/Pds1 protein which is specifically ubiquitinated at metaphase-anaphase transition (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996).

It is unclear exactly how proteins are specifically selected for ubiquitin-mediated degradation. Targeting may in some cases involve a coincidence of selected ubiquitination and specific activation of the proteasome. However, in general, the selectivity of protein degradation is determined mainly at the stage of ligation to ubiquitin (Hershko and Ciechanover, 1998), by two distinct groups of proteins (E3s and accessory proteins) (Ciechanover, 1998). Within the ubiquitin system, substrates are recognised by the different E3s, such as the APC, and it may be that a single E3 recognises a subset of similar, but not identical, structural motifs. Accessory proteins bound to substrate proteins also play an important role in the E3 recognition process (Ciechanover, 1998). As well as substrate-specific regulation, the large number of proteins which are involved in ubiquitination and deubiquitination of proteins are subject to temporal and spatial regulation (Wilkinson, 2000). For example, regarding the degradation of cell cycle proteins, E2s are constitutively active, yet the APC has a complex temporal cell cycle-dependent pattern of activity, which is generated by reversible phosphorylation of several of the APC subunits (Lahav-Baratz *et al.*, 1995; Peters *et al.*, 1996; Shteinberg *et al.*, 1999). Selectivity of proteolysis thus depends strongly on the exact combination of ubiquitinating and deubiquitinating enzymes present at any time and location (Wilkinson, 2000).

However, regulation of proteasome activity may also be important in the selective degradation of some proteins. Although experiments in *Xenopus* eggs have suggested that the degradation of cyclin at fertilisation is a consequence of its recognition by the ubiquitin-conjugating system (Glotzer *et al.*, 1991), it has been reported in the same species that Ca^{2+} induces assembly and activation of the 26S proteasome at egg activation (Aizawa *et al.*, 1996). In this study, pretreatment of *Xenopus* eggs with BAPTA-AM, prior to egg activation in the absence of extracellular Ca^{2+} , completely blocked the activation of the proteasome (Aizawa *et al.*, 1996). In fact, proteasome activation triggered by intracellular Ca^{2+} release appears to be a common event at least in embryonic meiotic and mitotic cell cycles of *Xenopus* and ascidian (Aizawa *et al.*, 1996; Kawahara *et al.*, 1992; Kawahara and Yokosawa, 1994).

The numerous substrates of the proteasome include cell cycle and growth regulators, components of signal transduction pathways, enzymes of house-keeping and cell-specific metabolic pathways, and mutated or post-translationally damaged proteins. The proteasome is also responsible for processing major histocompatibility complex class I antigens. The ubiquitin-proteasome system targets cytosolic and nuclear proteins and also membrane-anchored and secretory pathway compartmentalised proteins. These proteins must be first translocated in a retrograde manner into the cytosol, as components of the pathway have not been identified in the ER lumen (Ciechanover, 1998). An example of an integral ER

transmembrane protein which is degraded by the ubiquitin-proteasome pathway is the yeast Sec61p complex (Biederer *et al.*, 1996). Sec61p is an integral multispanning ER membrane protein and an essential component of the translocon (protein translocation apparatus) that delivers nascent proteins into the ER.

The other major pathway of cellular protein degradation is lysosomal proteolysis, which is particularly involved in the destruction of transmembrane proteins. Lysosomes are organelles which contain hydrolytic enzymes used for the controlled intracellular digestion of macromolecules. A third mechanism of protein degradation which may be relevant to this study is proteolysis by caspases. Caspases form a family of cysteine proteases which are critical mediators of programmed cell death (Cryns and Yuan, 1998), with 14 members so far identified. It has been shown in mice that caspase-12 is localised to the ER and is activated by ER stress, such as disruption of ER Ca^{2+} homeostasis using thapsigargin and Ca^{2+} ionophore A23187 (Nakagawa *et al.*, 2000).

1.7 Role of Ca^{2+} in the cell cycle

In order to provide a clear picture of what is known about the role of Ca^{2+} in the cell cycle, the regulation of the cell cycle by cell cycle proteins will first be discussed. This will be followed by a review of the well documented role of Ca^{2+} at fertilisation. Finally, the role of Ca^{2+} during mammalian oocyte maturation and during mitosis will be discussed.

1.7.1 Cell cycle kinases: MPF and MAP kinase

The metaphase state is characterised by the activity of two kinases. These are MPF (M phase/maturation-promoting factor) and mitogen-activated protein (MAP) kinase, although evidence for the role of MAP kinase at mitotic metaphase is limited (see below). MPF is composed of a regulatory cyclin B subunit (Evans *et al.*, 1983; Lohka *et al.*, 1988) and cyclin-dependent kinase (CDK1) (Gautier *et al.*, 1988; Labbe *et al.*, 1989; Nurse and Thuriaux, 1980). The activation of cyclin B1-CDK is dependent upon cyclin binding and a series of phosphorylation and dephosphorylation reactions (Morgan, 1997). Cyclin B1-CDK inactivation is triggered by the proteolytic destruction of ubiquitin-tagged cyclin B via the APC (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). It has been shown that cyclin B destruction is dependent on the N-terminal 90 amino acids (Murray and Kirschner, 1989), which contain the destruction box sequence (see above) that confers the ability to become ubiquitinated (Glutzer *et al.*, 1991).

Progression of the meiotic cell cycle is controlled by the activity of MPF (Masui and Markert, 1971; Murray and Kirschner, 1989; Newport and Kirschner, 1984). Primary oocytes have low levels of MPF activity. Progesterone secretion (stimulated by LH) induces the activation of MPF, leading to meiosis I (MI). MPF activity peaks at metaphase of MI, falls briefly between the two meiotic divisions, rises again as the MII spindle is

assembled, and then remains high during the MII arrest (Gerhart *et al.*, 1984). The rapid transition from MI to MII and the MII arrest are maintained by an activity named cytotstatic factor (CSF). CSF stabilises cyclin (by blocking the ability of active MPF to induce cyclin degradation) and maintains MPF levels (Masui, 1974) and involves the Mos/MAP kinase pathway (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Verlhac *et al.*, 1996). Experiments have shown that MAP kinase activity is capable of maintaining the meiotic metaphase state, characterised by condensed chromosomes and the absence of a nuclear envelope (Moos *et al.*, 1996b; Verlhac *et al.*, 1994).

Fertilisation leads rapidly to inactivation of MPF and interphase of the first mitotic cell cycle. MPF activity is no longer stabilised by the MAP kinase pathway (Palmer and Nebreda, 2000). The activity of MPF declines just before second polar body formation and that of Mos/MAP kinase decreases just before formation of the pronuclei (Verlhac *et al.*, 1994). Inactivation of CSF was originally believed to be due to degradation of the c-mos product by the Ca^{2+} -dependent cysteine protease calpain (Watanabe *et al.*, 1989) but is now thought to be caused by degradation of the c-mos product by the 26S proteasome (Ishida *et al.*, 1993). Although CSF stabilises MPF at the MII arrest, MPF inactivation and CSF inactivation at egg activation are independent events, at least in *Xenopus* eggs (Watanabe *et al.*, 1991).

After fertilisation, each mitosis is initiated by the activation of MPF and terminated by its inactivation. MPF activity thus rises each time the embryo enters mitosis and then falls as it enters the next interphase (Gerhart *et al.*, 1984). However, the role of the MAP kinase cascade in mitotic cell cycles is unclear. Some studies have shown that MAP kinase activity is elevated during mitosis, at least in mammalian cell lines (Shapiro *et al.*, 1998) and early sea urchin embryos (Chiri *et al.*, 1998; Philipova and Whitaker, 1998). However, MAP kinase activity is thought to be low in mouse oocytes during mitosis (Verlhac *et al.*, 1994).

It has been demonstrated that cyclin B turns over during the metaphase II arrest in mouse oocytes. A constant protein level is maintained for several hours through the continuous balanced synthesis and degradation of cyclin (Kubiak *et al.*, 1993). The equilibrium between these two processes is thought to be dependent in part upon CSF. The degradation of cyclin B during metaphase arrest, unlike stimulated degradation and release from metaphase arrest at egg activation (see below), is not dependent on a Ca^{2+} increase. The presence of the intact metaphase spindle was originally found to be required for cyclin destruction both during the metaphase arrest and at exit from M-phase in mouse oocytes (Kubiak *et al.*, 1993). An intact metaphase spindle has since been shown to be necessary for cyclin destruction in many other cell types. An exception is the cycling extracts of fertilised *Xenopus* egg, in which MPF cycles independently of an intact spindle (Gerhart *et al.*, 1984), although at very high densities of sperm nuclei, microtubule depolymerisation arrests extracts in mitosis (Minshull *et al.*, 1994). In cells other than the *Xenopus* oocyte,

exposure to the microtubule-depolymerising drug nocodazole is associated with a reduced rate of cyclin B degradation and it has been proposed that cyclin B and the proteins that degrade it must physically interact on the spindle to allow effective degradation and entry into interphase (Kubiak *et al.*, 1993). This is supported by the finding that, after oocyte bisection, MPF activity is predominantly associated with the spindle (Kubiak *et al.*, 1993). Furthermore, oocytes fertilised or parthenogenetically activated in the presence of nocodazole, fail to progress to interphase, despite responding with normal Ca^{2+} transient patterns (Winston *et al.*, 1995). The reason for this spindle “checkpoint” is that the chromosomes must be distributed accurately to the daughter cells (fertilised oocyte and second polar body) upon egg activation and errors will arise if chromosomes are improperly attached to the meiotic spindle. Arresting fertilised oocytes at metaphase of the second meiotic division by disrupting the spindle can be an important tool used to investigate the effect of the cell cycle on processes associated with sperm-egg fusion.

1.7.2 Role of Ca^{2+} at fertilisation

The Ca^{2+} signal induced by the sperm at fertilisation in mammals is described above in section 1.3.1. I will now give a broader review of Ca^{2+} signals at fertilisation across species and discuss the probable mechanism of action of Ca^{2+} oscillations. The induction by sperm of a Ca^{2+} explosion at fertilisation in deuterostome eggs was first described over 20 years ago (Jaffe, 1980; Jaffe, 1983). Sustained Ca^{2+} oscillations at fertilisation were later observed in mouse oocytes by using the Ca^{2+} indicator aequorin (Cuthbertson and Cobbold, 1985). It has been demonstrated that this global Ca^{2+} signal is necessary and sufficient for reactivation of the cell cycle and onset of development (Lorca *et al.*, 1991; Steinhardt *et al.*, 1974; Whitaker and Patel, 1990; Whitaker and Steinhardt, 1982). The Ca^{2+} signal is also established as being critical for development to proceed normally (Epel, 1990; Nuccitelli, 1991; Whitaker and Patel, 1990).

There is an abrupt and transient increase in Ca^{2+} concentration at fertilisation in most deuterostome eggs so far studied. This takes the form of a single transient in the echinoderms sea urchin (Wilding *et al.*, 1996) and starfish (Stricker, 1995) and in the medaka fish (Gilkey *et al.*, 1978) and in *Xenopus* (Busa and Nuccitelli, 1985). On the other hand, multiple transients are induced in mammals (Swann and Ozil, 1994) and in ascidians (Speksnijder *et al.*, 1989). As described earlier, oocytes of different species arrest and await fertilisation at different stages of maturation. It is thought that the number of Ca^{2+} transients induced at fertilisation may be related to the stage of the cell cycle at which the oocyte is arrested (Stricker, 1999). For example, in cases where meiosis either precedes fertilisation (sea urchin) or lacks a natural arrest point once GVBD has been triggered (starfish), a single transient may be sufficient, given that a resumption from meiotic arrest is not required. In the cases of fish and *Xenopus*, fertilisation triggers a rapid (10-30 minutes) transition from MII arrest to pronuclear formation prior to first cleavage. In contrast, 4-6 hours elapse during the same transition in mammalian zygotes (Jones, 1998).

Therefore the short transition to interphase in fish and *Xenopus* zygotes may make a repeated series of Ca^{2+} transients such as those found in mammals (Jones, 1998), unnecessary. The generation of repetitive oscillations in mammals and other species, rather than a sustained Ca^{2+} increase, may simply reflect the need for a long-term Ca^{2+} response without the continuous elevation of Ca^{2+} that can be toxic to cells (Dowd, 1995). Alternatively, oscillations could provide a more specific trigger of cellular processes, with both amplitude-related and frequency-encoded information (Gu and Spitzer, 1995; Tang and Othmer, 1995).

The role of repetitive Ca^{2+} increases at fertilisation of the mammalian MII egg has been well documented (Kline and Kline, 1992a; Lawrence *et al.*, 1998) (see section 1.3.1). How these Ca^{2+} oscillations stimulate reactivation of the cell cycle remains to be entirely explained. Meiotic resumption, which defines egg activation, is permitted by the inactivation of MPF, which in turn is caused by the destruction of its regulatory subunit, cyclin B (Draetta *et al.*, 1989; Gerhart *et al.*, 1984; Labbe *et al.*, 1989; Lohka *et al.*, 1988; Nurse and Bissett, 1981). Cyclin B is degraded by the ubiquitin-proteasome pathway (Glotzer *et al.*, 1991) and its degradation is therefore stimulated by the activation of the APC and possibly the proteasome itself. It appears that Ca^{2+} brings about egg activation at fertilisation and at parthenogenetic activation by stimulating the ubiquitin-mediated destruction of cyclin B. It has been shown that cyclin B degradation is driven by the sperm-induced Ca^{2+} transients (Kline and Kline, 1992a; Murray *et al.*, 1989; Newport and Kirschner, 1984; Watanabe *et al.*, 1991) and, furthermore, that assembly of the active 26S proteasome can be stimulated by Ca^{2+} in *Xenopus* (Aizawa *et al.*, 1996) and ascidian (Kawahara and Yokosawa, 1994). Thus Ca^{2+} appears to drive MPF inactivation at fertilisation.

Cell cycle transitions other than meiotic resumption at fertilisation also appear to be regulated by Ca^{2+} . This is suggested by the presence of endogenous cell cycle Ca^{2+} transients in various types of somatic cells (Hepler, 1994; Kao *et al.*, 1990; Poenie *et al.*, 1986; Ratan *et al.*, 1988; Wahl and Gruenstein, 1993) and in oocytes and early embryos of a range of species including the sea urchin (Ciapa *et al.*, 1994; Poenie *et al.*, 1985; Steinhardt and Alderton, 1988; Whitaker and Patel, 1990), *Xenopus* (Snow and Nuccitelli, 1993), the medaka fish (Fluck *et al.*, 1991) and the mouse (Carroll and Swann, 1992; Kono *et al.*, 1996; Stachecki and Armant, 1996b; Tombes *et al.*, 1992). In the sea urchin, the initial large increase in intracellular Ca^{2+} at fertilisation is followed by further smaller increases associated with nuclear envelope breakdown (NEBD), chromosome separation at metaphase-anaphase transition and cytokinesis (Poenie *et al.*, 1985; Whitaker and Patel, 1990). In the mouse, there have been several studies on cell cycle-associated Ca^{2+} release and sensitivity to agonists which stimulate Ca^{2+} release. These are discussed below.

1.7.3 Role of Ca^{2+} during mammalian oocyte maturation

The roles of intracellular and extracellular Ca^{2+} during meiotic maturation, reinitiation of meiosis at parthenogenetic activation (in mouse oocytes) and during first mitosis (of fertilised mouse embryos) were thoroughly investigated in a study in 1992 (Tombes *et al.*, 1992). The study found that GVBD was not associated with Ca^{2+} transients, was not blocked by Ca^{2+} chelators or depletion of extracellular Ca^{2+} and was not induced by treatment with ionomycin. In contrast, a different study (Carroll and Swann, 1992) observed a series of Ca^{2+} oscillations that continued for 1-3 hours in the majority of oocytes undergoing GVBD. However, in agreement with Tombes *et al.* (1992) and a more recent study (Coticchio and Fleming, 1998), GVBD did not appear to be regulated by Ca^{2+} as it was not inhibited by Ca^{2+} chelators (Carroll and Swann, 1992). Therefore, it appears from this data that GVBD is independent of internal and external Ca^{2+} . However, it should be noted that other studies suggest that phosphoinositide metabolism and Ca^{2+} transients are involved in the regulation of GVBD. One group not only employed lithium to block polyphosphoinositide metabolism and suggested that GVBD is dependent on PI turnover (Pesty *et al.*, 1994) but also detected spontaneous Ca^{2+} oscillations in most GV oocytes (Lefevre *et al.*, 1995) and used a type I InsP_3R antibody in the nucleus of the immature oocyte to inhibit GVBD (Pesty *et al.*, 1998). Also, BAPTA-AM was found by some studies to inhibit GVBD in cow (Homa, 1991) and pig (Kaufman and Homa, 1993) oocytes.

A possible explanation for the contrasting results on the involvement of Ca^{2+} in GVBD is that oocytes of different species are dependent on Ca^{2+} at different points in meiotic maturation. The mouse oocyte is arrested in the follicle at prophase of meiosis I and meiotic resumption does not require protein synthesis. In other mammalian species, oocytes are arrested in the follicle at an earlier stage of meiotic interphase and therefore cyclin synthesis is required to enter meiotic prophase (Homa, 1995). In these species it has been proposed that following cyclin synthesis, a Ca^{2+} signal is required for the activation of MPF and subsequent entry into metaphase of meiosis I (Homa, 1995), which may be blocked by Ca^{2+} chelators. An alternative explanation for why BAPTA may block GVBD in species in which cyclin synthesis is required for entry into meiotic metaphase at GVBD, is that BAPTA can inhibit protein synthesis (Lawrence *et al.*, 1998).

The Tombes *et al.* (1992) study also found that formation of the first polar body at meiosis I and the subsequent maturation to meiosis II metaphase arrest was dependent on external Ca^{2+} yet showed no Ca^{2+} changes during its 4-5 hour time-course (Tombes *et al.*, 1992). However, although first polar body formation was prevented by the absence of external Ca^{2+} , first meiotic spindle formation was only *delayed* (Tombes *et al.*, 1992). This study is in agreement with other studies (1) in which first polar body formation was dependent on external Ca^{2+} in mammalian oocytes (Jagiello *et al.*, 1982; Leibfried and First, 1979), (2) in which inhibiting transmembrane Ca^{2+} movements blocked first polar body formation but

not GVBD (Paleos and Powers, 1981), (3) in which inhibitors of calmodulin (Ca^{2+} -binding protein mediating Ca^{2+} -regulated processes) inhibited first polar body formation in rabbit oocytes (Henry *et al.*, 1997) (4) but in which first polar body extrusion was found to be InsP_3 -independent since it was not sensitive to lithium-induced inhibition of PI turnover (Pesty *et al.*, 1994).

1.7.4 Role of Ca^{2+} in mitotic cell cycles

The same study (Tombes *et al.*, 1992) also observed that NEBD of the first mitosis was dependent on internal Ca^{2+} stores and was associated with a Ca^{2+} transient in roughly 40% of eggs. In contrast, a different study (Kono *et al.*, 1996) observed single Ca^{2+} transients at NEBD in all fertilised 1-cell embryos examined. They could not, however, detect transients at NEBD in parthenogenetic embryos. The dispensability of global detectable transients combined with the fact that NEBD is inhibited in all embryos by Ca^{2+} chelators (Kono *et al.*, 1996; Tombes *et al.*, 1992) suggests that NEBD may be dependent on small *localised* Ca^{2+} transients. This is consistent with the observation that microinjection of Ca^{2+} into pronucleate sea urchin embryos advances NEBD (Steinhardt and Alderton, 1988). Detectable Ca^{2+} transients are also observed during the first mitosis *after* NEBD until cleavage to the 2-cell stage in fertilised embryos but not in parthenogenotes (Kono *et al.*, 1996). It is thus conceivable that transients following NEBD essential to mitosis may also be localised, and therefore undetectable in parthenogenotes.

There is much evidence for the existence of localised Ca^{2+} gradients (see section 1.5.8) and it has been pointed out that localised Ca^{2+} transients may be obscured when using whole-cell Ca^{2+} measurement techniques (Kono *et al.*, 1996; Whitaker and Patel, 1990). Support for a role for local Ca^{2+} transients in mouse embryos has been provided in studies on sea urchins. Firstly, the ER (Ca^{2+} store) localises to the nuclear area before mitosis in the sea urchin early embryo (Terasaki and Jaffe, 1991). Secondly, in the first mitotic cell cycle of the sea urchin egg global Ca^{2+} transients that appear to originate from the nuclear area are often observed just before NEBD. However, in the absence of global increases, confocal microscopy reveals localised Ca^{2+} transients in the region around the nucleus (Wilding *et al.*, 1996); in these experiments entry into mitosis was completely dependent on these Ca^{2+} increases.

In fact, the idea that mitotic Ca^{2+} transients originate from the perinuclear region is consistent with one explanation for why sperm-induced oscillations stop at entry into interphase. It has been found that the Ca^{2+} transient observed at NEBD appears to be associated with the nucleus since nuclear transfer experiments show that the presence of a nucleus from a fertilised embryo is essential (Kono *et al.*, 1995; Kono *et al.*, 1996). Furthermore, Ca^{2+} transients were detected only after the donor nucleus had undergone NEBD when nuclei were transferred to MII oocytes (in which the high MPF levels bring about NEBD) (Kono *et al.*, 1995). It was therefore hypothesised that the Ca^{2+} -releasing

activity introduced by the sperm may become localised in the pronucleus or associated with perinuclear material (ER). The alternative explanation is that sperm-induced oscillations cease at interphase due to inactivation of the mechanism of sperm-induced Ca^{2+} release during interphase. Thus mitotic Ca^{2+} transients may be generated by the Ca^{2+} -releasing activity being released from the nucleus or by the Ca^{2+} release mechanisms being reactivated to allow the generation of Ca^{2+} transients (Jones *et al.*, 1995a).

In addition to the role of Ca^{2+} at entry into mitosis (NEBD) (Hepler, 1994; Kono *et al.*, 1996; Lu and Means, 1993; Tombes *et al.*, 1992; Wilding *et al.*, 1996), it appears that Ca^{2+} is necessary at other key stages during the course of mitosis. In *Xenopus* oocyte extracts, onset of anaphase requires the ubiquitin-dependent proteolytic pathway to destroy both mitotic cyclins (see above) and an unknown protein responsible for preventing sister chromatid separation and maintaining metaphase arrest (Holloway *et al.*, 1993). However, chromatid disjunction and cyclin destruction can be dissociated (Holloway *et al.*, 1993; Surana *et al.*, 1993; Yamano *et al.*, 1996) and deciphering the role of Ca^{2+} in each event has proved controversial. A transient Ca^{2+} increase occurs as sea urchin embryos enter anaphase of the first cell cycle (Ciapa *et al.*, 1994; Poenie *et al.*, 1985) and at onset of anaphase in mammalian somatic cells (Ratan *et al.*, 1986). It was originally thought that Ca^{2+} signals were not required for metaphase-anaphase transition (Morin *et al.*, 1994). However, recent evidence from early sea urchin embryos has shown that while nuclear reformation/exit from mitosis (upon mitotic cyclin destruction) is not dependent on the anaphase Ca^{2+} increase, chromosome separation can be prevented by interfering with the anaphase Ca^{2+} signal (Groigno and Whitaker, 1998). This contrasts with the situation at fertilisation (*meiotic* metaphase), where the fertilisation Ca^{2+} signal has been shown to be essential for cyclin destruction and meiotic resumption in *Xenopus* (Lorca *et al.*, 1993). This may reflect the fact that the unfertilised egg is blocked in *meiotic* metaphase by CSF, which is absent in *mitotic* cell cycles (Sagata *et al.*, 1989).

1.7.5 Generation of cell cycle Ca^{2+} transients

The mechanism of generation of cell cycle Ca^{2+} transients appears to be via cyclic production of InsP_3 . During the early embryonic cell cycle in the sea urchin oscillations in the phosphoinositide messenger system cause cyclic increases in InsP_3 that drive cell cycle Ca^{2+} transients and mitosis (Ciapa *et al.*, 1994). These InsP_3 increases are observed at times corresponding to pronuclear migration and fusion, NEBD, anaphase onset and cytokinesis. These are the times at which Ca^{2+} transients have been reported in the sea urchin (Kao *et al.*, 1990; Poenie *et al.*, 1985). Furthermore, mitotic Ca^{2+} transients are absent in sea urchin eggs microinjected with heparin, an InsP_3R antagonist (Ciapa *et al.*, 1994), providing further evidence that InsP_3 -sensitive and not ryanodine-sensitive Ca^{2+} release regulates mitosis. Ca^{2+} increases associated with meiotic maturation of the egg are also likely to be generated by InsP_3 production. Cyclic production of InsP_3 is also likely to

regulate cell cycle Ca^{2+} transients in other animals. For example, InsP_3 levels increase at mitotic cleavage in *Xenopus* (Stith *et al.*, 1993).

1.7.6 Effect of cell cycle kinases on Ca^{2+} signaling

The above discussion suggests that Ca^{2+} regulates cell cycle proteins. The converse also appears to be true. In other words, Ca^{2+} signaling at fertilisation and during mitosis seems to be regulated by cell cycle kinases. The Ca^{2+} oscillations in oocytes and embryos occur during metaphase and terminate upon entry into interphase. It has so far not been determined which of the two candidate kinases, MAP kinase or cyclin B1-CDK (MPF), are involved in controlling either the sperm-induced Ca^{2+} oscillations or the mitotic Ca^{2+} signals.

After fertilisation in the mouse oocyte the Ca^{2+} oscillations terminate just before pronuclear envelope formation (Jones *et al.*, 1995a), when the MAP kinase activity falls to low levels (Verlhac *et al.*, 1994). This correlation suggests that MAP kinase is a more likely candidate for involvement in the generation of Ca^{2+} oscillations. However, experiments in ascidian oocytes suggest that cyclin B1-CDK is the most likely candidate for controlling the Ca^{2+} oscillations (McDougall and Levasseur, 1998). Sperm trigger two series of Ca^{2+} oscillations in metaphase I-arrested ascidian oocytes, which trigger meiotic exit (McDougall and Sardet, 1995; Yoshida *et al.*, 1998) and which terminate when the egg exits meiotic metaphase (Speksnijder *et al.*, 1989). Each series of Ca^{2+} oscillations correlates with elevated MPF activity, which is low during a gap phase equivalent to the time when the first polar body is extruded (McDougall and Levasseur, 1998). In addition to this correlative data there is recent evidence that the CDK activity of the ascidian oocyte acts as a positive regulator of sperm-induced Ca^{2+} oscillations (Levasseur and McDougall, 2000). It remains to be seen whether cyclin B1-CDK acts in the same way in mammalian oocytes, where there is a single series of sperm-induced Ca^{2+} oscillations which continue for 3-4 hours. As stated above, these oscillations appear to correlate not with polar body extrusion and MPF activity but with pronuclear formation and MAP kinase activity (Jones *et al.*, 1995a). However, Ca^{2+} oscillations can often cease before pronucleus formation (Cheung *et al.*, 2000), suggesting that oscillations will stop in the presence of active MAP kinase (Moos *et al.*, 1996b). Also, the continuation of fertilisation-induced Ca^{2+} oscillations in MII eggs treated with colcemid or nocodazole corresponds with the maintenance of MPF activity (Kubiak *et al.*, 1993; Moos *et al.*, 1995; Winston *et al.*, 1995). In support of a role for MPF, a recent study used the MPF inhibitor, roscovitine, to suppress fertilisation-induced Ca^{2+} oscillations in mouse eggs (Deng and Shen, 2000). The study suggested that Ca^{2+} oscillations were inhibited due to MPF inactivation causing disruption of Ca^{2+} release and refilling of Ca^{2+} stores. However, since the drug also suppressed thimerosal-induced Ca^{2+} oscillations (in Ca^{2+} - and magnesium-free medium) and thapsigargin-induced Ca^{2+} release (Deng and Shen, 2000), further work is needed to confirm the specificity of the effects of roscovitine. Nevertheless, a role for MPF in regulating Ca^{2+} oscillations also

seems likely when considering the biological significance of the long-lasting oscillations in fertilised mammalian eggs. Complete inactivation of MPF (permitting entry into interphase) may be an explanation for such an extensive duration of oscillations, as the high turnover of new cyclin B1 in MII eggs may lead to the oscillatory reactivation of MPF. For example, in rabbit oocytes a single Ca^{2+} stimulation initiates MPF inactivation, but multiple stimuli are necessary to maintain an MPF substrate (histone H1 kinase) inactive (Collas *et al.*, 1995). Furthermore, MPF activity has been shown to return after completion of meiosis II, resulting in the formation of a “metaphase III” (Kubiak, 1989). The idea that MPF (as well as MAP kinase) may remain above basal levels and support sperm-induced oscillations until their termination, represents a form of negative feedback regulation, since these same oscillations appear to be the trigger for MPF inactivation. Of course, oscillations would also depend upon MAP kinase, since the MAP kinase pathway stabilises MPF activity (Masui, 1974).

The underlying clock that controls the timing of Ca^{2+} transients during mitosis is also unclear. Nevertheless, it is established that these Ca^{2+} signals are cell cycle-regulated. One study hypothesised a model representing a cell cycle-associated change in the threshold for Ca^{2+} release, with the threshold low in mitosis and in metaphase of meiosis, and high in interphase (Kono *et al.*, 1996). The temporal association between increased Ca^{2+} releasing activity and activation of MPF at each mitosis suggested that Ca^{2+} spiking at mitosis is related to activation of MPF (Day *et al.*, 1998; Kono *et al.*, 1995). Recent work found that puromycin-arrested mouse zygotes (in which cyclin B1 synthesis and thus MPF activation is inhibited) showed no Ca^{2+} spikes at the time when control zygotes were entering first mitosis. However, Ca^{2+} spiking prior to NEBD did not occur in anucleate zygote fragments in which MPF is known to be active (Day *et al.*, 2000). Therefore, spiking may be caused by the signaling pathway downstream of MPF activation but also may involve factors associated with the nucleus. However, since the pattern of spiking and its relationship to the presence of a pronucleus is so different from that seen at fertilisation (Pn required for transients at NEBD, not at fertilisation), it is thought that the control of spiking at mitosis may differ from that at fertilisation (Day *et al.*, 2000). Despite the above evidence being consistent with a role for MPF in the regulation of mitotic Ca^{2+} transients, no study has directly investigated the cell cycle proteins (such as cyclin B1-CDK or MAP kinase) involved. However, it is likely that the same cell cycle kinases are involved in the generation of transients prior to NEBD and at other points of the mitotic cell cycle.

It is also unclear how the underlying clock directly causes the generation of mitotic Ca^{2+} transients, in other words which component(s) of the Ca^{2+} signaling pathway may be regulated by cell cycle kinase activity. Furthermore, the feedback effect of mitotic Ca^{2+} transients on cell cycle kinase activity requires further exploration; which event initially triggers the other and determines the timing of changes in the cell cycle is discussed below. Finally, it remains to be determined whether other factors, such as proteins involved in

Ca²⁺ signaling, contribute to the regulation of Ca²⁺ oscillations. An example of this level of control would be regulated expression of Ca²⁺ release channels such as InsP₃Rs.

1.7.7 Is Ca²⁺ release an endogenous clock regulating cell cycle progression?

What exactly regulates cell cycle progression and controls the timing of the mitotic cell cycle remains to be determined. It has been proposed, in early sea urchin embryos, that an endogenous oscillator, independent of the cyclin oscillator (Edgecombe *et al.*, 1991; Murray *et al.*, 1989), controls the timing of the cell cycle through InsP₃ production and subsequent intracellular Ca²⁺ release (Ciapa *et al.*, 1994). In the presence of protein synthesis inhibitors (to inhibit MPF activation), InsP₃ levels were significantly higher at times when controls were in mitosis than at times when controls were in interphase (Ciapa *et al.*, 1994). This indicated that, in the sea urchin, the InsP₃ cycles have an endogenous rhythm, with a period corresponding to the cell cycle time, and are independent of mitotic kinase activation and of the nuclear events of mitosis itself (Ciapa *et al.*, 1994).

The idea of InsP₃ being generated by an endogenous oscillator appears to be inconsistent with the idea of MPF activity being a requirement for mitotic Ca²⁺ spiking prior to NEBD (described above) (Day *et al.*, 2000). However, the two results can be reconciled in a simple speculative model (proposed for Ca²⁺ spikes at NEBD). This proposes that endogenous cycles of InsP₃ are generated by an unknown mechanism and bring about small, undetectable changes in Ca²⁺ levels. Ca²⁺-dependent pathways may stimulate the activity of MPF (Steinhardt and Alderton, 1988; Twigg *et al.*, 1988; Whitaker, 1995) (see section 1.8.1) which may in turn regulate the generation of InsP₃ (through PLC activation) and detectable Ca²⁺ transients in a positive feedback cycle. The model predicts that inhibition of MPF would not prevent the endogenous InsP₃ cycles (Ciapa *et al.*, 1994) but would prevent detectable MPF-amplified Ca²⁺ transients (Day *et al.*, 2000). This is also consistent with the observation in the former study (Ciapa *et al.*, 1994) that spikes of InsP₃ production were less defined when MPF activity was inhibited; Ca²⁺-dependent activation of sea urchin MPF may thus shape the wave-form of the endogenous oscillator to generate sharp spikes of InsP₃ and Ca²⁺ during mitosis. This model could also explain why Ca²⁺ transients, although necessary, are not detected at NEBD of first mitosis in parthenogenetic mouse embryos (Kono *et al.*, 1996; Tombes *et al.*, 1992): the global transients detected in fertilised embryos may be a consequence of the sperm factor (a PLC) being activated by MPF and generating excess InsP₃ and amplifying the local undetectable signal.

With respect to the nature of the endogenous oscillator which generates cycles of InsP₃, this may be linked to the centrosome cycle, as this can also be uncoupled from mitosis (Han *et al.*, 1992). It is likely that a kinase-independent InsP₃ cycle also exists in other species besides sea urchin. Indeed, one paper proposes a similar model to that proposed above, in which an endogenous Ca²⁺ oscillator controls early embryonic division in both

sea urchin and *Xenopus* embryos (Swanson *et al.*, 1997). The interaction of Ca^{2+} -dependent signaling pathways with proteins (such as MPF) involved in the regulation of the cell cycle, is discussed in detail below.

1.8 Ca^{2+} targets during the cell cycle

The targets of intracellular messengers such as Ca^{2+} are protein kinases and protein phosphatases, with few exceptions. The calmodulin protein binds Ca^{2+} ions, functioning as a multipurpose intracellular Ca^{2+} receptor and mediating most Ca^{2+} -regulated processes. Ca^{2+} -calmodulin complexes have no enzyme activity themselves but act by binding to other proteins. For example, CaM kinases (Ca^{2+} -calmodulin dependent protein kinases) and the type 2B-phosphatase calcineurin, are regulated by Ca^{2+} -calmodulin. On the other hand, protein kinase C (PKC) is regulated by Ca^{2+} and acyl lipids such as DAG. It has been shown that the activity of CaM kinase II (CaM KII) can be modulated by oscillatory Ca^{2+} transients (De Koninck and Schulman, 1998) and is thought to be essential for mitotic cell cycle progression both in sea urchin embryos and mammalian cell lines (Baitinger *et al.*, 1990; Dupont, 1998; Johnson *et al.*, 1998; Lorca *et al.*, 1993).

1.8.1 Regulation of MPF activity by CaM KII

In mammals, the Ca^{2+} changes at fertilisation are sufficient to induce many of the processes associated with egg activation such as cortical granule exocytosis and resumption of the cell cycle (Xu *et al.*, 1997). Facilitation of these processes presumably occurs via direct or indirect effects of Ca^{2+} transients on downstream effectors such as CaM kinases, CSF, MPF, MAPK and PKC (Gallicano *et al.*, 1993; Raz and Shalgi, 1998; Whitaker, 1995; Whitaker, 1996). In fact, there is convincing evidence that Ca^{2+} increases at parthenogenetic egg activation cause the activation of CaM KII. For example, in MII-arrested mouse oocytes CaM KII is inactive but following exposure to ethanol the kinase activity dramatically increases before returning to baseline activity within 15 minutes of removal from ethanol (Winston and Maro, 1995). CaM KII activation was unaffected by arresting oocytes at MII using nocodazole and therefore acts upstream of the microtubule-dependent cyclin destruction machinery (Winston and Maro, 1995).

A likely target effect of CaM KII activity at egg activation is activation of cyclin degradation. Consistent with this, there is data suggesting that CaM KII mediates inactivation of MPF and CSF upon meiotic resumption of *Xenopus* eggs. As discussed above, sperm-induced Ca^{2+} transients have been shown to drive the destruction of cyclin B (Kline and Kline, 1992a; Murray *et al.*, 1989; Newport and Kirschner, 1984; Watanabe *et al.*, 1991) and thus cause MPF inactivation and exit from M phase. A Ca^{2+} -calmodulin-dependent process is required for cyclin proteolysis (Lorca *et al.*, 1991) and microinjection of a constitutively active CaM KII mutant into unfertilised *Xenopus* eggs has been used to demonstrate firstly that cyclin B1-CDK and CSF inactivation occur even in the absence of

Ca²⁺ (Lorca *et al.*, 1993). Secondly, CaM KII inhibitors (in cell-free MII oocyte extracts) prevent cyclin degradation after Ca²⁺ addition, providing further proof that cyclin degradation depends on CaM KII (Lorca *et al.*, 1993). As described above, cyclin degradation at fertilisation has been shown in *Xenopus* to be carried out via the ubiquitin-dependent proteasomal pathway (Glutzer *et al.*, 1991). Consistent with this, Ca²⁺ has been shown to induce assembly of the active 26S proteasome from the inactive 20S proteasome during resumption of MII at *Xenopus* egg activation (Aizawa *et al.*, 1996). An equivalent situation exists in ascidians, in which MI resumes upon egg activation due to Ca²⁺ inducing assembly of the 26S proteasome (Kawahara and Yokosawa, 1994). Moreover, the Ca²⁺-dependent activation of the proteasome at fertilisation most probably involves stimulation of CaM KII (Lorca *et al.*, 1993; Morin *et al.*, 1994; Whitaker, 1993). In other species and in *Xenopus* (in addition to the above), CaM KII may stimulate the degradation of cyclin by stimulating activity of the APC which is responsible for the regulated ubiquitination of cyclin B for recognition by the proteasome (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995).

On the other hand, there is evidence in mammals for a role for CaM KII in causing the *activation* of MPF at entry into mitosis. A recent example comes from a study on HeLa cells (Patel *et al.*, 1999). Human cdc25-c (a tyrosine phosphatase) appears to be a target for CaM KII at the G2/M phase transition (mitosis entry) in HeLa cells. *In vitro* experiments showed that cdc25-c was phosphorylated and activated by CaM KII and the use of CaM KII inhibitors blocked the cell cycle in G2 phase (Patel *et al.*, 1999). Since activated cdc25-c in turn dephosphorylates and activates MPF, it appears that Ca²⁺ is able to initiate the accumulation of active MPF and cause entry into mitosis. This is consistent with the finding that NEBD (entry into mitosis) is dependent on internal Ca²⁺ release in mammals (Kono *et al.*, 1996; Tombes *et al.*, 1992).

The sea urchin provides further evidence for CaM kinase in the regulation of MPF at both egg activation and entry into mitosis. The fertilisation Ca²⁺ transient has been shown to trigger p34cdc2 tyrosine phosphorylation cycles in early sea urchin embryos, independent of the cycle of cyclin synthesis and destruction. p34cdc2 phosphorylation (MPF inactivation) increases at fertilisation and p34cdc2 dephosphorylation (MPF activation) increases at entry into mitosis, followed by reaccumulation of inactive MPF at entry into the next interphase (Edgecombe *et al.*, 1991). The role of Ca²⁺ in the accumulation of active dephosphorylated MPF at entry into mitosis in sea urchin may be analogous to its role in the accumulation of active MPF at entry into mitosis in human HeLa cells. Since CaM kinase has been shown to be involved in the regulation of NEBD of at least the first mitotic division in the sea urchin (Baitinger *et al.*, 1990), CaM kinase may activate the sea urchin equivalent of human cdc25-c. However, it is unclear how Ca²⁺ may stimulate the phosphorylation and thus inactivation of MPF in the sea urchin. As for other targets of Ca²⁺ in the sea urchin, the Ca²⁺ increase at fertilisation is somehow responsible for increases in

NAD(P)H (Epel, 1990) and, either alone or in concert with DAG, for activating the sodium/hydrogen exchange and increasing pH (Epel, 1990). Both changes are essential to normal sea urchin development (Whitaker and Steinhardt, 1981).

1.8.2 CaM KII at mitotic anaphase onset

With respect to Ca^{2+} targets at the metaphase-anaphase transition, Ca^{2+} -dependent degradation of cyclin in CSF extracts prepared from unfertilised *Xenopus* eggs (in a situation analogous to fertilisation) is stimulated by CaM KII (Lorca *et al.*, 1993; Morin *et al.*, 1994) and is discussed above. It may also be that the anaphase Ca^{2+} signal of mitosis and the fertilisation signal at meiotic resumption both target CaM KII and that CaM KII may stimulate the proteasomal degradation of the proteins involved in “glueing” chromatid pairs together. Mitotic cyclin destruction by the ubiquitin-proteasome pathway, on the other hand, is not dependent on the anaphase Ca^{2+} signal (Groigno and Whitaker, 1998).

1.9 Synopsis of chapters

Increases in the concentration of intracellular Ca^{2+} have been implicated as a major influence in the regulation of the cell cycle. In particular, Ca^{2+} increases are well documented as being both necessary and sufficient for egg activation and have been observed during the first mitosis. In connection with this, the work of this thesis concentrates on a major family of intracellular Ca^{2+} release channels, the InsP_3Rs , focusing on their expression in mammalian oocytes and early embryos. The subject of each chapter builds on the results of the previous chapter.

The first experimental chapter (Chapter 3) examines the change in expression of different InsP_3R isoforms in mouse oocytes and embryos in order to find out which subtypes are important for fertilisation and development of the blastocyst. Chapter 4 investigates how fertilisation brings about a change in the expression of type I InsP_3R and Chapter 5 deals with the mechanism of this change in expression. A change in the expression of different InsP_3R subtypes as well as a change in concentration of total InsP_3Rs will have consequences for the sensitivity of Ca^{2+} release to InsP_3 and thus influence Ca^{2+} signaling. The final experimental chapter (Chapter 6) investigates the role of InsP_3Rs and thus InsP_3 -induced Ca^{2+} release in meiotic and mitotic cell cycles in mouse oocytes and embryos.

Each experimental chapter begins with a specific introduction that builds upon what is discussed in the General Introduction. The results are then presented and discussed. Chapter 7 is a General Discussion that discusses my findings on a more general level and suggests work to investigate some areas which have not been completely explained.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Materials

Mice

I used sperm from (C57xCBA)F1 males and oocytes from MF1 females for *in vitro* fertilisation. For *in vivo* fertilisation, these strains were mated to produce fertilised eggs and embryos.

Antibodies

CT primary antibodies were a gift from Richard Wojcikiewicz (Department of Pharmacology, College of Medicine, SUNY Health Science Center at Syracuse, Syracuse, New York.) and are affinity-purified polyclonal type I InsP₃R (InsP₃R-I) antiserum (CT1), affinity-purified polyclonal type II InsP₃R (InsP₃R-II) antiserum (CT2), and affinity-purified polyclonal type III InsP₃R (InsP₃R-III) antiserum (CT3). All antibodies were raised in rabbit and were purified on peptide columns. CT1 is raised against a peptide corresponding to the C-terminal of the rat InsP₃R-I. CT2 is raised against the C-terminal of the rat InsP₃R-II and CT3 against the C-terminal of the rat InsP₃R-III. Previous work has characterised these antisera and shown that they are specific for the individual receptor isoforms (Wojcikiewicz, 1995). CT1 and CT2 label a single protein band on electrophoresis gels, corresponding in size to InsP₃R-I and InsP₃R-II, of about 260kDa. CT3 sometimes labels more than one protein band, one corresponding in size to the InsP₃R-III and another approximately 30kDa smaller. Ab40 is a type I-specific peptide polyclonal antibody (Zaidi *et al.*, 1995) which was a gift from Richard Tunwell (Department of Physiology, University College London.). The antisera were stored at 4°C.

2.2 Collection of oocytes and embryos

Mature ovulated oocytes were recovered from 21-24-day-old MF1 mice that had previously been administered pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) by intraperitoneal injection at an interval of 48 hours. Mice were killed by cervical dislocation 13-16 hours after hCG (1-4 hours after ovulation) and the oviducts were removed and placed into warm Hepes-buffered modified Kreb's ringer medium, M2 (Fulton and Whittingham, 1978), containing 4mg/ml BSA, pH7.4. The cumulus masses were released into M2 containing hyaluronidase (300µg/ml) and the cumulus-free oocytes were recovered and washed three times. Immature oocytes were recovered from the ovaries of mice that had been administered an intraperitoneal injection of 7IU of PMSG 48 hours previously. The ovaries were recovered from mice as described

above. Oocytes were released from the ovary by scraping the edge of the ovary with a 27 gauge needle. Oocytes with an intact layer of cumulus cells were collected before removing the cells by repeated pipeting.

2-cell embryos were obtained after the culture of *in vitro* fertilised embryos for 24 hours (see below) or from mated females 40-44 hours after hCG injection. To obtain embryos, superovulated female mice were mated with (C57xCBA)F1 males. After the required length of time, oviducts attached to the uterus were dissected out and blunted flushing needles were used to flush preimplantation embryos into M2/BSA medium. Morulae and blastocysts were flushed from the oviducts and uterus 68-72 and 92-96 hours after hCG injection, respectively.

If oocytes or embryos were to be used for analysis of protein levels, they were washed a further three times in 2ml of PBS, pH7.5, containing 1mg/ml PVA (polyvinyl alcohol) before being transferred to a sterile microcentrifuge tube in a minimal volume (5-10µl) of PBS/PVA and freezing in liquid nitrogen or on dry ice. The tubes were stored at -70°C until use.

2.3 *In vitro* fertilisation and parthenogenetic activation

IVF

The epididymi were removed from a (C57xCBA)F1 male of proven fertility and sperm were released into 1ml of T6 (pH7.6) containing 10mg/ml Fraction V BSA (Sigma, Poole, Dorset) which had been pre-equilibrated in a 37°C, 5%CO₂ incubator, and filtered. After 20 minutes the sperm suspension was diluted into 4ml of the same medium and incubated at 37°C, 5%CO₂ for 2 hours for sperm to capacitate. Just prior to the experiment, fertilisation drops were prepared by adding 3µl of sperm suspension to a 200µl insemination drop of T6/BSA under pre-equilibrated mineral oil. In order to synchronise the timing of fertilisation and ensure maximal rates of fertilisation, oocytes were fertilised zona-free. The zona pellucida was removed using a brief exposure to acidified Tyrode's medium, followed by extensive washing. The oocytes were placed into the sperm suspension and returned to the incubator for 10-15 minutes (see also section 3.6). At the end of this period, the oocytes were removed and washed in T6/BSA before further culture in the same medium. Aged oocytes were used to control for any changes that may occur in the oocyte between the time of ovulation and fertilisation. These oocytes were collected at the same time as those to be fertilised and were subsequently treated in the same manner, except that they were not exposed to sperm. After *in vitro* fertilisation, embryos were confirmed as fertilised by the presence of two pronuclei and a second polar body. Pronucleate embryos were frozen and stored at -70°C, as described above.

Parthenogenetic activation

Parthenogenetic activation of MII oocytes was stimulated using a number of different protocols. Oocytes were exposed to parthenogenetic agents 14-18 hours post hCG injection and maintained at 37°C throughout culture, first in M2 and then in T6 medium. The activated oocytes were sorted into those that had only extruded a polar body and those that had formed pronuclei. Only oocytes with pronuclei were used for analysis of InsP₃R levels by Western blotting. For Sr²⁺-induced egg activation, oocytes were incubated in Ca²⁺-free M2/BSA containing 10mM strontium chloride for 4 hours, followed by culture in T6 for 2 hours. Ethanol activation was performed by exposing oocytes to 7% ethanol in M2/BSA for 7 minutes, followed by culture in T6 for 6 hours. For activation in a Ca²⁺-independent manner, oocytes were incubated for 6 hours in T6 containing 10µg/ml cycloheximide (Sigma) or for 1 hour in T6 containing 10µM R0-31-8220 (Calbiochem), followed by culture in T6 for 5 hours. At low concentrations, R0-31-8220 is reported to be specific for PKC (IC₅₀≈10nM). At higher concentrations, it inhibits many kinases and it has previously been found that it activates mouse eggs at a concentration of 10µM (Jones and Carroll, unpublished observations). For aged-matched controls, oocytes were placed into T6 containing 1µl/ml DMSO for 6 hours. Pronucleate embryos were frozen and stored at -70°C, as described above.

2.4 Inhibition of proteases

In order to inhibit proteases, oocytes were treated with a number of protease inhibitors (Calbiochem) at fertilisation. Oocytes were preincubated for 90 minutes to 2 hours in 50µM (or concentration stated in Results) of ALLN or MG132 or lactacystin. The oocytes were then exposed to sperm for 15 minutes in inhibitor-free medium before returning to T6 containing the inhibitors. In the case of lactacystin, frozen stocks were diluted and used to make up fresh culture drops containing lactacystin, every 30 minutes. Other oocytes were preincubated for 1 hour in 100µM caspase inhibitor III before sperm addition, followed by continued culture in inhibitor. To inhibit lysosomal proteolysis, oocytes were transferred to T6 containing 20mM NH₄Cl or 200µM chloroquine, immediately after removal from sperm.

2.5 Microinjection

Mature or immature oocytes or 1-cell pronucleate embryos (fertilised or parthenogenetically activated) were pressure-injected with a micropipette and Narishige manipulators mounted on a Nikon Diaphot microscope. Oocytes were placed in a drop of M2 covered with mineral oil to prevent evaporation. Pipettes were back-filled with 1mM, 100µM or 10µM adenophostin A (Calbiochem) in a buffer containing 120mM KCl and 20mM Hepes. The oocytes were immobilised using a holding pipette and the injection pipette was pushed through the zona pellucida until it contacted the oocyte plasma membrane. A brief over-compensation of negative capacitance caused the pipette tip to penetrate the oocyte and a

dose of adenophostin A was applied using a pressure-injection system. Injection volumes were estimated to be between 1% and 5% of the oocyte volume, giving estimated final oocyte concentrations of 10-50 μ M, 1-5 μ M or 100-500nM, for pipette concentrations of 1mM, 100 μ M and 10 μ M, respectively. The injected oocyte was moved to the bottom of the field of view before starting the next injection. Survival rates were >90%.

2.6 ~~Ca²⁺ recording~~ Fura 2 imaging

Oocytes or 1-cell embryos were loaded with Fura-2-AM (Sigma) by a 15-20 minute incubation in 2 μ M of the acetoxymethyl ester and 0.025% Pluronic (Molecular Probes) in M2/BSA at 37°C. The zona pellucida was removed by a brief incubation in acidified Tyrode's solution (Sigma) (so that the oocyte or embryo adhered to the chamber coverslip) and the zona-free oocyte or embryo was transferred to 0.5ml of BSA-free M2 in a heated chamber on the stage of a Zeiss Axiovert microscope or Nikon Diaphot. 0.5ml of M2 containing 8mg/ml BSA was added. Regions of interest were drawn for each oocyte. Fura-2 was excited at 340nm and 380nm using a xenon lamp, and emission fluorescence was collected using a 20x or 40x 0.75NA objective lens, passed through a 520nm long-pass filter, and detected using a cooled CCD camera. Data was processed using IonVision software (ImproVision, Coventry, UK) or Newcastle photometrics software (Lawrence *et al.*, 1997). The data is presented as the ratio of the 340nm and 380nm fluorescence values. The absolute value of the ratio scales varied according to the microscope system used.

Ca²⁺ recording after exposure to parthenogenetic agents

With ethanol activation, oocytes were transferred to 0.5ml of M2 in the heated chamber and 0.5ml of 14% ethanol in M2/BSA was added, to achieve a final concentration of 7% ethanol. After imaging for a further 6.5 minutes, 10ml of M2/BSA was perfused through the chamber to remove the ethanol, and imaging continued. With Sr²⁺ activation, oocytes were transferred to 0.5ml of Ca²⁺-free M2 in the heated chamber and 0.5ml of 20mM strontium chloride/Ca²⁺-free M2/BSA was added and mixed in by pipeting. Note that intracellular Ca²⁺ imaging was started for a few minutes before addition of the activating agent (ethanol or Sr²⁺) to MII oocytes.

2.7 Western blotting

The oocytes, embryos or other tissue preparations were removed from the freezer and freshly-prepared loading buffer (125mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.02% bromophenol blue) was added to the frozen pellet before vortexing, heating at 95°C for 5 minutes and centrifugation at 13,000g for a few seconds. Aliquots containing the required number of oocytes or embryos (10-300) or quantity of tissue extract, were subjected to 5% SDS-PAGE, using a 3% stacking and 5% resolving minigel (Mini Protean II, Bio-Rad). The proteins were then subjected to a 2-hour, 15volt electrophoretic semi-dry transfer (Bio-Rad) to a PDVF membrane (Millipore), using a

freshly-prepared blotting buffer for high molecular weight proteins (3.03g/l Tris, 14.4g/l glycine, 0.6g/l SDS). Membranes were incubated with blocking buffer (PBS containing 0.05% Tween-20 and 1% BSA (Fraction V) or 5% milk (Marvel)) for 2 hours on a shaker at room temperature. To compare the relative levels of InsP₃R subtypes in oocytes and embryos, the proteins were immunoblotted with affinity-purified, subtype-specific antibodies, CT1 (for InsP₃R-I), CT2 (for InsP₃R-II) or CT3 (for InsP₃R-III) (Wojcikiewicz, 1995) at dilutions (1:80, 1:100, 1:50, respectively) that produced approximately equal amounts of chemiluminescence from equal amounts of the purified receptor standards (Wojcikiewicz, 1995). Other experiments were carried out using type I-specific antibodies, CT1 and Ab40 (Zaidi *et al.*, 1995). Primary antibodies were diluted in blocking buffer including 0.02% sodium azide, and incubated with the blot overnight at 4°C. Primary antibody incubation was followed by incubation in horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Dako), diluted 1:5000 in blocking buffer, for 2 hours on a shaker at room temperature. Immunoreactivity was detected using enhanced chemiluminescence (ECL kit, Amersham) and X-ray film (Hyperfilm ECL, Amersham). For densitometric analysis, blots were scanned and the total pixel intensity in each band was determined using NIH Image, as described in Results.

In order to reprobe a blot with a second primary antibody type, antibodies were stripped from membranes by incubating the membranes in stripping buffer (100mM β-mercaptoethanol, 2% w/v SDS, 62.5mM Tris pH 6.7) at 60°C for 30 minutes. The membrane was then washed in PBS/Tween three times for 30 minutes each, reblocked and incubated with the next primary antibody.

Preparation of mouse and rat tissues for Western blotting

Mouse brain, pancreas and testis (including *vas deferens*) and rat brain, each rich in at least one subtype of InsP₃R, were used to check the reactivity of antibodies CT1, CT2 and CT3 in immunoblotting experiments. Tissues were removed from 16-21g female mice and placed in ice-cold homogenisation buffer (pH 7.4, 0.32M sucrose, 5mM Hepes buffer, 100μM EGTA) containing 1μl/ml of each of PMSF, leupeptin and pepstatin A. After thorough washing with the buffer, tissues were homogenised on ice, with 20 strokes of a Dounce homogeniser. Homogenates were centrifuged at 4500rpm at 4°C, for 10 minutes. The supernatant was saved and centrifuged again at 4500rpm at 4°C for 10 minutes. This supernatant was saved and centrifuged at 30,000rpm for 1 hour in a vacuumed centrifuge at 4°C. The supernatant was discarded and an equal volume of resuspension buffer (pH 7.35, 40mM KCl, 60mM KPO₄, 10mM Hepes buffer, 1mM MgCl₂, 5mM NaCl) containing 1μl/ml of each of PMSF, leupeptin and pepstatin A, was added to the pellet and the pellet resuspended. Aliquots were stored at -70°C until use.

Protein assay

The protein content of each tissue sample was determined by adding 1µl of the sample to 1ml of a 1:50 solution of 4% CuSO₄ in bicinchoninic acid solution. A control was carried out in parallel, with a range of BSA standards of known concentrations in place of the tissue sample. After 10 minutes at 37°C, absorption at 562nm was measured for each sample in a spectrophotometer. The absorption values for tissue samples were compared to those for BSA standards. Thus the protein concentrations and therefore how much sample to load per gel lane, calculated.

2.8 Immunolocalisation of the InsP₃R

Oocytes and fertilised embryos were fixed in freshly-prepared 4% paraformaldehyde (in PBS pH7.4, 1mg/ml PVA) (or 100% cold methanol for 2-3 minutes for preadsorbed peptide antigen control) for 10 minutes, and permeabilised in blocking buffer (PBS, 3% BSA, 10% normal goat serum) containing 0.1% Triton X-100 for 45 minutes, before incubation with a 1:25 dilution of the CT1 antibody overnight at 4°C. The cells were washed extensively, incubated in fluorescein-conjugated secondary antibody (FITC-conjugated goat anti-rabbit IgG, Molecular Probes) for 1 hour at 37°C, and washed. Included in the first wash was 5µl/ml propidium iodide, a DNA-specific fluorochrome that allowed visualisation of the position of the meiotic spindle in eggs. Eggs and embryos were mounted in Citifluor antifade mountant on microscope slides. The immunostaining was visualised using a Leica confocal microscope. To ensure that comparisons of immunofluorescence could be made between developmental stages, the different samples were scanned and viewed with identical settings. Z-series images were generated through the egg or embryo and a maximum intensity projection image produced. A figure for mean pixel intensity was obtained using NIH Image.

CHAPTER 3:

EXPRESSION OF InsP₃Rs IN MATURE MOUSE OOCYTES AND EARLY EMBRYOS: THE TYPE I ISOFORM IS DOWNREGULATED AFTER FERTILISATION

3.1 INTRODUCTION

In this chapter I examine the change in expression of different InsP₃R isoforms during early development by examining mature mouse oocytes, fertilised eggs and early embryos. The fertilisation of mammalian oocytes stimulates a series of Ca²⁺ oscillations that drive the resumption of meiosis and entry into the first mitotic division (Kline and Kline, 1992a). These Ca²⁺ oscillations continue for 3-4 hours and in any single oocyte occur at a regular frequency of between 5 and 15 minutes (Swann and Ozil, 1994). The first few transients have been reported to occur initially as waves, after which the transients become global synchronous oscillations when monitored with a time resolution of 1-2 seconds (Miyazaki *et al.*, 1986). The generation of sperm-induced Ca²⁺ oscillations is strongly correlated with the meiotic and mitotic cell cycle (Carroll *et al.*, 1994; Jones *et al.*, 1995a; Jones *et al.*, 1995b; Kono *et al.*, 1996; Mehlmann and Kline, 1994). The Ca²⁺ oscillations cease during entry into the first mitotic interphase when the pronuclei form, and return later during mitosis (Jones *et al.*, 1995a; Kono *et al.*, 1996). Furthermore, arresting the oocyte in MII using spindle inhibitors leads to persistent oscillations that stop only when the oocyte is allowed to enter interphase (Jones *et al.*, 1995a). The mechanisms underlying such complex regulation of Ca²⁺ signaling may involve a number of factors including changes in the expression and regulation of the intracellular Ca²⁺ channels (Carroll *et al.*, 1996).

Ca²⁺ is released from intracellular stores in the endoplasmic reticulum through two families of Ca²⁺ release channels, the InsP₃Rs and ryanodine receptors (Berridge, 1993). They exist as tetramers with the C-terminal region providing the transmembrane and channel domains, while the bulk of the protein and N-terminus protrude into the cytoplasm. This large N-terminal provides ready access to cytoplasmic modulators of Ca²⁺ release including InsP₃ itself (Mikoshiba, 1993). Three isoforms of each family have been identified and their expression is tissue specific and developmentally regulated (De Smedt *et al.*, 1994; De Smedt *et al.*, 1997; Nakagawa *et al.*, 1991; Ross *et al.*, 1992). Recent studies have illustrated that any one cell type expresses multiple isoforms of InsP₃R (De Smedt *et al.*, 1994; De Smedt *et al.*, 1997; Nakagawa *et al.*, 1991; Newton *et al.*, 1994; Wojcikiewicz, 1995), although an exception is the nearly exclusive expression of type I in cerebellum. The simultaneous expression of different isoforms in the same cell type suggests some functional specialisation. In this respect it was found, in one study, that type I was the

predominant isoform in brain, type II was highly expressed in some epithelial cell types as well as in muscle cells and type III showed high expression in most of the cultured cells, and a link of the type III isoform with cell proliferation was suggested (De Smedt *et al.*, 1997). The expression of multiple, functionally distinct isoforms provides substantial variation for the regulation of Ca^{2+} release in any given cell type.

Both families of Ca^{2+} channels appear to be present in oocytes from different mammalian species, as shown by the ability of InsP_3 , ryanodine and cADP-ribose to release Ca^{2+} (Yue *et al.*, 1995). In some species this physiological evidence is supported by molecular and biochemical data which shows that mouse and hamster oocytes express the type I InsP_3R (Mehlmann *et al.*, 1996; Miyazaki *et al.*, 1992) and mouse oocytes express the type II and III ryanodine receptors (Ayabe *et al.*, 1995). The role of RyRs in the generation of Ca^{2+} transients at fertilisation in mammals is controversial (Ayabe *et al.*, 1995; Carroll *et al.*, 1996; Jones *et al.*, 1995b). However, there is strong evidence for a role for InsP_3Rs (Mehlmann and Kline, 1994; Miyazaki *et al.*, 1992). The functionally inhibitory type I InsP_3R antibody 18A10 inhibits fertilisation-induced Ca^{2+} transients in mouse and hamster oocytes (Miyazaki *et al.*, 1993; Miyazaki *et al.*, 1992). Despite this convincing evidence for InsP_3Rs in Ca^{2+} signaling at fertilisation, the identity of the isoforms expressed and whether they are developmentally regulated remains unknown.

In addition to the expression of different intracellular Ca^{2+} channels, Ca^{2+} release mechanisms during early development may be modulated by posttranslational modifications to the channels. Phosphorylation, ATP and cytoplasmic proteins such as FKBP12 and ankyrin are all known to modulate the sensitivity of Ca^{2+} release through InsP_3Rs (Bourguignon *et al.*, 1993b; Cameron *et al.*, 1995; Mikoshiba, 1993; Nakade *et al.*, 1994). More recent studies have revealed that Ca^{2+} release through the InsP_3Rs can also be downregulated via proteolysis of the receptor, with the effect of decreasing the sensitivity of Ca^{2+} release (Sipma *et al.*, 1998; Wojcikiewicz, 1995; Wojcikiewicz *et al.*, 1994).

The aim of the work of this chapter is to characterise the expression of the InsP_3R isoforms in mouse preimplantation development. This will be achieved by examining receptor levels and monitoring the time-course of changes in expression pattern, from the unfertilised oocyte through fertilisation to the formation of the blastocyst.

3.2 CHARACTERISATION OF ANTISERA: CT1, CT2 AND CT3 ANTIBODIES RECOGNISE InsP₃Rs IN MOUSE TISSUES

To examine expression levels of the three known InsP₃R isoforms, I used CT1, CT2 and CT3 which are previously characterised peptide polyclonal, affinity-purified, subtype-specific antibodies to type I, II and III InsP₃R, respectively (Wojcikiewicz, 1995). These antibodies had been raised against rat peptide sequences and had previously been shown, using quantitative immunoblotting, to recognise InsP₃Rs in a range of rat tissues and in rat and human cultured cell lines (Wojcikiewicz, 1995). In order to carry out Western analysis and immunocytochemistry on the three isoforms in mouse oocytes and embryos, I tested these antibodies on mouse pancreas and testis/*vas deferens* preparations. These mouse tissues were predicted, due to mouse mRNA (De Smedt *et al.*, 1997) and rat protein (Wojcikiewicz, 1995) relative abundance figures, to express significant levels of at least one InsP₃R isoform. Rat pancreas has almost equal proportions of types II and III InsP₃R with insignificant levels of type I, rat testis has roughly equal concentrations of all three and rat *vas deferens* has 80% type I (Wojcikiewicz, 1995).

Semiquantitative Western analysis was carried out on 50µg of whole rat brain, mouse pancreas or mouse testis/*vas deferens* preparations (prepared and assayed for protein content as described in Materials and Methods). Rat brain (96% type I InsP₃R) was included in the analysis to control for the electrophoresis and immunoblotting procedures. The antibodies were used at dilutions known to provide a similar staining intensity and thus chemiluminescence from equal amounts of purified InsP₃R (Wojcikiewicz, 1995). Rat brain showed a strongly immunoreactive band of the appropriate size with CT1 and a weaker band with CT2 (**Fig. 3.1**), confirming results of the previous analysis of rat brain (Wojcikiewicz, 1995). No band of the appropriate molecular mass was produced with CT3. However, CT3 recognised other bands relating to smaller proteins (largest of these is shown), presumably due to non-specific binding of the antibody rather than it recognising proteolytic products of the InsP₃R, since type III was not found in rat brain microsomes in the previous study (Wojcikiewicz, 1995). This is also consistent with observations in the previous study that CT1 and CT2 label a single band on gels corresponding in size to type I and II InsP₃Rs, whereas CT3 sometimes labels an additional band corresponding to a protein about 30kDa smaller (Wojcikiewicz, 1995).

Each antibody recognised a band of approximate molecular mass 260kDa, indicated by the arrow, in at least one mouse tissue, thereby confirming its ability to recognise mouse InsP₃R (**Fig. 3.1**). The analysis also confirmed that the relative protein expression levels of each subtype are similar between mouse and rat species; for both mouse tissue preparations tested, CT1, CT2 and CT3 behaved similarly with these tissues to as previously described in the rat (Wojcikiewicz, 1995). Immunoreactive bands of similar

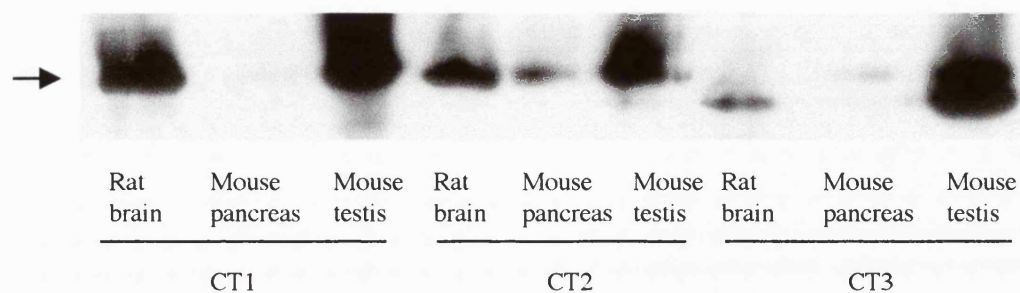


Figure 3.1 CT1, CT2 and CT3 isoform-specific peptide polyclonal antibodies recognise InsP₃Rs in mouse tissues. Whole rat brain, mouse pancreas or mouse testis (plus *vas deferens*) preparation (50 μ g protein per lane) was subjected to electrophoresis in a 5% gel and immunoblotted with CT1, CT2 or CT3, which specifically recognise type I, II and III InsP₃Rs, respectively. The antibodies were used at dilutions known to provide a similar staining intensity and thus chemiluminescence from equal amounts of purified InsP₃R standards (Wojcikiewicz, 1995). The arrow indicates the band position of the InsP₃R isoforms identified. Other immunoreactive bands probably represent non-specific binding of antibodies. Each antibody recognised a band of approximate molecular mass 260kDa in at least one mouse tissue, thereby confirming its ability to recognise mouse InsP₃R. Analysis is representative of 2 replicate experiments.

intensity for types II and III were found in mouse pancreas, with a lower level of type I, as in the rat pancreas. Also, in the combined sample of mouse testis and *vas deferens*, significant immunoreactivity was found for all three subtypes, especially type I, as predicted from the relative proportions in the same rat tissues. CT3 also recognised a smaller protein in mouse testis/*vas deferens*, of the same size as that recognised by this antibody in rat brain and to a very small extent in mouse pancreas, consistent with this particular antibody being less specific for the InsP₃R than CT1 and CT2. The relative expression of the InsP₃R isoforms at the protein level in the mouse pancreas and testis/*vas deferens* in my results are also in good general agreement with a determination of relative expression levels of InsP₃R mRNA isoforms in the same mouse cell types (De Smedt *et al.*, 1997).

3.3 EXPRESSION OF InsP₃R PROTEIN IN OOCYTES

3.3.1 The expression levels of InsP₃R isoforms in mature mouse oocytes

I first examined whether the levels of mRNA expression in the mature mouse oocyte reflected the relative abundance of each isoform of the protein. It has previously been shown that mouse oocytes express mRNAs for all three known InsP₃R subtypes (Parrington *et al.*, 1998). A semiquantitative ratio reverse-transcriptase PCR technique (De Smedt *et al.*, 1994; De Smedt *et al.*, 1997) shows that the type II isoform is the predominant message in mature MII oocytes, representing 67% of the InsP₃R mRNA but that after fertilisation and cleavage to the 2-cell stage types I and II mRNA constitute 40-50% each of total InsP₃R mRNA (Parrington *et al.*, 1998). In order to examine the expression of InsP₃R protein in oocytes I used semiquantitative Western analysis on 300 mature oocytes. Having confirmed that antibodies CT1, CT2 and CT3 were able to recognise mouse InsP₃Rs (section 3.2) I used these antibodies as well as Ab40, another type I-specific peptide polyclonal antibody (Zaidi *et al.*, 1995). Antibody concentrations of CT1-3 were used which would provide similar staining intensity and thus chemiluminescence from equal amounts of purified InsP₃R (Wojcikiewicz, 1995).

Both type I-specific antibodies recognised a single protein band corresponding to a molecular mass of approximately 260kDa (**Fig. 3.2**). This size is in agreement with a previous study in which CT1 recognised purified electrophoresed type I InsP₃R (Wojcikiewicz, 1995), therefore confirming that the band in my experiment corresponds to type I InsP₃R protein. In contrast, neither type II nor III InsP₃R was detected in 300 oocytes, despite CT2 and CT3 having proven immunoreactivity with mouse InsP₃Rs. Immunoreactivity remained undetected even after accumulated exposure of chemiluminescence to photographic film for one hour. This data supports previous work where similar analyses were carried out with only 185 oocytes per lane, in which type I was the only detectable isoform (Parrington *et al.*, 1998). My results, with a greater

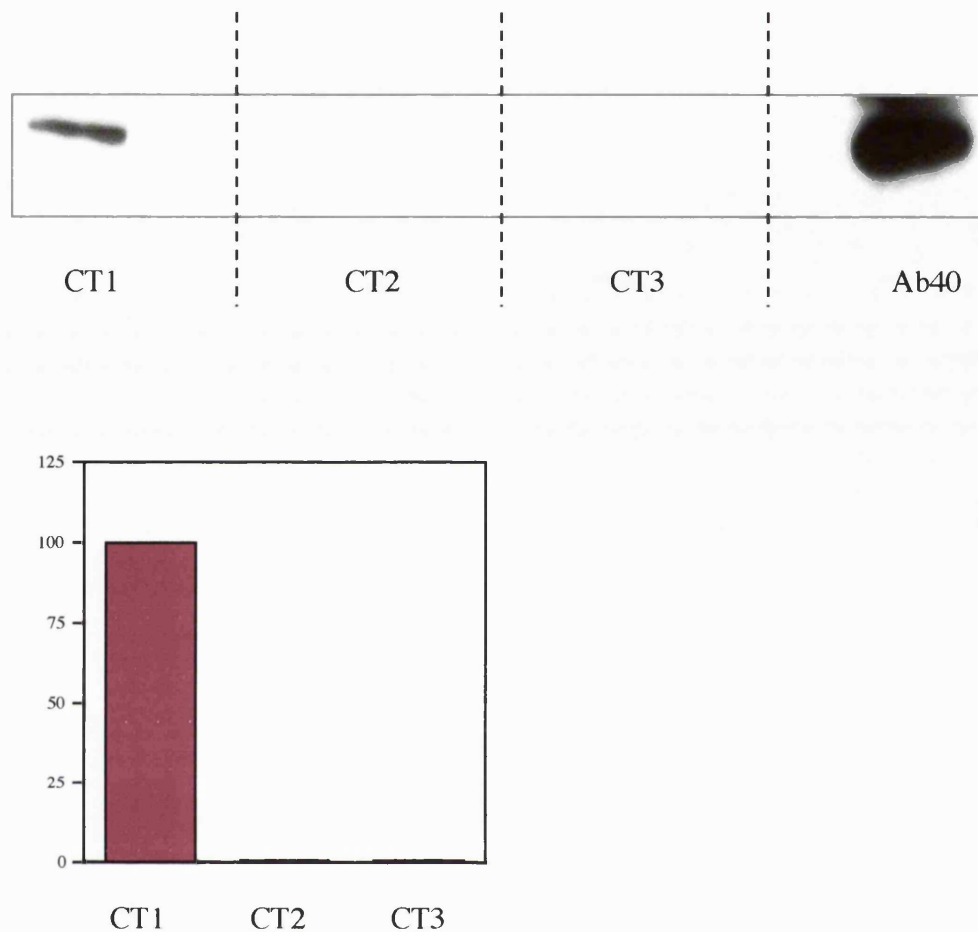


Figure 3.2 The type I InsP_3R is the only InsP_3R protein expressed in mature mouse oocytes. Semiquantitative Western analysis of InsP_3Rs in mature mouse oocytes. Protein from 300 mature mouse oocytes was subjected to electrophoresis. Replicate lanes on the protein blot were separated and immunoblotted with either CT1, CT2 or CT3, which specifically recognise the type I, II and III receptors, respectively. The antibodies were used at dilutions known to provide a similar staining intensity for equal amounts of purified InsP_3R . Ab40, another type I-specific antibody, was used as a control. Data are representative of 3 independent analyses. Both CT1 and Ab40 recognised a protein band of the appropriate molecular mass. Neither CT2 nor CT3 recognised InsP_3R protein in 300 oocytes.

number of oocytes, show that the type I InsP₃R is the predominant InsP₃R protein expressed in mature mouse oocytes.

3.3.2 The concentration of InsP₃Rs in mature mouse oocytes is similar between strains

In order to apply my results to mice as a species rather than confine them to the MF1 strain I carried out Western analysis on 60 MII oocytes of MF1 and F1 strains. Immunoreactivity with the type I specific antibody CT1 was similar between the strains, suggesting that the concentration of InsP₃Rs in mature mouse oocytes does not alter significantly between strains (**Fig. 3.3**).

3.3.3 The relative concentration of InsP₃Rs in mouse cerebellum, whole brain and mature oocytes

Having established that the type I InsP₃R is the only InsP₃R isoform significantly expressed in mature mouse oocytes I wanted to obtain a value for the relative abundance of InsP₃Rs (type I InsP₃R) in the mature oocyte. It is known that in rat cerebellum total InsP₃R protein concentration is over 10-fold higher than in other rat tissues examined and that InsP₃R protein is 99% type I (Wojcikiewicz, 1995). In agreement with this protein data, in *mouse* cerebellum over 90% of the total InsP₃R mRNA is of the type I InsP₃R (De Smedt *et al.*, 1997). Therefore I compared InsP₃R protein levels in whole mouse cerebellum with those in mature mouse oocytes using semiquantitative Western analysis. I used a type I-specific antibody (Ab40) since in both cerebellum and mature oocytes type I is the predominant isoform expressed. As an additional standard for comparison I used whole mouse brain which, due to it comprising a high proportion of cerebellum, should also express a significant level of type I InsP₃R. (Rat brain contains 96% type I protein (Wojcikiewicz, 1995) and mouse brain contains 69% type I mRNA (De Smedt *et al.*, 1997).)

In Western analyses with both cerebellum and whole brain a range of tissue preparation dilutions were electrophoresed next to lanes of oocytes (**Fig. 3.4**). The results from the first blot show that the level of immunoreactive type I InsP₃R in 210 MII oocytes lies between that for 0.5 and 0.1µg of cerebellum protein and that immunoreactivity in 47 oocytes is similar to that for 0.1µg of cerebellum protein (**Fig. 3.4.Aa and b**). Therefore I can estimate the relative concentration of type I InsP₃R in MII oocytes relative to that in cerebellum. Since each mouse oocyte contains about 25ng of protein (Gosden *et al.*, 1997), 47 oocytes contain about 1µg of total protein. Therefore, there is the same amount of InsP₃R in 0.1µg of cerebellum as 1µg of oocyte protein. Thus, there must be an approximately 10-fold higher concentration of type I InsP₃R in mouse cerebellum than in MII oocytes.

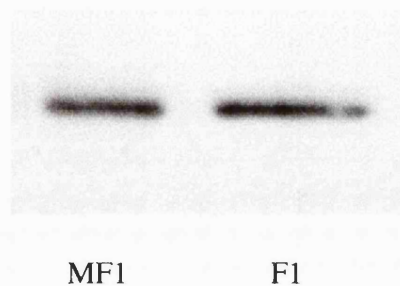


Figure 3.3 The concentration of InsP_3Rs in mature mouse oocytes is similar in MF1 and F1 strains. Protein from 60 MII oocytes of MF1 or F1 strains was subjected to electrophoresis and immunoblotted with CT1 antibody which specifically recognises type I InsP_3R . Immunoreactivity is similar in the two strains.

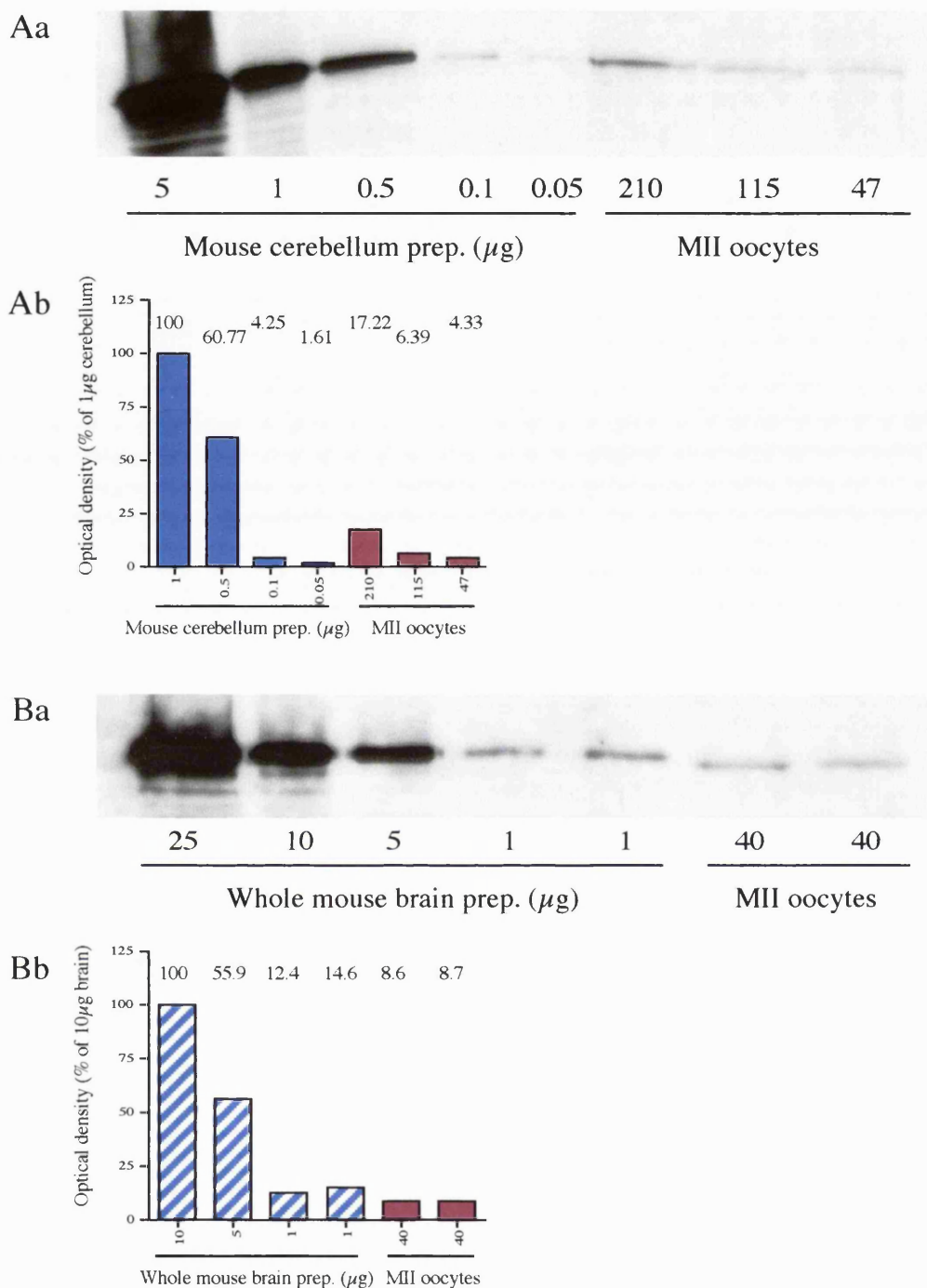


Figure 3.4 The relative concentration of InsP_3Rs in mouse cerebellum, whole brain and MII oocytes. A dilution series of mouse cerebellum preparation (0.05–5 μg total protein) (A) or mouse whole brain preparation (1–25 μg total protein) (B) was subjected to electrophoresis and immunoblotted with type I-specific Ab40 next to lanes of MII oocytes (A and B). Immunoreactive type I InsP_3R bands were quantified by densitometry using NIH image as described in section 3.5 (Ab and Bb). Optical density of each band is expressed as a percentage of that obtained with 1 μg of cerebellum protein (A) or 10 μg of whole brain protein (B); percentage values are also indicated above each column for ease of reference. Note that 5 μg of cerebellum and 25 μg whole brain are not used as reference samples due to the high amount of protein in each causing saturation of the ECL detection system so that corresponding optical density values do not relate to true amount of InsP_3R . (A) Immunoreactivity in 47 MII oocytes is similar to that for 0.1 μg of cerebellum protein. (B) Immunoreactivity in 40 MII oocytes is roughly two-thirds of that for 1 μg of total brain protein. Each immunoblot is representative of 2 replicate analyses.

In order to confirm this approximate relative concentration I compared mature oocytes with whole mouse brain. The results from the second blot show that the level of immunoreactive type I InsP₃R in 40 MII oocytes is roughly two-thirds of that for 1µg of mouse brain total protein (**Fig. 3.4.Ba and b**). Since the mass of protein in 40 oocytes is approximately 1µg (Gosden *et al.*, 1997), there must be a roughly 1.5-fold higher concentration of type I InsP₃R in whole mouse brain than in MII oocytes. Thus the ratio of InsP₃R concentration in mouse cerebellum to whole brain to MII oocytes is 10:1.5:1.

3.4 EXPRESSION OF InsP₃R ISOFORMS DURING PREIMPLANTATION DEVELOPMENT: LEVELS OF TYPE I PROTEIN DECREASE AFTER FERTILISATION

Having found that mature mouse oocytes express high levels of type I InsP₃R I next investigated how the pattern of expression of the three subtypes changes during preimplantation development. As described above, type II constitutes the majority and roughly half of InsP₃R mRNA present in the MII oocyte and in the fertilised egg, respectively. Thus I considered whether there would be an increase in types II and III protein after fertilisation resulting from translation of the abundant type II (and less abundant type III) message. It was conceived possible that the type II and III mRNAs were acting as stored maternal transcripts for expression during later development. To investigate this possibility Western analysis was carried out on MII oocytes alongside late 2-cell embryos and 8-cell embryos/early morulae (**Fig. 3.5.A**). A second analysis was carried out on MII oocytes alongside 8-cell embryos and blastocysts to achieve coverage of the whole of preimplantation development (**Fig. 3.5.B**). In both analyses 200 eggs or embryos were used per lane. To control for the electrophoresis and immunoblotting protocols, lanes of rat brain or mouse pancreas tissues were used with isoform-specific antibodies CT1-3 where they had been proven in earlier work to be immunoreactive with each antibody. To minimise the number of eggs and embryos required for these experiments the first blot was prepared and probed sequentially with CT2 and CT3. The blot was then stripped as described in Materials and Methods and probed with CT1. Both blots were probed with CT1 last, due to the higher predicted amount of type I InsP₃R, suggested after analysis of MII oocytes, which would be less affected by the blot-stripping conditions. To control for repeated washing of the blot causing loss of protein, the second blot was probed for type II and III isoforms in the reverse order.

Antibodies CT2 and CT3 confirmed that expression of types II and III could not be detected in oocytes and revealed that neither isoform was upregulated to detectable levels throughout preimplantation development (**Fig. 3.5**). Immunoreactivity remained undetected even after accumulated exposure of chemiluminescent blots to photographic film for one hour. Stripping the blots and reprobing with CT1 confirmed that the type I protein

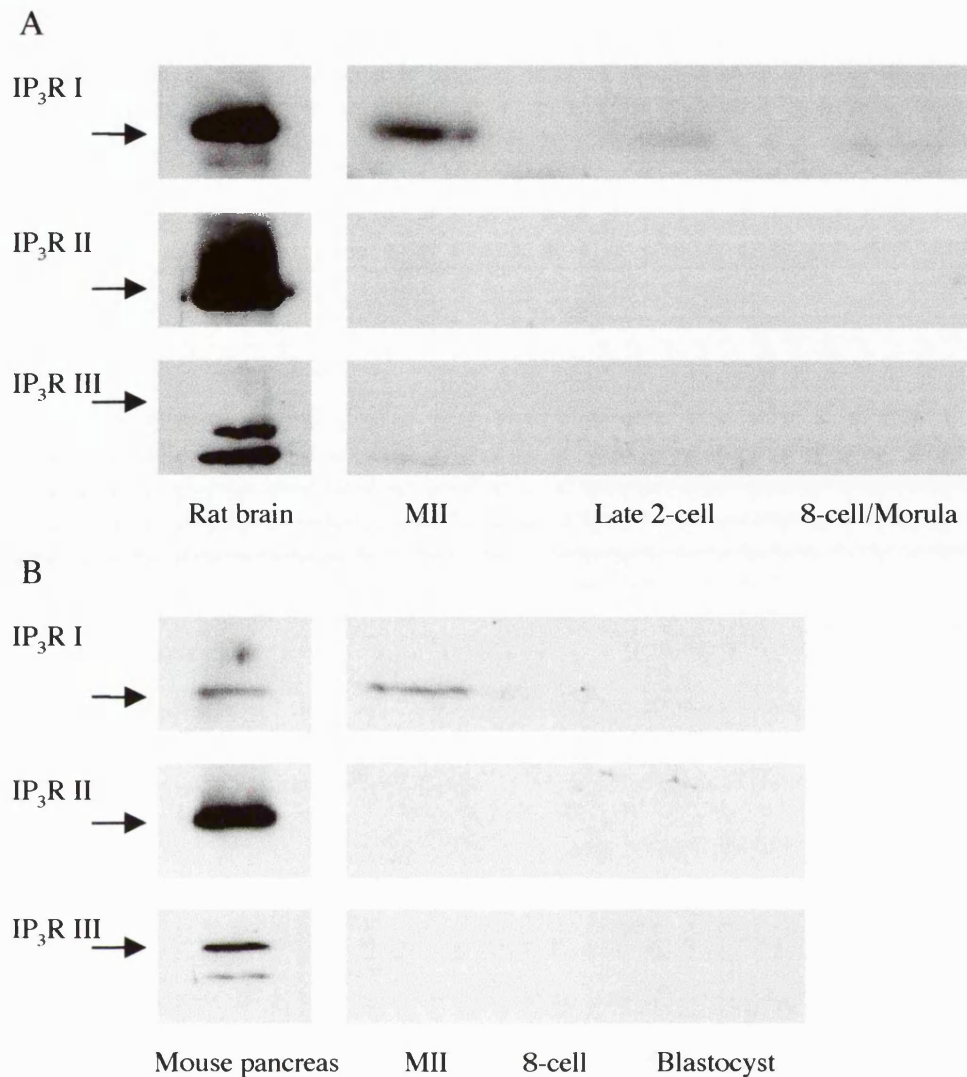


Figure 3.5 The expression of InsP₃Rs during preimplantation development. (A) Proteins from MII oocytes, late 2-cell embryos and 8-cell embryos/early morulae (200 of each) and whole rat brain (control) were subjected to electrophoresis and blotted with CT1, CT2 and CT3. The arrows indicate the InsP₃R isoforms identified by the subtype-specific antibodies. Note that my early results (Fig. 3.1) had confirmed that type III InsP₃R is not found in rat brain but had shown that CT3 recognises smaller non-specific proteins which are duplicated here to provide evidence that CT3 is recognising the usual pattern of rat brain proteins. The same gel was used for the analysis by probing the blot first with CT2 followed by CT3 and after stripping the blot, CT1. (B) A repeat Western analysis as in (A) but with MII oocytes, 8-cell embryos and blastocysts (200 of each) and mouse pancreas (control). The same gel was used for the analysis by probing the blot as for (A) but with CT2 and CT3 in the reverse order. There was no evidence for the expression of the type II or III InsP₃R isoforms in preimplantation development, even after exposure of blots to film for one hour. There was a dramatic decrease in the level of type I at the late 2-cell stage compared to mature oocytes, with no return to prefertilisation levels detected during preimplantation development.

was indeed present in MII oocytes but revealed a dramatic decrease in the level of type I at the late 2-cell stage compared to mature MII stage oocytes (**Fig. 3.5.A**). Furthermore, the type I protein was elevated in oocytes compared to all other stages, with no return to prefertilisation levels detected during preimplantation development (**Fig. 3.5.A and B**). It should be noted that *some* type I could be detected at the late 2-cell stage and the 8-cell/morula stage (not detectable in figure) but by the blastocyst stage type I protein was undetectable. Thus the type I InsP₃R is specifically upregulated in oocytes.

3.5 RELATIONSHIP BETWEEN WESTERN ANALYSIS BAND INTENSITY AND AMOUNT OF InsP₃R PROTEIN

In order to interpret my Western analyses semiquantitatively rather than simply qualitatively I needed to establish the relationship between the intensity of immunoreactive bands after ECL detection and the relative quantity of InsP₃R protein. This was investigated by analysing immunoreactive bands after Western blotting a series of samples of MII mouse oocytes of decreasing numbers of oocytes and probing with Ab40 (**Fig. 3.6.A**). NIH image was used to measure mean pixel intensity and total number of pixels within each band. After subtraction of background, the mean pixel intensity and total pixel number were multiplied to give a value for the summed pixel intensity (above background) of each band. This is referred to as optical density (O.D.). Each optical density value was then expressed as a percentage of the optical density obtained for the reference sample run on the same gel. This calculation was carried out on all Western blots in my subsequent experiments. In this particular experiment I used the sample of 30 oocytes as my reference sample (**Fig. 3.6.B and C**).

Samples of MII oocytes ranging in number from 30 to 3 resulted in immunoreactive bands which showed a gradual decrease in intensity (**Fig. 3.6.A**). The plot of relative optical density against number of oocytes could be fit with a straight line (**Fig. 3.6.C**) which demonstrated that a linear relationship exists between optical density and amount of InsP₃R protein over the range observed. Since the band corresponding to 10 oocytes was just detectable, I can infer that in order to analyse InsP₃R in oocytes using Ab40 I must use at least 10 oocytes per Western lane to detect an immunoreactive band. The same analysis was carried out using another type I InsP₃R-specific antibody, CT1, and also revealed a directly proportional relationship between band intensity and amount of InsP₃R loaded onto gels, over a range of 20 to 100 oocytes (data not shown). With this less sensitive antibody the minimum number of oocytes in which InsP₃R protein could be detected was 20.

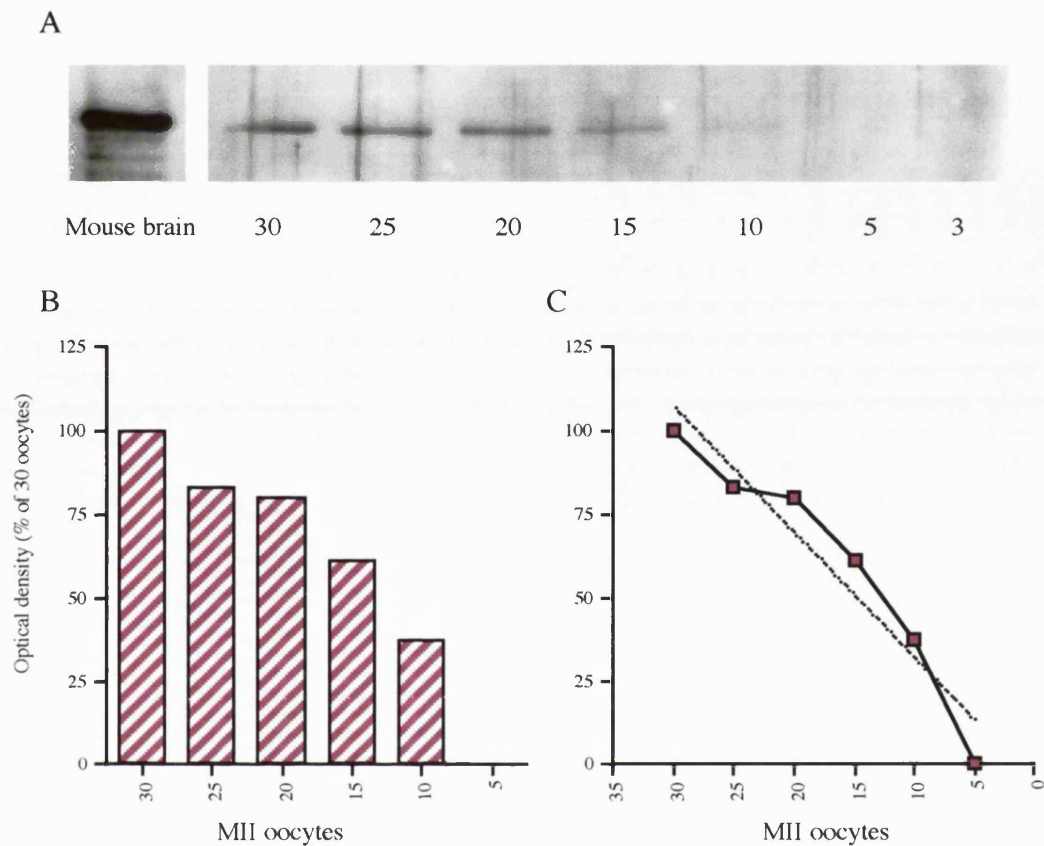


Figure 3.6 There is a linear relationship between optical density of immunoreactive bands and amount of InsP_3R protein. (A) Protein from a series of samples of MII mouse oocytes with 3 to 30 oocytes, was subjected to electrophoresis and blotted with Ab40 next to a lane of $5\mu\text{g}$ mouse brain protein. Immunoreactive type I InsP_3R bands were quantified by densitometry using NIH image as described in 3.5. Optical density of each band is expressed as a percentage of that obtained with 30 oocytes, in the form of a column chart (B) and a line graph with a line of best fit (C). Analysis shows a directly proportional relationship between band intensity and amount of InsP_3R , over this range of oocyte number. The minimum number of oocytes in which InsP_3R protein could be detected was 10.

3.6 IN VITRO FERTILISATION OF ZONA-FREE OOCYTES: REDUCING POLYSPERMY

In order to focus on the observed downregulation in the level of type I InsP₃R protein by the late 2-cell stage of development (see 3.4 and following sections) I needed to conduct experiments using fertilised eggs and embryos. The timing of fusion between oocyte and sperm at fertilisation can vary significantly between oocytes. Much of this variation in timing is due to the zona pellucida which acts as a temporary obstacle to the sperm, penetration through the zona taking between 30 minutes and 2 hours. In order to obtain synchrony in developmental timing I removed the zona of each oocyte just prior to fertilisation. However, this presented me with a new problem since the zona-reaction is an important mechanism used by the newly-fertilised egg to prevent fertilisation by additional sperm, known as polyspermy. I therefore conducted preliminary experiments to investigate conditions necessary to minimise polyspermy of zona-free oocytes. This was achieved using dilutions of capacitated sperm and varying the length of incubation with the sperm. 10µl of a 1ml suspension, containing sperm obtained from both epididymii of a single male mouse, was fixed in 90µl paraformaldehyde; sperm counts revealed a mean suspension concentration of 10-20 million sperms/ml. I used Hoechst staining and phase contrast microscopy to count pronuclei formed, 5 hours after sperm addition, for each sperm dilution and incubation length. It should be noted that a low level of polyspermy may not be detrimental to my analysis of InsP₃R levels since, in *Xenopus* eggs, conditions that produce various levels of polyspermy are associated with InsP₃ increases similar to those detected after monospermic fertilisation (Stith *et al.*, 1994). Also, experiments showed that polyspermic fertilisation does not apparently modify InsP₃R downregulation since a similar time-course of type I downregulation was observed after fertilisation of zona-intact oocytes, where 95% of fertilisation is monospermic (not shown).

Diluting 15µl of a 10-20 million sperms/ml suspension (of capacitated sperm) into 200µl to produce a final concentration of 0.7-1.4 million sperms/ml resulted in all 11 oocytes in the insemination dish being polyspermic with 3-4 pronuclei, with therefore 2-3 male pronuclei and the female pronucleus (**Table 3.1**). Polyspermy at this sperm concentration was unaffected by halving length of incubation with sperm, from 60 to 30 minutes. Diluting only 3µl of the initial sperm suspension to produce a final concentration of 0.15-0.3 million sperms/ml and using incubation lengths of 60 and 30 minutes resulted in 2 out of 4 and 5 out of 8 oocytes respectively being polyspermic. On the other hand, an incubation of only 15 minutes at this concentration resulted in only 1 out of 6 oocytes being polyspermic. 5 out of 6 of these oocytes had a single male pronucleus and a female pronucleus showing that they had been fertilised by a single sperm. Since all 6 oocytes were fertilised, I used this dilution and a 10-15 minute incubation period for subsequent experiments. This

Concentration of sperm (million sperms/ml)	Incubation length (min)	No. of oocytes in insemination dish	No. of oocytes with pronuclei (5h) (male and female)		
			No Pn	2 Pn	3-4 Pn
0.15-0.3	15	6	0	5	1
	30	8	2	1	5
	60	4	0	2	2
0.7-1.4	30	6	0	0	6
	60	5	0	0	5

Table 3.1 The rate of polyspermy in zona-free oocytes incubated with variable dilutions of sperm for variable time. Pn refers to pronucleus (male or female). Pronuclei were counted 5 hours after sperm addition using both Hoechst staining and phase contrast microscopy. Note that oocytes were zona-pellucida-free. The table indicates that a suitable protocol to minimise polyspermy would be incubation of zona-free oocytes with 0.15-0.3 million sperms/ml for 15 minutes.

allowed me to investigate the timing of the decrease in InsP₃Rs in a synchronous group of zona-free oocytes.

3.7 TIME-COURSE OF THE TYPE I InsP₃R DECREASE AFTER FERTILISATION

3.7.1 The level of type I InsP₃R decreases within eight hours of fertilisation

I investigated in more detail the downregulation of type I InsP₃R protein that I previously showed to take place by the late 2-cell stage. In order to determine at which point in development during progression to the 2-cell stage this decrease takes place, I initially carried out Western blotting on pronucleate embryos frozen for analysis 8 hours after exposure to sperm. 180 pronucleate embryos were subjected to Western analysis parallel to controls of MII oocytes and age-matched MII oocytes which had been incubated alone in T6 culture medium (**Fig. 3.7.A**). Analysis of type I InsP₃R levels indicated that the level of protein was similar in pronucleate and late 2-cell embryos, suggesting that downregulation was complete 8 hours after addition of sperm (**Fig. 3.7.B**). Similar results were obtained using two type I-specific antibodies, Ab40 (n=4) and CT1 (n=3). This decrease in type I InsP₃R protein was specific to fertilised embryos as unfertilised oocytes incubated (aged) in T6 medium for the same amount of time (8 h) showed roughly similar levels of protein as the freshly ovulated oocytes (**Fig. 3.7.A and B**). Collating results from between 6 and 14 Western analyses for aged eggs or fertilised embryos showed that the only significant change in InsP₃R levels occurred between MII stage oocytes and pronucleate embryos (8 hours after exposure to sperm) (**Fig. 3.7.C**).

3.7.2 Immunolocalisation of type I InsP₃R before and eight hours after fertilisation

To examine whether there was any spatial regulation in the loss of type I InsP₃R after fertilisation, I localised type I using the previously characterised CT1 antibody and confocal microscopy (**Fig. 3.8**). After staining with CT1, immunoreactive protein was distributed throughout the MII oocyte cytoplasm (**Fig. 3.8.A**). The meiotic spindle (section through spindle not shown) was devoid of any staining in all 12 of the mature oocytes examined. Individual bright spots, possible non-specific clustering of antibodies, overlay a network-like pattern of staining which may reflect the presence of type I InsP₃R distributed throughout the endoplasmic reticulum. No staining was detectable in the absence of the antibody, suggesting that the immunoreactivity was specific (**Fig. 3.8.C**). Furthermore, the staining was neutralised by the peptide antigen used to raise the antibody (not shown). Staining with CT2 antibody did not label the mature oocytes, consistent with my Western analysis result.

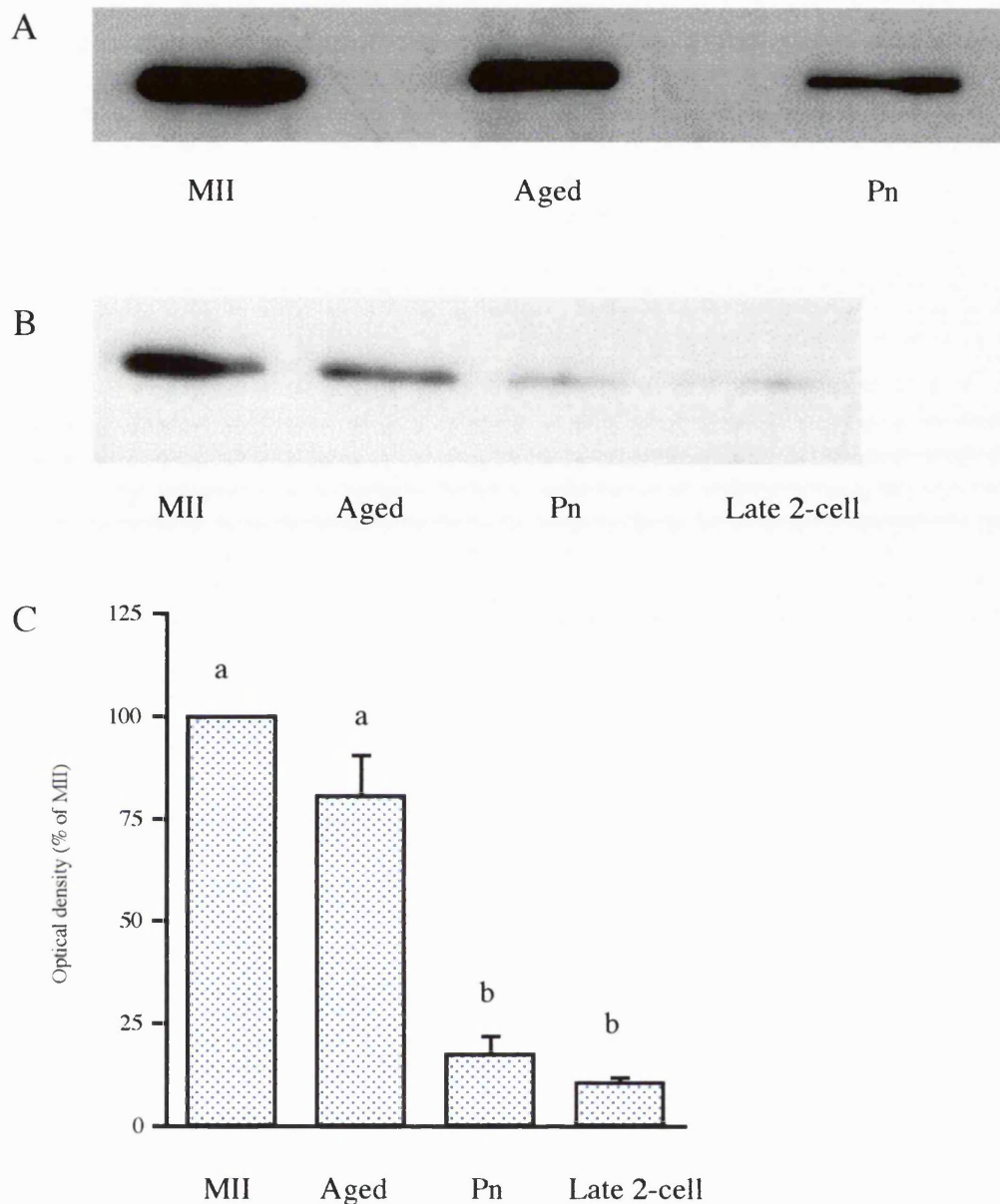
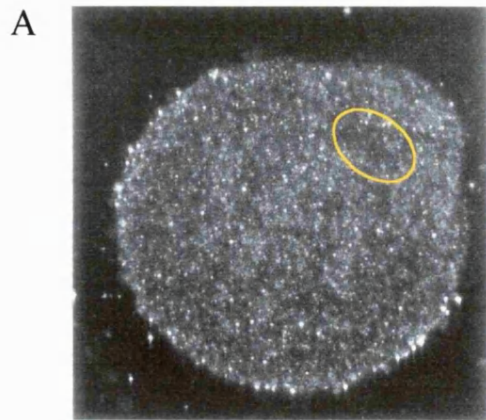
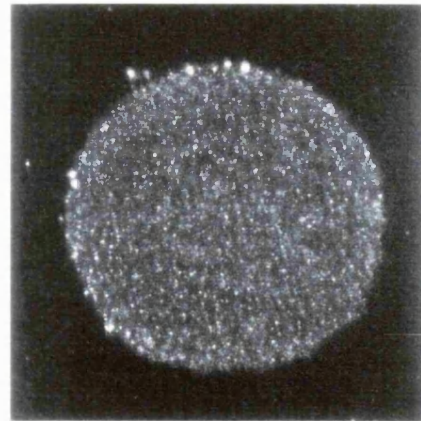


Figure 3.7 The level of type I InsP_3R decreases within eight hours of fertilisation. Western analysis of InsP_3Rs in fertilised pronucleate (Pn) embryos collected 8 hours after exposure to sperm, and late 2-cell embryos. Protein from 180 MII oocytes, age-matched MII oocytes (Aged), pronucleate (Pn) embryos or 2-cell embryos was subjected to electrophoresis and immunoblotted with the type I-specific antibody CT1. (A) Pn stage embryos show a decrease in type I InsP_3R levels but age-matched unfertilised control oocytes show similar levels of InsP_3R as freshly ovulated MII oocytes. (B) The decrease in type I levels by Pn stage is as extensive as that seen by the late 2-cell stage. The data in (A) and (B) is representative of at least 3 experiments for each stage of development, using both Ab40 and CT1 antibodies. (C) Optical densities of immunoreactive type I bands from up to 14 Western analyses for aged eggs or fertilised embryos were calculated using NIH image as described in section 3.5. Bars with common superscripts are not significantly different.

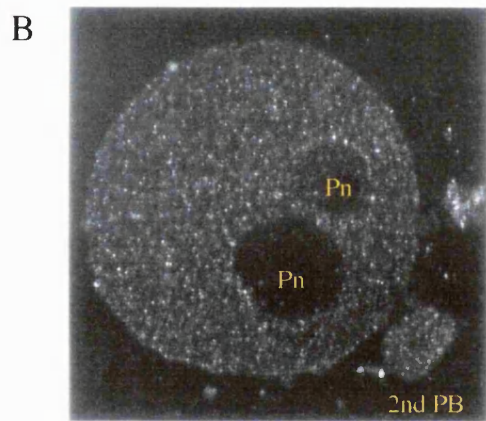
Figure 3.8 Immunolocalisation of type I InsP₃R before and after fertilisation. The distribution of type I InsP₃R was examined in ovulated MII oocytes and pronucleate (Pn) embryos fixed 8 hours after fertilisation, using the type I-specific antibody CT1 and confocal microscopy. (A) Subcortical and cortical sections are shown. Immunoreactive protein is distributed throughout the MII oocyte cytoplasm. The approximate position of the spindle (outside section) is indicated. (B) Immunoreactive protein remains dispersed throughout the fertilised Pn embryo cytoplasm and the second polar body but is absent from the pronuclei. In the absence of primary antibody (C) or after preadsorption with the peptide antigen (not shown), there was no detectable staining. (D) Mean pixel intensities taken from projected images of 5 MII and fertilised oocytes reveals a significant difference in staining intensity (t-test: $P < 0.01$).



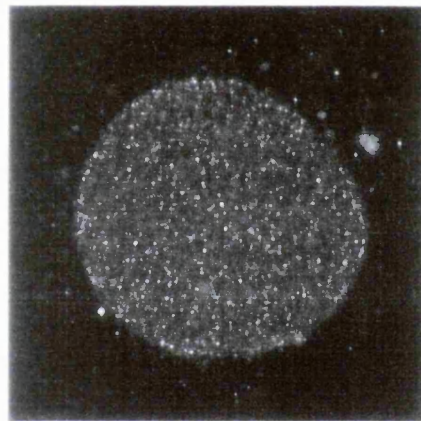
Metaphase II



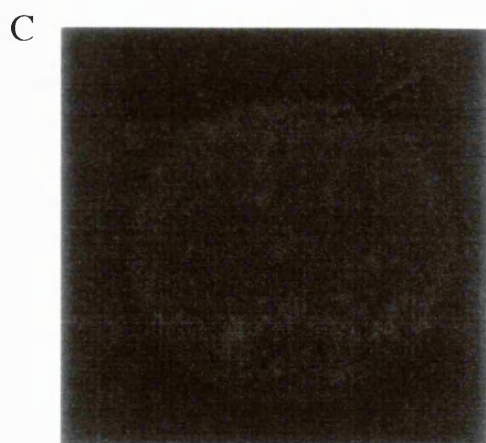
Metaphase II (cortex)



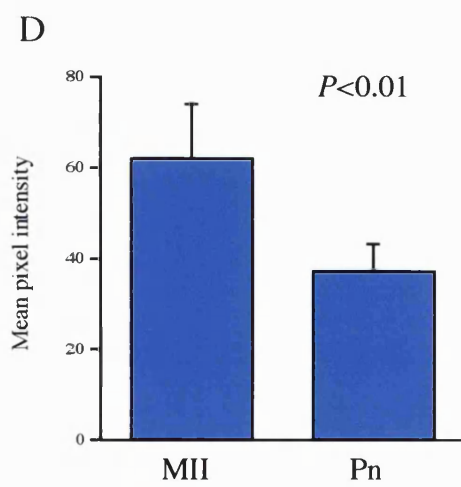
Fertilised Pn stage



Fertilised Pn stage (cortex)



Control



In fertilised pronucleate embryos (fixed 8 hours after exposure to sperm) the pattern of immunofluorescent staining remained dispersed throughout the cytoplasm and, as expected, was not present within the pronuclei (**Fig. 3.8.B**). After identical processing and at the same confocal settings it was observed that the staining intensity was consistently (9/9) decreased in fertilised embryos (8 hours after fertilisation), consistent with the data obtained from the Western blots (**compare Fig. 3.8.A and B**). Unfertilised but aged eggs had a level of staining of a similar intensity to that of freshly-ovulated eggs (not shown). Mean pixel intensities taken from projected images of 5 unfertilised and fertilised oocytes confirmed the difference in staining intensity (62 ± 12 and 37 ± 6 , respectively; t-test: $P < 0.01$) (**Fig. 3.8.D**).

3.7.3 Type I InsP₃R expression is downregulated over the four hours following sperm fusion

I had so far demonstrated that type I InsP₃Rs decrease in level within 8 hours of exposure to sperm. Since fusion of the sperm with the egg takes place very quickly in the absence of a zona pellucida this suggests that downregulation occurs within 8 hours of fertilisation itself. To determine whether the decrease correlates with any of the events of egg activation the time-course of the decrease in immunoreactivity was examined with greater accuracy. Oocytes were incubated for 15 minutes with capacitated sperm and returned to culture for 1-8 hours. At various time points throughout the culture period fertilised oocytes were removed and frozen in preparation for Western analysis.

The subsequent Western blots revealed that by 2 hours after exposure to sperm the levels of InsP₃R had decreased to approximately half the control levels. By 4 hours the levels were 20-30% and by 6 hours the maximal decrease to 20% of control levels of immunoreactivity had been achieved (**Fig. 3.9.A and B**). A separate blot confirmed that there was no significant further decrease in protein after 4 hours up until at least 7.5 hours post fertilisation (not shown). This indicates that the loss of InsP₃R protein occurs gradually over the 4 hours following fertilisation and that the protein level then plateaus at this lower level.

3.8 BOVINE TYPE I InsP₃R INCREASES DURING OOCYTE MATURATION AND DECREASES AFTER FERTILISATION AS IN THE MOUSE

In order to investigate whether the decrease in type I InsP₃R protein after fertilisation of the mouse oocyte also took place in other species I examined fertilised bovine eggs. I also investigated whether there was any change in the level of type I expression during bovine oocyte maturation. Western analysis was carried out on bovine GV stage and MII oocytes and pronucleate embryos (8 hours after fertilisation) (30 of each) parallel to a control of 80 mature mouse oocytes, using the CT1 antibody (**Fig. 3.10**). (This work was carried out

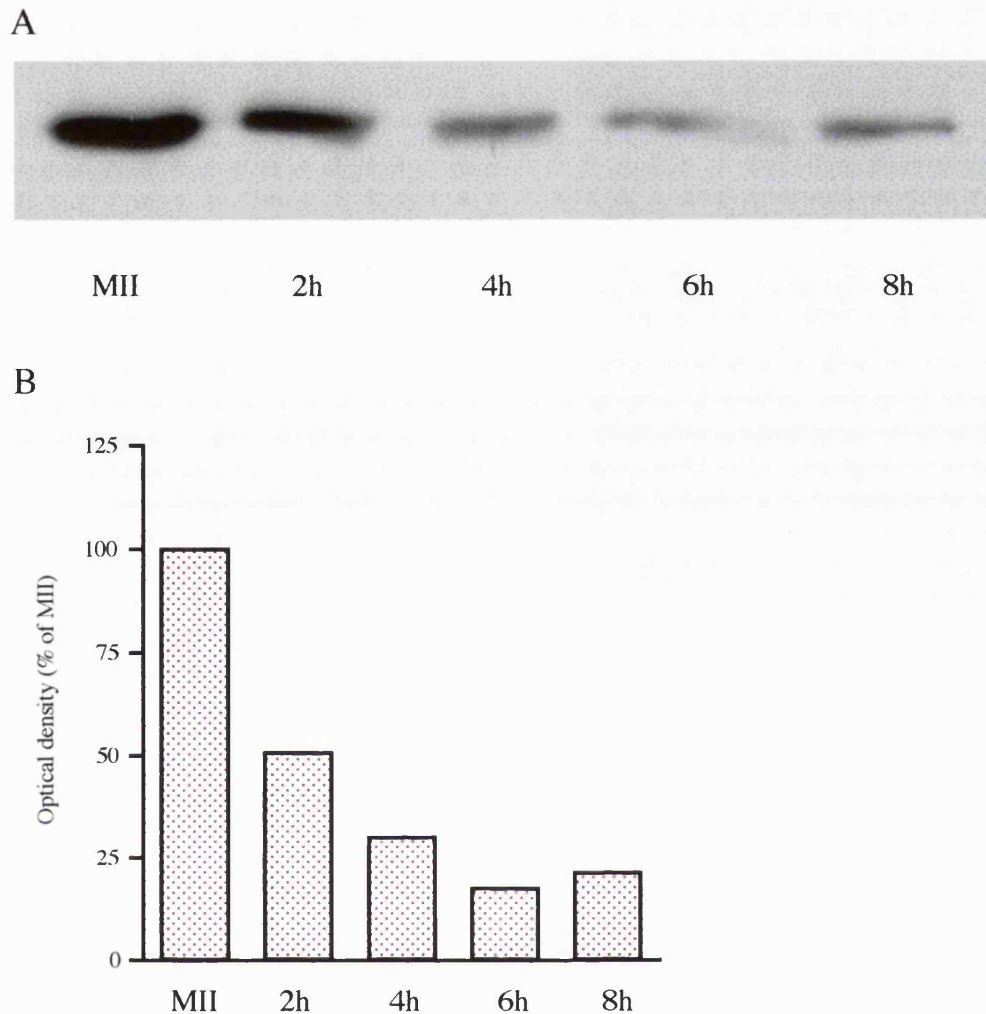
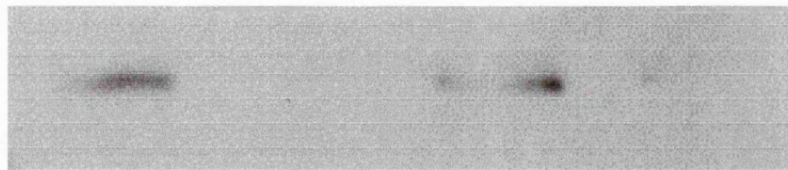


Figure 3.9 Type I InsP₃R expression is downregulated over the 4 hours after sperm fusion. (A) Western analysis of the InsP₃R in unfertilised (MII) oocytes and fertilised eggs frozen for analysis at 2-hour intervals after sperm addition to zona-pellucida-free eggs. Protein from 100 eggs was subjected to electrophoresis and immunoblotted with the type I-specific antibody CT1. (B) Immunoreactive type I InsP₃R bands were quantified by densitometry using NIH image as described in section 3.5. Data is representative of 3 independent analyses.



Mouse MII Bovine GV Bovine MII Bovine Pn

Figure 3.10 Bovine type I InsP_3R increases during oocyte maturation and decreases after fertilisation. Western analysis of type I InsP_3R during bovine oocyte maturation and after fertilisation. Protein from 30 bovine oocytes or embryos or 80 mouse MII oocytes was subjected to electrophoresis and immunoblotted with the type I-specific antibody CT1. During maturation from immature (GV) to mature (MII) oocytes there is an increase in type I InsP_3R immunoreactivity from undetectable to detectable levels. Pronucleate stage embryos (Pn) 8 hours after fertilisation show undetectable and therefore decreased levels of type I InsP_3R . Immunoreactivity for GV oocytes and Pn embryos remained undetectable even after a one hour exposure to film. Note that the level of immunoreactive protein is similar for 80 mouse MII oocytes and 30 bovine MII oocytes.

in collaboration with Michael Kane and Caroline Carolan, Department of Physiology, National University of Ireland, Galway, Ireland.)

The results show that since immunoreactivity is undetectable in both 30 bovine GV stage oocytes and Pn stage embryos (even after a one hour exposure) but is clearly detectable in 30 bovine MII oocytes, the level of type I InsP_3R protein increases during oocyte maturation and decreases by 8 hours after fertilisation. These preliminary findings are therefore consistent with the overall pattern of type I InsP_3R expression over this period in the mouse.

Furthermore, immunoreactivity in my analysis was similar for 80 mouse MII oocytes and 30 bovine MII oocytes (**Fig. 3.10**). Providing that CT1 has equal affinity for mouse and bovine InsP_3Rs , this suggests that bovine oocytes have 2-3 times as many InsP_3Rs as mouse oocytes, most likely due to the larger size of the bovine oocyte. The bovine oocyte is roughly 120 μm in diameter in contrast to the mouse oocyte which is roughly 80 μm across.

3.9 DISCUSSION

In this chapter I establish that mature mouse oocytes express predominantly the type I isoform of the InsP_3R . This isoform appears to be upregulated in oocytes and undergoes downregulation after fertilisation. This data suggests that the type I InsP_3R is regulated in a manner that reflects its critical role in fertilisation.

3.9.1 Antibodies CT1, CT2 and CT3 specifically recognise murine InsP_3Rs

Experiments were first undertaken in order to confirm that antibodies CT1, CT2 and CT3 were suitable for Western analysis and immunocytochemistry on mouse oocytes and embryos. These previously characterised affinity-purified polyclonal antibodies had been raised against rat peptide sequences and in Western analysis had been used to recognise InsP_3Rs in various human and rat cell lines and rat tissues (Wojcikiewicz, 1995). Nucleotide sequence identity between mouse and rat InsP_3R isoforms is 94-95% for all three known isoforms (De Smedt *et al.*, 1997). Therefore it was expected that CT1-3 would bind specifically to mouse as well as rat InsP_3Rs . My analysis of mouse pancreas and testis/*vas deferens* tissues, which are known to express significant levels of at least one InsP_3R isoform (Wojcikiewicz, 1995), revealed that each antibody was able to specifically recognise mouse InsP_3R .

The relative expression levels of InsP_3R isoforms are similar in mouse and rat species

There is literature on the relative abundance of InsP_3R isoforms in rat tissues in terms of protein expression (Wojcikiewicz, 1995) and in mouse tissues in terms of mRNA expression (De Smedt *et al.*, 1997) but not in mouse tissues in terms of protein expression. These previous two studies showed very good general agreement between a number of rat and mouse cell lines and tissues, suggesting that the mRNA expression is also relevant for protein levels of these isoforms. I applied antibodies at dilutions known to provide similar immunoreactivity from equal amounts of purified InsP_3R standards. Although the standards were purified from human (CT1) and rat (CT2 and CT3) cell lines, the antibodies were raised against C terminal peptides which are highly conserved between species (Wojcikiewicz *et al.*, 1994; Yamamoto-Hino *et al.*, 1994). It has been shown that the antibodies recognise rat and human InsP_3Rs with very similar or identical affinity (Wojcikiewicz, 1995) and it is probable that they bind mouse InsP_3Rs with similar affinity. Therefore I can interpret my analysis semiquantitatively in terms of relative protein expression of the three isoforms in these mouse tissues. I found that the relative protein expression levels of each InsP_3R subtype in pancreas and testis/*vas deferens* are similar between mouse and rat species and thus that mouse receptor protein expression is also consistent with mouse mRNA expression in the same cell types.

3.9.2 Expression of the three InsP₃R isoforms during mouse preimplantation development: mouse oocytes predominantly express type I InsP₃R

I began my investigation into InsP₃Rs in mouse development by examining the expression levels of the three isoforms in mature mouse oocytes. Semiquantitative Western analysis of 300 oocytes revealed that in terms of protein levels the type I InsP₃R is the predominant InsP₃R subtype expressed, with levels of type II and III protein being undetectable. This is consistent with a recent similar analysis using only 185 oocytes, in which type I was the only detectable isoform (Parrington *et al.*, 1998) and studies on *Xenopus* oocytes which indicated an exclusive or nearly exclusive presence of type I InsP₃R (Kume *et al.*, 1993; Parys and Bezprozvanny, 1995). My Western analysis results were supported by immunocytochemical staining, which detected type I InsP₃R but failed to detect any immunofluorescent type II in mature oocytes. In another study on mouse eggs, using different InsP₃R antibodies, only 20 eggs were required for a strong type I Western signal whereas a weak signal was obtained for type III from 1100 eggs (Fissore *et al.*, 1999a). Furthermore, in the same study, complete cytoplasmic immunofluorescence was found for type I but small punctate regions were found for type II (Fissore *et al.*, 1999a). These data suggest that very low levels of type II and III InsP₃Rs are in fact expressed in mature mouse oocytes, which may be too low to be detected in the 300 oocytes in my study. However, it is also possible, considering that I detected no type II immunoreactivity, that the punctate type II staining observed represented an artefact. This is made more likely by the fact that immunofluorescence and Western results did not agree in the latter study, since Western analysis using the same antibody failed to detect type II in mouse eggs. Further work is necessary to determine whether the reported punctate pattern of type II immunofluorescence (Fissore *et al.*, 1999a) is specific or a result of non-specific staining. Further work is also necessary to confirm the level of type III expression.

Finally, a study on InsP₃R isoforms in bovine oocytes and ovaries showed that in MII oocytes all three isoforms are expressed, but type I is present in overwhelmingly larger amounts (He *et al.*, 1999); whereas type I could easily be detected in as few as 10 oocytes, 400 oocytes were required to detect types II and III (He *et al.*, 1999). Thus it appears that in independent studies on mouse oocytes and in oocytes of different species, there appears to be a clear predominance of the type I isoform.

Expression levels of InsP₃R protein are inconsistent with those of InsP₃R mRNA

It is known that mouse oocytes express mRNAs for all three characterised InsP₃R subtypes (Parrington *et al.*, 1998) and the proportional expression of each subtype has been determined using a semiquantitative ratio rt-PCR technique (De Smedt *et al.*, 1994; De Smedt *et al.*, 1997). In the mature mouse oocyte the type II isoform is the predominant message, representing 67% of the InsP₃R mRNA with types I and III mRNAs making up

20% and 13%, respectively (Parrington *et al.*, 1998). Thus in the mature mouse oocyte the levels of mRNA for the different InsP₃R isoforms are clearly not representative of the respective protein levels. Such a complete discrepancy between the mRNA expression levels (mouse) and protein levels (rat) was not found after analysis of a number of other tissue types (De Smedt *et al.*, 1997; Wojcikiewicz, 1995). Also, in my study I found that InsP₃R isoform protein expression levels in the *mouse* pancreas and testis/*vas deferens* were also in good general agreement with the corresponding mouse mRNA expression levels (see above). The difference in mRNA and protein expression levels that I observe in the MII oocyte may reflect a peculiarity of RNA and protein processing in the oocyte. The high proportion of type II message may have been established during oocyte maturation either through transcription of type II during maturation or alternatively by specific degradation of the type I message. The latter seems most likely since transcription ceases soon after the initiation of oocyte maturation. Also, the type I mRNA is apparently translated during maturation since the levels of type I protein increase and it is known that mRNAs undergoing translation are more unstable than stored messages (Huarte *et al.*, 1987). Thus the instability of type I message following translation during maturation could be the explanation for the predominance of type II *mRNA*, despite there being a predominance of type I *protein*, in the mature oocyte.

A discrepancy between the mRNA expression levels and protein levels was also found after fertilisation, in 2-cell embryos. Western analysis on 200 late 2-cell embryos, 8-cell embryos and blastocysts revealed that neither type II nor III InsP₃R was upregulated to detectable levels throughout preimplantation development. However, after fertilisation and cleavage to the 2-cell stage, types I and II mRNA constitute 40-50% each of total InsP₃R mRNA with the type I/II ratio approaching one and type III constituting 10% (Parrington *et al.*, 1998). A proposed explanation for this change in I/II ratio is that after fertilisation degradation of type II mRNA occurs. However, since I have found no increase in type II protein it is likely that this post fertilisation mRNA decrease is not one associated with translation, but is probably associated with the general destruction of maternal mRNAs that occurs as the embryonic genome is activated at the 2-cell stage (Paynton *et al.*, 1988). It should, however, also be noted that the former study only examined relative proportions and not absolute levels of InsP₃R mRNAs at different stages of development (Parrington *et al.*, 1998). Therefore it may be that although type II mRNA is degraded to a greater extent, there being an abundance of type II in the unfertilised oocyte, all three mRNA subtypes are degraded to very low absolute levels after fertilisation, so that the total amount of InsP₃R mRNAs is significantly less than in the unfertilised oocyte. In this case, very low levels of InsP₃R mRNA would be consistent with the undetectable levels of types II and III protein I observed in 2-cell embryos. Quantitative PCR to determine the absolute levels of InsP₃R mRNA, would be important to resolve these issues.

The reason why oocytes express but do not translate the type II and III messages into protein is not clear. Oocytes are known to accumulate mRNAs, some of which are translated while others are masked until stimulation of translation by polyadenylation at specific stages of development (Bachvarova, 1992). Thus the mRNA for the types II and III isoforms may be stored mRNAs awaiting activation for a role in later development. However, my data does not support the idea that types II and III are regulated in such a manner as no corresponding protein could be detected from the oocyte through to the blastocyst stage. Comparing the 3' regions of the mRNAs may shed some light on possible translational control mechanisms that lead to this pattern of expression.

3.9.3 The concentration of InsP₃Rs in MII oocytes and other tissues

Having established that type I InsP₃R is the predominant InsP₃R protein expressed in mature mouse oocytes I compared InsP₃R levels firstly between oocytes of different mouse strains and oocytes of different species and secondly between mouse oocytes and other mouse tissues.

Firstly, the amount of InsP₃R appears to be well conserved between strains and species. Equal numbers of mature oocytes from MF1 and F1 strains gave similar levels of immunoreactivity in Western analysis with a type I antibody, thus suggesting that concentration of InsP₃Rs is conserved between strains. Furthermore, comparison of type I InsP₃R levels between mature mouse and bovine oocytes suggested that bovine oocytes have 2-3 times as many InsP₃Rs as mouse oocytes. However, this may well be a consequence of the bovine oocyte having a 1.5-fold larger diameter and thus being about 3-fold larger in volume than the mouse oocyte, taking the volume of an oocyte to be $\frac{4}{3}\pi r^3$. Thus concentration of InsP₃Rs also appears to be conserved between species.

The finding that only the type I InsP₃R is expressed in mature mouse oocytes and preimplantation embryos is not a unique phenomenon. In most previous studies on InsP₃R expression, type I has been found to be ubiquitously expressed, but found to be particularly predominant in the central nervous system (Furuichi *et al.*, 1990; Furuichi *et al.*, 1993) and, as described in section 3.9.2, in oocytes and eggs of mouse, cow and *Xenopus* (Fissore *et al.*, 1999a; He *et al.*, 1999; Kume *et al.*, 1993; Parys and Bezprozvanny, 1995; Parys *et al.*, 1994; Parys *et al.*, 1992). I chose to express the concentration of InsP₃Rs in the mature oocyte relative to that in the cerebellum owing to the exceptionally high receptor concentration in this tissue and to the fact that 99% of receptors are type I (Wojcikiewicz, 1995). The advantage of comparing two tissues with primarily type I InsP₃R was that a single subtype-specific antibody could be used both to compare the expression of type I and the expression of total InsP₃Rs. Comparison of the type I InsP₃R level in mature oocytes and cerebellum revealed that the concentration is roughly 10-fold higher in cerebellum. This places mature mouse oocytes alongside *vas deferens* and

pituitary in terms of similar total InsP₃R concentration (Wojcikiewicz, 1995). To confirm this expression ratio I used whole mouse brain which, due to it comprising a high proportion of cerebellum, should also express significant type I protein. Indeed it is known that *rat* brain contains 96% type I protein (Wojcikiewicz, 1995). I found a roughly 1.5-fold higher concentration of type I in whole brain than in mature oocytes. This is consistent with the figure obtained with cerebellum since the ratio of total InsP₃R concentration in whole brain to cerebellum is roughly 1.5:10 (Wojcikiewicz, 1995).

A similar comparison has been carried out in *Xenopus* where it was determined that the density of InsP₃Rs in Purkinje cells of the cerebellum was estimated to be about 20,000-fold higher than in *Xenopus* oocytes (Parys and Bezprozvanny, 1995). However, the InsP₃R density in other types of neurons was similar to, or up to 20-fold higher than, in *Xenopus* oocytes (Parys and Bezprozvanny, 1995). Since the cerebellum in my assay most likely contained granule as well as Purkinje cells, the overall concentration of InsP₃Rs in the cerebellar preparation may have been reduced.

3.9.4 Fertilisation stimulates downregulation of type I InsP₃R protein

I have demonstrated with both Western analysis and immunocytochemistry that by 8 hours after fertilisation there is a dramatic decrease in the level of immunoreactive type I InsP₃R protein. More specifically, I have shown using Western analysis that this downregulation is achieved over the 4 hours following sperm fusion and that it comprises the loss of about 80% of the level of protein found in MII oocytes. The fact that oocytes aged *in vitro* have levels of protein similar to freshly ovulated oocytes shows that fertilisation, or egg activation, is necessary for stimulating the decrease in InsP₃R protein. This suggests that the protein is tightly regulated during early development and may explain why fertilised pronucleate embryos are less sensitive to InsP₃ injection than age-matched unfertilised oocytes (Parrington *et al.*, 1998). However, it does not account for why there is a decrease in sensitivity to InsP₃ due to ageing alone (Jones and Whittingham, 1996; Parrington *et al.*, 1998). The 4-hour time-course of InsP₃R downregulation suggests that the loss of InsP₃R protein is not associated with any of the early events of egg activation such as cortical granule exocytosis, which occurs within the first 15 minutes of fertilisation (Kline and Stewart-Savage, 1994), but rather with events occurring over a longer time scale such as cell cycle resumption or repetitive Ca²⁺ transients that are known to last 3-4 hours (Jones *et al.*, 1995a; Kline and Kline, 1992a). As well as 4 hours after fertilisation being the approximate time that the sperm-induced Ca²⁺ transients stop, 4 hours also correlates with the time that the fertilised oocyte forms pronuclei and enters interphase. Since entering interphase has been shown to be directly related to the cessation of Ca²⁺ transients (Jones *et al.*, 1995a) InsP₃R downregulation may indeed be linked to either or both of these later events. (For discussion of the implications and role of downregulation see General Discussion.) This downregulation of 80% of InsP₃Rs should not be considered as leading

to the depletion of InsP₃Rs from oocytes. Instead, downregulation leaves the pronucleate embryo with a total InsP₃R concentration similar to tissues such as liver, testes and pancreas (Wojcikiewicz, 1995) (see 3.9.3).

Western analysis carried out on bovine GV stage and MII oocytes and pronucleate embryos 8 hours post fertilisation, revealed that type I InsP₃R increases during oocyte maturation and decreases by 8 hours after fertilisation. These preliminary findings are therefore consistent with the overall pattern of type I InsP₃R expression over this period in the mouse. However, I cannot infer from them whether this downregulation after fertilisation is as extensive as that seen in the fertilised mouse embryo, nor can I conclude whether it is completed in the 4 hours post fertilisation, as in the mouse. It should be noted that these results are also consistent with another recent study on InsP₃R isoforms in bovine oocytes which demonstrated a fertilisation-induced reduction of around 40% in the amount of type I InsP₃R protein by 16 hours post insemination (He *et al.*, 1999). It may be that this pattern of expression of the InsP₃R at fertilisation represents a conserved adaptation in all mammalian species.

The phenomenon of InsP₃R downregulation in cells is not unknown. Proteolytic degradation of the type I InsP₃R has been demonstrated in microsomal preparations (Magnusson *et al.*, 1993) and a number of cell lines in response to chronic exposure to agonists (Wojcikiewicz *et al.*, 1994). In these cell lines, it has been demonstrated that InsP₃R downregulation (1) is an adaptive response which occurs during activation of cell surface receptors that stimulate PIP₂ hydrolysis, (2) results from accelerated InsP₃R degradation and (3) leads to a reduction in the sensitivity of Ca²⁺ stores to InsP₃ (Wojcikiewicz *et al.*, 1994; Wojcikiewicz and Nahorski, 1991; Wojcikiewicz *et al.*, 1992).

The literature suggests that it is unlikely that the InsP₃R is just one of a broad range of substrates which are downregulated in parallel after fertilisation. In fact, it is likely that type I InsP₃R is specifically targeted for downregulation. For example, in both intact SH-SY5Y and AR4-2J cells stimulated respectively by carbachol or cholecystokinin (CCK), InsP₃Rs are specifically degraded (Wojcikiewicz and Oberdorf, 1996). Unlike the type I InsP₃R, ER Ca²⁺-ATPase and PKC in SH-SY5Y cells were unaffected by carbachol, suggesting that InsP₃R downregulation does not reflect a general increase in degradation of ER membrane proteins or PKCs. Similarly, the levels of the ER membrane enzyme HMG-CoA reductase in AR4-2J cells were unaffected by CCK (Wojcikiewicz and Oberdorf, 1996). Both PKC and HMG-CoA reductase can be downregulated in response to sterols in the above cell types during cell stimulation because of accelerated degradation; InsP₃R downregulation must be discrete from the processes that lead to this degradation.

The time-course of the type I downregulation may vary between cell type. For example, in both carbachol-stimulated SH-SY5Y cells (Wojcikiewicz *et al.*, 1994; Wojcikiewicz *et al.*,

1992; Wojcikiewicz and Oberdorf, 1996; Zhu *et al.*, 1999) and CCK-stimulated AR4-2J cells (Wojcikiewicz, 1995), type I InsP_3Rs are downregulated by over 90% with a half-time of approximately one hour with maximal effect at around 6 hours. This indicates that these cell types and these cell surface receptors utilise very similar downregulatory mechanisms. In contrast, chronic stimulation of WB rat liver epithelial cells by angiotensin II results in downregulation of type I by about 80% and with a half-time of about two hours, with maximal effect at around 6 hours (Bokkala and Joseph, 1997). This time-course is more similar to the downregulation of type I that I observed after fertilisation, which entailed a loss of 80% of protein with a half-time of roughly two hours and with maximal effect at 4 hours. Thus downregulation in rat WB cells by Ang II and in mouse oocytes after fertilisation may be via similar mechanisms. This presents us with the possibility that the 4-hour time-course in fertilised eggs may not be tightly linked to events such as exit from meiosis or the termination of sperm-induced Ca^{2+} transients, which occur at around 4 hours, but may simply be the time taken for this particular mechanism of downregulation to be completed.

3.9.5 Type I InsP_3R downregulation is matched by a decrease in sensitivity of Ca^{2+} release

The levels of InsP_3R protein I have observed generally correlate well with the ability to generate sperm-induced Ca^{2+} transients. During oocyte maturation the sensitivity of Ca^{2+} release increases in concert with the increase in InsP_3R protein (Jones *et al.*, 1995b; Mehlmann and Kline, 1994; Parrington *et al.*, 1998). As described above, it has also recently been demonstrated that the sensitivity of InsP_3 -induced Ca^{2+} release decreases after fertilisation. Aged-matched unfertilised oocytes release more Ca^{2+} in response to InsP_3 injection than fertilised pronucleate oocytes (Parrington *et al.*, 1998). I have shown that this decrease in sensitivity coincides with a decrease in the level of InsP_3R protein. However, ageing unfertilised oocytes alone has also been shown to decrease sensitivity to InsP_3 (Jones and Whittingham, 1996; Parrington *et al.*, 1998), although there is no significant decrease in the level of InsP_3Rs (this study) and the mechanism(s) involved remain unclear. Lastly, since the cell cycle is also known to influence Ca^{2+} release (see General Discussion), further experiments are necessary to investigate the specific effect of InsP_3R downregulation on Ca^{2+} release.

3.9.6 The type I InsP_3R is specifically upregulated in oocytes

During oocyte maturation there is a 1.8-fold increase in immunoreactive mass of the InsP_3R (Fissore *et al.*, 1999a; Mehlmann *et al.*, 1996; Parrington *et al.*, 1998). This suggests that during maturation there is specific synthesis of the type I isoform since these studies employed type I-specific antibodies and I have shown that no other isoforms are detectable in mature oocytes. Also, a different study using subtype-specific InsP_3R antibodies microinjected into the germinal vesicle detected type I InsP_3Rs at the GV membrane

whereas type III InsP₃Rs were found to be absent from the GV (Pesty *et al.*, 1998). This suggests that, like in the mature oocyte, the predominant InsP₃R isoform expressed in the GV stage oocyte is the type I InsP₃R. The finding, in this study, that after downregulation the type I InsP₃R did not return to prefertilisation levels during preimplantation development, suggests that it is specifically upregulated in oocytes, presumably reflecting its requirement for fertilisation (Carroll *et al.*, 1996; Mehlmann *et al.*, 1996). Thus, upregulation of the InsP₃R can be considered an important contribution to the final maturation of the oocyte cytoplasm that ensures that sufficient Ca²⁺ is released at fertilisation in order to initiate embryogenesis. In this case, the requirement to maintain InsP₃R levels after fertilisation may not be necessary.

It should be noted that in other systems InsP₃Rs have been found to upregulate again after downregulation. For example, chronic stimulation of WB rat liver epithelial cells by Ang II causes type I and III InsP₃R downregulation (Bokkala and Joseph, 1997). However, continuous incubation of the WB cells with Ang II for over 6 hours resulted in partial recovery of both subtypes (Bokkala and Joseph, 1997), which is not seen at fertilisation. However, it is likely that after fertilisation, as the development of the embryo progresses, the stimulus for downregulation of type I is not maintained at full strength for as long as 6 hours, making the two situations incomparable. (See Chapters 4 and 5 for discussion of the stimulus and mechanism of InsP₃R downregulation after fertilisation.)

Immunocytochemical staining revealed that the type I InsP₃R protein is evenly distributed throughout the MII oocyte cytoplasm and that after fertilisation the protein is evenly lost from all regions of the cytoplasm. This result contrasts with that of another study, which proposed the existence of large cortical clusters of type I InsP₃Rs in the mature mouse oocyte and detected little immunofluorescent staining in the interior cytoplasm (Mehlmann *et al.*, 1996). However, a more recent study has reported immunoreactive type I InsP₃R to have a reticular staining pattern *throughout* the mouse egg (Fissore *et al.*, 1999a). Furthermore, in the mature hamster egg, small patches of InsP₃Rs have been observed throughout the cytoplasm (Shiraishi *et al.*, 1995). My result is also consistent with a sea urchin study in which the InsP₃R was found to be present throughout the egg cytoplasm in a pattern compatible with the distribution of ER (Parys *et al.*, 1994). A correlation of type I staining with the ER in the mouse egg may be confirmed by using DiI to visualise the ER. Note that in the unfertilised *Xenopus* oocyte immunocytochemical studies revealed a less homogeneous staining pattern than observed in my experiments. The InsP₃R was densely enriched in the cortical region of both hemispheres with a weaker, polarised distribution in the interior cytoplasm of the animal hemisphere in an ER-like reticular network (Kume *et al.*, 1993). In this study (Kume *et al.*, 1993), although there is no data on change in actual expression levels, InsP₃R localisation underwent a dramatic reorganisation at fertilisation and seemed to disappear. This suggests a similar downregulation of receptor levels to that observed in the mouse. The inhomogeneous InsP₃R distribution observed in *Xenopus*

oocytes may be owing to the use of a different antibody in that study or may reflect differences between animal species.

Implications of almost exclusive and global type I distribution for Ca^{2+} release at fertilisation

As described above, my Western and immunofluorescence data suggests that the type I InsP_3R is the most prevalent and functionally important isoform in mouse eggs. Given that successful fertilisation requires a global all-or-nothing Ca^{2+} signal, it may be that the type I InsP_3R is best suited to this role. Some of the observed properties of the type I InsP_3R support this idea. Firstly, cerebellar purkinje cells, which express almost exclusively the type I InsP_3R , are relatively insensitive to InsP_3 but release more Ca^{2+} once activated (Cardy *et al.*, 1997). Secondly, compared to cells expressing the type III isoform, it has been suggested that cells expressing the type I InsP_3R are less sensitive to InsP_3 at resting Ca^{2+} levels and more sensitive once the cytosolic Ca^{2+} begins to rise (Cardy *et al.*, 1997). These properties would favour fertilisation since as well as filtering out any small signals, thereby preventing spontaneous parthenogenetic activation, they would ensure that the stimulus delivered by the sperm induced a maximal release of Ca^{2+} .

As well as protein expression levels, the spatial localisation of InsP_3R subtypes also has profound implications for Ca^{2+} release at fertilisation. It has been shown that Ca^{2+} wave propagation through the inner cytoplasm of hamster eggs is dependent on InsP_3Rs (Miyazaki, 1995; Miyazaki *et al.*, 1992; Shiraishi *et al.*, 1995). The ubiquitous expression of type I InsP_3R , possibly with extremely low expression of types II and III, is consistent with these isoforms having particular functional roles in the initiation and spread of the fertilisation-associated Ca^{2+} wave. It has previously been suggested that one or more isoforms at the cortex may play a role in initiating Ca^{2+} release, in conjunction with the Ca^{2+} -mobilising sperm factor (Carroll *et al.*, 1994; Parrington *et al.*, 1996; Swann and Lai, 1997). The predominance of type I at the cortex as well as in the interior cytoplasm implies firstly that this subtype plays an important role in Ca^{2+} wave initiation. Secondly, because the type I InsP_3R and ER are present throughout the cytoplasm, type I is also likely to provide a continuous network for the spread of the global Ca^{2+} wave and oscillations which are seen in fertilised MII mouse eggs (Nakano *et al.*, 1997). Since the type I-specific monoclonal function-blocking antibody 18A10 has been shown to nearly completely inhibit Ca^{2+} release at fertilisation and in CICR in hamster and mouse oocytes (Miyazaki *et al.*, 1993; Miyazaki *et al.*, 1992), the other isoforms may not release significant amounts of Ca^{2+} relative to the total released, but they may play a more indirect role. For example, distinct foci of the type II InsP_3R as observed in one study (Fissore *et al.*, 1999a), may assist type I in the propagation of the Ca^{2+} wave or may play a role in initiating Ca^{2+} release at the site of sperm-egg fusion. Alternatively, types II and III may constitute minor components of a heterotetrameric InsP_3R (Joseph *et al.*, 1995; Monkawa *et al.*, 1995; Nucifora *et al.*, 1996).

The contribution of the other major family of intracellular Ca^{2+} release channels, the ryanodine receptors (RyRs), to Ca^{2+} oscillations at fertilisation of mammalian oocytes, remains controversial. They appear to be present in most mammalian species, although at very low levels (Ayabe *et al.*, 1995; Carroll *et al.*, 1996; He *et al.*, 1997), and modulators of RyR function have been shown to modify Ca^{2+} release in mouse oocytes at fertilisation (Jones *et al.*, 1995b; Swann, 1992). Further studies are required to clarify the role of RyRs at fertilisation in mammals but my findings clearly show that, of the InsP_3R family, only the type I isoform is required. In Chapter 6 I provide more direct and thus conclusive evidence for an essential role for InsP_3Rs in Ca^{2+} oscillations at fertilisation.

CHAPTER 4:

DOWNREGULATION OF THE TYPE I InsP_3R : ACTIVATION OF THE DOWNREGULATORY MECHANISM AFTER FERTILISATION

4.1 INTRODUCTION

In Chapter 3 I described the downregulation of type I InsP_3R which takes place during the 4 hours following fertilisation of mouse eggs. In this chapter I investigate the nature of the stimulus which activates the downregulatory mechanism at fertilisation. Downregulation of the InsP_3R has been demonstrated in several cell lines in response to activation of PLC-linked cell surface receptors (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999; Sipma *et al.*, 1998; Wojcikiewicz, 1995; Wojcikiewicz *et al.*, 1994; Wojcikiewicz and Nahorski, 1991). Furthermore, InsP_3Rs have been shown to downregulate in pancreatic acinar cells in response to secretagogues that activate PLC (Wojcikiewicz *et al.*, 1999). The downregulation of type I InsP_3Rs in mouse oocytes in response to sperm represents, besides the response of acinar cells to secretagogues, the only physiological example of InsP_3R downregulation. It has been indicated in recent studies that InsP_3R downregulation in somatic cell lines is stimulated only by those agonists that elevate InsP_3 concentration persistently (Oberdorf *et al.*, 1999; Zhu *et al.*, 1999; Zhu and Wojcikiewicz, 2000). Also, it has been shown using binding-defective mutant InsP_3Rs that InsP_3R downregulation requires the InsP_3 -binding domain (Zhu *et al.*, 1999). These data suggest that InsP_3 binding to the InsP_3R may act as the signal that causes InsP_3Rs to be downregulated.

Alternative candidates for the stimulus of InsP_3R downregulation at fertilisation are, firstly, the sperm-induced Ca^{2+} oscillations, which may activate Ca^{2+} -dependent proteolysis. Secondly, one of the developmental processes associated with activation of the egg may be responsible for inducing downregulation. The possibility also exists that activation of protein kinases C by the diacylglycerol (DAG) that is generated simultaneously with InsP_3 , may stimulate downregulation. However, this is less likely as previous studies have shown that activation of protein kinases C with phorbol ester fails to elicit downregulation in cell lines (Bokkala and Joseph, 1997; Wojcikiewicz and Nahorski, 1991).

With respect to the ability of sperm-induced Ca^{2+} oscillations to cause InsP_3R downregulation, it is interesting to note that these Ca^{2+} increases are well established as being both necessary and sufficient for complete egg activation (Kline and Kline, 1992a; Lawrence *et al.*, 1998). Exit from meiotic metaphase arrest (egg activation) depends on the destruction of the regulatory subunit of MPF, cyclin B (Draetta *et al.*, 1989; Gerhart *et al.*, 1984; Labbe *et al.*, 1989; Lohka *et al.*, 1988; Nurse and Bissett, 1981). The destruction of cyclin B has been shown to be driven by the sperm-induced Ca^{2+} transients (Kline and

Kline, 1992a; Murray *et al.*, 1989; Newport and Kirschner, 1984; Watanabe *et al.*, 1991). Activation of the 26S proteasome is thought to be necessary for the degradation of cyclin B at egg activation (Glotzer *et al.*, 1991) and, at least in *Xenopus* and ascidians, activity of the 26S proteasome may be stimulated by these Ca^{2+} increases (Aizawa *et al.*, 1996; Kawahara and Yokosawa, 1994). Sperm-induced Ca^{2+} increases therefore stimulate cyclin B downregulation but it is not known whether these same increases have any effect on InsP_3R levels.

Parthenogenetic activation of mouse eggs can be induced by several agonists that stimulate single or multiple Ca^{2+} rises or inhibit protein synthesis. I investigated the effects of these agonists on type I InsP_3R levels. As described above, the destruction of cyclin B at fertilisation is driven by the sperm-induced Ca^{2+} transients, and parthenogenetic agents which generate Ca^{2+} increases in MII oocytes are also able to induce egg activation. It is well established that single Ca^{2+} responses, like those induced by ethanol and ionomycin, trigger high rates of activation and initiation of development in aged oocytes (Cuthbertson, 1983; Shiina *et al.*, 1993; Susko-Parrish *et al.*, 1994; Swann and Ozil, 1994). Exposure to Sr^{2+} , on the other hand, results in a series of oscillations of $\text{Ca}^{2+}/\text{Sr}^{2+}$, more closely resembling fertilisation, and rapidly activates even freshly-ovulated mouse eggs (Fraser, 1987; Kline and Kline, 1992b). Inhibiting protein synthesis in MII mouse eggs using cycloheximide also causes egg activation but bypasses a requirement for Ca^{2+} transients by decreasing the levels of both cdc2/cyclin B kinase (MPF) and MAP kinase, mimicking the fall in levels of these proteins at fertilisation (Kubiak *et al.*, 1993; Moos *et al.*, 1996a). Therefore, I used parthenogenetic activation in order to examine whether increases in intracellular Ca^{2+} concentration, or egg activation itself, could stimulate InsP_3R downregulation.

In this chapter I show that receptor downregulation occurs after egg activation in response to sperm but not to Sr^{2+} , ethanol or cycloheximide. It was found that the only means of mimicking the response was by microinjecting the InsP_3R agonist adenophostin A and, interestingly, exposing oocytes to agents which disrupt the cytoskeleton - nocodazole and cytochalasin D. I also show that fertilisation and adenophostin can downregulate InsP_3R s in immature oocytes.

4.2 THE EFFECT OF PARTHENOGENETIC ACTIVATION ON InsP_3R LEVELS

In Chapter 3 I demonstrated that by 4 hours after fertilisation type I InsP_3R protein is downregulated to approximately 20% of the level found in MII oocytes. In order to establish the “trigger” of the mechanism leading to the decrease in InsP_3R immunoreactivity I examined the level of InsP_3R protein in oocytes after artificial activation. MII oocytes can be parthenogenetically activated using Ca^{2+} -dependent or Ca^{2+} -independent mechanisms so that completion of meiosis is achieved without a sperm. Sr^{2+} -containing medium and ethanol were chosen as the Ca^{2+} -dependent means of activation as they produce very different Ca^{2+} responses: in the case of Sr^{2+} , a series of oscillations similar to those seen at fertilisation, while ethanol induces a monotonic Ca^{2+} transient that is sustained for the duration of ethanol exposure (Kline and Kline, 1992a; Swann and Ozil, 1994). To confirm the pattern of Ca^{2+} increases in MII oocytes after exposure to the Ca^{2+} -dependent activating agents I imaged ^{Fig. 2} Ca^{2+} in MII oocytes using the Ca^{2+} -sensitive dye Fura-2-AM. In all oocytes exposed to 7% ethanol (n=12), the global Ca^{2+} increase was immediate and approximately 1.5-fold larger than the first Ca^{2+} increase of the series of oscillations at fertilisation, before gradually returning to basal levels as soon as ethanol was removed after 7 minutes. All oocytes (n=10) exposed to 10mM Sr^{2+} exhibited the series of Ca^{2+} oscillations characteristic of this treatment. For the Ca^{2+} -independent methods of activation I used the protein synthesis inhibitor, cycloheximide and a protein kinase inhibitor, R0-31-8220, which presumably act by inhibiting the synthesis of cyclin B (Kubiak *et al.*, 1993; Moos *et al.*, 1996a) and the activity of MPF, respectively. Note that during egg activation caused by exposure to cycloheximide, intracellular Ca^{2+} concentration does not change (Bos-Mikich *et al.*, 1995; Moses and Kline, 1995).

In the case of Sr^{2+} , a preliminary set of experiments was carried out to optimise egg activation and subsequent development to the 2-cell stage. In one representative experiment, whereas 88% of MII oocytes (n=56) exposed to Sr^{2+} activated and developed to 2-cells, only 4% of control MII oocytes (n=75) cultured in parallel underwent spontaneous activation and developed to 2-cell embryos. In a representative ethanol activation the pronuclear formation rate was 69% (n=175) and in a representative activation experiment by cycloheximide, 60% (n=150). Note that only eggs which activated and formed pronuclei were analysed by Western blotting.

Surprisingly, despite the wide-ranging mechanisms of action, Western analysis of InsP_3R levels in eggs with obvious pronuclei 6 hours after activation revealed that with all of the parthenogenetic stimuli employed, no effect on InsP_3R levels was observed (**Fig. 4.1**). This suggests that cell cycle progression itself or the generation of increases in intracellular Ca^{2+} is not sufficient to cause the loss of InsP_3R protein. Also, since cycloheximide inhibits

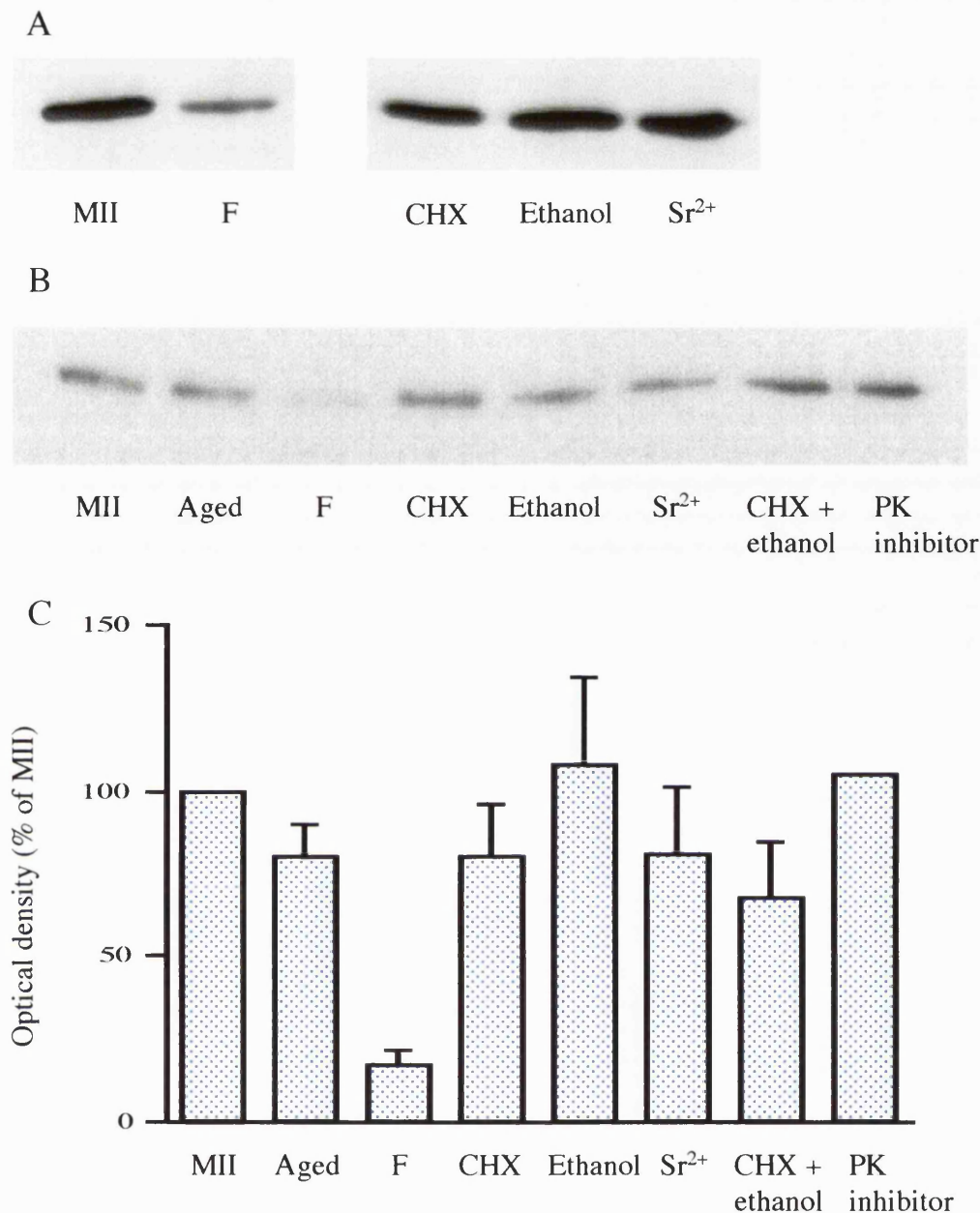


Figure 4.1 Parthenogenetic egg activation does not cause type I InsP_3R downregulation. Mature oocytes were exposed to 7% ethanol for 7 minutes, Sr²⁺ (10mM), cycloheximide (10 $\mu\text{g}/\text{ml}$) or kinase inhibitor RO-31-8220 (10 μM), all of which bring about parthenogenetic activation. Activated eggs were collected after 6 hours (100 per treatment) and protein was electrophoresed and immunoblotted with CT1 next to control lanes of MII and fertilised eggs (A and B). B shows a repeat analysis including an additional control lane of MII eggs aged in culture medium for 6 hours. The Ca²⁺ transient and oscillations caused in the egg by ethanol and Sr²⁺, respectively, were insufficient as triggers for downregulation. Total protein synthesis inhibition by cycloheximide and kinase inhibition were also insufficient, as was egg activation *per se* (C). The blots are representative of independent experiments. Sr²⁺, ethanol and cycloheximide were repeated at least 5 times and the PK inhibitor and cycloheximide/ethanol combination at least twice.

protein synthesis, its inability to downregulate type I InsP₃R tells us that sperm-induced downregulation must be an active mechanism of increased protein degradation.

4.3 THE EFFECT OF BAPTA ON THE FERTILISATION-INDUCED LOSS OF InsP₃R PROTEIN

The lack of an effect on InsP₃R levels of Ca²⁺-dependent activation suggested that Ca²⁺ itself is insufficient to stimulate the loss of InsP₃R protein. To investigate whether Ca²⁺ increases are in fact necessary for downregulation at fertilisation, oocytes were preloaded with BAPTA-AM (10µM for 30 minutes) (Molecular Probes) prior to fertilisation. First, I confirmed previous observations (Kline and Kline, 1992a) that BAPTA treatment abolishes Ca²⁺ signaling at fertilisation. Direct measurement of Ca²⁺ in BAPTA-loaded oocytes demonstrated that fertilisation-induced Ca²⁺ transients were completely inhibited (**Fig. 4.2.1**). In order to confirm that the lack of Ca²⁺ response was not due to inhibition of sperm-egg fusion oocytes were stained with the DNA-specific fluorochrome Hoechst (Sigma). In all oocytes examined (n=12), sperm heads were present in the cytoplasm (**Fig. 4.2.2B and C**) and the maternal chromosomes remained together at the spindle (indicating the inhibition of egg activation), although they appeared to be partially decondensed (**Fig. 4.2.2B**). Note that BAPTA alone also appeared to cause a partial decondensation of the chromosomes (**Fig. 4.2.2A**). Western analysis of BAPTA-loaded oocytes revealed that, even in the absence of Ca²⁺ transients and egg activation, fertilisation remained a potent stimulator of InsP₃R downregulation (**Fig. 4.2.3**). Furthermore, control experiments revealed that BAPTA treatment in the absence of fertilisation was not sufficient to induce downregulation, although it did lead to a partial loss of InsP₃R immunoreactivity to about 60% of control levels (t-test: $P < 0.05$) (**Fig. 4.2.3**). This experiment suggests that cytosolic increases in Ca²⁺ are not necessary for InsP₃R downregulation at fertilisation. Since BAPTA also prevents egg activation (**Fig. 4.2.2B and C**), this result also suggests that the downregulation of InsP₃Rs is independent of egg activation.

4.4 THE EFFECT OF NOCODAZOLE AND CYTOCHALASIN D ON InsP₃R LEVELS

In order to confirm that oocyte activation is not required for InsP₃R downregulation after fertilisation I fertilised MII oocytes which were locked in metaphase II arrest. Oocytes were arrested at MII by addition of the microtubule depolymerising drug nocodazole during incubation with sperm. Fertilised oocytes treated with 10µM (or 20µM) nocodazole remained arrested at MII due to disruption of the metaphase spindle, which must be intact for cyclin B degradation and thus exit from metaphase (Kubiak *et al.*, 1993; Winston *et al.*, 1995). The results showed that, as expected, preventing egg activation did not inhibit

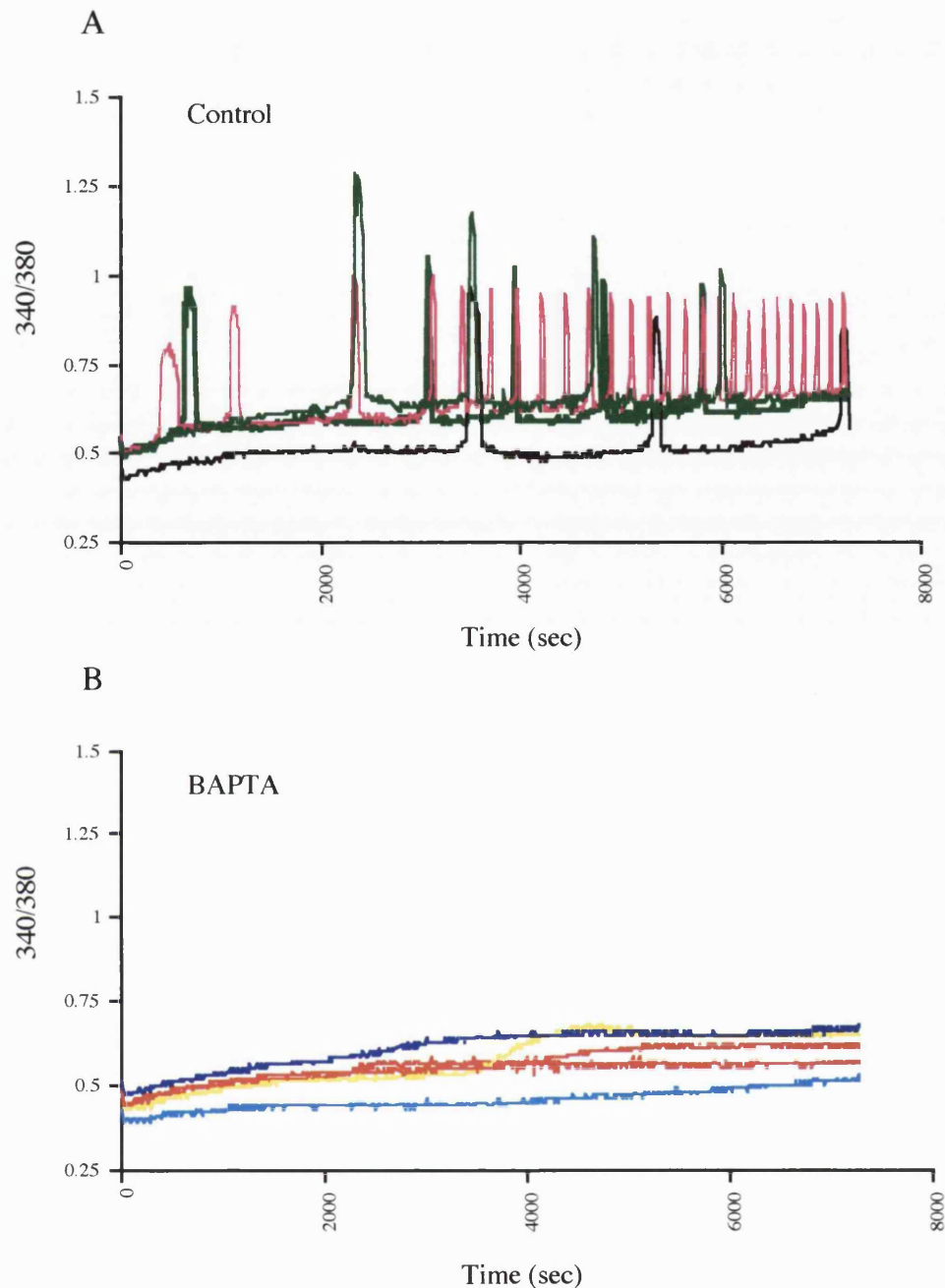


Figure 4.2.1 BAPTA inhibits fertilisation-induced Ca^{2+} transients. MII oocytes were loaded for 30 minutes with $10\mu\text{M}$ BAPTA-AM to chelate intracellular Ca^{2+} . Control age-matched and BAPTA-loaded oocytes were loaded with Fura-2 and intracellular Ca^{2+} was monitored at fertilisation. Traces are shown from all 4 (Control) or 5 (BAPTA) oocytes in which Ca^{2+} was imaged. Ca^{2+} is presented as the ratio of 340/380nm. (A) Control oocytes generated characteristic sperm-induced Ca^{2+} oscillations at fertilisation. (B) Imaging Ca^{2+} in BAPTA-loaded oocytes demonstrated that fertilisation-induced Ca^{2+} transients were completely inhibited.

A

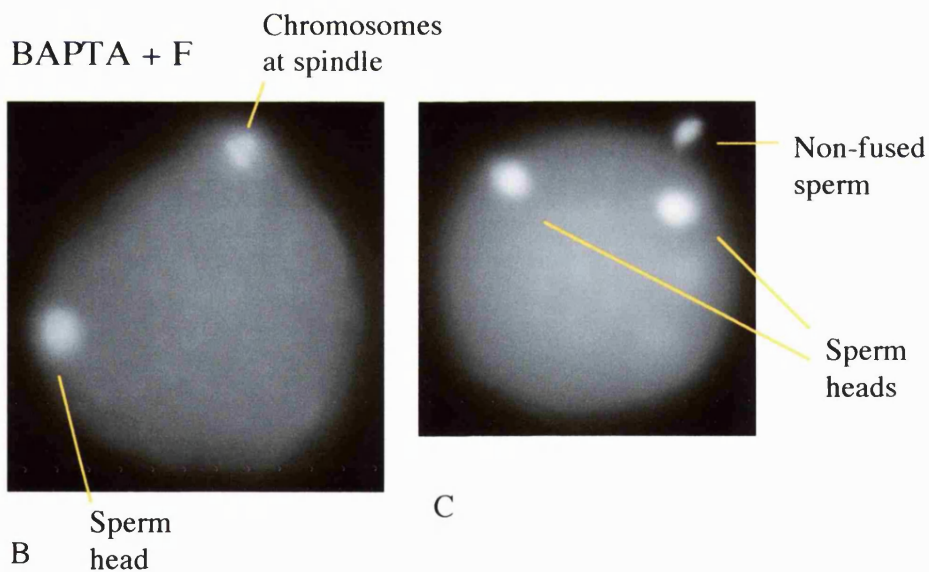
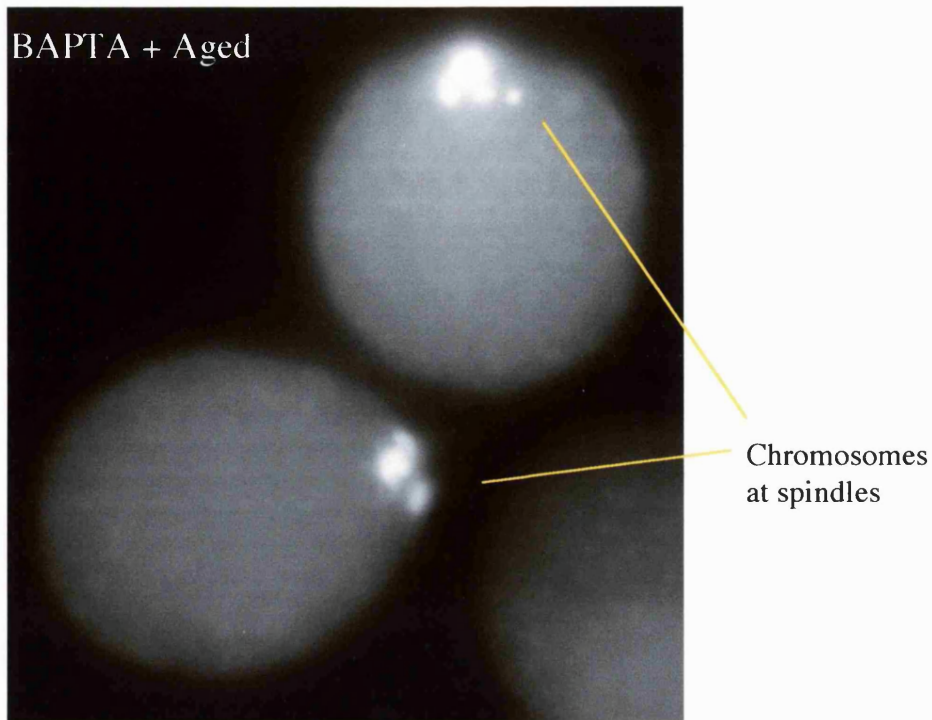


Figure 4.2.2 BAPTA-AM does not prevent fusion between egg and sperm. MII oocytes were loaded for 30 minutes with 10 μ M BAPTA-AM to chelate intracellular Ca^{2+} , and were exposed to sperm or cultured alone (Controls). BAPTA-loaded aged (A) and fertilised (B and C) oocytes were fixed and stained with Hoechst after 6 hours. The presence of one (B) or two (C) sperm heads inside oocytes indicates fertilisation has taken place despite the maternal metaphase chromosomes remaining together at the spindle (B, not in section in C). Maternal chromosomes appear partially decondensed in both controls and fertilised oocytes (A and B).

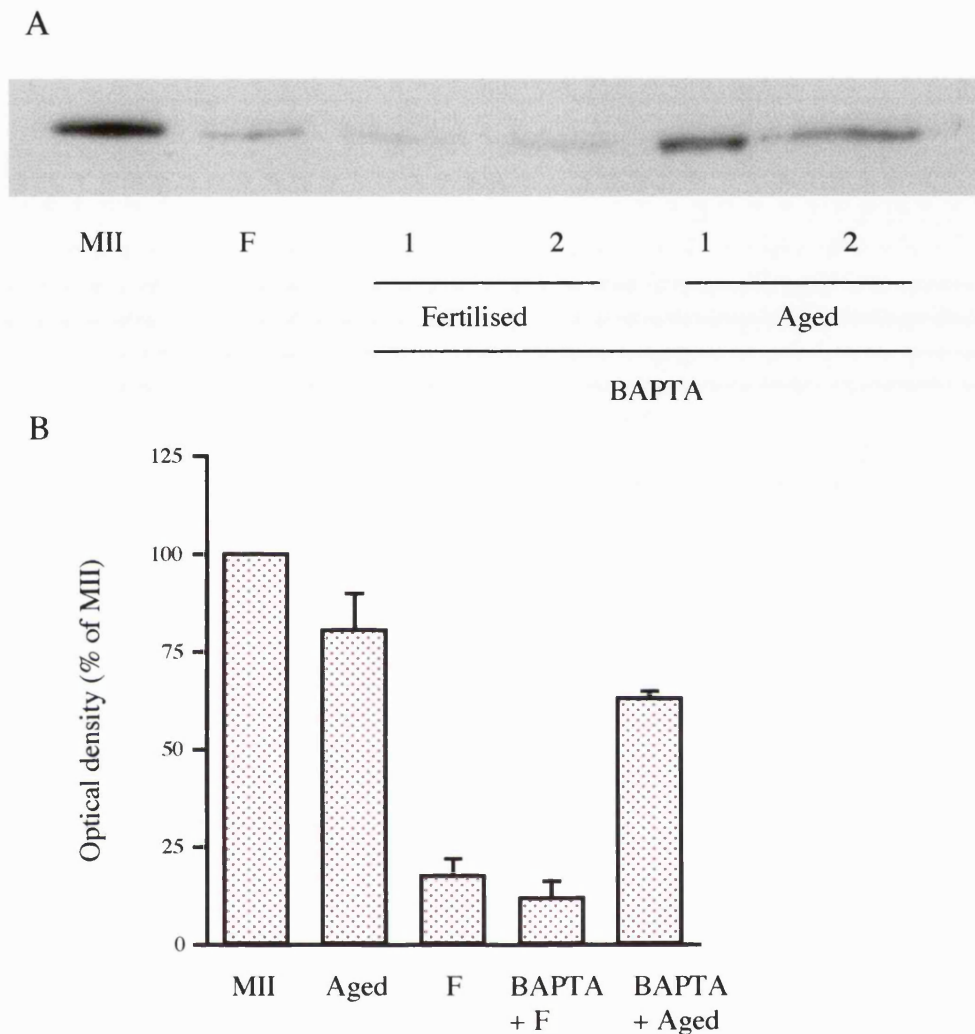


Figure 4.2.3 BAPTA does not inhibit InsP₃R-I downregulation at fertilisation. MII eggs were incubated with BAPTA-AM (10 μ M) for 30 minutes prior to sperm addition, to abolish the Ca²⁺ oscillation at fertilisation. Eggs were collected after 6 hours (100 per treatment) and protein was electrophoresed and immunoblotted with CT1 next to control lanes of MII and fertilised eggs and MII eggs aged for 6 hours after BAPTA pretreatment (A). (A and B) The absence of both Ca²⁺ transients and egg activation did not prevent InsP₃R-I downregulation at fertilisation. BAPTA treatment alone caused a partial decrease in expression to approximately 60% of control levels. The blot is representative of 3 independent experiments.

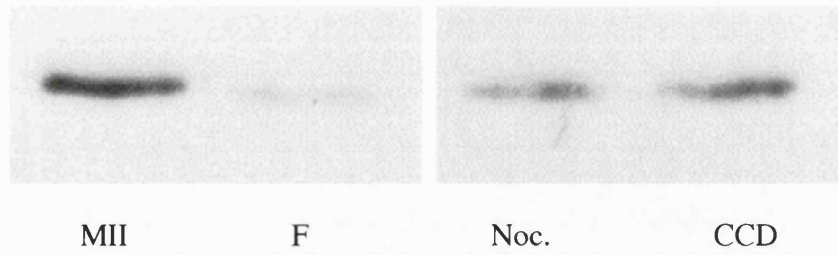
fertilisation-induced InsP_3R downregulation (not shown). However, the control experiment of MII oocytes incubated for 5.5 hours in nocodazole alone surprisingly revealed that nocodazole itself stimulated downregulation to 20-25% of control levels, therefore similar in extent to the downregulation stimulated by sperm (**Fig. 4.3**). A similar amount of InsP_3R protein was depleted after incubation in either 10 μM (data not shown) or 20 μM nocodazole. To explore this finding further I also examined the effect on InsP_3R levels of cytochalasin D (CCD), which depolymerises actin microfilaments and breaks down the actin cytoskeleton. MII oocytes incubated for 5.5 hours in 10 $\mu\text{g/ml}$ CCD also showed a decrease in InsP_3Rs to 20-25% of control levels (**Fig. 4.3**). In order to control for the vehicle (Dimethyl sulfoxide, DMSO) in which nocodazole and CCD were dissolved, oocytes were incubated in parallel in DMSO alone. Western analysis revealed that DMSO had no effect on InsP_3R levels (not shown).

In order to propose possible mechanisms by which nocodazole stimulates InsP_3R downregulation it was necessary to confirm that the spindle was indeed disrupted. To check for spindle disruption MII oocytes were incubated simultaneously in 10 μM or 20 μM nocodazole and 10mM Sr^{2+} and were monitored for oocyte activation. In 5 out of 5 oocytes nocodazole (both 10 μM and 20 μM) inhibited activation by Sr^{2+} and oocytes did not develop a second polar body or clear pronucleus, indicating that the spindle checkpoint had been activated. To observe MII arrest more directly I applied Hoechst to stain the chromosomes. Hoechst staining after 4 hours revealed that 3 out of 5 oocytes exposed to Sr^{2+} in nocodazole (both 10 μM and 20 μM) still contained individual, condensed chromosomes. Also, rather than being arranged as an MII plate, chromosomes were scattered due to spindle disruption. The remaining 2 out of 5 oocytes in both concentrations of nocodazole contained a very small pronucleus with a “ball” of partially decondensed chromatin, although the second polar body was absent. These experiments tell me that both concentrations of nocodazole to which MII oocytes were subjected in the above experiments, disrupted the meiotic spindle.

4.5 THE EFFECT OF PROTEIN KINASE C ACTIVATION ON InsP_3R LEVELS

Since the data suggests that Ca^{2+} is not the stimulus for InsP_3R downregulation I investigated whether the other arm of the PIP_2 hydrolysis pathway is involved. MII oocytes were treated with phorbol ester (PMA) (100nM) to activate the diacylglycerol (DAG)-sensitive PKCs and to investigate a role for PKCs in InsP_3R downregulation. (Note that PMA simulates the action of DAG). The activity of PMA was confirmed by assaying germinal vesicle breakdown (GVBD) in immature oocytes. After 6 hours of continuous incubation in PMA only 6% (n=17) of oocytes had undergone GVBD compared to 83% (n=12) of controls. MII oocytes were incubated in PMA for 1.5 hours followed by culture in control medium for 4.5 hours (2 experiments), or incubated in PMA continuously for 6

A



B

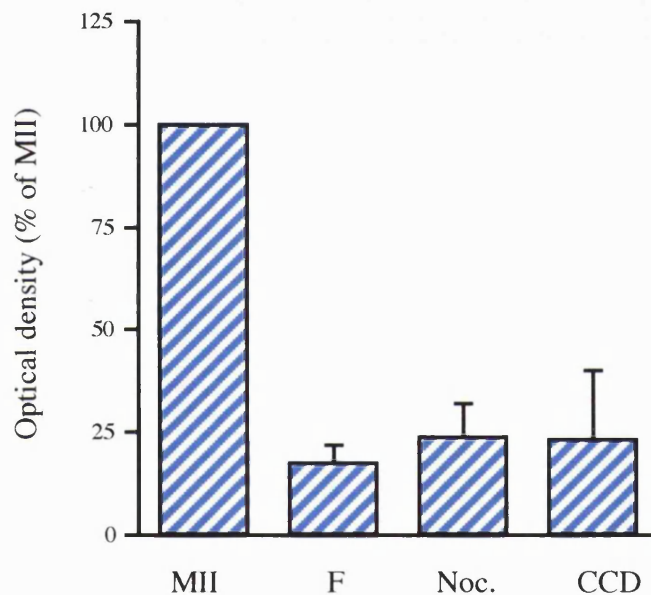


Figure 4.3 Nocodazole and cytochalasin D stimulate type I InsP₃R downregulation in MII oocytes. MII oocytes were incubated for 5.5 hours in 20 μ M nocodazole or 10 μ g/ml cytochalasin D and collected for Western analysis of type I InsP₃R. Protein from 110 oocytes per treatment was electrophoresed and immunoblotted with CT1 next to control lanes of MII and fertilised eggs (A). (A and B) Both nocodazole and cytochalasin D induced downregulation of the type I InsP₃R to 20-25% of control levels. The blot is representative of 5 independent experiments for nocodazole (both 10 μ M and 20 μ M) and 3 for cytochalasin D.

hours (2 experiments), before Western analysis of InsP₃R levels. Despite its proven activity, PMA failed to induce InsP₃R downregulation in all four experiments, with either a 1.5 hour or a continuous incubation (**Fig. 4.4**). This suggests that DAG-sensitive (“conventional”) PKCs do not play a role in InsP₃R downregulation.

4.6 SPERM-INDUCED InsP₃R DOWNREGULATION OCCURS IN IMMATURE OOCYTES

Experiments so far have been performed using mature metaphase II oocytes. To determine whether InsP₃R downregulation was dependent on the stage of oocyte maturation I carried out experiments using immature oocytes. It has previously been shown that the Ca²⁺ response of immature mouse oocytes to sperm is variable and reduced compared to the response of mature MII oocytes (Jones *et al.*, 1995b; Jones and Nixon, 2000; Mehlmann and Kline, 1994). Maturing oocytes were fertilised within 2 hours of release from the follicle. Western analysis of oocytes collected 4 hours after fertilisation revealed that InsP₃R downregulation takes place similar to that in mature oocytes (**Fig. 4.5**). Thus loss of InsP₃Rs is independent of the stage of maturation. Also, these experiments confirm that downregulation does not require the normal events associated with egg activation, since immature oocytes do not activate upon fertilisation.

4.7 ADENOPHOSTIN STIMULATES EXTENSIVE InsP₃R DOWNREGULATION

4.7.1 Carbachol is unable to stimulate loss of InsP₃Rs in mouse oocytes

The absence of any apparent role for Ca²⁺ or PKC or even oocyte activation *per se* in the downregulation of InsP₃Rs suggests that the sperm directly stimulates the loss of InsP₃Rs or that it is caused by a different aspect of cell signaling at fertilisation. The one stimulus not yet investigated is the activation of the InsP₃R itself. To determine whether InsP₃ production could stimulate downregulation I first applied carbachol to activate muscarinic acetylcholine receptors present on MII oocytes. Carbachol stimulates InsP₃ production in rat striatum (de la Vega *et al.*, 1997) and is known to induce a short-lived series of Ca²⁺ transients in mouse MII eggs (Swann, 1992) which can last for up to 8 minutes (Lawrence *et al.*, 1997). However, oocytes that were incubated for 30 minutes in 100µM carbachol followed by 6 hours in culture and Western analysis showed no sign of a decrease in InsP₃R levels (**Fig. 4.6.C**). Due to insufficient size and duration of the carbachol-induced transients, the oocytes remained unactivated after carbachol stimulation.

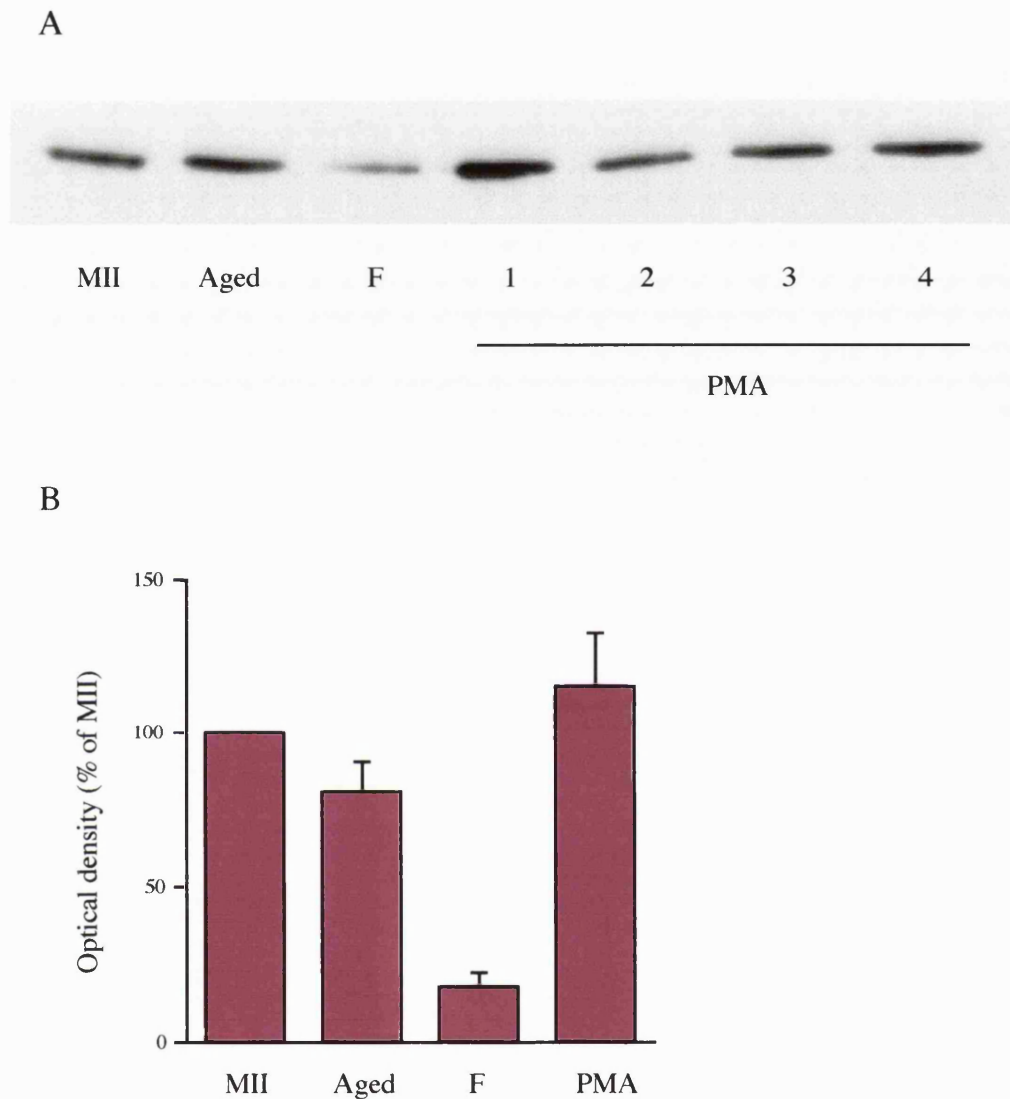


Figure 4.4 Protein kinase C activation does not cause InsP₃R-I downregulation. MII oocytes were incubated with 100nM phorbol ester (PMA) for 6 hours and protein was electrophoresed (100 oocytes per lane) and immunoblotted with CT1 next to control lanes of MII and fertilised eggs and eggs aged for 6 hours in culture medium alone. In all 4 independent experiments PMA had no effect on InsP₃R-I levels (A and B).

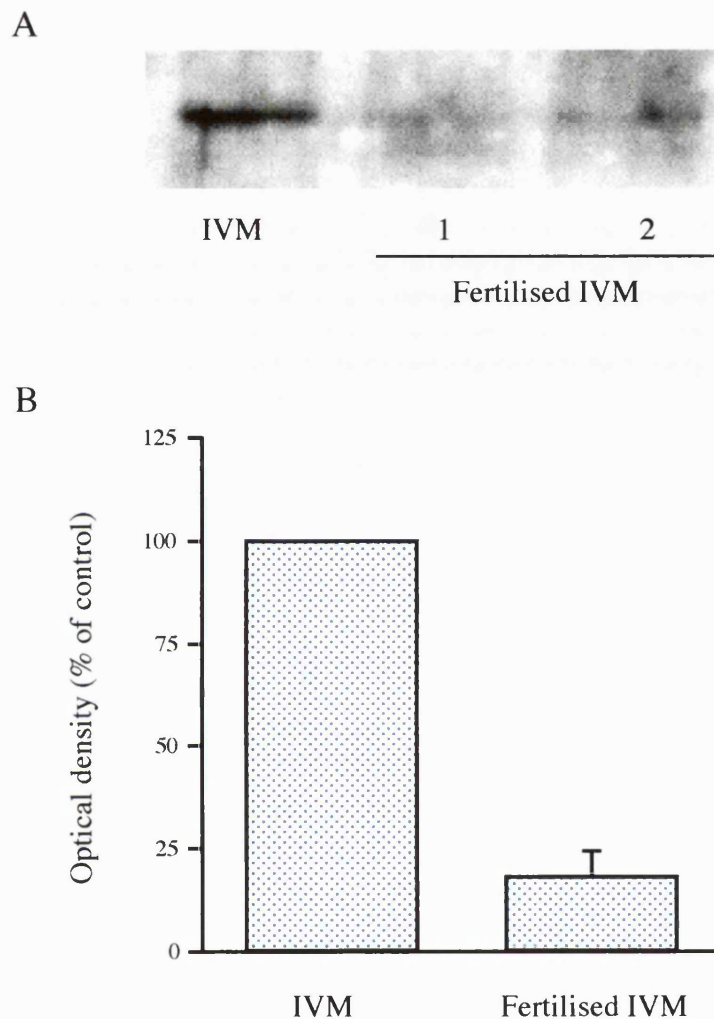


Figure 4.5 InsP₃R-I expression is downregulated at fertilisation of immature oocytes undergoing *in vitro* maturation (IVM). Immature oocytes were fertilised and collected for Western analysis of InsP₃R-I 6 hours later. Protein from 40 fertilised immature oocytes (Fertilised IVM) was electrophoresed and immunoblotted with Ab40 antibody next to a control lane of 40 age-matched control oocytes (IVM) (A). After fertilisation, InsP₃R-I levels had decreased to around 20% of control levels (A and B). The blot is representative of 3 separate experiments.

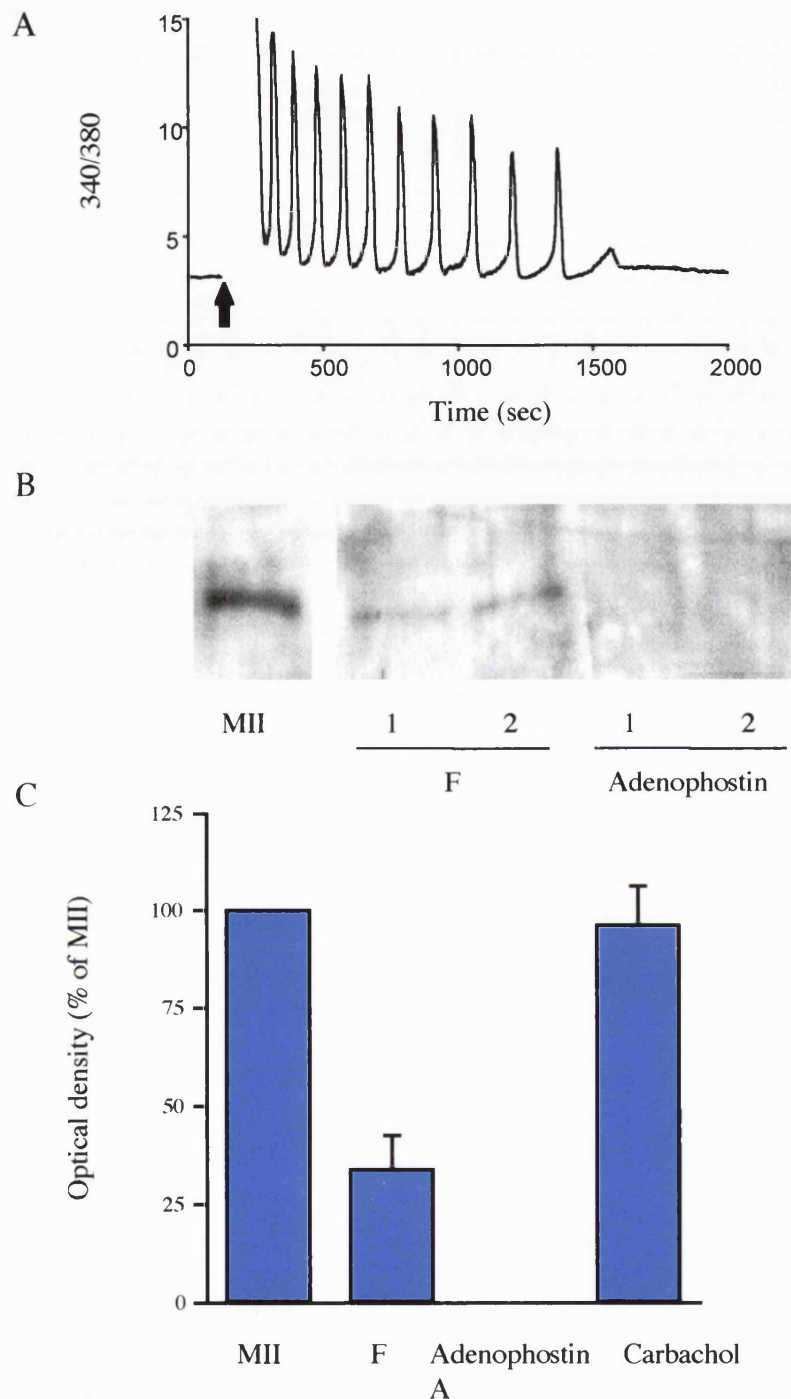


Figure 4.6 Microinjection of adenophostin A into MII oocytes causes extensive $\text{InsP}_3\text{R-I}$ downregulation. MII oocytes were microinjected with adenophostin to produce a final concentration of 100-500nM, or were pretreated with 100 μM carbachol for 30 minutes. (A) An example Ca^{2+} record during microinjection of adenophostin into a single Fura-2-loaded MII oocyte. (In this experiment microinjection was performed by Karl Swann, Department of Anatomy and Developmental Biology, UCL). Ca^{2+} oscillations were induced immediately after injection, which is indicated by the arrow. Ca^{2+} is presented as the ratio of 340/380nm. After 6 hours adenophostin-injected pronucleate eggs (B) or carbachol-treated MII-arrested eggs were collected and protein was electrophoresed (20 eggs per lane) and blotted with Ab40 type I-specific antibody. Adenophostin A caused downregulation of the protein to below detectable levels (B and C), whereas carbachol had no effect on $\text{InsP}_3\text{R-I}$ levels (C). Blots are representative of 4 experiments for each treatment.

4.7.2 Adenophostin A stimulates loss of InsP₃Rs in mature mouse oocytes

It has recently been found that InsP₃R downregulation in somatic cell lines happens only in the presence of *persistent* InsP₃ production (Oberdorf *et al.*, 1999) and an InsP₃R with an InsP₃-binding domain (Zhu *et al.*, 1999). To examine whether prolonged stimulation of the InsP₃R causes downregulation in mature mouse oocytes, adenophostin A, a potent non-metabolisable InsP₃R agonist, was microinjected. I first examined the ability of three concentrations of adenophostin A to effectively activate InsP₃Rs in mouse eggs. The ability of adenophostin A to stimulate ^{Fura2}Ca²⁺ oscillations in mouse eggs (He *et al.*, 1999; Sato *et al.*, 1998) was investigated using ^{Fura2}Ca²⁺ imaging. Microinjection of 100-500nM (final oocyte concentration) adenophostin A stimulated Ca²⁺ oscillations in 7 out of 10 MII eggs (**Fig. 4.6.A**) and a single large Ca²⁺ transient in the remaining 3 MII eggs. With a higher concentration of adenophostin I examined the ability of the InsP₃R agonist to stimulate oocyte activation. Scoring for pronucleus formation revealed that injection of 1-5μM adenophostin (oocyte concentration) resulted in the activation and formation of a pronucleus in 18/19 oocytes.

Having confirmed that adenophostin A was able to activate InsP₃R channels I examined the effect of a series of oocyte concentrations of adenophostin on the level of InsP₃R protein. MII oocytes were microinjected with final oocyte concentrations of 10-50μM, 1-5μM and 100-500nM adenophostin A. Within 6 hours all three concentrations of adenophostin caused extensive downregulation of InsP₃Rs such that InsP₃R protein could no longer be detected on a Western blot of 20 oocytes (**Fig. 4.6.B and C**). Adenophostin A is therefore the only agonist of Ca²⁺ release tested that can downregulate InsP₃Rs in a manner similar to that of fertilisation.

4.7.3 Adenophostin A stimulates loss of InsP₃Rs in immature mouse oocytes

In order to explore further the stimulus for InsP₃R downregulation I next examined the ability of adenophostin A to downregulate InsP₃Rs in *immature* oocytes. Monitoring Ca²⁺ release after adenophostin A injection (100-500nM) into germinal vesicle stage immature oocytes revealed that in all nine oocytes only one single Ca²⁺ transient was generated (**Fig. 4.7.Aa**). However, Western analysis showed that adenophostin, like fertilisation, leads to the loss of InsP₃Rs in immature oocytes (**Fig. 4.7.Ab**). This result suggests that adenophostin-induced InsP₃R downregulation in mouse oocytes is independent of both repetitive Ca²⁺ transients and egg activation.

4.7.4 Adenophostin A stimulates loss of InsP₃Rs in interphase embryos

Finally, I investigated whether in addition to triggering downregulation of InsP₃Rs in immature (prophase of meiosis I) and mature (metaphase of meiosis II) oocytes, adenophostin A was able to stimulate loss of InsP₃Rs in interphase embryos. I examined the ability of adenophostin to downregulate receptors in Sr²⁺-generated parthenogenotes at

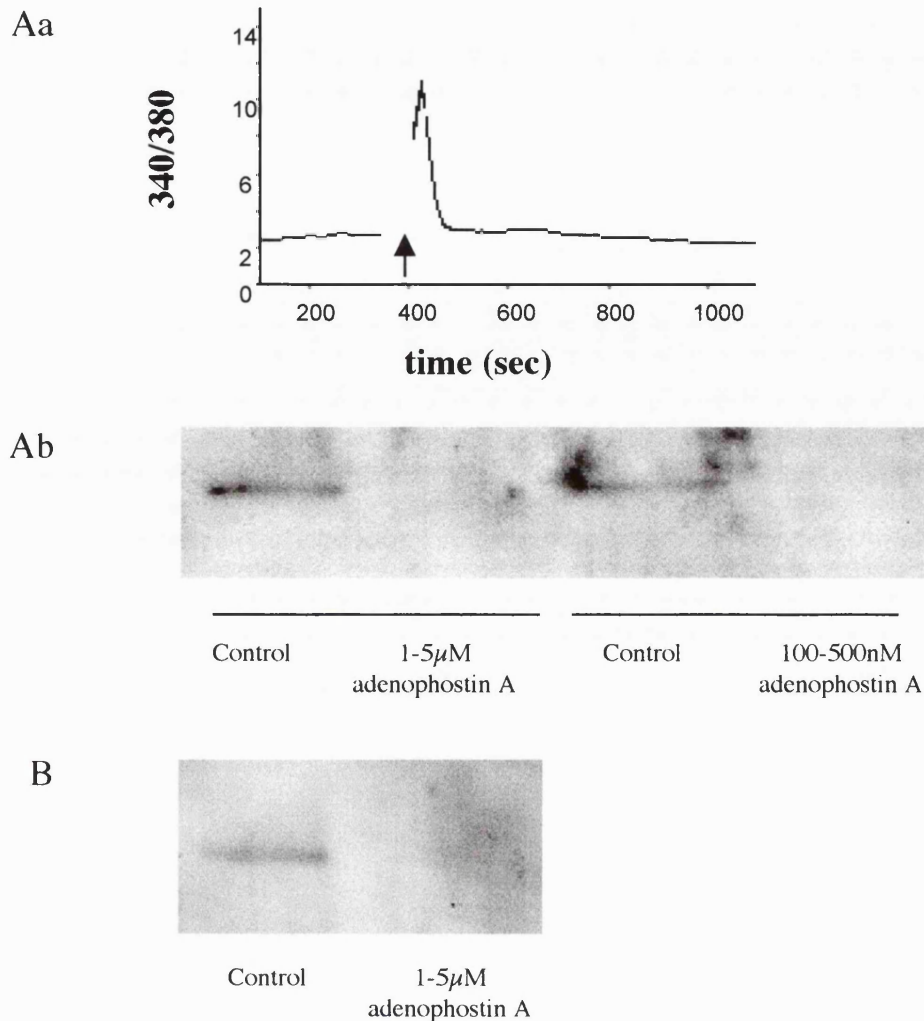


Figure 4.7 Microinjection of adenophostin A into a) immature oocytes and b) interphase 1-cell embryos causes $\text{InsP}_3\text{R-I}$ downregulation. (A) Immature oocytes were microinjected with adenophostin to produce a final concentration of 1-5 μM or 100-500 nM. (Aa) A representative Ca^{2+} record during microinjection of adenophostin (100-500 nM final) into a single Fura-2-loaded immature oocyte. A single Ca^{2+} transient was induced immediately after injection of the drug at the arrow (typical of 9). Injected oocytes were allowed to mature over 14 hours to the MII stage before collection for Western analysis. (Ab) Protein from 20 MII eggs was electrophoresed and immunoblotted with Ab40 next to a control lane of uninjected *in vitro*-matured oocytes. Both concentrations of adenophostin induced extensive InsP_3R downregulation. These data are representative of 3 experiments. (B) Sr^{2+} -activated eggs were cultured to the pronucleate (interphase) stage and microinjected with adenophostin (1-5 μM final) at 8 hours after Sr^{2+} addition. The 1-cell embryos were cultured for a further 4 hours and collected for Western analysis. Protein from 30 Pn embryos was electrophoresed and immunoblotted with Ab40 next to a control lane of uninjected parthenogenotes which had been cultured in parallel. Adenophostin induced extensive InsP_3R downregulation to below our limits of detection. Analysis is representative of 2 experiments.

the pronucleate stage. Sr^{2+} -activated eggs were cultured to the pronucleate stage and injected with 100 μM adenophostin A (final oocyte concentration of 1-5 μM) at 8 hours after Sr^{2+} addition. Western analysis 4 hours after injection of adenophostin revealed that the level of InsP_3Rs was undetectable in 30 embryos (**Fig. 4.7.B**). Therefore it appears that adenophostin A can induce InsP_3R downregulation in both M-phase and interphase of the cell cycle.

4.8 DISCUSSION

In this chapter I explore the observations described in Chapter 3 that InsP₃Rs undergo downregulation within the 4 hours following fertilisation. I establish that InsP₃R downregulation is independent of an increase in cytosolic Ca²⁺ and of egg activation. The strict requirements for the stimulation of downregulation appear to involve prolonged activation of the InsP₃R, a condition that provides an insight into the mechanism of Ca²⁺ signaling at fertilisation.

4.8.1 The stimulus of InsP₃R downregulation at fertilisation: implications for the mechanism of Ca²⁺ signaling at fertilisation

Egg activation and Ca²⁺ increases are neither sufficient nor necessary for InsP₃R downregulation

Fertilisation stimulates a number of events that may be important in the initiation of InsP₃R downregulation. The main candidates for a role in InsP₃R downregulation include (1) the structural reorganisation of the oocyte at fertilisation, (2) the sperm-induced Ca²⁺ changes and (3) the activation of proteolytic pathways that are normally associated with cell cycle progression by degrading cyclin B (Glotzer *et al.*, 1991) and *c-mos* (Ishida *et al.*, 1993). However, my results indicate that none of the above mechanisms can account for sperm-induced InsP₃R downregulation. The finding that parthenogenetic activating agents ethanol, Sr²⁺ and cycloheximide failed to downregulate InsP₃Rs shows that egg activation itself is not sufficient for downregulation. To determine whether egg activation at fertilisation is necessary for downregulation, BAPTA was used to inhibit Ca²⁺ transients and therefore egg activation. BAPTA treatment had no effect on the ability of fertilisation to stimulate downregulation. Other experiments provided more evidence that oocyte activation is not necessary because fertilisation of immature oocytes also induced downregulation. Together, these experiments show that egg activation is neither sufficient nor necessary for InsP₃R downregulation. Since cyclin B and *c-mos* are degraded in all activated mouse eggs (Kubiak *et al.*, 1993; Moos *et al.*, 1996a), the mechanism that marks cyclin B and *c-mos* for proteolysis appears to operate independently of the mechanism responsible for InsP₃R downregulation.

These same experiments rule out a role for increases in Ca²⁺. Sr²⁺ induces persistent Ca²⁺ oscillations, probably by sensitising a CICR mechanism, although the actual mechanism is not yet known (Cheek *et al.*, 1993). Ethanol induces a single monotonic Ca²⁺ increase. However, both Sr²⁺ and ethanol fail to downregulate InsP₃Rs. Furthermore, downregulation occurs in the absence of Ca²⁺ transients, or in the presence of limited Ca²⁺ transients (BAPTA-treated eggs and immature oocytes, respectively). These observations are in agreement with a recent study in AR4-2J cells in which the downregulation of types

I, II and III InsP₃Rs was shown not to correlate with changes in intracellular Ca²⁺ concentration (Oberdorf *et al.*, 1999). In this study, it was found that bombesin, pituitary adenylate cyclase-activating peptide (PACAP) and cholecystokinin (CCK) were all able to downregulate InsP₃Rs. However, whereas these stimuli all maintained InsP₃ concentration at significantly elevated levels for longer than agonists which were unable to downregulate InsP₃Rs, the effect on Ca²⁺ levels was similar amongst all agonists (Oberdorf *et al.*, 1999). Thus, like egg activation, changes in cytosolic Ca²⁺ are neither necessary nor sufficient for inducing InsP₃R downregulation at fertilisation.

Although downregulation appears to be independent of changes in cytosolic Ca²⁺, studies have indicated that this process does require ER Ca²⁺ stores to be intact (Oberdorf *et al.*, 1999; Wojcikiewicz *et al.*, 1994). Evidence supporting this comes from treating cells with thapsigargin, which inhibits the ER Ca²⁺-ATPase and causes intracellular Ca²⁺ stores to empty, including InsP₃-sensitive stores (Thastrup *et al.*, 1990). Thapsigargin on its own does not cause InsP₃R downregulation (Wojcikiewicz *et al.*, 1999; Wojcikiewicz *et al.*, 1994). However, thapsigargin blocks (1) carbachol-induced InsP₃R downregulation in SH-SY5Y cells (Wojcikiewicz *et al.*, 1994), (2) CCK-induced InsP₃R downregulation in AR4-2J cells (Oberdorf *et al.*, 1999) and (3) the physiological downregulation of InsP₃Rs by secretagogues in rat pancreatic acinar cells (Wojcikiewicz *et al.*, 1999). At least in the cultured cell types, thapsigargin does not affect InsP₃ formation (Oberdorf *et al.*, 1999; Wojcikiewicz *et al.*, 1994), indicating that inhibition of downregulation is due to its effects on the Ca²⁺ stores, rather than inhibiting InsP₃ production. Intact Ca²⁺ stores may be needed for InsP₃R downregulation due to a requirement for physiological Ca²⁺ flux through the channel (Wojcikiewicz *et al.*, 1999; Wojcikiewicz *et al.*, 1994) (discussed in more detail below) or due to an effect of ER luminal Ca²⁺ on InsP₃ binding. Alternatively, inhibition of downregulation by thapsigargin may be mediated by the elevated cytosolic Ca²⁺ concentration inhibiting InsP₃ binding, since binding of InsP₃ to its receptor is reversibly inhibited by high Ca²⁺ concentration (Cardy *et al.*, 1997; Taylor, 1998). This may be possible since in some cell types thapsigargin stimulates capacitative Ca²⁺ entry, leading to a sustained increase in cytoplasmic Ca²⁺. Although it would be interesting to examine the role of intracellular Ca²⁺ stores in InsP₃R downregulation in oocytes, thapsigargin treatment is unlikely to be informative. Previous studies have revealed that the ability of thapsigargin to inhibit fertilisation-induced Ca²⁺ transients is variable (Jones *et al.*, 1995b; Kline and Kline, 1992b).

InsP₃R downregulation at fertilisation is a result of increased protein degradation

There are at least two possible mechanisms that may explain the decrease in InsP₃R protein after fertilisation. Fertilisation may switch off the synthesis of any new receptor protein or may lead to the active degradation of the receptor. The estimated half-life of the InsP₃R in WB rat liver epithelial cells is about 11 hours (Joseph, 1994). Therefore, by 4 hours after

fertilisation (time taken for InsP₃R protein to maximally decrease), at least 75% of the *original* protein should remain; if the decrease in protein levels is a result of inhibited protein synthesis alone, it follows that at least 75% of control protein levels should be present at this time. Since fertilisation downregulates receptors to 20% of control levels within 4 hours it seems likely that the sperm stimulates active proteolysis of InsP₃Rs. As described above, fertilisation leads to the active proteolysis of other proteins including cyclin B (Glotzer *et al.*, 1991) and *c-mos* (Ishida *et al.*, 1993). Furthermore, the inability of cycloheximide, which inhibits protein synthesis, to significantly downregulate protein levels of type I InsP₃R (this study) provides additional evidence for sperm-induced downregulation being an active mechanism of increased protein degradation. In fact, the inability of cycloheximide to affect InsP₃R levels over a duration of 6 hours suggests that the half-life in oocytes may be longer than the published half-life estimate of 11 hours (Joseph, 1994). The concept that sperm-induced downregulation is a result of enhanced protein degradation is in agreement with previous studies. These established that downregulation of InsP₃Rs induced by carbachol in SH-SY5Y cells and by angiotensin II in WB cells, is mediated by stimulation of InsP₃R degradation and does not involve an inhibition of InsP₃R biosynthesis (Bokkala and Joseph, 1997; Wojcikiewicz *et al.*, 1994). However, there is some evidence for reduced type I InsP₃R synthesis contributing towards downregulation at certain times. For example, in one study in SHSY-5Y cells, type I InsP₃R mRNA levels fell after 3 hours of muscarinic receptor stimulation but then returned to basal levels (Wojcikiewicz *et al.*, 1994). Another study in a murine mesangial cell line showed that TGF-β1 downregulates type I InsP₃R protein expression and also causes a reduction in type I mRNA expression which precedes the reduction in protein (Sharma *et al.*, 1997). A third study in rat A7r5 smooth muscle cells transiently expressed a luciferase coding sequence, placed under control of the rat InsP₃R-I promoter, to examine the effect of agonists on transcription-initiation from the InsP₃R promoter. They found that stimulation of cells with agonists which caused downregulation of InsP₃Rs, also caused reduced luciferase activity, indicating a reduction of type I InsP₃R promoter activity (Sipma *et al.*, 1998). Nevertheless, when half-life and the observed rate of downregulation are taken into account, it is expected that an enhanced rate of degradation of the protein is the major determinant causing downregulation of InsP₃Rs even in these cell types. (See Chapter 5 for investigation into the protease responsible for fertilisation-induced InsP₃R degradation).

Prolonged activation of the InsP₃R may stimulate downregulation

It is generally regarded that fertilisation stimulates Ca²⁺ release by the hydrolysis of PIP₂ by PLC (Carroll *et al.*, 1997; Jones *et al.*, 1998; Shearer *et al.*, 1999). For the reasons described above, the Ca²⁺-releasing arm of this signaling pathway appears ineffective in the stimulation of InsP₃R downregulation and my experiments also make unlikely a role for the PKC arm. The finding that PKC activation had no effect on InsP₃R levels is consistent with other studies on cell lines, which have also shown that activation of protein kinases C

with phorbol ester does not elicit downregulation (Bokkala and Joseph, 1997; Wojcikiewicz and Nahorski, 1991). The lack of any role for egg activation, Ca^{2+} or PKC suggests that the mechanism must be highly specific to the process of fertilisation, being either a sperm-derived protease that degrades InsP_3Rs or a distinct feature of cell signaling at fertilisation.

A recent study employing mutant InsP_3Rs demonstrated that the InsP_3 -binding domain of the receptor was necessary for receptor downregulation in SH-SY5Y cells stimulated with carbachol (Zhu *et al.*, 1999), and suggested that InsP_3 binding directly activates InsP_3R downregulation. This supports my conclusion that Ca^{2+} is not necessary or sufficient for downregulation since the downregulation-resistant InsP_3Rs are also exposed to Ca^{2+} increases. Work by the same group has also established a correlation between the ability of an agonist to cause sustained InsP_3 production and InsP_3R downregulation (Oberdorf *et al.*, 1999). The concept that InsP_3 binding is necessary to downregulate InsP_3Rs appears to explain why the activation methods described above failed to stimulate downregulation, since none of these methods of parthenogenetic activation have been reported to increase the production of InsP_3 . The failure of carbachol to stimulate downregulation in mouse oocytes, despite mobilising Ca^{2+} , may be because it does not generate prolonged InsP_3 production in mouse oocytes (Lawrence *et al.*, 1997; Swann, 1992). In contrast, when I activated InsP_3Rs using the potent InsP_3R agonist, adenophostin A, I found that, other than sperm, it was the only stimulus capable of inducing InsP_3R downregulation. Adenophostin A caused a decrease in the levels of InsP_3Rs to an even greater extent than sperm, such that the InsP_3Rs were no longer detectable in Western analysis of 20 oocytes (protein levels <15% of levels in unfertilised oocytes). This increased efficiency for the stimulation of downregulation is probably due to adenophostin having an 100-fold greater potency than InsP_3 , demonstrated by a K_i value of 0.18nM compared with a value of 15nM for InsP_3 (Takahashi *et al.*, 1994b). Adenophostins also exhibit a resistance to phosphorylation and dephosphorylation by InsP_3 -metabolising enzymes and show prolonged activation of the InsP_3R (Takahashi *et al.*, 1994a). The ability of injected adenophostin to downregulate InsP_3Rs also demonstrates that the plasma membrane receptor pathway is not necessary for the loss of InsP_3Rs . Previously, experiments using cell lines employed cell-surface receptor agonists and were not able to rule out a contribution from the receptor-mediated signaling (Bokkala and Joseph, 1997; Sipma *et al.*, 1998; Wojcikiewicz, 1995; Wojcikiewicz *et al.*, 1994). The results of this study suggest that occupation of the InsP_3R is sufficient to induce its downregulation (Zhu *et al.*, 1999).

Prolonged activation of InsP_3Rs stimulates downregulation independent of stage of oocyte maturation or cell cycle

In addition to stimulating loss of InsP_3Rs in mature mouse oocytes, adenophostin A was also able to stimulate downregulation in immature mouse oocytes and interphase 1-cell embryos. The finding that both adenophostin-induced and sperm-induced InsP_3R

downregulation can take place in immature oocytes and thus do not require either repetitive Ca^{2+} oscillations or egg activation, strongly implies that downregulation takes place via a similar mechanism in the two situations. Thus the trigger for InsP_3R downregulation may well be persistent stimulation of the InsP_3R by adenophostin or, in the case of fertilisation, by a persistent production of InsP_3 .

The ability of prolonged binding to and activation of the InsP_3R (by adenophostin) to cause downregulation in both immature and mature and in both M-phase and interphase of the cell cycle also suggests that the downregulatory mechanism does not require any additional factors specific to either “fully-grown” oocytes or to the stage of the cell cycle. As described above, fertilisation also is able to stimulate loss of InsP_3Rs in immature oocytes. Thus, unlike the ability to generate normal Ca^{2+} transients in response to sperm or the capacity to decondense sperm chromatin, both of which develop during the final stages of oocyte growth and maturation (Carroll *et al.*, 1996; Cheung *et al.*, 2000; Perreault *et al.*, 1988), the pathway which results in the downregulation of InsP_3Rs appears to be set up independently of the stage of oocyte maturation or of the cell cycle. This is consistent with the downregulation of the InsP_3R at fertilisation being an inherent property of Ca^{2+} signaling pathways that involve persistent InsP_3 production. In other words, it is likely that the capacity to downregulate InsP_3Rs does not *develop* during maturation, like the ability to generate Ca^{2+} oscillations, in order that the mature oocyte can support fertilisation and early development.

If InsP_3R downregulation can be stimulated in the immature oocyte, this suggests that persistent production of InsP_3 must be stimulated. However, only a few Ca^{2+} transients are generated in response to either sperm or adenophostin in the immature oocyte. Since persistent InsP_3 production in both immature and mature oocytes indicates that PIP_2 availability is not limiting, the ability of oocytes to generate normal Ca^{2+} transients may be limited by the InsP_3R itself or by the Ca^{2+} store.

Binding of InsP_3 induces a conformational change in the InsP_3R which may signal downregulation

Another recent study has also investigated the stimulus responsible for inducing type I InsP_3R downregulation at fertilisation of mouse eggs (Jellerette *et al.*, 2000). In support of my results, exposure to ethanol or ionomycin, which, like ethanol, induces a single intracellular Ca^{2+} rise, or Sr^{2+} , failed to stimulate downregulation of InsP_3Rs . In addition, injection of boar sperm fractions, which probably stimulate production of InsP_3 (Jones *et al.*, 2000), and adenophostin A, induced InsP_3R downregulation. However, a further finding was that downregulation was initiated by exposure to thimerosal, an oxidising agent that modifies the InsP_3R without stimulating production of InsP_3 (Jellerette *et al.*, 2000). In this study the concentration of thimerosal used (200 μM) stimulated a series of low frequency Ca^{2+} oscillations. Thimerosal has been shown to increase the affinity of type

I InsP₃R for InsP₃ (Kaplin *et al.*, 1994; Poitras *et al.*, 1993; Vanlingen *et al.*, 1999), making the channel open at lower InsP₃ concentrations (Sayers *et al.*, 1993). Thus thimerosal may induce downregulation of the InsP₃R by causing the same end effect as adenophostin, that is a much more potent interaction between ligand and receptor. Alternatively, thimerosal has been demonstrated to oxidise critical cysteine residues in the receptor, inducing a change in the conformational state of the InsP₃R (Sayers *et al.*, 1993), which may be sufficient to induce downregulation. In fact, a conformational change has been demonstrated in the InsP₃R following binding of InsP₃, which may be the mechanism by which ligand binding causes opening of the channel (Mignery and Sudhof, 1990; Miyawaki *et al.*, 1991). This structural change may also be required for the downregulation of the receptor (Zhu *et al.*, 1999; Zhu and Wojcikiewicz, 2000) (see Chapter 5). Another alternative explanation for how thimerosal stimulates InsP₃R downregulation is that thimerosal-induced disruption of the meiotic spindle (Cheek *et al.*, 1993) stimulates receptor degradation as I find for nocodazole (discussed in more detail in 4.8.2).

InsP₃R downregulation: implications for Ca²⁺ signaling at fertilisation

The observation that sperm and adenophostin act in a similar manner to downregulate InsP₃Rs has obvious implications for our understanding of the mechanism of Ca²⁺ signaling at fertilisation. The properties of adenophostin A outlined above may allow this agonist to remain bound to the receptor for longer periods of time than InsP₃. Therefore a major implication is that sperm-induced Ca²⁺ signaling is achieved through prolonged and persistent production of InsP₃. At present there is a general agreement in the field that a PLC is involved at fertilisation (Carroll *et al.*, 1997; Jones *et al.*, 1998; Shearer *et al.*, 1999). However, it is not clear how the PLC is activated. Clearly, my data support the involvement of a PLC and suggest that the PLC remains active for the duration of the Ca²⁺ signaling period. There are at least two models that can support the generation of InsP₃ for prolonged periods. One is a persistent, chronic activation of PLC resulting in sustained elevation of InsP₃ while the other is a mechanism that leads to transient activation of PLC which in turn leads to pulses of InsP₃ being produced at each Ca²⁺ transient (Meyer and Stryer, 1988). A recent study has found evidence to support a continuous low level generation of InsP₃ during mammalian fertilisation (Jones and Nixon, 2000). In this work the photolysis of caged InsP₃ at low amounts for extended periods was able to closely mimic fertilisation-induced Ca²⁺ oscillations. Consistent with these findings a much earlier study found that in mouse pancreatic acinar cells, pulsatile intracellular Ca²⁺ release could be stimulated by a constant intracellular concentration of InsPS₃ (a nonmetabolisable InsP₃ analogue) (Wakui *et al.*, 1989).

The fact that a constant elevated InsP₃ level can cause changes in Ca²⁺ similar to those at fertilisation does not necessarily mean that this is the mechanism that physiologically occurs but it is strongly consistent with my data. A current hypothesis is that a sperm PLC generates InsP₃-driven Ca²⁺ oscillations following sperm-egg fusion (Jones *et al.*, 2000;

Jones and Nixon, 2000; Mehlmann *et al.*, 1998; Parrington *et al.*, 2000). My data appears to argue against a mechanism of InsP_3 production involving Ca^{2+} -dependent activation of PLC. BAPTA-treated eggs demonstrate InsP_3R downregulation but do not generate Ca^{2+} transients and therefore lack the capacity to periodically activate PLC in a Ca^{2+} -dependent manner. A similar finding has been reported in *Xenopus* eggs in which fertilisation stimulates an increase in InsP_3 mass which is not affected by the presence of BAPTA (Stith *et al.*, 1994). A recent study appears, at first, to challenge the idea that a continuous generation of InsP_3 is required for InsP_3R downregulation. In this study it was found that the ability of the mammalian sperm-derived PLC to cause InsP_3 production in sea urchin egg homogenate (Jones *et al.*, 1998; Jones *et al.*, 2000) is Ca^{2+} -dependent (Rice *et al.*, 2000). This would suggest that InsP_3 -driven Ca^{2+} oscillations in turn cause pulsatile activation of the sperm-derived PLC and generate pulses of InsP_3 , rather than a continuous InsP_3 production. However, the sperm-derived PLC is active at resting Ca^{2+} concentrations as sperm extract was able to trigger Ca^{2+} release from a baseline Ca^{2+} concentration of $<50\text{nM}$ (Rice *et al.*, 2000). One hypothesis, therefore, is that the sperm-derived PLC is persistently active in the egg cytosol, generating a continuous low level production of InsP_3 during fertilisation (Jones and Nixon, 2000), which binds to and may trigger the downregulation of, the InsP_3R . Superimposed upon this constant InsP_3 generation may be pulses of increased InsP_3 concentration as a result of the Ca^{2+} spikes activating the Ca^{2+} -dependent PLC. However, although the mammalian *sperm*-derived PLC activity described above (Rice *et al.*, 2000) is Ca^{2+} -sensitive, there is no evidence to exclude a role (downstream of sperm factor addition) for a Ca^{2+} -insensitive *egg*-derived PLC activity, in generating InsP_3 at fertilisation. This scenario could also lead to the persistent generation of InsP_3 (independent of Ca^{2+}) necessary for InsP_3R downregulation.

The existence in this model of “spikes” of increased InsP_3 production upon an elevated baseline is compatible with other studies on the temporal dynamics of InsP_3 in the generation of Ca^{2+} oscillations. Firstly, monitoring the translocation of a GFP-PH domain to analyse changes in intracellular InsP_3 concentration provided evidence for InsP_3 oscillations accompanying Ca^{2+} oscillations in single MD canine kidney epithelial cells (Hirose *et al.*, 1999). Secondly, a different study in fibroblasts showed that Ca^{2+} oscillations were accompanied by oscillations in InsP_3 concentration. It also found evidence to suggest a feedback mechanism, involving Ca^{2+} stimulation of a PLC, for the Ca^{2+} oscillations initiated by agonists stimulating the PI signaling pathway (Harootunian *et al.*, 1991).

An alternative explanation for InsP_3R downregulation in the presence of BAPTA (other than a persistent Ca^{2+} -independent production of InsP_3) is that the combination of BAPTA and fertilisation leads to depletion of Ca^{2+} stores, which can induce ER stress (Llewellyn *et al.*, 1996) and a resultant increase in proteolysis of ER-associated proteins (Haze *et al.*, 1999; Nakagawa *et al.*, 2000). This is unlikely, however, since in other cell types,

artificially emptying the Ca^{2+} stores does not affect InsP_3R levels. For example, thapsigargin blocks agonist-induced type I InsP_3R downregulation in SH-SY5Y, AR4-2J and pancreatic acinar cells and is unable to alter receptor levels alone (Oberdorf *et al.*, 1999; Wojcikiewicz *et al.*, 1999; Wojcikiewicz *et al.*, 1994). One explanation for this is that physiological release (rather than artificial release such as that caused by thapsigargin) of ER Ca^{2+} , combined with receptor activation by InsP_3 , might mediate downregulation (Wojcikiewicz *et al.*, 1999). Ca^{2+} store depletion therefore may be a factor contributing to InsP_3R downregulation at fertilisation when combined with InsP_3 binding (in the absence or presence of BAPTA) and requires further investigation. Another possible explanation for the ability of sperm-induced downregulation to take place in the presence of BAPTA is that BAPTA treatment may interfere with InsP_3 metabolism resulting in prolonged elevation of InsP_3 (Stith *et al.*, 1994).

Further work is required to identify the properties and isoform of the PLC involved at fertilisation; my data indicate that it is continuously activated for several hours. A different study has even suggested that PLC activity is maintained up to the first mitotic metaphase (Kono *et al.*, 1996). It was shown that the sperm provides a Ca^{2+} -releasing activity that becomes associated with the pronuclei and leads to the generation of Ca^{2+} transients during mitosis of the first cell cycle.

BAPTA stimulates InsP_3R downregulation

It is interesting to note that incubation of MII oocytes in 10 μM BAPTA-AM alone leads to downregulation of InsP_3Rs to about 60% of control levels. 10 μM BAPTA-AM has been shown to inhibit protein synthesis (a 2/3 decrease in leucine incorporation) in fertilised mouse eggs (Lawrence *et al.*, 1998). However, as described above, the protein synthesis inhibitor, cycloheximide, does not have a significant effect on InsP_3R protein levels. Together with the low turnover rate for the InsP_3R (see above) this suggests that BAPTA stimulates an active, Ca^{2+} -independent, degradation of InsP_3Rs .

The BAPTA-stimulated and sperm-stimulated downregulation of InsP_3Rs , which presumably act simultaneously in the BAPTA-loaded and fertilised oocyte, may involve the same or completely different pathways. One explanation for how BAPTA stimulates degradation of InsP_3Rs is that it may inhibit metabolism of basal InsP_3 (Stith *et al.*, 1994), causing an increase in InsP_3 levels and thus an increase in InsP_3R activation. An alternative explanation is that BAPTA may have an effect on the cytoskeleton. I have shown that both nocodazole and CCD (which disrupt the spindle and the actin cytoskeleton, respectively) also induce the degradation of InsP_3Rs (see 4.8.2). Evidence for a direct effect of BAPTA on the spindle comes from a paper on the requirement of an intact spindle during oocyte activation (Winston *et al.*, 1995). Unfertilised oocytes were treated with nocodazole and then washed free of nocodazole, after which the chromosomes were condensed and dispersed around the cortex in clumps, each of which had recruited polymerised

microtubules into a miniature meiotic spindle. On the other hand, when fertilised, nocodazole-treated oocytes were removed to a medium containing BAPTA, they contained multiple groups of condensed chromosomes, but failed to form complete spindles (Winston *et al.*, 1995). The action of BAPTA in preventing the reformation of miniature spindles indicates that it has an inhibitory effect on tubulin polymerisation. In addition, it has been shown that actin polymerisation during the inflammatory response in human eosinophils depends on intracellular Ca^{2+} mobilisation and is inhibited by BAPTA (Elsner *et al.*, 1996). Thus it is conceivable that the polymerisation of the actin cytoskeleton or microtubules of the spindle in the MII oocyte may be dependent on local (and therefore undetected) Ca^{2+} gradients and that these gradients may be buffered by BAPTA. Further experiments are required to investigate whether inhibited polymerisation of actin or tubulin does induce degradation of InsP_3Rs and to discover the exact cause of the effect of BAPTA on InsP_3R levels.

4.8.2 Nocodazole and cytochalasin D stimulate InsP_3R downregulation: implications for the mechanism of downregulation at fertilisation

The observation that both nocodazole and cytochalasin D (CCD) are able to induce the downregulation of InsP_3Rs in MII mouse oocytes may be important in deducing the stimulus and actual mechanism of the downregulation seen at fertilisation. Nocodazole depolymerises microtubules and disrupts the metaphase spindle of the MII oocyte and CCD depolymerises actin microfilaments and thus breaks down the actin cytoskeleton. Western analysis showed that both nocodazole and CCD stimulated InsP_3R downregulation to 20-25% of control protein levels (untreated MII oocytes), similar to the decrease to 20% of control levels observed after fertilisation (Chapter 3). Due to the lack of an effect of cycloheximide on InsP_3R protein levels (see above), I propose that nocodazole- and CCD-induced InsP_3R downregulation is due to active protein degradation rather than an inhibition of protein synthesis.

Why would disruption of microtubules or microfilaments induce InsP_3R degradation?

There is substantial evidence in the literature to suggest some form of interaction between microtubules and microfilaments and InsP_3Rs (Guillemette *et al.*, 1990; Rossier *et al.*, 1991; Tasaka *et al.*, 1991). One theory is that disruption of this interaction between cytoskeleton and InsP_3R may lead to an increase in InsP_3 binding, which in turn may stimulate receptor degradation. However, there is contrasting evidence in the literature, suggesting that disrupting the cytoskeleton causes InsP_3 - InsP_3R binding to be inhibited in some cell types but increased in others. On the one hand, CCD and colchicine (a microtubule inhibitor) inhibit InsP_3 binding and InsP_3 -mediated Ca^{2+} release during activation of human blood platelets (Bourguignon *et al.*, 1993a). Also, latrunculin B (disrupts actin cytoskeleton) reduces the rate of Ca^{2+} release and interferes with propagation

of Ca^{2+} waves in intact pancreatic acinar cells (Shin *et al.*, 2000). On the other hand, disrupting the cytoskeleton in other cell types may lead to an increase in InsP_3 binding. Ankyrin is an adaptor protein which connects plasma membrane proteins with the spectrin-based cytoskeleton (Bennett, 1992; Bennett and Lambert, 1991). A population of InsP_3Rs in rat cerebellum may be attached to an isoform of ankyrin, which might mediate interactions of the receptor with the cytoskeleton (Joseph and Samanta, 1993). Furthermore, high affinity binding has been found between ankyrin and the InsP_3R on Ca^{2+} -storage vesicles in mouse T-lymphoma cells (Bourguignon and Jin, 1995; Bourguignon *et al.*, 1993b). However, the binding of ankyrin to the InsP_3R inhibits InsP_3 binding and InsP_3 -mediated Ca^{2+} release (Bourguignon *et al.*, 1993b).

The role of the cytoskeleton in the regulation of InsP_3 -mediated Ca^{2+} release during lymphocyte activation (Bourguignon *et al.*, 1993b) thus appears to contrast with its role during platelet activation, as discussed above (Bourguignon *et al.*, 1993a). This discrepancy may arise from differences between cell types or experimental conditions. It is clear that the cytoskeleton can regulate InsP_3 -mediated Ca^{2+} release but the effects of nocodazole and CCD on InsP_3 binding in MII oocytes remain to be determined. However, if CCD and nocodazole inhibit InsP_3 binding in mouse oocytes, this suggests that sperm-induced and CCD/nocodazole-induced degradation are stimulated by different pathways. This is because sperm-induced InsP_3R degradation appears to be stimulated by prolonged InsP_3 binding (see 4.8.1).

A recent study offers a more plausible explanation for nocodazole and CCD-induced InsP_3R degradation. An investigation into the failure of PDGF to induce Ca^{2+} release in cytoskeleton-disrupted NIH 3T3 cells, found that CCD pretreatment alone increased InsP_3 levels and that CCD pretreatment augmented InsP_3 production stimulated by PDGF (Ribeiro *et al.*, 1997). Although the CCD-induced increases in InsP_3 are likely to be very small (Ribeiro *et al.*, 1997), over a period of 5.5 hours (used in my study) it is conceivable that a persistently raised InsP_3 level may be a sufficient stimulus for the downregulation that I observed.

Work investigating the intracellular distribution of proteasomes has suggested a different effect of nocodazole, which may be a requirement for nocodazole-induced InsP_3R degradation. Proteasomes may carry out nocodazole-induced receptor degradation since they have been shown to be responsible for InsP_3R degradation in other cell types (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999) (see Chapter 5). Rat oocytes arrested at MII exhibit a colocalisation of the proteasome with the MII spindle apparatus (detected using an antibody against the 20S proteasome) (Josefsberg *et al.*, 2000). Therefore, depolymerising the microtubules of the MII spindle using nocodazole may release the proteasomes into the cytosol. Released proteasomes may diffuse throughout the cytoplasm (Reits *et al.*, 1997) and degrade ubiquitinated InsP_3Rs on the ER.

To summarise, it is conceivable that nocodazole may bring about InsP₃R downregulation by causing an increase in InsP₃-InsP₃R binding in conjunction with an increased level of proteasomes in the cytosol. Further experiments are necessary to examine the effects of both nocodazole and CCD on InsP₃ levels, on InsP₃ binding and on proteasome distribution in mouse oocytes. These experiments may help to determine the mechanism of nocodazole- and CCD-induced InsP₃R degradation.

It is unlikely that a release of proteasomes into the cytosol is part of the mechanism leading to InsP₃R degradation at fertilisation. Although the meiotic spindle undergoes structural changes, it remains intact until after second polar body formation in order for the two haploid maternal chromosome sets to be separated successfully (Howlett *et al.*, 1985). I have observed a loss of at least 50% of InsP₃Rs by the time of second polar body extrusion. The integrity of the spindle over this period suggests that degradation cannot depend on the release of proteasomes from depolymerising microtubules.

Finally, one explanation for InsP₃R downregulation stimulated by thimerosal (described above) (Jellerette *et al.*, 2000) is that thimerosal-induced disruption of the spindle triggers receptor degradation via the same mechanism as nocodazole. Consistent with this notion, an independent study found that thimerosal caused complete destruction of the spindle in mouse oocytes, and therefore prevented progression into interphase (Cheek *et al.*, 1993). Indeed, the thimerosal-induced Ca²⁺ responses and InsP₃R downregulation in the former study were not accompanied by egg activation (Jellerette *et al.*, 2000).

4.8.3 Conclusion

In conclusion, it may be that in the case of fertilisation, persistent InsP₃ production and InsP₃R activation causes a conformational change in the receptor which may label it for degradation. In the case of CCD, nocodazole (and BAPTA), elevated InsP₃ levels due to drug-induced InsP₃ production or inhibition of InsP₃ metabolism, and/or an increase in InsP₃ binding *per se*, combined with an increased cytosolic concentration of proteasomes, may be sufficient to cause InsP₃R degradation. In the latter case, further experiments are needed to determine which of the given hypotheses are correct.

CHAPTER 5:

MECHANISM OF InsP₃R DEGRADATION AT FERTILISATION

5.1 INTRODUCTION

In this chapter I investigate the identity of the protease responsible for InsP₃R degradation at fertilisation. The ubiquitin-proteasome pathway is the principal mechanism for the turnover of short-lived proteins in eukaryotic cells. Furthermore, this pathway is thought to be largely responsible for the selective degradation of ER membrane proteins (Biederer *et al.*, 1996; Ciechanover, 1998; Sommer and Wolf, 1997). Indeed, there is convincing data in somatic cells that InsP₃R downregulation is carried out by the 26S proteasome. InsP₃Rs undergo ubiquitination and downregulation can be inhibited effectively with both the specific proteasome inhibitor lactacystin and with ALLN, which inhibits both cysteine proteases (e.g. calpain) and the proteasome (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999). The process of ubiquitination and degradation by the proteasome is stimulated at oocyte activation in order to degrade cyclin B (Glotzer *et al.*, 1991) and *c-mos* (Ishida *et al.*, 1993). However, as discussed in Chapter 4, although the time-course of InsP₃R degradation is similar to that of these proteins, it is independent of stimulated cyclin B destruction at egg activation, and *c-mos* destruction. InsP₃R degradation is also dissimilar to cyclin B destruction at egg activation since it is not dependent on Ca²⁺ (Chapter 4).

The 26S proteasome is composed of a 20S catalytic subunit and a regulatory subunit complex. The 26S proteasome inhibitors used in this study are lactacystin, *N*-acetyl-Leu-Leu-norleucinal (ALLN) and MG132. Lactacystin is reported to inhibit all three activities that are associated with the 20S proteasome. Inhibition is irreversible, since the inhibitor covalently inactivates the active site threonine residue of the mammalian 20S subunit. In aqueous solutions, lactacystin, which itself does not react with the proteasome, undergoes a spontaneous conversion to the active proteasome inhibitor, clasto-lactacystin β -lactone. ALLN and MG132 are both reversible peptide aldehyde inhibitors and are termed "transition state analogues", meaning that they compete with substrates of the proteasome. ALLN was previously known as Calpain Inhibitor I and also inhibits cysteine proteases.

The other major pathway of cellular protein degradation is lysosomal proteolysis, which is particularly involved in the destruction of transmembrane proteins. Lysosomes are organelles containing hydrolytic enzymes used for the intracellular digestion of macromolecules. It is known that Ca²⁺ mobilising agents can cause ER fragmentation and can regulate lysosome-mediated ER autophagy (Booth and Koch, 1989; Dunn, 1990; Gordon *et al.*, 1993). Furthermore, it has been shown that chloroquine and NH₄Cl (which inhibit lysosomal proteases) increase the basal levels of InsP₃Rs in WB rat liver epithelial

cells by 2-fold, suggesting that the basal turnover of InsP₃Rs occurs via a lysosomal pathway (Bokkala and Joseph, 1997). NH₄Cl is thought to dispel the acidic pH in lysosomes, thereby inhibiting lysosomal proteases, which are optimally active at pH 5. Chloroquine also inhibits lysosomal proteases by raising lysosomal pH, blocking the action of cathepsins and many other lysosomal enzymes including phospholipases.

A third mechanism of protein degradation which I investigate is proteolysis by caspases. Caspases form a family of cysteine proteases which are essential mediators of programmed cell death (Cryns and Yuan, 1998), with 14 members so far identified. Caspase-12 is localised to the ER and is activated by ER stress, such as disruption of ER Ca²⁺ homeostasis using thapsigargin and Ca²⁺ ionophore A23187 (Nakagawa *et al.*, 2000). It is thus conceivable that periodic emptying of ER Ca²⁺ stores for a prolonged period at fertilisation, may lead to the activation of ER-localised caspases and proteolysis of ER membrane proteins such as the InsP₃R.

5.2 THE EFFECT OF PROTEASE INHIBITORS ON InsP₃R DEGRADATION

In Chapter 4 I investigated the stimulus which activates the downregulation of type I InsP₃R at fertilisation of mouse eggs. It was concluded that the likely trigger for sperm-induced downregulation is prolonged binding to and activation of the receptor by InsP₃ itself. It was also shown that a 6-hour incubation in cycloheximide, a protein synthesis inhibitor, has no significant effect on InsP₃R protein levels. This indicated that the half-life of the protein in mouse oocytes is significantly longer than 6 hours and that the basal rate of protein turnover is unable to explain the loss of InsP₃R immunoreactivity. The mechanism responsible for InsP₃R downregulation at fertilisation must therefore be one of increased protein degradation and in this chapter I investigate the nature of the protease involved. In somatic cells InsP₃R downregulation appears to be carried out by the ubiquitin-proteasome pathway as there is evidence for both ubiquitination of the receptor and inhibition of downregulation by proteasome inhibitors (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999). In order to identify the mechanism of degradation of InsP₃Rs in oocytes I have inhibited the different pathways of protein degradation.

5.2.1 Effect of proteasome inhibitors on egg activation

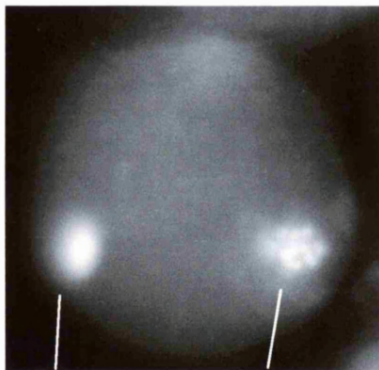
I first investigated the effect of inhibiting the proteasome in MII mouse eggs. It has long been established that release from metaphase arrest at fertilisation is induced by the degradation of cyclin B, which in turn leads to the inactivation of MPF (Murray *et al.*, 1989). Fertilisation also triggers the inactivation of CSF (Watanabe *et al.*, 1989), which is responsible for preventing cyclin destruction in the unfertilised vertebrate egg. Both degradation of cyclin B and of the *c-mos* product (component of CSF) in *Xenopus* have been shown to involve the 26S proteasome (Glutzer *et al.*, 1991; Ishida *et al.*, 1993), although an earlier study in *Xenopus* eggs found evidence for proteolysis of *c-mos* by the cysteine protease, calpain (Watanabe *et al.*, 1989). In the mouse egg, it has also been shown that degradation of both cyclin B (Kubiak *et al.*, 1993) and the *c-mos* product (Weber *et al.*, 1991) take place at fertilisation. Therefore, I was able to test the efficiency of proteasome inhibitors used in this study by observing whether they prevented egg activation and caused the fertilised egg to remain arrested at MII.

The results show that ALLN, MG132 and lactacystin effectively inhibited polar body extrusion and activation of fertilised eggs (**Fig. 5.1**), presumably by blocking the proteasome-dependent degradation of cyclin B (Glutzer *et al.*, 1991). Preincubation of oocytes for 2 hours in 10µM lactacystin did not prevent oocytes undergoing activation (n=6). However, preincubation in 20µM lactacystin prevented 100% of oocytes from activating (n=12). Although 10µM lactacystin is the concentration recommended in the literature for use in somatic cells (Bokkala and Joseph, 1997), the larger volume of the mouse oocyte may mean that a higher concentration (20µM) is necessary for complete

A

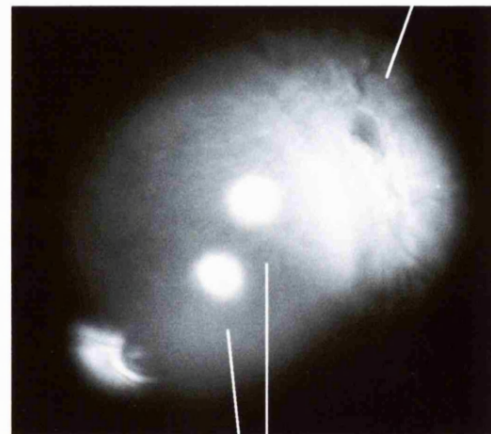
	No. oocytes exposed to sperm	No. oocytes activated after 4 hours*
Control	24	24 (100)
10 μ M lactacystin	6	6 (100)
20 μ M lactacystin	12	0 (0)
Control	24	24 (100)
10 μ M MG132	38	0 (0)
Control	10	10 (100)
50 μ g/ml ALLN	110	0 (0)

B



SH Condensed chromosomes at metaphase plate

C



Male and female pronuclei

Figure 5.1 The effect of proteasome inhibitors on egg activation. (A) MII oocytes were preincubated for 2 hours in lactacystin (10 or 20 μ M), MG132 (10 μ M) or ALLN (50 μ g/ml) and exposed to sperm with inhibitor maintained in the medium. Control oocytes of a similar age post superovulation were exposed to sperm from the same sperm preparation. *Activated eggs were scored at 4 hours after first exposure to sperm. Activation was defined as second polar body extrusion. Numbers (n) in table are from a single representative experiment on a single day. Values in parentheses are percentages. Results show that 100% of egg activation was prevented by 20 μ M lactacystin, 10 μ M MG132 and 50 μ g/ml ALLN. MG132-incubated (B) and control (C) fertilised oocytes were fixed and stained with Hoechst 6 hours after first exposure to sperm. (B) Presence of sperm head (SH) inside oocyte indicates fertilisation has taken place but metaphase plate appears to remain intact.

proteasome inhibition. Likewise, a 2-hour incubation in 10 μ M MG132 or 50 μ g/ml ALLN was able to prevent activation in 100% of fertilised oocytes (n=38 and n=110, respectively). Hoechst staining fertilised eggs revealed that oocytes were arrested at MII with chromosomes aligned on the metaphase plate. Sperm penetration was not inhibited in these oocytes as indicated by the presence of sperm heads in the oocyte cytoplasm (shown for MG132). Therefore inhibition of fertilisation was not the reason behind lack of egg activation. Sperm penetration was also confirmed by the ability to generate Ca²⁺ oscillations (see 5.2.2). In contrast to the proteasome inhibitors, neither 100 μ M caspase inhibitor III nor 20mM NH₄Cl or 200 μ M chloroquine inhibited egg activation. This is consistent with what is known about the mechanism of cyclin destruction.

All proteasome inhibitors also blocked Sr²⁺-mediated egg activation, providing more evidence that these inhibitors were functional in mouse eggs and also supporting evidence that Sr²⁺-containing medium, like sperm, utilises proteasomal degradation to bring about egg activation. A 2-hour incubation in 20 μ M lactacystin, 10 μ M MG132 or 50 μ g/ml ALLN was sufficient to prevent 100% of oocytes from activating in Sr²⁺-containing medium (n=7, n=19 and n=13, respectively).

5.2.2 Effect of proteasome inhibitors on sperm-induced and Sr²⁺-induced Ca²⁺ oscillations

I examined the effect of using proteasome inhibitors on Ca²⁺ oscillations. It was necessary to confirm that the pattern of sperm-induced oscillations was not modified/inhibited by a non-specific (or specific) effect of the inhibitors. I also examined the effect of using proteasome inhibitors on Sr²⁺-induced oscillations.

The results show that none of the three proteasome inhibitors employed affect the generation of Ca²⁺ oscillations in response to sperm, when compared to controls (**Fig. 5.2**). The highest concentrations of drug used were 50 μ g/ml ALLN, 50 μ M MG132 and 20 μ M lactacystin. Control oocytes, not incubated in drug, were prepared in parallel to oocytes incubated in inhibitor and were imaged either immediately before or after the treated oocytes. The mean number of oscillations in the 2 hours after sperm fusion was similar in oocytes incubated in ALLN and in controls (t-test: $P=0.83$). Similar results were obtained with MG132 and lactacystin (not shown). In contrast, the mean number of oscillations in the first 2 hours following addition of Sr²⁺-containing medium was significantly higher in oocytes incubated in ALLN than in controls (t-test: $P<0.01$) (**Fig. 5.3**). Similar results were obtained with MG132 and lactacystin. The oocytes which were subjected to ~~Ca²⁺~~^{Fig. 2} imaging at fertilisation or Sr²⁺ addition, were also scored for activation (using Hoechst staining and phase contrast microscopy) as described in 5.2.1. Despite proteasome inhibitors having no inhibitory effect on oscillations induced by either sperm or Sr²⁺, oocytes which were exposed to proteasome inhibitor did not undergo activation. In the case

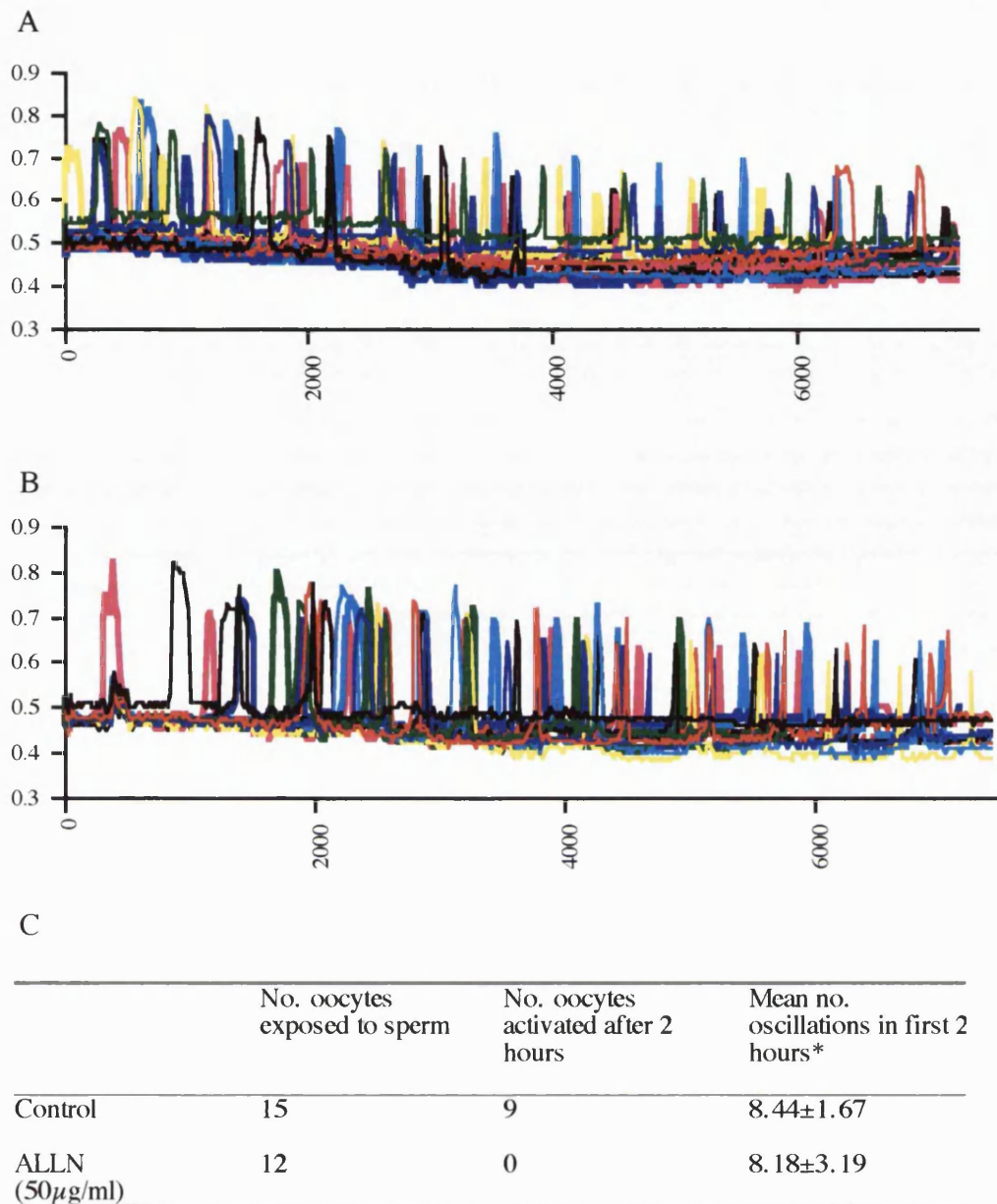


Figure 5.2 The effect of proteasome inhibitors on sperm-induced Ca^{2+} oscillations. (A) MII oocytes were preincubated for 2 hours in ALLN (50µg/ml). Oocytes were loaded with Fura-2 and intracellular Ca^{2+} was monitored at fertilisation, with ALLN maintained in the medium. (B) Control oocytes were loaded with Fura-2 and Ca^{2+} was monitored at fertilisation. Traces shown are from oocytes from 2 separate recordings on the same day (A and B). Ca^{2+} is presented as the ratio of 340/380nm. Results show that ALLN had no effect on the pattern of Ca^{2+} oscillations in response to sperm. (C) Fertilised eggs were scored for second polar body extrusion (egg activation) and the number of oscillations in the first 2 hours following the start of the first transient was counted. Numbers in table are from one experiment. Data shows means \pm s.d. The mean number of oscillations in the first 2 hours was not significantly different between oocytes incubated in ALLN and controls (t-test: $P=0.83$).

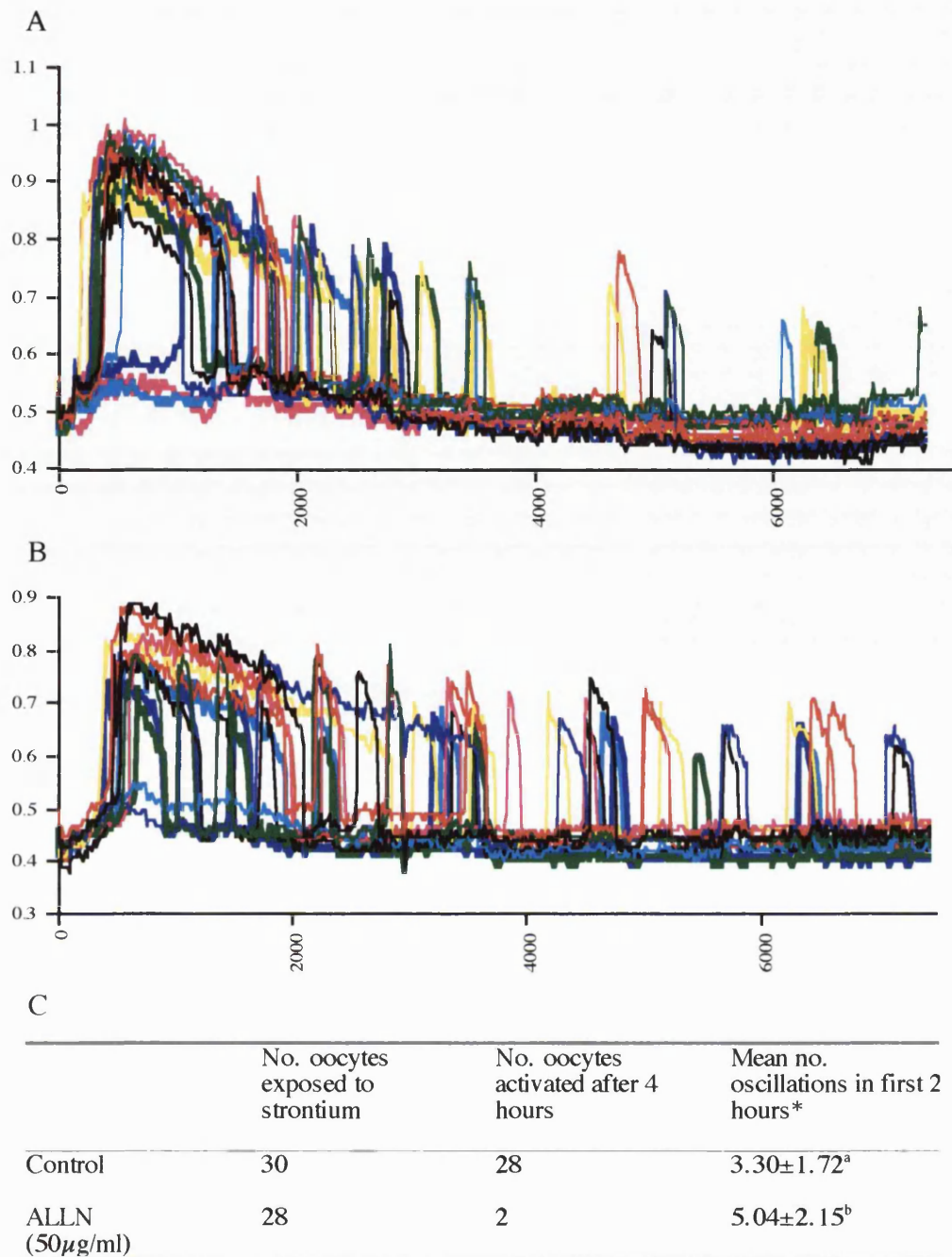


Figure 5.3 The effect of proteasome inhibitors on oscillations induced by Sr^{2+} -containing medium. (A) MII oocytes were preincubated for 2 hours in ALLN (50µg/ml). Oocytes were loaded with Fura-2 and intracellular Ca^{2+} was monitored after the addition of 10mM Sr^{2+} , with ALLN maintained in the medium. (B) Control oocytes were loaded with Fura-2 and Ca^{2+} monitored upon Sr^{2+} addition. Traces shown are from oocytes from 2 separate recordings on the same day (A and B). Ca^{2+} is presented as the ratio of 340/380nm. Results show that ALLN had no effect on the pattern of $\text{Ca}^{2+}/\text{Sr}^{2+}$ oscillations in response to Sr^{2+} . (C) Oscillating eggs were scored for egg activation and the number of oscillations in the first 2 hours following the start of the first transient was counted. Numbers refer to pooling of 2 independent experiments. Data shows means \pm s.d. Treatments with superscripts were significantly different (t-test: $P < 0.01$).

of ALLN, 2 hours after fertilisation, 9/15 control oocytes had a second polar body whereas none of 12 oocytes incubated in ALLN showed any signs of activation (chi²-test: $P < 0.001$). Similarly, 4 hours after the first transient in Sr²⁺, 28/30 control oocytes and 2/28 ALLN oocytes had a second polar body (chi²-test: $P < 0.001$). The presence of a sufficient Ca²⁺ stimulus at fertilisation suggests that inhibition of egg activation is due to specific inhibition of the proteasome. Consequently any InsP₃R degradation which takes place (see 5.2.3) must be taking place via a mechanism independent of the proteasome.

5.2.3 Effect of proteasome inhibitors on InsP₃R degradation at fertilisation

The results show that ALLN (50µg/ml) was effective at inhibiting InsP₃R degradation (t-test: $P < 0.01$), although there was considerable variation between experiments. In contrast, MG132 (10µM), a different peptide aldehyde, failed to inhibit downregulation (t-test: $P = 0.64$), as did the more specific proteasome inhibitor, lactacystin (50µM) (t-test: $P = 0.25$) (**Fig. 5.4**). Despite lactacystin being an irreversible inhibitor and ALLN and MG132 being reversible inhibitors, oocytes were incubated in all drugs continuously from 2 hours before sperm addition until the end of the experiment. Lactacystin has been reported to be highly unstable in aqueous solution (Dick *et al.*, 1996) and can be inactivated by glutathione (Dick *et al.*, 1997) which is present at 10mM in eggs (Perreault *et al.*, 1988). However, using a number of different batches and fresh lactacystin every 30 minutes of culture failed to show any convincing inhibition of downregulation. Incubation of MII oocytes in either ALLN, MG132 or lactacystin alone for 6 hours had no effect on InsP₃R levels.

5.2.4 Effect of inhibitors of other proteases on InsP₃R degradation at fertilisation

In order to examine other possible routes of InsP₃R degradation I used caspase inhibitor III (100µM) to inhibit caspases, and NH₄Cl (20mM) and chloroquine (200µM) (data not shown) to inhibit lysosomal proteases. Western analysis revealed that none of these inhibitors had any significant effect on the ability of sperm to induce InsP₃R downregulation (**Fig. 5.5**).

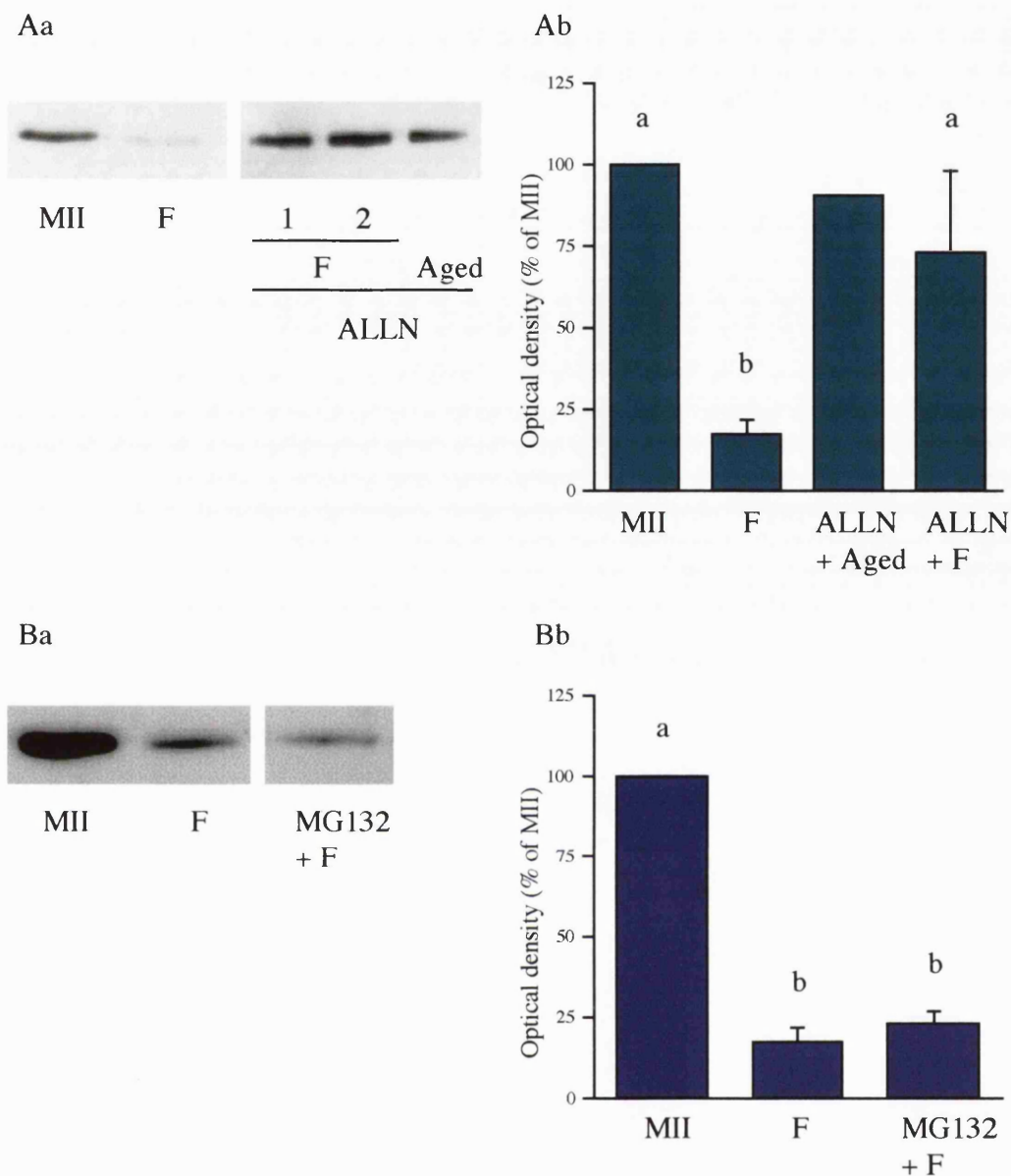
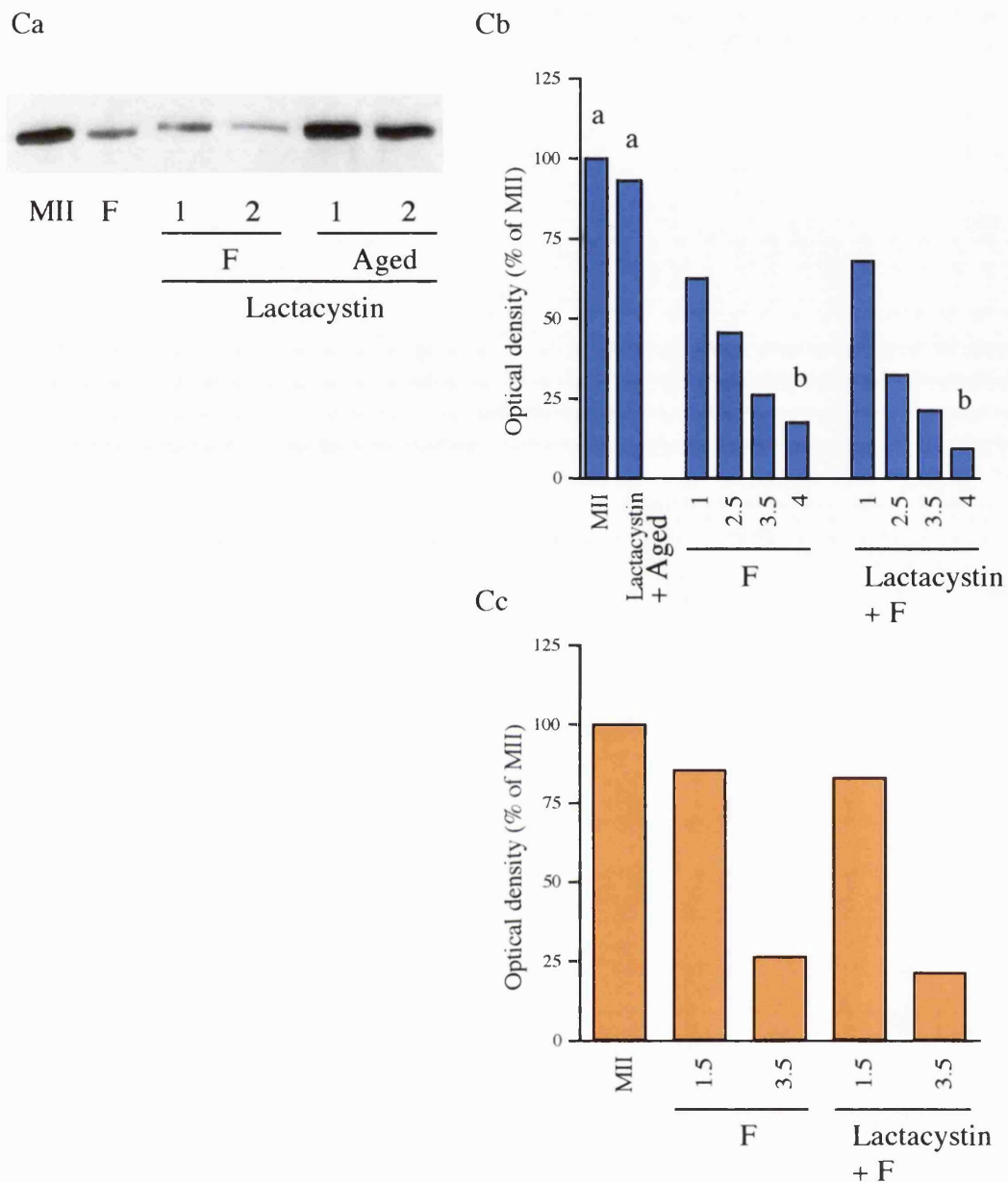


Figure 5.4 The effect of proteasome inhibitors on $\text{InsP}_3\text{R-I}$ downregulation at fertilisation. MII oocytes were preincubated for 2 hours in ALLN ($50\mu\text{g/ml}$) or MG132 ($10\mu\text{M}$) before sperm addition and continued culture in inhibitor. Other oocytes were preincubated in fresh lactacystin ($50\mu\text{M}$) for 30 minutes before sperm addition and in fresh lactacystin every 30 minutes. Fertilised eggs were collected after 6 hours (A, B and Ca) or after 1, 2.5, 3.5 and 4 hours (Cb) or after 1.5 and 3.5 hours (Cc). Cb and Cc represent experimental protocols which are identical except for time points of collections. Protein from 100 eggs (A, B and Ca) was electrophoresed and immunoblotted with CT1 next to a control lane of eggs aged in inhibitor over the same time period (A and Ca). (continued over page)



For the earlier time points with lactacystin, protein from 40 eggs was electrophoresed and immunoblotted with Ab40 next to a control fertilisation lane for each time point (Cb and Cc) in addition to the control for inhibitor alone (Cb). Error bars indicate positive standard error. Treatments under bars that share a common superscript are not significantly different (t-test: $P>0.05$). Results show that ALLN was effective at inhibiting downregulation (A) but there was substantial variation between experiments (Ab). Both MG132 and lactacystin failed to inhibit downregulation (B and C). Numbers in Aa and Ca refer to replicate lanes. The blots are representative of at least 3 collections at each time point for each inhibitor.

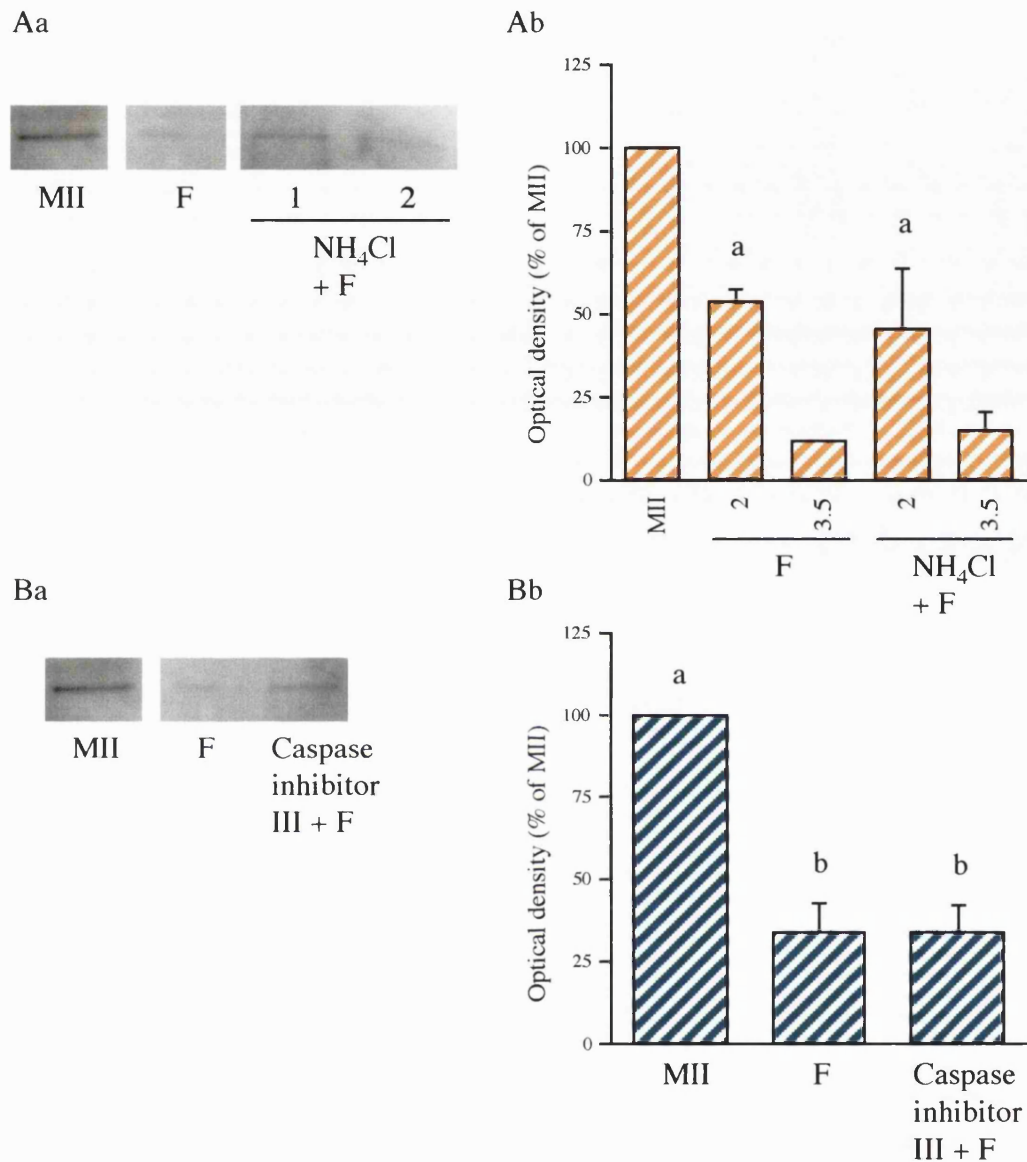


Figure 5.5 The effect of inhibiting lysosomal proteases and caspases on InsP₃R-I downregulation at fertilisation. MII oocytes were incubated with sperm for 20 minutes before culture in medium containing 20mM NH₄Cl or were preincubated in 100μM caspase inhibitor III for one hour before sperm addition and continued culture in inhibitor. Fertilised eggs were collected after 2 hours (Ab) and after 3.5 hours (A and B). Protein from 40 eggs was electrophoresed and immunoblotted with Ab40 next to a control fertilisation lane for the respective time point. Error bars indicate positive standard error. Treatments under bars that share a common superscript are not significantly different (t-test: $P>0.05$). Results show that neither inhibitor prevented InsP₃R-I downregulation. Numbers refer to replicate lanes. The blots are representative of 3 collections at each time point for each inhibitor.

5.3 DISCUSSION

5.3.1 The nature of the protease responsible for InsP₃R degradation at fertilisation

Proteasome inhibitors are effective in mammalian eggs

In order to draw any conclusions from experiments using proteasome inhibitors, it was first necessary to prove that these inhibitors were active in mouse oocytes. It is well known that release from meiotic arrest is stimulated by destruction of cyclin B, the regulatory subunit of MPF. This is thought to be mediated by the proteasome (Glotzer *et al.*, 1991) and therefore provided an ideal assay for the effectiveness of the proteasome inhibitors. As predicted, the proteasome inhibitors inhibited oocyte activation. The inhibitors had no inhibitory effect on the ability of sperm to generate the Ca²⁺ transients responsible for stimulating cyclin B degradation and egg activation (see 5.3.3). The presence of normal oscillations indicates that the proteasome inhibitors prevented egg activation by specifically inhibiting the proteasome. Since these inhibitors were effective in mouse oocytes in blocking egg activation, I used them to investigate the role of the proteasome in InsP₃R degradation.

The role of the proteasome in InsP₃R degradation at fertilisation

The results of my experiments with protease inhibitors have not conclusively identified any one protease responsible for InsP₃R degradation. ALLN is the only inhibitor to have shown any significant inhibition of InsP₃R downregulation (t-test: $P < 0.01$), with the specific proteasome inhibitors, lactacystin and MG132, having no significant effect. The inability of all three proteasome inhibitors to completely block downregulation means that it is not possible to conclude decisively from my experiments that it is the proteasome that causes the degradation of InsP₃Rs at fertilisation. Although ALLN is known to inhibit both the proteasome and cysteine proteases (Groll *et al.*, 1997; Mellgren, 1997; Rock *et al.*, 1994), Ca²⁺-dependent cysteine proteases such as calpain are unlikely to be responsible for InsP₃R degradation since I have demonstrated that degradation is independent of Ca²⁺ increases (Chapter 4). Preliminary experiments have been carried out to examine the role of ubiquitin-dependent proteolysis by the proteasome, by trying to detect ubiquitination of InsP₃Rs at fertilisation. Experiments were performed (in collaboration with Richard Wojcikiewicz, Department of Pharmacology, College of Medicine, SUNY Health Science Center at Syracuse, Syracuse, New York.) to try to immunoprecipitate InsP₃Rs from fertilised mouse oocytes using an anti-ubiquitin antibody. However, no signal has been detected using protein from 500 mouse oocytes. The transient nature of ubiquitination means that many more oocytes may be required.

Inconclusive results using proteasome inhibitors necessitated the investigation of a role for caspases and lysosomal proteolysis in InsP_3R degradation. Caspases are a family of cysteine proteases which are involved in programmed cell death (Cryns and Yuan, 1998). Lysosomal proteolysis (in addition to proteasomal degradation) is a major pathway of cellular protein degradation, particularly involved in the destruction of transmembrane proteins. It has been shown that chloroquine and NH_4Cl (inhibitors of lysosomal proteases) increase the basal levels of InsP_3Rs in WB rat liver epithelial cells by 2-fold, which indicates that basal turnover of InsP_3Rs occurs via lysosomal proteolysis (Bokkala and Joseph, 1997). However, the same study found that stimulated degradation of InsP_3Rs (by angiotensin II) occurs via a non-lysosomal pathway, since it was not affected by these inhibitors. My results show that alternative pathways such as those involving caspases and lysosomal degradation of InsP_3Rs , do not appear to be necessary for stimulated InsP_3R downregulation at fertilisation.

The results obtained using proteasome inhibitors at fertilisation are inconsistent with those of studies on InsP_3R degradation in cell lines. There is convincing data in somatic cells that InsP_3R downregulation is carried out by the 26S proteasome. InsP_3Rs undergo ubiquitination and downregulation is inhibited by the specific proteasome inhibitor lactacystin and by ALLN, which inhibits both cysteine proteases and the proteasome (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999). Furthermore, secretagogues such as CCK stimulate InsP_3R ubiquitination and downregulation in rat pancreatic acinar cells, and proteasome inhibitors have been shown to cause the accumulation of ubiquitinated species in these cells (Wojcikiewicz *et al.*, 1999). This indicates that the ubiquitin-proteasome pathway is involved in InsP_3R downregulation in a physiological signaling pathway.

The lack of a general effect of proteasome inhibitors is even more surprising given that the mechanism for stimulating downregulation appears to be similar in oocytes and somatic cells. Work on somatic cells has suggested a hypothesis in which InsP_3R activation signals InsP_3R degradation via the ubiquitin-proteasome pathway. Thus the proposal (in Chapter 4) that prolonged activation of InsP_3Rs stimulates receptor degradation at fertilisation as well, is consistent with this degradation also being carried out by the ubiquitin-dependent proteasome. The most likely hypothesis for how InsP_3R activation may signal recruitment of the proteasome at fertilisation is that a conformational change in the InsP_3R induced by InsP_3 binding (Mignery and Sudhof, 1990) facilitates ubiquitination (Oberdorf *et al.*, 1999). This is supported by studies showing that mutant InsP_3Rs that are unable to bind InsP_3 are not downregulated (Zhu *et al.*, 1999) or ubiquitinated (Zhu and Wojcikiewicz, 2000). It would also explain why InsP_3Rs are downregulated in a specific manner, at least in cell lines (Bokkala and Joseph, 1997; Sipma *et al.*, 1998; Wojcikiewicz, 1995; Wojcikiewicz *et al.*, 1994; Wojcikiewicz and Oberdorf, 1996). It is not clear how a conformational change might lead to ubiquitination, as it is mostly unknown how ubiquitin-conjugating enzymes select their targets (Haas and Siepmann, 1997; Varshavsky, 1997). In

any case, it appears that if InsP₃R activation is indeed responsible for stimulating degradation at fertilisation, a conformational change and recruitment of ubiquitin followed by proteasomal proteolysis is a likely sequence of events.

To summarise, InsP₃R degradation (1) appears to be stimulated by InsP₃R activation and executed by the proteasome in cell lines and mammalian tissues and (2) is stimulated at fertilisation of mouse oocytes by persistent InsP₃R activation. Hence it seems reasonable that the proteasome is also involved in InsP₃R degradation at fertilisation of mouse oocytes and that, for one or more of the reasons laid out below, the proteasome inhibitors employed in this study were not completely effective in blocking InsP₃R degradation, despite successfully blocking egg activation.

(1) The proteasome may only be partially inhibited

If I assume that the proteasome is solely responsible for InsP₃R degradation at fertilisation, I need to explain how proteasome inhibitors could inhibit the proteasome sufficiently to prevent cyclin B degradation (and thus egg activation) but not InsP₃R degradation. One possibility is that, in the presence of inhibitors, a low residual activity of the proteasome in oocytes may have different effects on the levels of proteins with different turnover rates. Cyclin B turns over at a high rate ($t_{1/2}$ =1.9 hours in the unactivated MII oocyte (Winston, 1997)) and there is a large maternal store of cyclin B in mouse oocytes (Kanatsu-Shinohara *et al.*, 2000). As such, a low residual proteasome activity is unlikely to suppress cyclin B levels below the threshold that permits egg activation. In contrast, InsP₃Rs are turning over at a much slower rate ($t_{1/2}$ ≈11 hours in other cell types (Joseph, 1994)) and are therefore not rapidly replaced, after stimulated degradation, by protein synthesis or from maternal stores. It is possible then that low residual activity of the proteasome is sufficient to degrade and lower the level of InsP₃Rs while not having any impact on the role of cyclin B in maintaining meiotic arrest.

It is not known whether the inhibitors completely or partially blocked the proteasome. In the case of lactacystin, evidence in the literature suggests that it is possible that inhibition was not complete. Lactacystin itself does not react with the proteasome but undergoes a spontaneous conversion in aqueous solutions to the active proteasome inhibitor, *clasto*-lactacystin β-lactone. However, the active compound can react with glutathione, a compound present at 10mM in mature oocytes (Perreault *et al.*, 1988), to form a second product which is inactive (Dick *et al.*, 1997). It may therefore be difficult to completely inhibit the proteasome with lactacystin. This may also be true of ALLN and MG132 which act as competitive inhibitors of the proteasome. The concentrations used were effective at inhibiting InsP₃R downregulation in other cell types (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999; Wojcikiewicz and Oberdorf, 1996). However, the large cytoplasmic volume and low surface area/volume ratio of the mouse oocyte means that higher concentrations of inhibitors may be necessary to fully inhibit the proteasome and block InsP₃R

downregulation, than in the smaller somatic cells with much higher surface area/volume ratios.

It should also be pointed out that, perhaps for the reasons speculated above, inhibitors of the proteasome have been shown to vary in their ability to block protein degradation in different cell types. For example, degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) in a mammalian cell-free system has been shown to be significantly inhibited by proteasome inhibitors hemin and MG132, partially inhibited by *clasto*-lactacystin β -lactone and ALLN and relatively unaffected by lactacystin (Xiong *et al.*, 1999). Like the InsP₃R, the CFTR is a transmembrane polytopic protein. Although residing in the plasma membrane, 80% of CFTR proteins are degraded while associated with the ER. Despite the variable effects of proteasome inhibitors, degradation of this protein requires an intact ubiquitin-conjugation pathway and is thought to involve the 26S proteasome (Jensen *et al.*, 1995; Ward *et al.*, 1995).

There is another case in the literature of ER-membrane protein degradation which, like InsP₃Rs at fertilisation, appears resistant to a range of inhibitors. The bioluminescent protein apoaquorin can be targeted to the ER in HeLa cells where it undergoes degradation in response to release of Ca²⁺ from the ER (the ER stress response) (Jeffery *et al.*, 2000). The study found that degradation could not be inhibited by the proteasomal inhibitor lactacystin, serine protease inhibitors or zinc, a known caspase-3 inhibitor. There is another potential similarity between the degradation of apoaquorin and InsP₃Rs. Aequorin undergoes a conformational change in the absence of Ca²⁺ (Ray *et al.*, 1985) and it has been suggested that this may reveal a structural site sensitive to attack by proteases (Jeffery *et al.*, 2000). As described in Chapter 4, the binding of InsP₃ to its receptor induces a conformational change (Mignery and Sudhof, 1990) which may be sufficient to induce downregulation. The similarities between the aequorin case and degradation of InsP₃Rs at fertilisation points to the mechanisms of degradation being similar. However, in contrast to the degradation of InsP₃Rs in mouse eggs and cultured cells, loss of apoaquorin is stimulated by thapsigargin and ionomycin, which cause only a single, sustained release of ER Ca²⁺ (Jeffery *et al.*, 2000). Thus the mechanisms causing degradation of apoaquorin and InsP₃Rs are probably not identical.

(2) Degradation at fertilisation may involve multiple proteolytic mechanisms

Another explanation for why proteasome inhibitors fail to block InsP₃R degradation is that multiple proteolytic pathways are involved. An interesting case in the literature suggests that it may be possible for more than one proteolytic mechanism to function simultaneously and result in the downregulation of InsP₃Rs. Secretagogues, which activate PLC-linked cell surface receptors (e.g. CCK), acting on rat pancreatic acinar cells, stimulate InsP₃R ubiquitination and downregulation that is not inhibited by lactacystin or ALLN

(Wojcikiewicz *et al.*, 1999). Receptor ubiquitination and the finding that proteasome inhibitors cause the accumulation of ubiquitinated species (Wojcikiewicz *et al.*, 1999) indicate that the ubiquitin-proteasome proteolytic pathway contributes to the downregulation. However, to explain why proteasome inhibitors have no effect on downregulation, it has been suggested that an additional proteolytic mechanism functions in parallel in these cells. Indeed, such a system has previously been described in acinar cells. It may be that fertilisation stimulates more than one mechanism of proteolysis for the degradation of InsP₃Rs, as is proposed for secretagogues. This may explain why the use of proteasome inhibitors alone appears to have a variable effect in blocking InsP₃R degradation.

It is not unreasonable to make a comparison between the downregulation of InsP₃Rs in pancreatic acinar cells and oocytes. Secretagogue-stimulated degradation (*in vitro* and *in vivo*) is as yet the only example, besides fertilisation, of InsP₃R degradation as part of a physiological pathway. In acinar cells, InsP₃R downregulation takes place as a way of regulating the cellular response to CCK exposure. In the case of fertilisation as well, downregulation may modulate the sperm-induced Ca²⁺ oscillations, although it is probable that entry into interphase has a more dominant effect on oscillations (see General Discussion). Therefore, in both physiological instances of InsP₃R degradation, it is conceivable that Ca²⁺ release is accompanied by the activation of more than one proteolytic pathway. For example, fertilisation may stimulate activation of both the ubiquitin-proteasome pathway as well as an additional, as yet unspecified protease. There are, however, differences between InsP₃R degradation in these two cell types. Degradation is more rapid in acinar cells than in oocytes, with a half-life of 30 and 15 minutes *in vitro* and *in vivo*, respectively (Wojcikiewicz *et al.*, 1999). Also, downregulation of some other signaling proteins (a PLC and a PKC) occurs simultaneously with InsP₃R degradation in acinar cells (Wojcikiewicz *et al.*, 1999). Thus the rapid downregulation of both InsP₃Rs and other proteins in acinar cells would appear to have a greater requirement for multiple simultaneous proteolytic mechanisms. In cultured cells, similar doses of ALLN and lactacystin completely blocked InsP₃R downregulation (Bokkala and Joseph, 1997; Oberdorf *et al.*, 1999), implying that in these cultured cell types, non-physiological stimulation with agonists of PLC-linked cell surface receptors activates only the proteasomal pathway of degradation.

The idea of multiple proteolytic pathways being activated at fertilisation appears to be consistent with another study using mouse oocytes. Evidence was found for the role of the proteasome in InsP₃R degradation after injection of sperm factor into mouse oocytes (Jellerette *et al.*, 2000). The proteasome inhibitor lactacystin was found to inhibit the degradation of type I InsP₃R. However, there are differences between sperm factor-induced and fertilisation-induced degradation in the amount of InsP₃ generated and in the extent to which InsP₃Rs are downregulated. Sperm factor (SF) preparation was

microinjected into oocytes to produce an intracellular concentration of 2.5-5 sperm equivalents (Wu *et al.*, 1998) and this produced more rapid and extensive degradation than seen at fertilisation (Jellerette *et al.*, 2000). One explanation for why lactacystin appears to inhibit InsP₃R degradation induced by SF but not by a single sperm at fertilisation may be related to the more rapid time-course of degradation induced by SF. This explanation also relies on the notion of simultaneous activation of more than one proteolytic pathway, as proposed above. At fertilisation, the rate of receptor degradation may be sufficiently slow for inhibition of proteasomal degradation to be compensated for by a second parallel pathway of degradation. However, after the more potent stimulus of SF, the putative second pathway of degradation may be unable to compensate for the proportion of degradation usually carried out by proteasomes, and this may bring about the observed delay in downregulation two hours after SF injection (Jellerette *et al.*, 2000). In the latter study, no data was presented on the final InsP₃R levels, which may have been unaffected by lactacystin, if a second pathway eventually compensated for all proteasomal InsP₃R degradation. An alternative explanation for how lactacystin inhibits SF-induced InsP₃R degradation (Jellerette *et al.*, 2000) is that the high concentration of lactacystin which was used (100µM) may have had a non-specific effect on non-proteasomal degradation pathways, which may be operating in parallel to proteasomal degradation. A final consideration is that 100µM, but not 50µM lactacystin (highest concentration used in my experiments) may be sufficiently high for some to remain after inactivation by glutathione (see above). In this case, the proteasome may only be inhibited and its role made apparent, at the higher concentration.

Proteasome distribution is consistent with a role in InsP₃R degradation

Clearly, further work is necessary to clarify the mechanism of InsP₃R degradation at fertilisation. Ubiquitin-mediated degradation by the proteasome appears to be involved, whether alone or in conjunction with other degradation pathways. In addition to the evidence from other cell types, described above, the subcellular distribution of the proteasome appears to be consistent with a role in InsP₃R degradation. In mammals, 20S proteasomes are localised in the nucleus and in the cytoplasm with a percentage of the total proteasomes (<20%) loosely associated with the cytoplasmic surface of the ER (Palmer *et al.*, 1996; Rivett *et al.*, 1992), which is the location of InsP₃Rs. In Chapter 4 it was described that rat oocytes arrested at MII exhibit a colocalisation of the proteasome with the MII spindle apparatus (Josefsberg *et al.*, 2000). However, proteasomes can diffuse rapidly throughout the cytoplasm in mammalian cells (Reits *et al.*, 1997) and could be conceived to redistribute from the spindle to the ER upon meiotic resumption at fertilisation. Further work is required to determine the distribution of the proteasomes after fertilisation.

5.3.2 The role of the proteasome in cyclin B degradation at egg activation

Sperm- and Sr^{2+} -induced oscillations stimulate proteolysis of cyclin B by activating the proteasome

The finding that all proteasome inhibitors employed effectively inhibited egg activation has important implications for the mechanism of activation. As well as confirming the functional activity of my inhibitors in mouse eggs, this result with proteasome inhibitors also provides direct supporting evidence that the proteasome is the protease complex responsible for the degradation of cyclin B at egg activation. Previous work indirectly indicated the proteasome by demonstrating that during the course of degradation, cyclin conjugates with ubiquitin (Glotzer *et al.*, 1991). All proteasome inhibitors also blocked Sr^{2+} -mediated egg activation, supporting evidence that Sr^{2+} -containing medium, like sperm, utilises proteasomal degradation to bring about egg activation. It is well established that repetitive sperm-induced Ca^{2+} oscillations at mammalian fertilisation are essential for complete oocyte activation (Kline and Kline, 1992a; Lawrence *et al.*, 1998). It has also been demonstrated in *Xenopus* and ascidians that activation of the 26S proteasome may be regulated by these Ca^{2+} increases (Aizawa *et al.*, 1996; Kawahara and Yokosawa, 1994). It is conceivable that Sr^{2+} , which causes Ca^{2+}/Sr^{2+} oscillations in unfertilised oocytes (Bos-Mikich *et al.*, 1995; Kline and Kline, 1992a), may activate oocytes via the same mechanism. Sr^{2+} and cycloheximide act synergistically to cause parthenogenetic activation in freshly-ovulated oocytes, and incubation in Sr^{2+} -containing medium alone does not affect protein synthesis (Bos-Mikich *et al.*, 1995). The absence of an effect on protein synthesis implies that Sr^{2+} causes a decrease in cyclin B levels, and thus egg activation, by triggering active proteolysis. My results with proteasome inhibitors support the idea that Sr^{2+} -generated oscillations in Ca^{2+}/Sr^{2+} bring about this proteolysis by activating the proteasome.

5.3.3 The generation of sperm-induced Ca^{2+} oscillations does not require the action of the proteasome

I have examined the effect of proteasome inhibitors on Ca^{2+} oscillations induced by sperm. The experiments confirmed that the pattern of oscillations was not modified by the inhibitors, thereby preventing egg activation. These results also indicated that proteolysis by the proteasome is not required for the generation of these oscillations. However, it has been suggested by a different study that proteolysis is involved in generating sperm-induced Ca^{2+} oscillations. It was found that the induction of sperm-induced Ca^{2+} transients in mouse oocytes appeared to involve proteolytic processing of a "SOAF" (sperm-borne oocyte-activating factor) from sperm head submembrane compartments (Perry *et al.*, 2000). The finding that in the presence of proteasome inhibitors, sperm-induced oscillations were not inhibited, implies that the generation of oscillations does not involve proteolytic activation of a sperm factor or PLC by the proteasome. However, this is consistent with the finding that processing of SOAF may involve sperm-derived serine

proteases (Perry *et al.*, 2000). My experiments also indicate that proteasome inhibitors do not interfere with the production of InsP_3 , which I have proposed is a requirement for InsP_3R downregulation.

While proteasome inhibitors did not inhibit Sr^{2+} -induced oscillations, the frequency of oscillations was in fact increased, at least with ALLN. A possible explanation for the higher frequency of oscillations in ALLN-treated oocytes may be related to the fact that these oocytes were prevented from progressing through the cell cycle and remained arrested at MII. Alternatively, these oocytes may have been more sensitive to the generation of oscillations due to being older than controls at the time of Sr^{2+} addition.

CHAPTER 6:

ROLE OF THE InsP₃R IN MEIOTIC AND MITOTIC CELL CYCLES OF MOUSE OOCYTES AND EMBRYOS

6.1 INTRODUCTION

In this chapter I investigate the role of the InsP₃R at various points during oocyte meiotic maturation, the completion of meiosis, and mitotic cell cycles of preimplantation embryos. The overall pattern of InsP₃R protein expression over this time is a 180% increase in protein expression levels during meiotic maturation (Mehlmann *et al.*, 1996; Parrington *et al.*, 1998), peaking in the MII egg, followed by a decrease to 20% of MII levels 4 hours after fertilisation (Chapter 3). I have shown that this low level of InsP₃Rs is maintained in subsequent preimplantation development up to the formation of the blastocyst (Chapter 3). This change in expression levels may be expected to be reflected in a corresponding change in the requirement for InsP₃Rs and InsP₃-induced Ca²⁺ release. Interestingly, the levels of InsP₃R protein in the mouse egg generally correlate well with the ability to generate Ca²⁺ transients. During oocyte maturation the sensitivity of Ca²⁺ release increases (Jones *et al.*, 1995b; Mehlmann and Kline, 1994; Parrington *et al.*, 1998). Similarly, following degradation of InsP₃Rs after fertilisation this sensitivity decreases as clearly demonstrated by the fact that unfertilised oocytes are more sensitive to microinjected InsP₃ than are pronucleate stage fertilised embryos (Parrington *et al.*, 1998).

The regulation of cell cycle transitions by Ca²⁺ is suggested by the presence of endogenous cell cycle Ca²⁺ transients in various types of somatic cells (Hepler, 1994; Kao *et al.*, 1990; Poenie *et al.*, 1986; Ratan *et al.*, 1988; Wahl and Gruenstein, 1993) and in oocytes and early embryos of a range of species including the sea urchin (Ciapa *et al.*, 1994; Steinhardt and Alderton, 1988; Whitaker and Patel, 1990), *Xenopus* (Snow and Nuccitelli, 1993), the medaka fish (Fluck *et al.*, 1991) and the mouse (Carroll and Swann, 1992; Kono *et al.*, 1996; Stachecki and Armant, 1996b; Tombes *et al.*, 1992). In the mouse, there have been several studies on cell cycle-associated Ca²⁺ release and on the sensitivity of oocytes and embryos to agonists which stimulate Ca²⁺ release. These are discussed in the General Introduction and below.

In mouse oocytes, the role of Ca²⁺ in meiotic maturation is controversial (see General Introduction). In summary, GVBD (Carroll and Swann, 1992; Coticchio and Fleming, 1998; Tombes *et al.*, 1992) and formation of the first polar body (Tombes *et al.*, 1992) are apparently not dependent on intracellular Ca²⁺ transients since BAPTA has no effect on the timing of these events. However, other data, showing that lithium treatment (Pesty *et al.*, 1994) or injection of anti-InsP₃R antibodies (Pesty *et al.*, 1998) inhibits GVBD, suggest

that intracellular Ca^{2+} release may play a role in the regulation of GVBD. Species differences are also apparent in that GVBD in cow (Homa, 1991) and pig (Kaufman and Homa, 1993) oocytes does appear to be inhibited by BAPTA.

The role of Ca^{2+} increases at fertilisation of the MII egg has been well documented (Kline and Kline, 1992a; Lawrence *et al.*, 1998 and see General Introduction). A role for the InsP_3R as the predominant Ca^{2+} release channel involved in these oscillations has been implicated by using a functionally inhibitory type I InsP_3R antibody (18A10) to inhibit sperm-induced Ca^{2+} transients in mouse and hamster oocytes (Miyazaki *et al.*, 1993; Miyazaki *et al.*, 1992). Although ryanodine receptor Ca^{2+} release channels types II and III are expressed in mouse oocytes (Ayabe *et al.*, 1995), the role of RyRs in the generation of Ca^{2+} transients at fertilisation in mammals is controversial (Ayabe *et al.*, 1995; Carroll *et al.*, 1996; Jones *et al.*, 1995b).

Evidence for a role for intracellular Ca^{2+} release at NEBD of mitosis is strong but not yet conclusive. NEBD of the first mitosis can be blocked by BAPTA and is associated with a Ca^{2+} transient in 40-100% of fertilised embryos (Kono *et al.*, 1996; Tombes *et al.*, 1992). However, Ca^{2+} transients cannot be detected at NEBD in parthenogenetic embryos (Kono *et al.*, 1996). The dispensability of global detectable transients, combined with the fact that NEBD is inhibited in all embryos by Ca^{2+} chelators, suggests that NEBD may be dependent on small *localised* Ca^{2+} transients. Support for a role for local Ca^{2+} transients in mouse embryos has been provided in studies on sea urchins. In the first mitotic cell cycle of the sea urchin egg, global Ca^{2+} transients that appear to originate from the nuclear area are often observed just before NEBD. However, in the absence of global increases, confocal microscopy reveals localised Ca^{2+} transients in the perinuclear region (Wilding *et al.*, 1996).

The mechanism of generation of cell cycle Ca^{2+} transients appears to be via cyclic production of InsP_3 . There is evidence in sea urchins for a role for increases in InsP_3 during early cell cycles. During the early embryonic cell cycle in the sea urchin, oscillations in the phosphoinositide messenger system cause cyclic increases in InsP_3 that drive cell cycle Ca^{2+} transients and mitosis (Ciapa *et al.*, 1994). Furthermore, mitotic transients are absent in sea urchin eggs microinjected with heparin, an InsP_3R antagonist (Ciapa *et al.*, 1994).

These previous studies have primarily investigated the role of Ca^{2+} release *per se* and InsP_3 increases in development. I will focus specifically on the role of the InsP_3R and thus of InsP_3 -induced Ca^{2+} release in these cell cycle transitions and in subsequent development. The obvious way to elucidate the role of the InsP_3R is to study InsP_3R knockout mice, in which receptors are completely absent. However, it has not been possible to use such mice to study Ca^{2+} signaling at fertilisation and early development. The type I $\text{InsP}_3\text{R}(-/-)$ mouse

dies prior to maturity (Matsumoto *et al.*, 1996) and oocytes generated from the crosses of heterozygous $\text{InsP}_3\text{R}(+/-)$ parents are endowed with maternal InsP_3Rs . The heterozygotes themselves have similar levels of InsP_3R protein to the wildtype, as reflected in their normal growth and absence of obvious defects (Matsumoto *et al.*, 1996). As a result it has not been possible to test, in a knockout oocyte or oocyte with reduced InsP_3R levels (via genetic means), the role of InsP_3Rs at fertilisation and in preimplantation development. The technique employed in my experiments will involve downregulating InsP_3R protein expression in order to produce oocytes and embryos with reduced levels of InsP_3Rs .

6.2 ROLE OF THE InsP_3R DURING OOCYTE MATURATION

I carried out experiments to investigate the role of the InsP_3R during germinal vesicle breakdown and later meiotic maturation. A role for the InsP_3R in oocyte maturation was not expected since maturation does not appear to depend upon intracellular Ca^{2+} increases (see Introduction of this chapter).

To examine the role of InsP_3Rs in GVBD I microinjected 100 μM adenophostin A (final oocyte concentration of 1-5 μM) into GV stage oocytes maintained in M2 medium containing dibutyryl cAMP (dbcAMP) to cause arrest at the GV stage. After culture for 4 hours to allow for the adenophostin to downregulate InsP_3Rs , the oocytes were washed out of dbcAMP and their ability to undergo GVBD monitored over the next 3 hours. The timing of GVBD in adenophostin-injected oocytes was no different from that seen in both KCl-buffer-injected and uninjected controls (**Fig. 6.1**). The final proportion of oocytes undergoing GVBD was also not significantly different between the groups. 48/52 adenophostin-injected, 29/36 KCl-injected and 52/55 uninjected oocytes underwent GVBD (92%, 81% and 95% respectively).

To examine the role of InsP_3Rs in completion of meiosis I and progression to metaphase II arrest, maturing oocytes were microinjected with 100 μM adenophostin A within two hours of release from the follicle. The oocytes were cultured for a further 14 hours to allow *in vitro* maturation to take place. (Note that immature oocytes arrested at the germinal vesicle stage undergo spontaneous maturation under culture conditions.) Presuming that adenophostin depleted InsP_3Rs within 6 hours of injection, these oocytes would have undergone *in vitro* maturation through completion of meiosis I to MII arrest with downregulated levels of InsP_3Rs . However, adenophostin had no effect on the ability of oocytes to mature through these stages *in vitro*. The proportion of oocytes reaching MII, assayed by first polar body formation, was 82% in both adenophostin-injected oocytes (n=148) and in uninjected controls (n=120).

6.3 ROLE OF InsP_3R IN SPERM-INDUCED Ca^{2+} OSCILLATIONS AT FERTILISATION

Experiments were performed to investigate the role of the InsP_3R in the generation of Ca^{2+} oscillations at fertilisation. One method of achieving this was to examine the Ca^{2+} response to sperm of MII oocytes expressing different levels of InsP_3Rs . I have previously shown that fertilisation stimulates a decrease in InsP_3R expression levels which results in only 50% of receptors remaining after 2 hours. However, the time resolution I have used for imaging does not reveal any obvious change in the pattern of Ca^{2+} transients during the

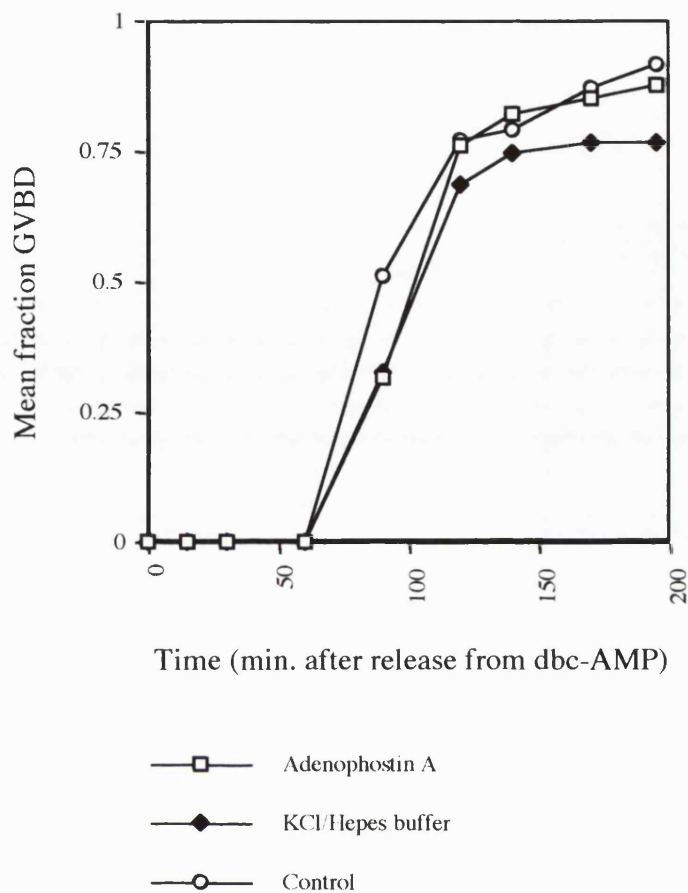


Figure 6.1 The effect of InsP_3R -depletion on the ability to undergo GVBD. GV stage oocytes were collected in $250\mu\text{M}$ dibutyryl cAMP and thus arrested at GV before microinjection of $1\text{-}5\mu\text{M}$ adenophostin A (oocyte concentration) or of KCl/Hepes injection buffer alone. After a culture period of 4 hours to allow for the adenophostin to downregulate receptor levels the oocytes were washed out of dbc-AMP and scored for GVBD. The number of oocytes which had undergone GVBD was counted at 20- or 30-minute intervals depending on the individual experiment, for the duration of 3 hours. Data represents mean fractions for 3 replicate experiments and a total of 52 (A), 36 (B) and 55 (C) oocytes. The rate of GVBD in InsP_3R -depleted oocytes was no different from that seen in both buffer-injected and uninjected controls.

course of the sperm-induced oscillations, to reflect this halving of receptors. Alternative approaches were therefore used to deplete MII oocytes of InsP₃Rs.

6.3.1 Effect of nocodazole-induced InsP₃R downregulation on fertilisation and carbachol-induced Ca²⁺ transients

Previous work has shown that 10µM nocodazole is able to induce a downregulation of InsP₃R expression levels within 4 hours to 20-25% in MII eggs, confirmed by Western analysis (Chapter 2). Therefore I used nocodazole to downregulate the receptor levels whilst maintaining the oocyte at MII. Monitoring intracellular Ca²⁺ at fertilisation of these oocytes revealed that the pattern of oscillations was very similar to that seen in controls (**Fig. 6.2**). Neither the number of oscillations in 60 minutes nor the amplitude of the first transient was significantly different between the groups (**Table 6.1**). There was a small but statistically significant decrease in the mean amplitude of the next 3 transients in the nocodazole-treated eggs (t-test: $P < 0.05$) (**Table 6.1**).

Ca²⁺ release through the muscarinic acetylcholine receptor pathway was shown to be more severely compromised as the Ca²⁺ response to carbachol was completely inhibited in the nocodazole-preincubated eggs. Whereas 14/17 control oocytes responded to carbachol, only 1/18 responded after nocodazole-induced InsP₃R downregulation (**Table 6.2**). Ionomycin was used to confirm that all oocytes that did not respond to carbachol were able to release Ca²⁺ (data not shown), indicating that internal Ca²⁺ stores were intact.

6.3.2 Effect of adenophostin A-induced InsP₃R downregulation on fertilisation-induced Ca²⁺ transients

6.3.2.1 Preparation of InsP₃R-deficient MII oocytes using adenophostin A

Since the Ca²⁺ response to fertilisation appeared to be unaffected by a nocodazole-induced decrease in InsP₃R levels of 75-80%, I aimed to induce a more extensive downregulation. This was performed by microinjecting adenophostin A (see Chapter 4) into maturing oocytes within two hours of release from the follicle. The oocytes were then cultured for a further 14 hours to allow *in vitro* maturation to take place. Adenophostin had no effect on the ability of oocytes to mature *in vitro* (see 6.2). I confirmed using Western analysis that InsP₃Rs were downregulated and that the receptors were not replaced by new protein synthesis during oocyte maturation. With three concentrations in the injection pipette of 1mM, 100µM and 10µM adenophostin A (final oocyte concentrations of 10-50µM, 1-5µM and 100-500nM respectively), mature metaphase II oocytes were produced in which the level of InsP₃R protein was below the level of detection on a Western blot of 40 oocytes (**Fig. 6.3.A**). Therefore 100µM and 10µM adenophostin were the pipette concentrations used to deplete InsP₃Rs for the examination of Ca²⁺ release in InsP₃R-deficient MII eggs.

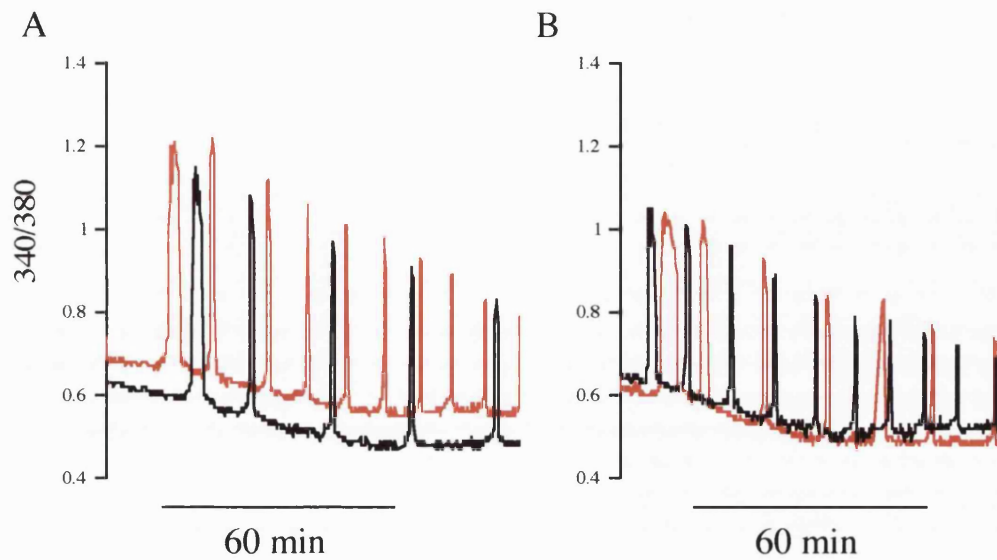


Figure 6.2 The pattern of Ca^{2+} oscillations in response to sperm in MII oocytes with reduced InsP_3R levels as a result of downregulation by **nocodazole**. MII oocytes were incubated in $10\mu\text{M}$ nocodazole for 4 hours to induce downregulation of InsP_3Rs to 20-25% of control levels (Chapter 4). Control age-matched (A) and treated (B) oocytes were loaded with Fura-2 and intracellular Ca^{2+} was monitored at fertilisation. Traces from 2 oocytes in each group are shown which are typical of recordings from 10 fertilised oocytes per group. The data is representative of 2 independent experiments. Ca^{2+} is presented as the ratio of 340/380nm. Reduction of InsP_3R levels had no effect on the fundamental pattern of oscillations (see Table 6.1).

	Mean no. of oscillations in 60 minutes	Mean amplitude of 1st transient *	Mean amplitude of next 3 transients *
Control	6.29±2.69	0.44±0.08	0.41±0.04 ^a
Post-nocodazole incubation (4 h)	4.33±1.58	0.40±0.08	0.36±0.04 ^b

Table 6.1 Analysis of the pattern of Ca²⁺ oscillations in response to sperm in MII oocytes with reduced InsP₃R levels as a result of downregulation by nocodazole. Experimental methods are as described in Fig. 6.2. The number of oscillations seen over 60 minutes from the time of fertilisation, the peak Ca²⁺ increase of the first transient (amplitude) and the mean amplitude of the following 3 transients were determined in control age-matched and nocodazole pre-treated MII oocytes. Data is pooled from 2 replicate experiments with a total of 10 oocytes in each group. The mean amplitude of the 2nd-4th transients (a and b) was significantly smaller in the treatment group (t-test: $P<0.05$). Data shows means ± s.d. *measured as peak fluorescence ratio value (340nm-background/380nm-background) after subtraction of basal value.

	Mean amplitude of Ca ²⁺ rise *	No. of oocytes showing Ca ²⁺ rise
Control	0.33±0.22 ^c	14/17 ^a
Post-nocodazole incubation (4 h)	0.01±0.05 ^d	1/18 ^b

Table 6.2 Analysis of intracellular Ca²⁺ increase in response to carbachol in MII oocytes with reduced InsP₃R levels as a result of downregulation by nocodazole. MII oocytes were incubated in 10μM nocodazole for 4 hours to induce downregulation of InsP₃Rs to 20-25% (Chapter 4). Control age-matched (A) and treated (B) oocytes were loaded with Fura-2 and intracellular Ca²⁺ was monitored upon addition of 100μM carbachol. The mean amplitude of the carbachol-induced Ca²⁺ rise and the fraction of oocytes showing this rise was determined in control age-matched and nocodazole pre-treated MII oocytes. Data is pooled from 2 replicate experiments. The number of oocytes showing a Ca²⁺ transient (a and b) was significantly lower in the treatment group (chi-squared test (Yates' correction): $P<0.0001$). The amplitude of the Ca²⁺ increase (c and d) was found to be significantly smaller in the treatment group (t-test: $P<0.0001$) (only one oocyte showing a Ca²⁺ response). Data shows means ± s.d. *measured as peak fluorescence ratio value (340nm-background / 380nm-background) after subtraction of basal value.

6.3.2.2 Effect of InsP₃R depletion in MII oocytes on fertilisation-induced Ca²⁺ transients

Measuring intracellular Ca²⁺ at fertilisation of InsP₃R-depleted eggs revealed a dramatic inhibition of Ca²⁺ release. Oocytes injected with a pipette concentration of 100µM adenophostin, giving a final oocyte concentration of 1-5µM (n=9), showed no sperm-induced Ca²⁺ transients (**Table 6.3**). Of oocytes injected with a pipette concentration of 10µM adenophostin, giving a final oocyte concentration of 100-500nM, 6/13 generated a single sperm-induced Ca²⁺ transient and no spikes were generated in the others (**Figs. 6.3.Bb and c; Table 6.3**).

This inhibition of Ca²⁺ release was reflected in a failure of the adenophostin-injected oocytes to activate, as shown by lack of formation of second polar bodies and pronuclei after fertilisation. I used two assays to confirm that fusion of the sperm with the egg had taken place in adenophostin-injected eggs. Firstly, in one experiment Hoechst staining revealed sperm heads in the cytoplasm of 6/6 eggs. Secondly, 8/12 Ca²⁺ traces from oocytes that failed to generate a Ca²⁺ spike showed small changes in baseline Ca²⁺ which could be interpreted as abortive sperm-induced Ca²⁺ transients (**Fig. 6.3.Bc**). Therefore Ca²⁺ release was inhibited in adenophostin-injected oocytes despite normal sperm-egg fusion.

6.3.2.3 Effect of InsP₃R depletion in MII oocytes on Sr²⁺-induced Ca²⁺ transients

Having examined the effect of depletion of InsP₃Rs on the Ca²⁺ oscillations at fertilisation I used the receptor-deficient MII oocytes (1-5µM adenophostin in oocyte) to examine the role of the InsP₃R in the generation of Sr²⁺-induced oscillations. Imaging intracellular Ca²⁺/Sr²⁺ in 10mM Sr²⁺-containing medium revealed a similar effect to that seen at fertilisation. In 6/9 adenophostin-injected oocytes Sr²⁺-containing medium induced a single Ca²⁺ transient. 1/9 showed three oscillations and 2/9 showed none (**Fig. 6.4.Ab; Table 6.3**). However, 11/13 uninjected control oocytes showed the repetitive Ca²⁺ transients typical of Sr²⁺ stimulation (**Fig. 6.4.Aa; Table 6.3**). Therefore the InsP₃R-deficient oocytes have a significantly inhibited response to both fertilisation and Sr²⁺-induced Ca²⁺ oscillations.

In order to ensure that this effect was due to InsP₃R downregulation and not some non-specific effect of adenophostin A on the Ca²⁺ stores, the release of intracellular Ca²⁺ stores by thapsigargin was examined. Thapsigargin caused an increase in intracellular Ca²⁺ in all 15 adenophostin-injected (1-5µM adenophostin in oocyte) and all 16 uninjected control oocytes examined (**Fig. 6.4.B**). The peak amplitude of the response was not significantly different between the two groups (t-test: $P < 0.08$; **Table 6.4**). However, the rate of rise was significantly slower in adenophostin-injected oocytes compared to controls (t-test: $P < 0.0001$; **Table 6.4**). Thus it appears that the depletion of InsP₃Rs does not

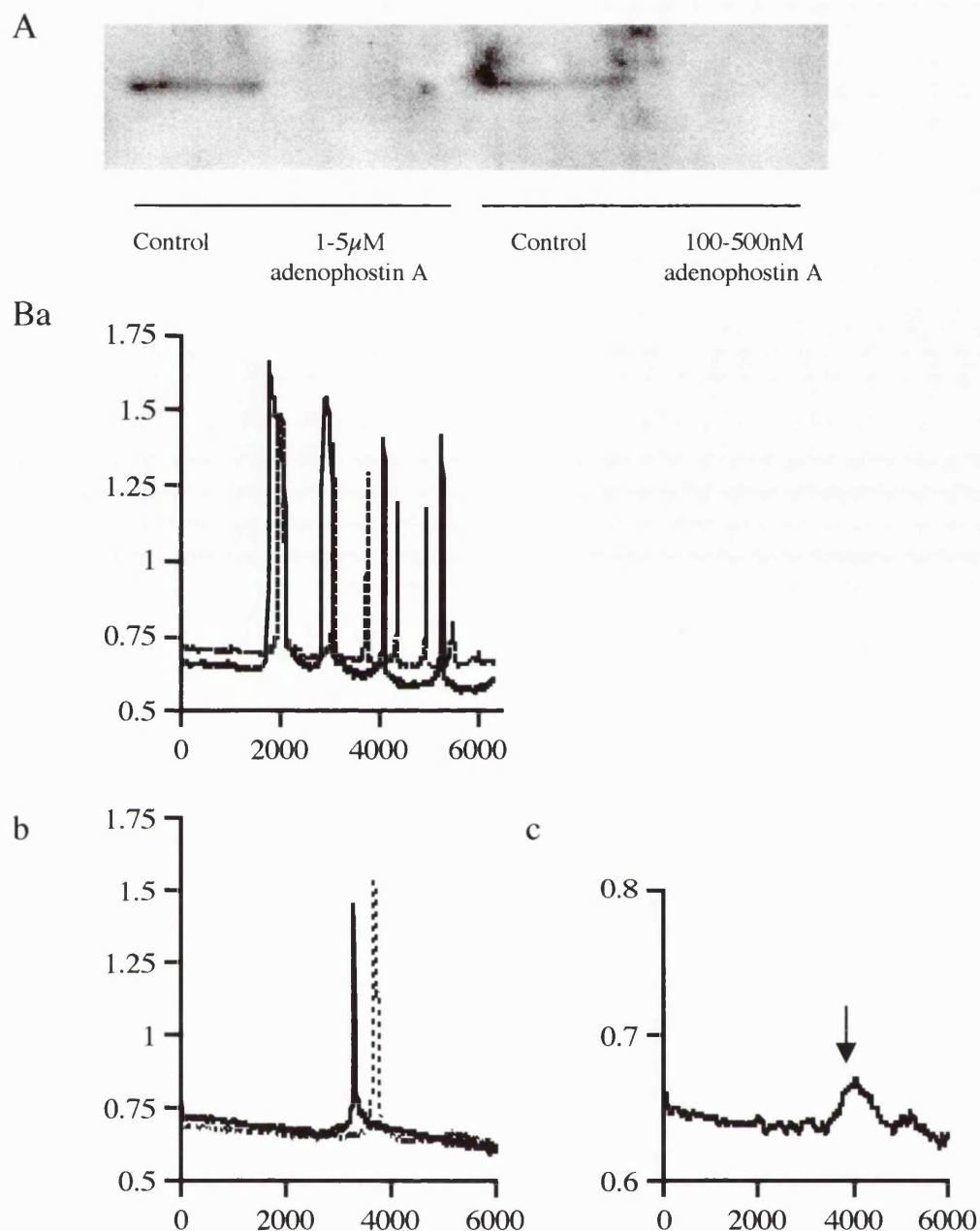


Figure 6.3 Ca^{2+} oscillations at fertilisation can be inhibited by downregulating InsP_3Rs . Immature oocytes were microinjected with adenophostin to produce a final concentration of 1-5 μM or 100-500nM. Injected oocytes were allowed to mature over 14 hours to the MII stage before collection for Western analysis. Protein from 40 MII eggs matured after treatment with an oocyte concentration of 1-5 μM adenophostin was electrophoresed and blotted with Ab40 (A lane 2) next to a control lane of non-injected *in vitro* matured oocytes (A lane 1). This was repeated with an oocyte concentration of 100-500nM adenophostin (A lanes 3 (control) and 4). Both concentrations of adenophostin induced extensive InsP_3R downregulation to below our levels of detection. This data is representative of 4 experiments. Ca^{2+} -imaging of InsP_3R -depleted MII oocytes at fertilisation revealed an almost complete inhibition of Ca^{2+} release (B). Control *in vitro* matured oocytes generated repetitive Ca^{2+} oscillations as illustrated by the representative records of two oocytes (Ba). Representative traces of 3 100-500nM adenophostin-injected *in vitro* matured oocytes are shown in Bb and Bc. Oocytes generate at most a single Ca^{2+} transient in response to sperm (Bb) while others show an abortive Ca^{2+} spike (Bc, arrow). With 1-5 μM adenophostin sperm-induced transients were completely inhibited (data not shown). Ca^{2+} is presented as the ratio of 340/380nm. The data are typical examples of recordings from 18 adenophostin-injected oocytes and 19 control oocytes.

Table 6.3 Sperm and Sr^{2+} -induced Ca^{2+} signaling is inhibited in InsP_3R -depleted oocytes. *1-5 μM adenophostin (oocyte concentration) was injected into immature oocytes which were then matured *in vitro* for 14 hours. The mature oocytes that result are depleted of InsP_3Rs . Controls were treated similarly but were not injected.

Stimulus	Treatment*	n	Multiple Ca^{2+} transients	Single Ca^{2+} transients	No Ca^{2+} transients
Fertilisation	Control	19	17	0	2
	Adenophostin				
	1-5 μM	9	0	0	9
	100-500nM	13	0	6	7
Strontium	Control	7	5	0	2
	Adenophostin	9	1	6	2

Table 6.4 The effect of InsP_3R depletion on thapsigargin-induced Ca^{2+} release. * InsP_3Rs were depleted by microinjecting immature oocytes with 1-5 μM adenophostin (oocyte concentration) before maturation *in vitro*. Controls were treated similarly except that they were not injected. The rate of Ca^{2+} increase (a and b) was significantly slower in the treatment group (t-test: $P < 0.0001$).

Response to thapsigargin	Controls (n=15)	Adenophostin (n=16)
Peak ratio	0.95 ± 0.1	0.89 ± 0.08
Rate of Ca^{2+} increase (sec / 0.1 ratio unit)	13.4 ± 5.4^a	71 ± 33.9^b

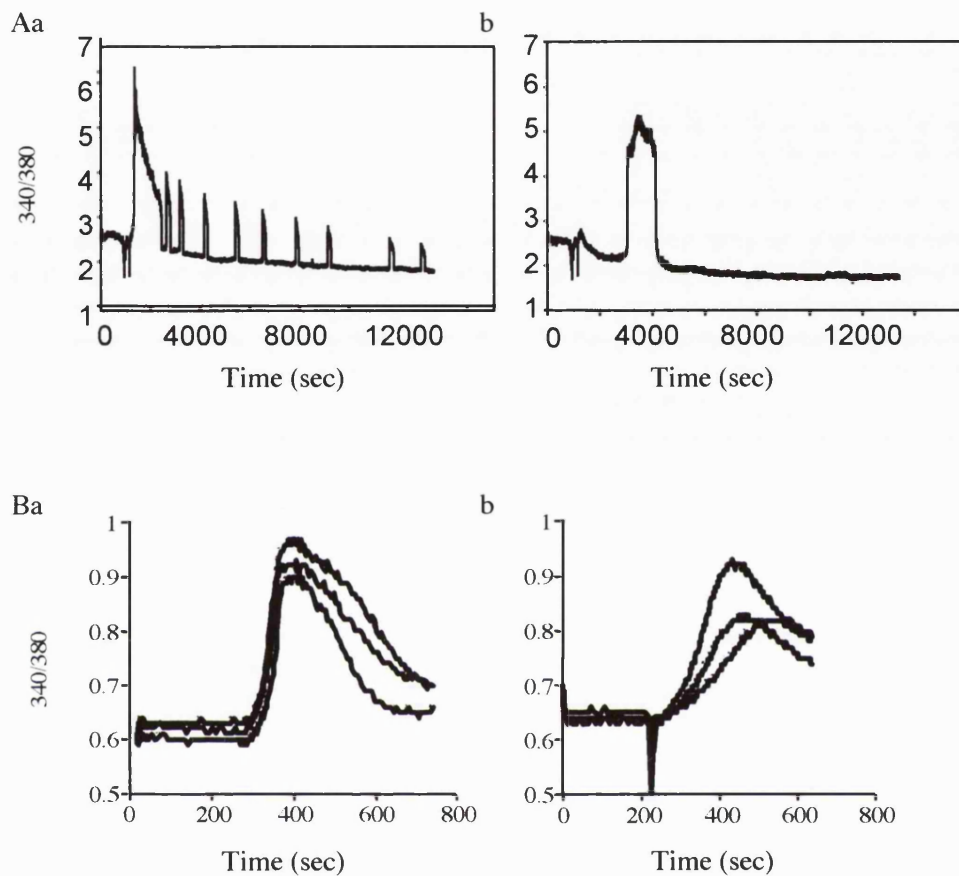


Figure 6.4 Ca^{2+} release in InsP_3R -depleted eggs in response to Sr^{2+} and thapsigargin. M2 containing 10mM Sr^{2+} was perfused onto Fura-2-loaded oocytes (A). A representative trace from 7 *in vitro* matured controls (Aa) and 9 adenophostin-injected (1-5 μM in oocyte), *in vitro* matured oocytes (Ab) are shown. Note the inability of Sr^{2+} to stimulate more than one transient. The application of 10 μM thapsigargin in Ca^{2+} -free medium caused an increase in intracellular Ca^{2+} in both control (Ba) and adenophostin-injected (1-5 μM in oocyte) (Bb) oocytes. Representative traces are from a total of 15 control and 16 adenophostin-injected oocytes. Adenophostin-injected oocytes show a significantly reduced rate of Ca^{2+} release (see Table 6.4).

deplete Ca^{2+} stores but does dramatically slow the loss of Ca^{2+} from the endoplasmic reticulum when the Ca^{2+} -ATPases are inhibited with thapsigargin.

6.4 ROLE OF InsP_3R DURING PREIMPLANTATION DEVELOPMENT

Having examined the role of the InsP_3R during oocyte maturation and at fertilisation, I carried out experiments to investigate its role in mitotic divisions during preimplantation development. Previous studies reveal that NEBD at first mitosis in fertilised mouse eggs is often associated with a brief Ca^{2+} transient, and that first mitosis can be blocked by intracellular Ca^{2+} chelators (Kono *et al.*, 1996; Tombes *et al.*, 1992) and thus is dependent on internal Ca^{2+} . Clearly a requirement for InsP_3Rs in preimplantation development would be strongly consistent with there being a role for intracellular Ca^{2+} increases during this period.

6.4.1 Role of the InsP_3R in the timing of NEBD and of the first mitotic cleavage

This work has so far established that the full complement of InsP_3Rs is not necessary for the first meiotic division but that a certain level is critical for the generation of Ca^{2+} oscillations at fertilisation which stimulate completion of the second meiotic division. I now investigated the role of the InsP_3R in NEBD and cytokinesis of the first mitotic division. I investigated the timing of NEBD and first cytokinesis in Sr^{2+} -activated parthenogenotes, which have the same level of InsP_3Rs as MII oocytes, and in the same type of embryo after adenophostin-induced InsP_3R downregulation.

Sr^{2+} -activated eggs were cultured to the pronucleate stage and microinjected with $100\mu\text{M}$ adenophostin A (final oocyte concentration of $1\text{-}5\mu\text{M}$) at 8 hours after Sr^{2+} addition. The embryos were cultured for a further 8 hours and were scored both for NEBD and cleavage to the 2-cell embryo stage 60-80 minutes later. Surprisingly, the timings of both NEBD and first cleavage in adenophostin-injected embryos were no different from those in KCl-buffer-injected and uninjected embryos (Fig. 6.5).

6.4.2 Role of the InsP_3R in subsequent development of the 2-cell embryo

Having established that depletion of InsP_3Rs has no effect on the time-course of NEBD and the first mitotic division, I examined its effect on subsequent mitotic divisions in both Sr^{2+} -activated parthenogenotes and fertilised embryos.

6.4.2.1 Mitotic divisions in InsP_3R -deficient parthenogenetic embryos

I first examined the role of InsP_3Rs in the subsequent mitotic divisions of the 2-cell parthenogenetic embryo. Embryos subjected to the same protocol (see 6.4.1) were scored

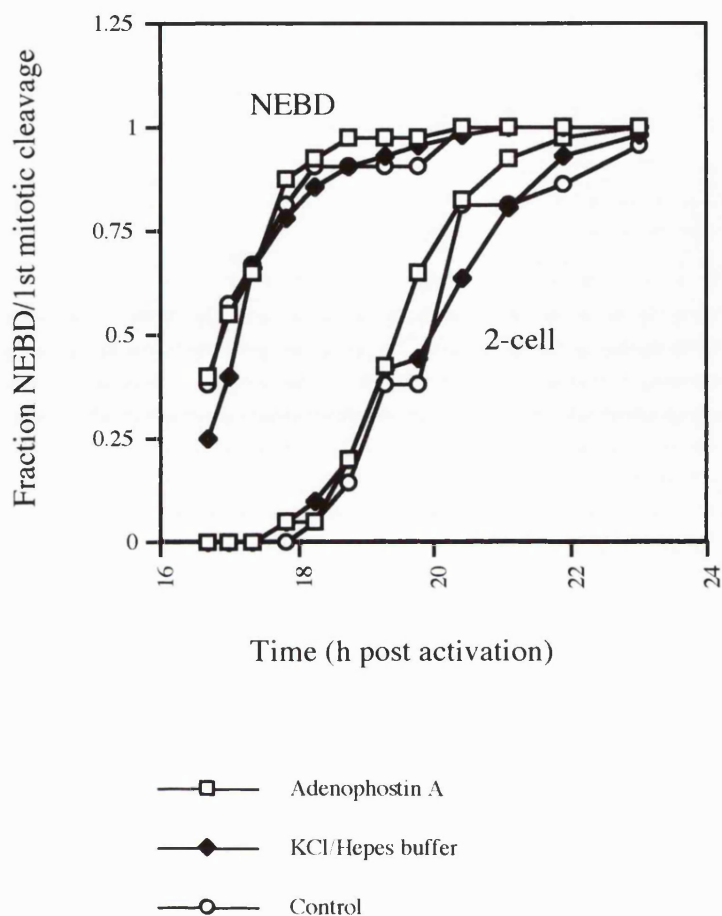


Figure 6.5 The effect of InsP_3R depletion on the ability to undergo NEBD and cleavage to the 2-cell stage. Sr^{2+} -activated eggs were cultured to the pronucleate stage and microinjected with $100\mu\text{M}$ adenophostin A (final oocyte concentration of $1\text{--}5\mu\text{M}$) or with KCl/Hepes injection buffer alone 8 hours after Sr^{2+} addition. The embryos were cultured for a further 8 hours and were scored both for NEBD and cleavage to 2-cell embryos. The number of 1-cell embryos which had undergone NEBD and cleavage was counted at 20- or 30-minute intervals for the duration of 6-8 hours. Data is from one experiment and is representative of 6 experiments each with 30-40 1-cell embryos per treatment group. The rates of both NEBD and 1st cleavage in adenophostin-injected embryos were no different from those in KCl-buffer-injected and uninjected embryos.

for the final proportion reaching 2-cells by 24 hours after Sr^{2+} addition. They were then cultured further and scored at 48 hours for the proportion reaching 4-cells and at 72 hours for the proportion reaching 8-cells/early morulae. 5mM glucose (final concentration) was added into the culture medium at the 4-cell stage to support the development of the later embryonic stages (Chatot *et al.*, 1990). Total numbers of embryos reaching each stage were not significantly different between InsP_3R -depleted and control groups (**Fig. 6.6**). The one exception was the statistically significant (t-test: $P < 0.05$) difference between the fraction of InsP_3R -depleted and uninjected embryos reaching 3/4-cells. However, the difference in this fraction between InsP_3R -depleted and the more rigorous control of KCl-injected embryos was insignificant (**Fig. 6.6**).

6.4.2.2 Analysis of adenophostin A-induced downregulation in parthenogenetic embryos

To determine whether the expression of InsP_3Rs remains downregulated during preimplantation development, after adenophostin injection at the pronucleate stage, I examined receptor levels at various stages. Western analysis of 30-40 adenophostin-injected and uninjected or buffer-injected embryos collected 4 hours after injection, at the 2-cell stage and early morula stage revealed that the level of InsP_3Rs was undetectable at all three stages and therefore that once downregulated by adenophostin, InsP_3Rs were not significantly upregulated again (**Figs. 6.7.A, 6.7.Ba and 6.7.C**). Furthermore, Sr^{2+} -activated parthenogenotes maintained a constant level of InsP_3Rs at all stages, confirming that activation by Sr^{2+} does not stimulate even a gradual loss of receptors (**Fig. 6.7**) (see Chapter 4).

Although the level of InsP_3Rs after adenophostin injection was undetectable with my analysis protocol using 40 embryos it was unlikely that stimulation by even such a potent agonist would result in the degradation of 100% of receptors. Therefore I examined the level of InsP_3Rs remaining after injection of adenophostin, using Western analysis of 327 embryos. Levels of InsP_3Rs were found to be similar in MII oocytes and 2-cell uninjected parthenogenotes (**Fig. 6.7.Bb**). However, NIH image analysis of immunoreactivity from the adenophostin-injected 2-cell parthenogenotes revealed a decrease in band intensity of 85-90%, suggesting that 10-15% of receptors were remaining (see Chapter 3 for relationship between band intensity and quantity of InsP_3R) (**Fig. 6.7.Bb**).

6.4.2.3 Mitotic divisions in InsP_3R -deficient fertilised embryos

In order to investigate the requirement for InsP_3Rs during the later stages of preimplantation development, *in vivo* fertilised 1-cell embryos were depleted of InsP_3Rs using adenophostin and cultured up to the blastocyst stage. Although less synchronous in their rate of development than parthenogenotes, the use of fertilised diploid embryos allowed me to culture more advanced embryos with greater success. 1-cell embryos were

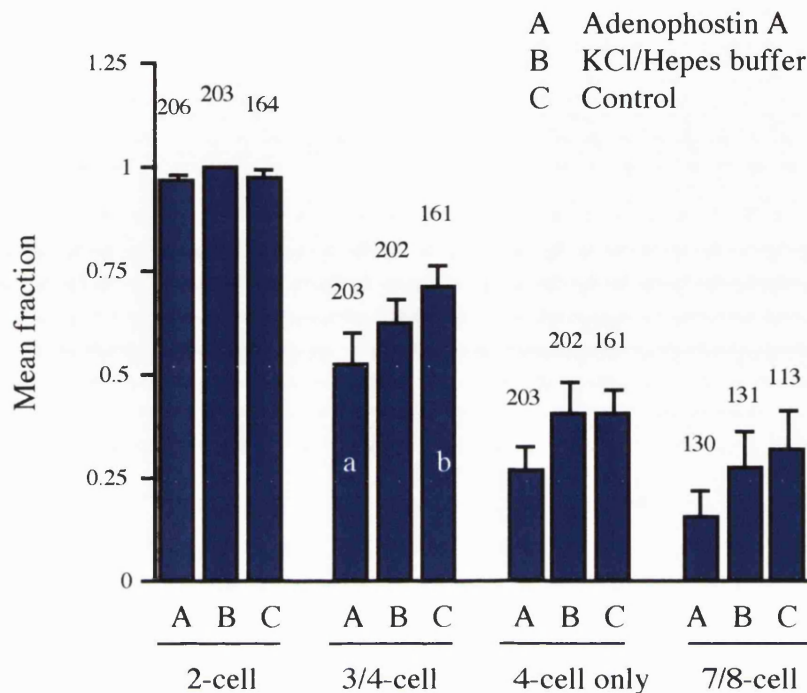


Figure 6.6 The effect of InsP_3R depletion on first and subsequent mitotic divisions of the parthenogenetic embryo. Sr^{2+} -activated eggs were cultured to the pronucleate stage and injected with $100\mu\text{M}$ adenophostin A (final oocyte concentration of $1\text{--}5\mu\text{M}$) or with KCl/Hepes injection buffer alone 8 hours after Sr^{2+} addition. The embryos were cultured *in vitro* and scored for final proportion (of total activated) reaching the 2-cell stage by 24 hours after Sr^{2+} addition. The combined proportion reaching 3- or 4-cells and proportion reaching 4-cells only (48 hours) and the proportion of 7/8-cell embryos (72 hours) were scored as a fraction of the number of 2-cells in each treatment group. Numbers above columns indicate the total number of embryos scored (see Table 6.5). Data represents mean fractions from 6 replicate experiments except in the case of data for 7/8-cell embryos which represents mean fractions from 4 replicate experiments. Error bars indicate positive standard error. Treatment groups marked a and b are significantly different (t-test: $P < 0.05$). In other groups total numbers of embryos reaching each stage did not differ significantly between InsP_3R -depleted and control groups.

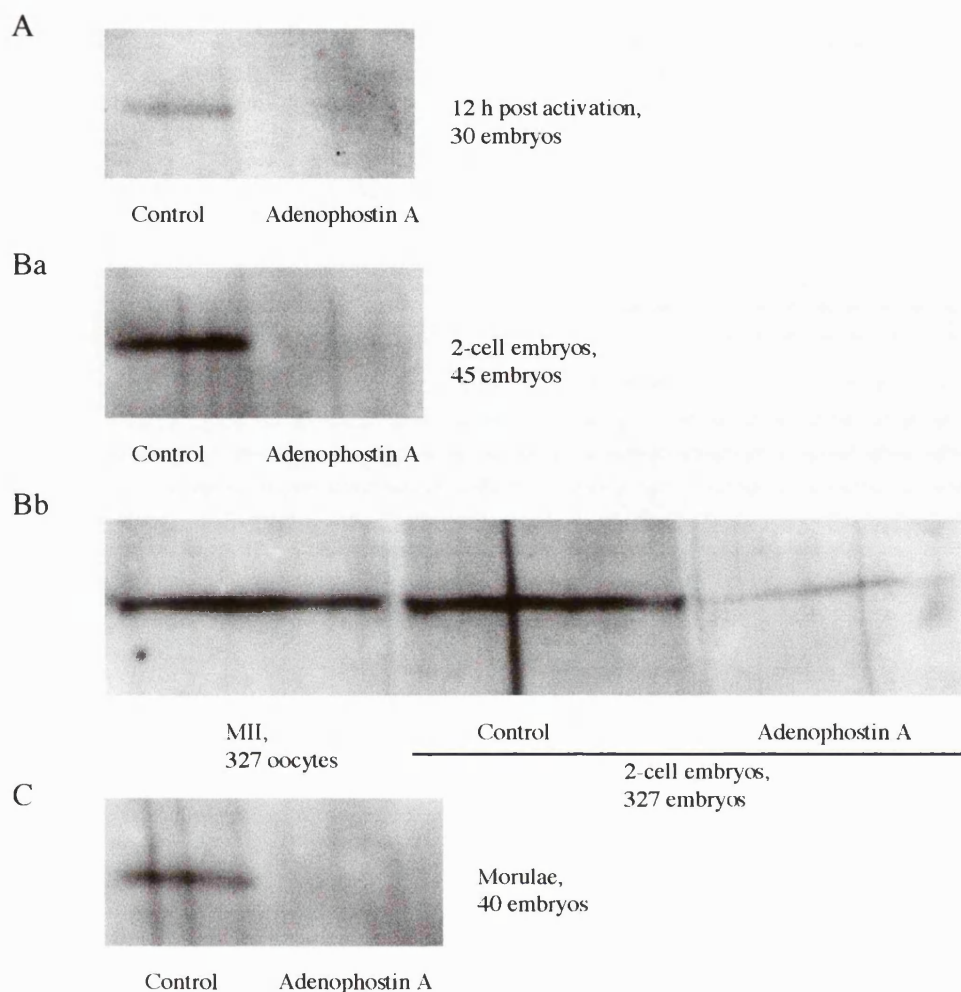


Figure 6.7 Effects of adenophostin A on InsP_3R levels in preimplantation development.

Sr^{2+} -activated eggs were cultured to the pronucleate stage and microinjected with $100\mu\text{M}$ adenophostin A (final oocyte concentration of $1\text{--}5\mu\text{M}$) 8 hours after Sr^{2+} addition. (A) The 1-cell embryos were cultured for a further 4 hours and collected for Western analysis. Protein from 30 PN eggs was electrophoresed, immunoblotted and probed with Ab40 (A lane 2) next to a control lane of uninjected parthenogenotes which had been cultured in parallel (A lane 1). Adenophostin A induced extensive InsP_3R downregulation to below the detection limit within 4 hours. Analysis representative of 2 experiments. (B) In other experiments the embryos were cultured for a further 14–16 hours after microinjection and collected for Western analysis as 2-cell embryos. (Ba) Protein from 45 2-cell embryos was analysed using Ab40 (Ba lane 2) next to a control lane of uninjected parallel cultured 2-cells (Ba lane 1). Analysis representative of 3 experiments. Following adenophostin-induced downregulation the expression of InsP_3Rs was maintained at an undetectable level in 45 2-cell embryos. (Bb) Protein from 327 2-cell embryos was analysed using Ab40 (Bb lane 3) next to control lanes of uninjected parallel cultured 2-cells (Bb lane 2) and MII unactivated oocytes (Bb lane 1). InsP_3Rs were detected when 327 2-cell embryos were analysed. NIH image analysis of immunoreactive bands revealed a decrease in band intensity of 85–90%. (C) Injected embryos were cultured to the morula stage and subjected to Western analysis. Protein from 40 morulae was analysed using Ab40 (C lane 2) next to a control lane of KCl/Hepes buffer-injected parthenogenetic morulae cultured in parallel (C lane 1). Following adenophostin-induced downregulation InsP_3R expression was maintained at an undetectable level in 40 early morulae.

injected with adenophostin 30 hours after hCG priming (approximately 18 hours after fertilisation). They were scored at 48 hours post hCG for the proportion reaching 2-cells, at 102 hours post hCG for the proportion of morulae and at 126 hours post hCG for the proportion of blastocysts. 5mM glucose (final concentration) was added into the culture medium at the 4-cell stage to support the development of the later embryonic stages (Chatot *et al.*, 1990). As seen with the earlier stages of preimplantation development, total numbers of embryos reaching each stage did not alter significantly between InsP₃R-depleted and control groups (**Fig. 6.8**). However, due to less than optimal culture conditions the proportion of control embryos making the transition from morulae to blastocysts was very low and further work is needed to investigate the requirement for InsP₃Rs at this transition.

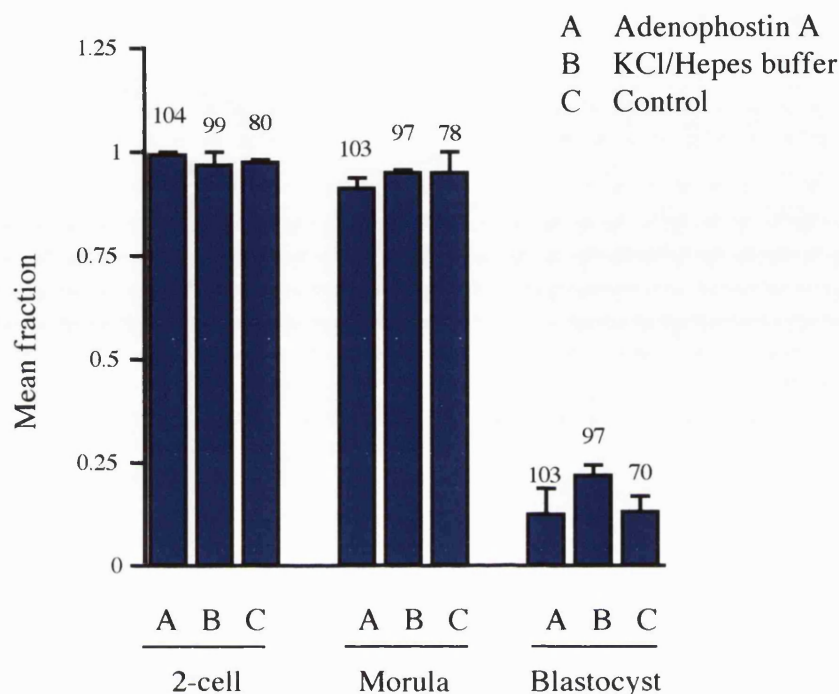


Figure 6.8 The effect of InsP_3R depletion on first and subsequent mitotic divisions of the fertilised embryo. *In vivo* fertilised 1-cell embryos were microinjected with $100\mu\text{M}$ adenophostin A (final oocyte concentration of $1\text{--}5\mu\text{M}$) or with KCl/Hepes injection buffer alone 30 hours after hCG priming (about 18 hours after fertilisation) and cultured *in vitro* up to the blastocyst stage. Embryos were scored at 48 hours after hCG for proportion at 2-cells. The proportion of morulae 102 hours after hCG and the proportion of blastocysts 126 hours after hCG were scored as a fraction of the number of 2-cells at 30 hours in each treatment group. Numbers above columns indicate the total number of embryos scored (see Table 6.6). Data represents mean fractions from 3 replicate experiments except in the case of data for uninjected controls which represents mean fractions from 2 replicate experiments. Error bars indicate positive standard error. Numbers of embryos reaching each stage did not differ significantly between InsP_3R -depleted and control groups (t-test). (But note that proportion of control embryos making the transition from morulae to blastocysts is below optimal.)

6.5 DISCUSSION

6.5.1 The role of the InsP_3R during oocyte maturation

Experiments carried out to examine the role of the InsP_3R during oocyte maturation suggested that the endogenous levels of InsP_3Rs are not required for both GVBD and the first meiotic division.

Firstly, the depletion of InsP_3Rs in GV-arrested oocytes had no effect on the timing and rate of subsequent GVBD. Secondly, in order to generate mature InsP_3R -deficient MII oocytes, I injected immature oocytes with adenophostin within two hours of release from the follicle. Downregulation of the receptors within 4 hours of injection would have meant that the final 6-8 hours of progression to metaphase II arrest would have taken place with very low receptor numbers. Despite this, the ability of oocytes to mature *in vitro* was unaffected. Thus the normal maturation of immature oocytes, despite adenophostin-depleted levels of InsP_3Rs , implies that InsP_3 -induced Ca^{2+} release is not necessary for oocyte maturation. An alternative explanation is that low levels of receptors are sufficient to create local Ca^{2+} gradients and thereby support potentially critical local InsP_3 -induced Ca^{2+} transients. However, the former is more likely due to several lines of evidence from previous studies investigating a role for Ca^{2+} in GVBD and meiosis I.

Firstly, in one study spontaneous global Ca^{2+} oscillations were observed in roughly 80% of mouse oocytes undergoing GVBD (Carroll and Swann, 1992). These oscillations could be mimicked by injection of exogenous InsP_3 and blocked by heparin, suggesting that they were caused by an increase in InsP_3 . However, in another study, global Ca^{2+} transients were not observed around the time of GVBD (Tombes *et al.*, 1992). Moreover, in both studies the use of intracellular Ca^{2+} chelators showed that GVBD is Ca^{2+} -independent. The completion of meiosis I and ensuing maturation to metaphase of meiosis II is dependent on external Ca^{2+} (Jagiello *et al.*, 1982; Leibfried and First, 1979; Paleos and Powers, 1981; Tombes *et al.*, 1992). Nevertheless, the literature suggests that these steps occur independently of internal Ca^{2+} release and this is consistent with my results. Whereas intracellular Ca^{2+} chelators block Ca^{2+} increases from both internal and external sources, I have provided a means of drastically inhibiting release from internal stores whilst leaving increases from the external Ca^{2+} source unaffected. I therefore presume that completion of meiosis I is likely to be independent of internal Ca^{2+} release from InsP_3 -gated stores.

There is much data in the literature on the development of increased sensitivity to InsP_3 during oocyte maturation. The concept that GVBD is Ca^{2+} -independent and that completion of meiosis I and progression to MII arrest is only dependent on external and not internal Ca^{2+} , is consistent with this increase in sensitivity being exclusively a means of preparing for the state of maximum sensitivity to InsP_3 required for fertilisation. For example, the

peak Ca^{2+} increase after sperm fusion or injection of InsP_3 in GV stage oocytes is significantly less than in mature eggs in both hamster and mouse (Fujiwara *et al.*, 1993; Mehlmann and Kline, 1994). The near maximum response to InsP_3 in hamster immature oocytes was 80% of that in mature eggs (Fujiwara *et al.*, 1993), which suggests that while immature oocytes are less sensitive to InsP_3 , most of the InsP_3 -sensitive Ca^{2+} stores are already present in the immature hamster oocyte. Secondly, immature mouse oocytes release significantly less Ca^{2+} from stores than mature oocytes in response to ionomycin (Jones *et al.*, 1995b; Tombes *et al.*, 1992) and thapsigargin (Jones *et al.*, 1995b). Thirdly, there is a 1.8-fold increase in immunoreactive InsP_3R protein observed following oocyte maturation and a function-blocking antibody has been used to show that this immunoreactive increase represents a greater number of *functional* InsP_3Rs (Mehlmann *et al.*, 1996). My results support the idea that the role of the changes described above is in preparation for fertilisation rather than for a functional role of InsP_3 -induced Ca^{2+} release during oocyte maturation, *per se*.

6.5.2 The role of the InsP_3R in the Ca^{2+} oscillations at fertilisation and in response to Sr^{2+}

I have used a variety of means of downregulating InsP_3Rs and have revealed that there is a threshold level of InsP_3Rs necessary for generating Ca^{2+} changes in response to sperm or Sr^{2+} .

The MII oocyte contains a large functional reserve of InsP_3Rs

Pretreating MII oocytes with nocodazole to downregulate InsP_3Rs to 20-25% of control receptor levels (see Chapter 4) had no significant effect on the pattern of oscillations at fertilisation, suggesting that a large functional reserve in receptor numbers exists. This is consistent with the fact that by two hours after fertilisation in control oocytes 50% of receptors have been degraded, yet the same basic pattern of oscillations is maintained for a further 1-2 hours. However, in the same nocodazole-pretreated oocytes the response to carbachol was completely inhibited.

Nocodazole pretreatment inhibits the Ca^{2+} response of MII oocytes to carbachol

One way in which nocodazole may inhibit the Ca^{2+} response to carbachol is related to its effect of disrupting the tubulin cytoskeleton. Evidence to support the idea that disruption of the cytoskeleton may also disrupt the Ca^{2+} response pathway to carbachol, is variable. It has been shown in one study that nocodazole has no effect on carbachol-induced Ca^{2+} increases via a G protein-coupled receptor, the human muscarinic acetylcholine receptor 1, expressed in Jurkat T cells (Huby *et al.*, 1998). However, in a separate study, a 30 minute preincubation of NIH 3T3 cells in nocodazole resulted in complete inhibition of agonist-induced Ca^{2+} signaling by hormones coupled to $\text{PLC}\beta$ or $\text{PLC}\gamma$ activation (ATP and

PDGF respectively) (Ribeiro *et al.*, 1997). In the same study, neither agonist-activated PLC nor the ability of InsP_3 to mobilise Ca^{2+} was affected by disruption of the tubulin network. It was suggested that the observed inhibition of Ca^{2+} release was due to nocodazole changing the spatial-temporal relationship between PLC activation at the plasma membrane and Ca^{2+} release sites (Ribeiro *et al.*, 1997). The same group had previously implicated that InsP_3R -containing organelles may be physically linked to the plasma membrane through both the actin and tubulin cytoskeletons (Rossier *et al.*, 1991). This data (Ribeiro *et al.*, 1997) is also in agreement with another study in which microtubule inhibitors, including nocodazole, reduced Ca^{2+} elevations stimulated by agonists which work via Gi proteins (Michel *et al.*, 1996). The discrepancy between the Huby *et al.* (1998) and Ribeiro *et al.* (1997) studies could be due to the shorter length of nocodazole preincubation in the first study (3 minutes) being too short to alter the proposed relationship between PLC and Ca^{2+} release sites sufficiently to inhibit signaling.

I have discussed above the evidence for nocodazole treatment disrupting the carbachol signaling pathway. I now summarise possible reasons for why carbachol-induced, but not sperm-induced signaling, is inhibited by nocodazole pretreatment. One explanation makes the assumption that a 4-hour preincubation in nocodazole specifically inhibits G protein-muscarinic receptor interaction. In this case, the finding that nocodazole has no effect on Ca^{2+} release at fertilisation supports the idea that sperm do not act through a pathway involving G proteins (Williams *et al.*, 1998), and is consistent with the fact that carbachol stimulates G protein-coupled receptors. An alternative explanation assumes that nocodazole acts further downstream in the signaling pathway, disrupting communication between PLC activation and InsP_3Rs . The evidence for this is discussed above. In addition to that evidence, a functional link has been described between the InsP_3R and PIP_2 (Lupu *et al.*, 1998), which may provide efficient coupling between PLC activation, PIP_2 hydrolysis and Ca^{2+} release. If this communication is disrupted by nocodazole, there are two reasons why sperm-induced, but not carbachol-induced signaling may be possible. Firstly, a sperm may be able to mobilise Ca^{2+} by introducing a diffusible sperm-derived PLC (Jones *et al.*, 1998) which does not depend on the cytoskeleton to cause InsP_3R activation. Secondly, if fertilisation depends upon the activation of egg PLCs at the plasma membrane, sperm, but not carbachol, may be a sufficiently potent stimulus to overcome impaired communication to the InsP_3R and mobilise Ca^{2+} . Finally, the other possible explanation for why nocodazole pretreatment may block the response to carbachol but not fertilisation is related to the effect of nocodazole on InsP_3R levels. Carbachol may be a weaker stimulus than the sperm and may therefore require a greater concentration of InsP_3Rs to generate a signal.

Fertilisation-induced oscillations require a threshold level of InsP_3Rs

Although the nocodazole-induced depletion of 75-80% of receptors did not affect Ca^{2+} signaling at fertilisation, it remained the case that a minimum level of receptors must be essential for generation of these oscillations. Indeed, there was a significant decrease in the

mean amplitude of 2nd, 3rd and 4th transients in the nocodazole-treated eggs, which suggests that less Ca^{2+} was released due to the lower density of InsP_3Rs . I therefore used the potent InsP_3R agonist adenophostin A as a tool to extensively downregulate receptor levels in immature oocytes and create mature MII oocytes which were almost entirely depleted of InsP_3Rs . Note that in 1-cell parthenogenotes (see 6.5.3) 85-90% of InsP_3R immunoreactivity was eliminated after treatment with $1\mu\text{M}$ adenophostin (oocyte concentration) and it is highly probable that the loss of InsP_3Rs is to a similar extent in immature oocytes. Thus it is likely that 10-15% of control levels of InsP_3Rs were present in the InsP_3R -depleted MII oocytes prepared with $1\mu\text{M}$ adenophostin and a slightly higher receptor level in oocytes prepared with 100nM adenophostin. As predicted, the generation of oscillations at fertilisation of these oocytes was dramatically inhibited, suggesting that despite at least 50% of receptors being *excess* to requirement, these oscillations were dependent on a minimum critical level of receptors.

The Ca^{2+} response at fertilisation of InsP_3R -depleted oocytes appeared to be graded in a manner reflecting the dose of adenophostin A, in support of the idea that a lower concentration of drug will cause less receptor downregulation. The injection of approximately $1\mu\text{M}$ adenophostin (oocyte concentration) completely abolished Ca^{2+} signaling at fertilisation. Oocytes injected with approximately 100nM adenophostin (oocyte concentration) in some cases failed to generate a Ca^{2+} spike but showed small increases in baseline Ca^{2+} . In other cases 100nM -injected oocytes were able to support the generation of one Ca^{2+} spike, yet in still more cases oocytes showed no change in Ca^{2+} at all. Note that 100nM adenophostin stimulates a Ca^{2+} increase in immature oocytes (this study) and stimulates long-lasting Ca^{2+} oscillations in mouse MII oocytes (He *et al.*, 1999; Sato *et al.*, 1998). However, the inhibition of Ca^{2+} signaling at fertilisation of adenophostin-injected MII oocytes was not the result of Ca^{2+} stores being depleted, since thapsigargin produced a Ca^{2+} increase that reached a similar peak to in uninjected controls. I can thus attribute the results of these experiments to the effect of adenophostin on InsP_3R protein levels. Western analysis of 40 oocytes has shown that in MII oocytes prepared with both $1\mu\text{M}$ and 100nM adenophostin (oocyte concentrations) the level of InsP_3R protein is below the level of detection. Therefore, further experiments using larger numbers of oocytes will be necessary to determine if $1\mu\text{M}$ and 100nM adenophostin downregulate InsP_3Rs to different levels in a dose-dependent manner.

The variety of Ca^{2+} responses at fertilisation of oocytes injected with 100nM adenophostin (oocyte concentration) can be explained by the models proposed in Figure 6.9. (**Fig. 6.9**). The models assume that there are small differences between individual oocytes in density and distribution of InsP_3Rs after adenophostin-induced downregulation and in state of sensitivity to Ca^{2+} release. I suggest that in cases where oocytes did not generate any detectable global change in Ca^{2+} , these oocytes completely failed to propagate the local InsP_3 -induced Ca^{2+} rise triggered by the fertilising sperm, due to InsP_3R density being too

Figure 6.9 Proposed models to explain Ca^{2+} responses of InsP_3R -depleted MII oocytes at fertilisation.

A. At fertilisation in the control MII oocyte InsP_3 releases Ca^{2+} via InsP_3Rs from the internal stores. The released Ca^{2+} acts via CICR to stimulate regenerative Ca^{2+} release from InsP_3Rs throughout the oocyte. This results in an emptying of the Ca^{2+} stores which stimulates Ca^{2+} influx, possibly through store-operated Ca^{2+} channels in the plasma membrane. The conformational coupling model of capacitative Ca^{2+} entry proposes that information on store depletion is transferred to the plasma membrane via the InsP_3R (Berridge, 1990; Berridge, 1995). The Ca^{2+} -ATPase pumps on the ER membrane pump Ca^{2+} back into the stores thus enabling the generation of repetitive global Ca^{2+} oscillations.

B (i). At fertilisation of InsP_3R -depleted MII oocytes InsP_3 releases Ca^{2+} from the ER via InsP_3Rs which are at a much lower density. This model proposes that released Ca^{2+} has a much reduced influence on neighbouring InsP_3Rs since the distance between adjacent InsP_3Rs has increased. Therefore CICR is drastically reduced and the initial Ca^{2+} rise, instead of regenerating to produce a full-blown spike, is followed by a return to basal Ca^{2+} levels as the Ca^{2+} -ATPase returns Ca^{2+} back to the stores. Since Ca^{2+} stores remain almost full capacitative Ca^{2+} influx is not stimulated.

(ii). At fertilisation of InsP_3R -depleted MII oocytes InsP_3 releases Ca^{2+} from the ER via InsP_3Rs which are at a much lower density. This model proposes that released Ca^{2+} is able to act via CICR to stimulate regenerative Ca^{2+} release despite neighbouring InsP_3Rs being much further apart. Therefore the initial Ca^{2+} rise is propagated to produce a single full-blown spike. However, information on the resulting emptying of the stores cannot be transmitted to the SOCCs to stimulate capacitative Ca^{2+} influx. This is due to there being too few InsP_3Rs mediating this information transfer. Without influx of external Ca^{2+} to replenish internal stores further Ca^{2+} spikes cannot be generated. Although the Ca^{2+} -ATPases pump Ca^{2+} back into the stores causing Ca^{2+} levels to fall back to basal, the stores remain partially empty due to loss of Ca^{2+} from the oocyte with no countering Ca^{2+} uptake.
(continued overleaf)

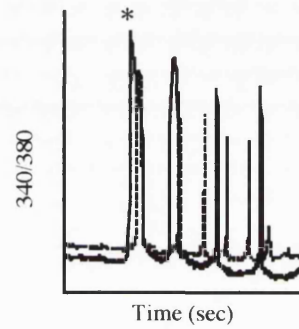
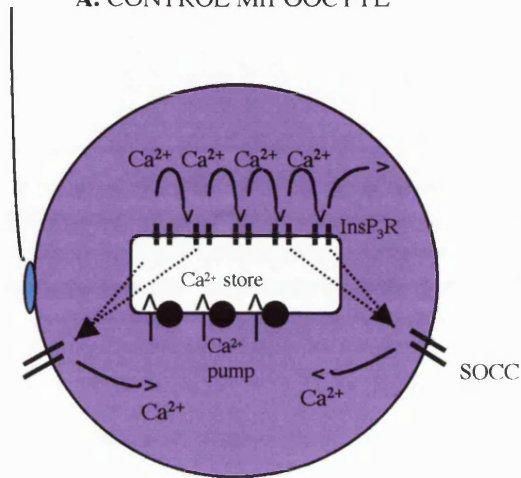
In InsP_3R -depleted oocytes produced after injection of 100nM adenophostin, Ca^{2+} responses at fertilisation included some oocytes generating a single transient as in (ii), some showing an aborted spike as in (i) and some showing no change in Ca^{2+} . This may be explained by differences between individual oocytes in density and distribution of InsP_3Rs after adenophostin downregulation and other variation in state of sensitivity to Ca^{2+} release. For example, some oocytes failed to propagate the local InsP_3 -induced Ca^{2+} rise at all so that a detectable global transient was not generated. Some oocytes could support limited CICR and thus exhibited a brief, but aborted, Ca^{2+} transient. Some oocytes, however, could support CICR sufficiently to generate a global Ca^{2+} spike but further spikes were limited by InsP_3R density being too low to support capacitative Ca^{2+} influx to refill the stores.

Note that the asterisks refer to the point in time during the Ca^{2+} transient which is represented by the model.

- Low $[Ca^{2+}]$ §
- Medium $[Ca^{2+}]$ §
- High $[Ca^{2+}]$ §

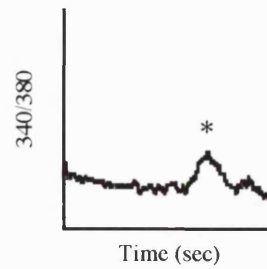
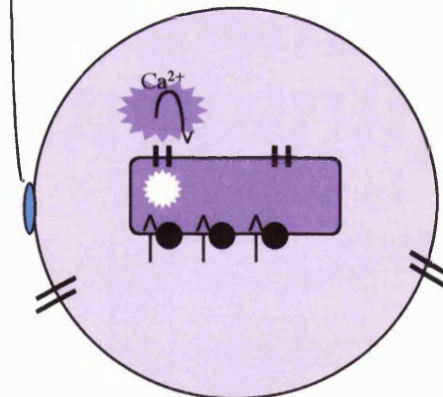
§ Note that at rest $[Ca^{2+}]$ in store (10-100 μM) is 100-2000-fold higher than $[Ca^{2+}]$ in cytosol (50-100 nM); the resting situation would be represented by "high" $[Ca^{2+}]$ in store and "low" $[Ca^{2+}]$ in cytosol

A. CONTROL MII OOCYTE

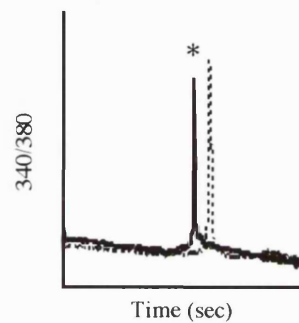
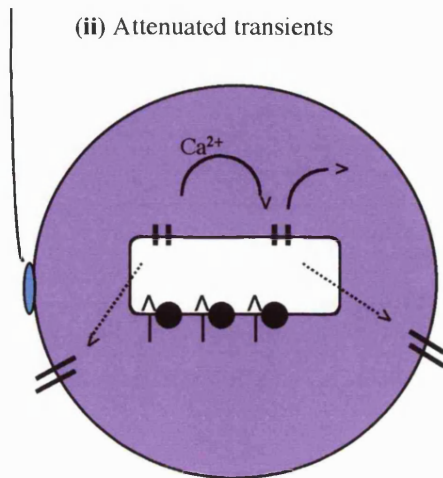


B. InsP₃R-DEPLETED MII OOCYTES:

(i) Aborted spikes



(ii) Attenuated transients



low. I further suggest that the small but global increases in baseline Ca^{2+} exhibited by some other oocytes are attempted transients which are initially propagated by positive feedback (CICR) but subsequently aborted as a result of InsP_3Rs being too few and far apart to maintain CICR.

It is more difficult to explain the generation of a single global transient in some oocytes injected with 100nM adenophostin. One possible explanation for the inability to generate more than one transient is that store-refilling is compromised. The conformational coupling model of capacitative Ca^{2+} entry proposes that information on store depletion is transferred to the plasma membrane via the InsP_3R (Berridge, 1990; Berridge, 1995). In fact, there is evidence that the type III InsP_3R can function as a capacitative Ca^{2+} entry channel (Putney, 1997) and that there is a functional interaction between InsP_3Rs in the ER and store-operated plasma membrane Htrp3 channels in HEK293 cells (Kiselyov *et al.*, 1998). Furthermore, inhibiting InsP_3R function in HEK293 cells prevents store-induced store-operated- Ca^{2+} -channel (SOCC) activation, showing that the InsP_3R is essential for maintaining coupling between store emptying and physiological activation of SOCCs (Ma *et al.*, 2000). This coupling may be mediated by Ca^{2+} -dependent changes in conformation of ER lumen-resident proteins that couple to the InsP_3R , thereby influencing its interaction with the SOCC (Berridge, 1995). Therefore, in the case of those InsP_3R -depleted oocytes in which CICR is sufficient to generate a single Ca^{2+} spike, the number of InsP_3Rs linked to SOCCs may be too low to support the capacitative Ca^{2+} influx required for the generation of subsequent oscillations. This result is therefore analogous to that of Ma *et al.* (2000), described above, in which an InsP_3R inhibitor was used to lower the number of functional InsP_3Rs , rather than downregulation of InsP_3R expression as used here (Ma *et al.*, 2000). The idea that capacitative influx of Ca^{2+} is required to refill the internal stores for second and subsequent Ca^{2+} spikes is supported by a study in which evidence was found for a capacitative entry mechanism in mouse oocytes (McGuinness *et al.*, 1996). This study also directly demonstrated, using the manganese-quench technique, that there is a stimulation of cation influx associated with the onset of the first and subsequent fertilisation Ca^{2+} spikes. This lead them to propose that Ca^{2+} influx at fertilisation is controlled by a capacitative mechanism and sets the interspike period by determining the time taken to refill the internal stores to a point where they are again sensitive enough to initiate the next spike (McGuinness *et al.*, 1996).

What level of InsP_3Rs are required for fertilisation-induced oscillations?

To establish the minimum level of InsP_3Rs required to support sperm-induced oscillations I can compare the ability of sperm to generate oscillations in MII oocytes after InsP_3R downregulation by nocodazole and by adenophostin A. I have shown that in 1-cell parthenogenotes the extent of degradation of the InsP_3R after the injection of 1-5 μM adenophostin (oocyte concentration), is 85-90%. Presuming that adenophostin acts in the same way in maturing oocytes as in interphase 1-cell embryos and using the knowledge

that in both, the extent of downregulation is to below detectable levels in 40 oocytes or embryos, I can infer that fertilisation-stimulated oscillations require a level of InsP_3Rs greater than 10-15% of that in the MII oocyte. I have also shown that with nocodazole-induced downregulation to 20-25%, there was no notable effect on the pattern of oscillations at fertilisation. I predict that the minimum level of InsP_3Rs required to support oscillations at fertilisation must lie in-between 15% and 25% of control levels.

The InsP_3R may act as a Ca^{2+} “leak” channel

As noted above, the inhibition of Ca^{2+} signaling was not the result of adenophostin depleting Ca^{2+} stores, since thapsigargin produced a Ca^{2+} increase in InsP_3R -depleted MII oocytes similar to that in uninjected controls. Depletion of InsP_3Rs did, however, have a dramatic slowing effect on the rate at which Ca^{2+} was released from the ER after thapsigargin treatment. This result may provide us with an insight into the mechanism of Ca^{2+} cycling between the ER lumen and the cytosol. It is known that thapsigargin inhibits the ER membrane Ca^{2+} -ATPase pump. Following exposure to thapsigargin there is a net cytosolic Ca^{2+} increase indicating a significant Ca^{2+} “leak” from the internal stores, which is normally countered by re-uptake into the stores by pump activity. However, there is little data on the mechanism of this Ca^{2+} leak. Ca^{2+} leak channels may include characterised Ca^{2+} release channels, other transmembrane channels and even transient “holes” in the ER membrane. My result suggests that since depletion of InsP_3Rs substantially slowed down the rate of Ca^{2+} leak, the InsP_3R must be a major component of the leak pathway (**Fig. 6.10**). This is consistent with data showing that the rate of Ca^{2+} release following Ca^{2+} -ATPase inhibition is sensitive to heparin, a competitive InsP_3 antagonist (Guillemette *et al.*, 1989), in both intact HL-60 granulocytes (Favre *et al.*, 1994) and in mouse lacrimal acinar cells (Smith and Gallacher, 1994). Furthermore, in permeabilised HL-60 cells the addition of low InsP_3 concentrations before thapsigargin increases the rate of thapsigargin-induced Ca^{2+} release 4-fold (Favre *et al.*, 1994) while in mouse lacrimal acinar cells caffeine, which suppresses basal InsP_3 levels in exocrine acinar cells (Toescu *et al.*, 1992), inhibits the response to subsequent thapsigargin exposure (Smith and Gallacher, 1994). The involvement of the InsP_3R could be as a “leaky” channel itself or, alternatively, Ca^{2+} leaked via other pathways could activate InsP_3Rs by CICR, thus amplifying the original Ca^{2+} leak. Both mechanisms would be dependent on resting levels of InsP_3 bound to the InsP_3R and thus would be inhibited by heparin. However, in mouse eggs the former possibility is more likely as CICR is difficult to activate prior to fertilisation. This has been demonstrated in hamster eggs where the critical Ca^{2+} injection current necessary to activate CICR was over 10-fold lower after fertilisation than in unfertilised oocytes (Igusa and Miyazaki, 1983). Further work is necessary to determine the contributions of Ca^{2+} leak and CICR through the InsP_3R to the dynamics of thapsigargin-induced Ca^{2+} release.

Figure 6.10 Proposed model to explain Ca^{2+} response of InsP_3R -depleted MII oocytes to thapsigargin.

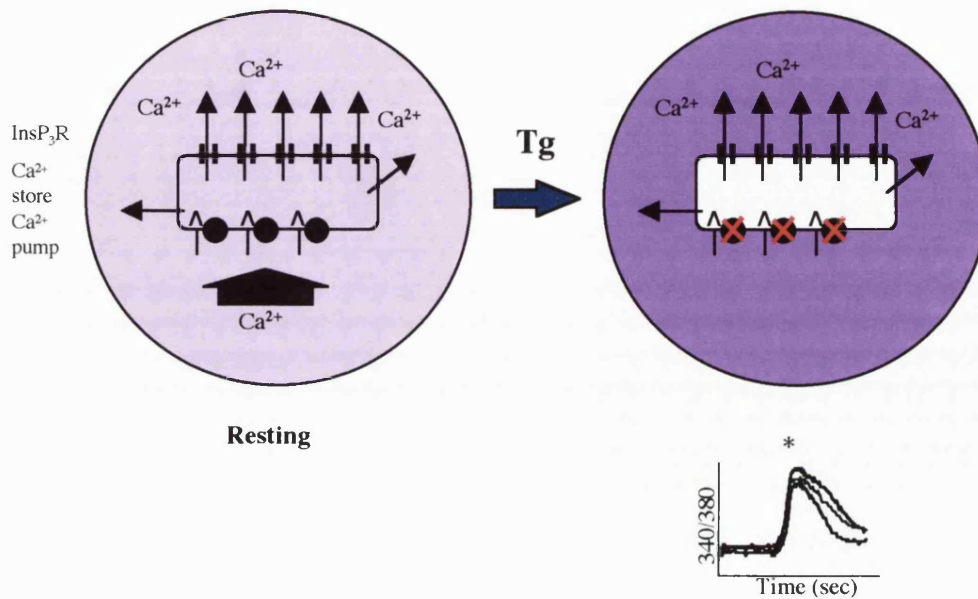
In the resting cell, Ca^{2+} is continuously cycling between the ER lumen and the cytosol, maintaining a constant level of Ca^{2+} within each compartment. (Note that Ca^{2+} concentration is higher within the ER Ca^{2+} store.) To maintain equilibrium, cycling must involve a balance between Ca^{2+} leak from the internal stores and re-uptake into the stores by the Ca^{2+} -ATPase pump on the ER membrane. However, Ca^{2+} leak channels remain uncharacterised; candidates include known Ca^{2+} -release channels, other transmembrane ER channels and transient gaps in the ER membrane. This model proposes that the InsP_3R is a major component of the leak pathway by allowing Ca^{2+} to escape through the receptor channel in the presence of basal levels of InsP_3 .

A. In the resting control MII oocyte, Ca^{2+} leak from the ER through InsP_3Rs and other leak pathways is countered by re-uptake by pump activity. Thapsigargin (Tg) is an inhibitor of the Ca^{2+} -ATPase. Upon application of Tg, re-uptake is prevented, thus causing a net cytosolic Ca^{2+} increase (and ER lumen Ca^{2+} decrease) due to the maintained leak from internal stores (see graph inset).

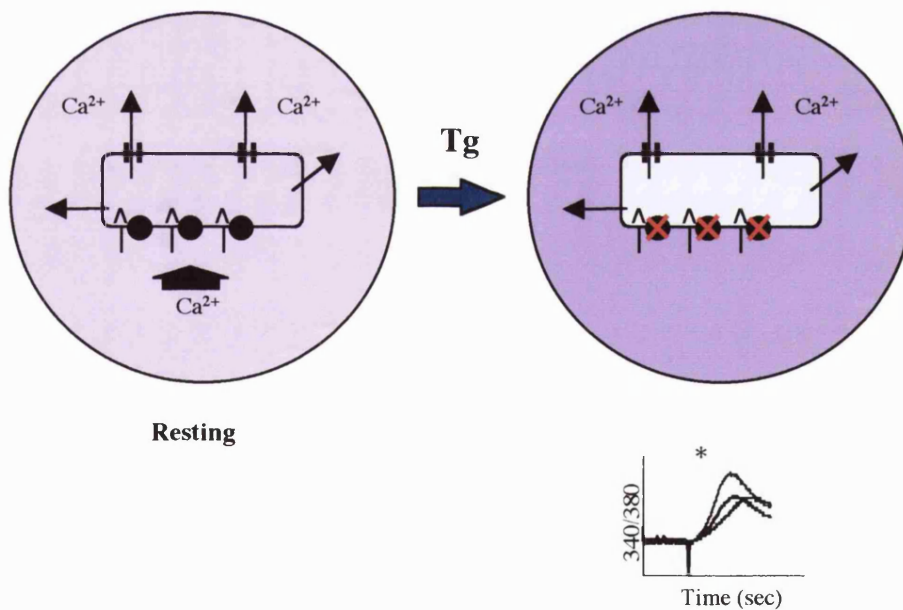
B. In the resting InsP_3R -depleted MII oocyte, Ca^{2+} leak from the ER is smaller due to there being fewer InsP_3R leak channels. This leak is countered by a similarly reduced re-uptake due to pump activity. Upon application of Tg, re-uptake is prevented, thus causing a net cytosolic Ca^{2+} increase (see graph inset). However, since the leak from internal stores is smaller due to fewer InsP_3Rs the rate of Ca^{2+} increase is lower. Nevertheless, the peak amplitude of the increase is as in control oocytes due to the equal size of the Ca^{2+} stores at equilibrium.

Note that the asterisks refer to the point in time during the Ca^{2+} transient which is represented by the model, at which the Ca^{2+} concentration in the control oocyte is at a peak but in the InsP_3R -depleted oocyte is still increasing.

A. CONTROL MII OOCYTE



B. InsP₃R-DEPLETED MII OOCYTE



- Lower $[\text{Ca}^{2+}]$ §
- Low $[\text{Ca}^{2+}]$ §
- Resting $[\text{Ca}^{2+}]$
- High $[\text{Ca}^{2+}]$ §
- Higher $[\text{Ca}^{2+}]$ §

§ Note that at rest $[\text{Ca}^{2+}]$ in store (10-100 μM) is 100-2000-fold higher than $[\text{Ca}^{2+}]$ in cytosol (50-100 nM); the colour categories used in this model indicate a lower or higher $[\text{Ca}^{2+}]$ than resting $[\text{Ca}^{2+}]$ for either store or cytosol

Sr²⁺-mediated oscillations are also generated through the InsP₃R

To determine whether InsP₃R-depleted oocytes were specifically affected in fertilisation-induced Ca²⁺ signaling I applied Sr²⁺-containing medium to oocytes injected with 1μM adenophostin. Sr²⁺ produced a response very similar to that of the fertilised oocyte with only one oocyte showing multiple oscillations. I can thus infer that Sr²⁺-stimulated oscillations require a level of InsP₃Rs at least greater than 10-15% of control levels (see above). It has been shown that Sr²⁺ binds to and activates an activating Ca²⁺ binding site on the InsP₃R (Marshall and Taylor, 1994) but my data is the first to directly show that Sr²⁺-mediated oscillations are generated through the InsP₃R in mouse oocytes. InsP₃Rs therefore appear to be the sole Ca²⁺ release channels necessary for both fertilisation and Sr²⁺-induced oscillations.

A role for the RyR in fertilisation-induced oscillations?

Although functional ryanodine receptors have been found in mouse oocytes (Ayabe *et al.*, 1995) the inhibition of sperm-induced oscillations in InsP₃R-depleted oocytes suggests that, contingent on adenophostin having no effect on RyR levels, RyRs play little role in the generation of oscillations at fertilisation and that the sole Ca²⁺ release channel involved is the InsP₃R. This is in agreement with experiments showing that sperm can fully activate mouse eggs after microinjection with concentrations of ryanodine thought to inhibit Ca²⁺ release from the ryanodine-sensitive store (Ayabe *et al.*, 1995). These experiments suggest that if the ryanodine-sensitive store is mobilised following fertilisation, the resulting Ca²⁺ released is not essential for any event of egg activation (Ayabe *et al.*, 1995). In contrast, inhibiting Ca²⁺ release from the InsP₃-sensitive store using adenophostin (this study) or the 18A10 InsP₃R antibody, inhibits sperm-induced Ca²⁺ transients (Miyazaki *et al.*, 1992; this study) and egg activation (Xu *et al.*, 1994; this study). However, the role of the RyR in the generation of sperm-induced oscillations is controversial. In other studies ryanodine has been shown to block Ca²⁺ oscillations induced by sperm or a sperm factor in mouse oocytes, under conditions in which ryanodine induced an immediate Ca²⁺ increase (Jones *et al.*, 1995b; Swann, 1992). This suggests that ryanodine-sensitive stores may modulate the Ca²⁺ oscillations at fertilisation in the mouse but further experiments are required to clarify this point.

6.5.3 The role of the InsP₃R in the mitotic divisions of preimplantation development

The previous experiments showed that the InsP₃R has an essential role in the generation of Ca²⁺ oscillations in the MII oocyte. Consistent with these oscillations being critical for the activation of mammalian embryonic development, InsP₃R-depleted oocytes failed to activate upon fertilisation. I next examined the role of the InsP₃R in NEBD and cytokinesis of the first mitotic division. As in the case of oocyte maturation, the use of adenophostin to

downregulate receptor levels did not point to a requirement for a full complement of InsP_3Rs .

The depletion of InsP_3Rs in interphase pronucleate parthenogenotes had no effect on either the occurrence or timing of NEBD 8-9 hours later or of subsequent cleavage to 2-cell embryos. Furthermore, the proportions of parthenogenetic embryos developing to 4-cells and 8-cells were not significantly different from the proportions in two control groups. Fertilised embryos depleted of InsP_3Rs also showed no difference from controls in development to the 2-cell and morulae stages. Although the proportion of fertilised embryos progressing from morulae to blastocysts was also similar in all three groups, my culture conditions did not support high rates of blastocyst development even in control groups. Therefore I cannot conclude that there is no requirement for InsP_3Rs at this transition. In fact, it is probable that InsP_3 -induced Ca^{2+} release plays a role in the regulation of blastocyst development. Exposure of mouse morulae to ethanol or ionomycin to elevate intracellular Ca^{2+} levels accelerates the rate of cavitation and cell division (Stachecki and Armant, 1996b). In addition, mouse morulae exposed to Ca^{2+} chelators show delayed blastocoele formation and Ca^{2+} release in these embryos occurs predominantly through the InsP_3R (Stachecki and Armant, 1996b). Furthermore, exposure to PLC inhibitors results in a reversible inhibition of cavitation, suggesting that PLC-mediated signaling is critical for blastocoele formation (Stachecki and Armant, 1996a).

6.5.4 Implications for InsP_3 -induced Ca^{2+} release in mitotic divisions

My results suggest that NEBD of the 1-cell embryo and the mitotic divisions which lead to the formation of a morula are independent of InsP_3 -induced Ca^{2+} release. This is surprising given the observation that fertilised embryos generate Ca^{2+} transients at NEBD and during the first mitosis up until cleavage to the 2-cell stage (Kono *et al.*, 1996). Moreover, NEBD in both fertilised (Kono *et al.*, 1996; Tombes *et al.*, 1992) and parthenogenetic (Kono *et al.*, 1996) embryos is inhibited by the intracellular Ca^{2+} chelator BAPTA-AM. A possible cause of NEBD inhibition by BAPTA-AM is a non-specific effect. For example, 10 μM BAPTA-AM has been shown to inhibit protein synthesis (a 2/3 decrease in leucine incorporation) in fertilised mouse eggs (Lawrence *et al.*, 1998) and thus in pronucleate stage embryos may prevent the accumulation of MPF necessary for NEBD at the onset of mitosis. However, there is evidence to suggest that maximum levels of cyclin B are attained by as much as 2-3 hours prior to NEBD since onset of mitosis is unaffected if the protein synthesis inhibitor puromycin is added after this time (Howlett, 1986). Therefore, inhibited MPF synthesis seems unlikely since in the Kono *et al.* (1996) (Kono *et al.*, 1996) experiment BAPTA-AM was added within the 3 hours preceding NEBD.

There is, however, a more plausible explanation for inhibition of NEBD by BAPTA-AM. It should first be noted that mitotic Ca^{2+} transients are not always detected in some studies

(Tombes *et al.*, 1992; Whitaker and Patel, 1990; Wilding *et al.*, 1996) and that, unlike fertilised embryos, parthenogenotes do not generate detectable transients at NEBD or in mitosis (Kono *et al.*, 1996). Despite the lack of detectable Ca^{2+} transients, parthenogenetic embryos develop normally, suggesting that the generation of *global* Ca^{2+} transients is not necessary for normal development. Rather than it having a non-specific effect, BAPTA-AM may be buffering *localised* Ca^{2+} transients, which are too small to be detected using conventional imaging systems, but which may be responsible for NEBD and progression through mitosis (Kao *et al.*, 1990; Kono *et al.*, 1996; Snow and Nuccitelli, 1993; Whitaker and Patel, 1990). Indeed, in one study on the sea urchin, global Ca^{2+} transients originating from the nuclear area were often detected just before NEBD of first mitosis. In the absence of global increases, however, confocal microscopy using Ca^{2+} Green-1 dextran revealed localised Ca^{2+} transients in the nuclear region (Wilding *et al.*, 1996). The possibility that local Ca^{2+} transients in the nuclear region may induce NEBD and progression through mitosis is further supported by the finding that the nuclear envelope releases Ca^{2+} (Gerasimenko *et al.*, 1995). These putative localised transients may be analogous to the InsP_3 -induced transient “puffs” (peak free Ca^{2+} 100-200nM) which have been evoked by photorelease of caged InsP_3 in *Xenopus* oocytes, and are thought to arise from the concerted opening of several clustered InsP_3Rs (Parker and Yao, 1996).

This study has shown that after downregulation by adenophostin in parthenogenetic pronucleate 1-cell embryos, 10-15% of InsP_3Rs remain. If local, undetectable Ca^{2+} transients *are* responsible for progression through mitosis, this study also suggests that 10-15% of control levels of InsP_3Rs are sufficient to support these transients. This would permit the normal rate of development that I have seen, at least up to the morula stage, in these embryos.

InsP₃Rs may concentrate at the pronuclei prior to NEBD

One explanation for *how* 10-15% of InsP_3Rs would be able to support local Ca^{2+} release is that receptors concentrate around the pronucleus prior to NEBD of mitosis. In embryos with depleted levels of InsP_3Rs , either via fertilisation or adenophostin, this may generate a limited area of receptors sufficiently dense to generate a Ca^{2+} gradient which would be located near the mitotic apparatus. Clustering of InsP_3Rs may be achieved either by (1) reorganisation of the ER by a cell cycle-dependent mechanism or by (2) a change in distribution of the InsP_3Rs themselves, independent of ER distribution. (1) In support of a reorganisation of the ER, there is evidence in the sea urchin for cell cycle changes in ER distribution during early development (Terasaki, 2000; Terasaki and Jaffe, 1991). The ER is uniformly distributed at interphase but gradually accumulates at the mitotic poles before NEBD and remains there during mitosis (Terasaki, 2000). (2) Alternatively, clustering may result from an aggregation of InsP_3Rs , independent of ER reorganisation. It has been shown that activation of AR4-2J cells with CCK stimulates receptor clustering in addition to InsP_3R downregulation (Wojcikiewicz, 1995). Another study demonstrated Ca^{2+} -

dependent InsP₃R clustering in RBL-2H3 cells, which was independent of ER vesiculation (Wilson *et al.*, 1998). Clusters were dispersed along the ER network and concentrated along the nuclear envelope (Wilson *et al.*, 1998). Whether by (1) or (2), clustering of InsP₃Rs in the pronucleate egg may explain how a low number of receptors could support local Ca²⁺ gradients.

Whether or not receptor clustering is stimulated in the fertilised egg is not clear from my immunolocalisation studies. I have shown using immunocytochemistry that InsP₃Rs appear to be distributed fairly evenly throughout the MII oocyte before fertilisation *and* the pronucleate 1-cell embryo after fertilisation, with no apparent receptor clustering taking place (Chapter 3). This data is supported by the finding that the ER of mature mouse eggs does not become disrupted during the first few Ca²⁺ oscillations at fertilisation (Kline *et al.*, 1999). The distribution of receptors and ER throughout the cytoplasm may be beneficial to the fertilised oocyte: global InsP₃-mediated Ca²⁺ oscillations continue for up to 4 hours until pronuclear formation (see General Introduction). However, InsP₃Rs may still increase in concentration around the pronucleus prior to NEBD in the fertilised egg. This may not have been detected using immunocytochemistry because (1) the low resolution of antibody staining in this study may have obscured any receptor clustering at the pronuclear stage and (2) pronucleate eggs were fixed for staining 8 hours after fertilisation. Thus staining could not reveal any clustering which may occur at a later stage preceding NEBD. The idea that InsP₃Rs become clustered around the pronuclei is supported by preliminary work tracking the ER, which suggests that after fertilisation there is an increase in ER density around the pronuclei.

It remains to be determined whether or not there is a requirement for InsP₃R clustering to generate the proposed local Ca²⁺ signals. Further experiments (using immunocytochemistry or a GFP-tagged InsP₃R in conjunction with an ER-specific dye) are required to examine whether clustering of InsP₃Rs does occur in pronucleate mouse eggs.

6.5.5 A role for alternative sources of intracellular Ca²⁺ increase in preimplantation development?

An alternative explanation for the inhibition of preimplantation development by BAPTA yet normal development after InsP₃R depletion is that Ca²⁺ transients which are essential to mitosis are generated independently of InsP₃Rs. Alternative sources of Ca²⁺ include extracellular Ca²⁺ and ryanodine-sensitive Ca²⁺ stores which could be employed to establish a Ca²⁺ gradient.

It is unlikely, however, that external Ca²⁺ plays a role in the mitotic divisions of preimplantation development. BAPTA-AM will inhibit Ca²⁺ increases in the cytosol from both internal and external sources. In a study on 1-cell mouse embryos it has been shown

that BAPTA alone does not completely inhibit NEBD and is more effective in combination with Ca^{2+} -free medium (Tombes *et al.*, 1992). This *could* be explained by inefficient buffering by BAPTA in this study enabling Ca^{2+} gradients at plasma membrane Ca^{2+} channels to temporarily build up and suggests a possible role for external Ca^{2+} influx creating local Ca^{2+} gradients. However, simply removing extracellular Ca^{2+} alone neither blocks NEBD nor any other mitotic event, including anaphase and cytokinesis (Tombes *et al.*, 1992), therefore ruling out a dependency on external Ca^{2+} stores. It is more likely that local Ca^{2+} increases around the ER Ca^{2+} release channels are permitted by inefficient buffering. Furthermore, nuclear and cytoplasm transfer experiments revealed that the Ca^{2+} transient observed at NEBD by Kono *et al.* (1996) (Kono *et al.*, 1996) appeared to be associated with the pronucleus, pointing at Ca^{2+} release from internal stores.

Alternatively, one conceivable way in which external Ca^{2+} could be involved in preimplantation development is as a Ca^{2+} source working in a compensatory mode alongside internal Ca^{2+} stores. Inhibition of internal Ca^{2+} release alone by InsP_3R depletion, or removal of extracellular Ca^{2+} alone, may have no effect on development as the other mechanism may be functioning simultaneously. However, inhibition of influx into the cytoplasm from both sources at once by chelating intracellular Ca^{2+} may inhibit development (Kono *et al.*, 1996; Tombes *et al.*, 1992). To explore this idea it would be necessary to carry out further experiments looking at the effect of removing extracellular Ca^{2+} on the development of InsP_3R -depleted eggs.

Regarding the role of ryanodine-sensitive Ca^{2+} stores in preimplantation development, it has been shown that mouse oocytes express the type II and III ryanodine receptors (Ayabe *et al.*, 1995; Jones *et al.*, 1995b). However, RyR mRNA is not expressed in the mouse blastocyst (Rosemblit *et al.*, 1999) and I have demonstrated that InsP_3Rs are the sole Ca^{2+} release channels necessary for Ca^{2+} oscillations at fertilisation. Despite this, I cannot rule out a role for RyRs in the Ca^{2+} transients generated at NEBD and during mitosis. Ryanodine induces a small biphasic release of Ca^{2+} in mouse morulae, suggesting that ryanodine-sensitive Ca^{2+} stores may exist in mouse embryos (Stachecki and Armant, 1996b). However, the elevation in Ca^{2+} stimulated by thimerosal and InsP_3 in mouse morulae is significantly greater (Stachecki and Armant, 1996b), suggesting that InsP_3 -sensitive Ca^{2+} stores are more important at this developmental stage. On the other hand, Sousa *et al.* (1996) (Sousa *et al.*, 1996) found that, in the human, after the appearance of pronuclei, ryanodine-sensitive Ca^{2+} oscillations of low amplitude and frequency were observed until pronuclear breakdown. They also found that ryanodine increased the amplitude and frequency of the Ca^{2+} oscillations observed in human blastocysts. Further work is necessary to assess any possible contribution of ryanodine-sensitive Ca^{2+} stores to mitotic divisions of preimplantation development.

6.5.6 InsP₃R-deficient oocytes: adenophostin versus InsP₃R(-/-) mice

Besides downregulating InsP₃R expression to produce oocytes and embryos with reduced levels of InsP₃Rs, another potential way to examine the role of the InsP₃R at fertilisation and in preimplantation development would be to study InsP₃R knockout mice. However, for the reasons described in the introduction of this chapter, it is not possible to obtain oocytes from these mice which are completely free of both embryonic and maternal InsP₃Rs. Even the heterozygotes produce InsP₃R(-/-) oocytes which probably have the wildtype complement of maternal InsP₃R protein (Matsumoto *et al.*, 1996). The technique of adenophostin-induced InsP₃R downregulation which I have used is an effective alternative way of addressing the role of the InsP₃R at fertilisation and in preimplantation development. This technique also has an advantage over the use of function-blocking InsP₃R antibodies, such as 18A10 (Miyazaki *et al.*, 1992), since these may have non-specific effects in addition to inhibiting the InsP₃R.

6.5.7 Conclusion

In conclusion, GVBD and meiosis I of oocyte maturation as well as NEBD of the 1-cell embryo and subsequent mitotic divisions to form the morula, can take place with very low levels of InsP₃Rs. I propose that InsP₃-induced Ca²⁺ release is not necessary for oocyte maturation. However, previous studies implementing Ca²⁺ chelators strongly suggest that NEBD of the first mitosis is dependent on Ca²⁺ release from internal stores (Kono *et al.*, 1996; Tombes *et al.*, 1992). Therefore, I also propose that local Ca²⁺ increases, which can be generated through this low number of receptors in InsP₃R-depleted eggs, may be essential regulators of NEBD and early mitotic cell cycles. Such transients may be revealed by confocal microscope imaging of adenophostin-treated embryos during these stages. Alternatively, sources of Ca²⁺ release other than InsP₃-sensitive stores may be involved. In contrast, egg activation and subsequent completion of meiosis are blocked by the removal of the majority of InsP₃Rs, as the sperm-induced Ca²⁺ oscillations critical for activation are inhibited.

CHAPTER 7:

GENERAL DISCUSSION

This thesis has presented the results of work investigating the expression and the role of the inositol 1,4,5-trisphosphate receptor in mammalian oocytes and early embryos. The research has resulted in several principal conclusions. Firstly, mature mouse oocytes exclusively express the type I isoform of the InsP_3R . Secondly, type I InsP_3R is degraded after fertilisation. Thirdly, InsP_3R degradation at fertilisation may be stimulated as a result of prolonged activation of the InsP_3R , and there are indications, although not conclusive proof, that the proteasome is the protease responsible for this degradation. Fourthly, the results provide direct evidence that the proteasome is responsible for the degradation of cyclin B at egg activation. Fifthly, the generation of sperm-induced Ca^{2+} oscillations does not appear to require the action of the proteasome. Finally, I have shown that GVBD and meiosis I as well as mitotic divisions to form the morula, can take place at low levels (≈ 10 -15% of control) of InsP_3Rs . These conclusions have been discussed in the Discussion sections of each experimental chapter. This chapter of the thesis will discuss the experiments which should be undertaken to follow up this work and investigate areas which have not been completely explained.

7.1 The mechanism of InsP_3R downregulation at fertilisation

I have described the downregulation of the expression of the type I InsP_3R which takes place at fertilisation. The results showed that proteasome inhibitors, with the exception of ALLN, were not effective in inhibiting this degradation of InsP_3Rs . However, I have provided substantial evidence from the literature to suggest that this degradation does involve ubiquitin-mediated proteolysis by the proteasome. Having examined the effect of proteasome inhibitors on degradation, there are other techniques which can be used to investigate the role of the proteasome. Firstly, preliminary work has attempted to detect ubiquitination of InsP_3Rs at fertilisation. Experiments were performed (in collaboration with Richard Wojcikiewicz) to try to immunoprecipitate InsP_3Rs from fertilised mouse oocytes using an anti-ubiquitin antibody. However, no signal was detected using protein from 500 mouse oocytes; the transient nature of ubiquitination means that many more oocytes will be required in future attempts. Secondly, another approach to investigate the role of the proteasome would be to microinject methylated ubiquitin protein into unfertilised mouse oocytes prior to fertilisation. Since methylated ubiquitin cannot form polyubiquitin links with other ubiquitin molecules, it can prevent the formation of ubiquitin chains and thus ubiquitin-mediated proteolysis.

Thirdly, InsP_3R proteolysis could be investigated using oocytes microinjected with Green Fluorescent Protein (GFP)-tagged InsP_3R mRNA. The expression of a functional GFP

fusion protein with the InsP₃R would firstly enable the distribution of the receptor in the ER, before and after fertilisation, to be confirmed. This may be a more reliable technique to use than the immunocytochemistry presented in this thesis. The latter involves fixing and staining with antibodies and is subject to non-specific staining of other proteins, whereas fluorescence arising from GFP proteins is specific for the tagged protein and also allows observation of changes in protein distribution in living cells. Secondly, single oocytes expressing a GFP-tagged InsP₃R could be used in an assay for InsP₃R downregulation. Western blotting requires the analysis of a minimum of 20 oocytes and the results represent the mean loss of protein across the whole sample, forcing the assumption that the loss of InsP₃Rs is synchronous across the sample. Using a GFP protein would make more efficient use of mouse oocytes and would allow the time-course of InsP₃R degradation in individual oocytes, to be followed. Thirdly, a GFP-tagged InsP₃R may be informative for examining the mechanism of receptor degradation. This is because if degradation is undertaken by the proteasome, then the complete fusion protein including the GFP will be broken down into short peptide fragments, thus eliminating GFP fluorescence. However, if the InsP₃R is cleaved by other proteases at specific sites, the GFP may remain intact. In this case, GFP fluorescence may be maintained after InsP₃R degradation but may no longer be localised to the ER. Furthermore, if experiments conclusively prove that degradation is carried out by the proteasome, then monitoring the GFP fluorescence during the process of downregulation may also reveal the precise location of proteasomal action. For example, ubiquitin-mediated proteolysis of the ER membrane-integrated cystic fibrosis transmembrane conductance regulator (CFTR) is physically localised to the ER (Xiong *et al.*, 1999). In other words, the cytosolic proteasomal machinery is recruited to the ER membrane by the polyubiquitinated protein and degradation occurs simultaneous to retrograde translocation and removal of the polypeptide from the ER membrane. The degradation of the multispinning transmembrane InsP₃R protein may also involve membrane-bound ubiquitination and recruitment of the proteasome to the ER membrane, rather than the formation of a cytosolic intermediate. This could be tested using the GFP-tagged InsP₃R.

An alternative use for GFP technology would be the expression of both functional GFP fusions of proteasomal subunits and nuclear envelope-ER marker proteins to follow the change in distribution of 26S proteasomes upon fertilisation. A correlation of proteasome and InsP₃R distribution would be consistent with the proteasome being responsible for degrading the InsP₃R.

7.2 The role of InsP₃R downregulation at fertilisation

This thesis has concentrated on the regulation of expression of the InsP₃R and its role in early mouse development. Clearly, the next step should be to investigate the role or functional consequence of type I InsP₃R downregulation after fertilisation. The literature

provides evidence to suggest that the level of InsP₃Rs has an effect on Ca²⁺ signaling and that downregulation may play a role in modulating Ca²⁺ release following fertilisation.

Fertilisation-induced Ca²⁺ transients continue for 3-4 hours. Two candidates for causing the oscillations to terminate are the transition into interphase of the cell cycle and the downregulation of Ca²⁺ release channels (InsP₃Rs). There is a discussion in the General Introduction of this thesis on the effect of cell cycle kinases and thus stage of the cell cycle, on Ca²⁺ signaling. The downregulation of InsP₃R expression at fertilisation (this study) may provide another form of regulation of Ca²⁺ signaling. I have shown that fertilisation stimulates a gradual decrease in InsP₃R expression levels which results in only 50% receptors remaining after 2 hours. Maximal downregulation to about 25-35% of control levels is achieved by 4 hours after fertilisation. However, at the imaging resolution I have used there is no notable change in the amplitude, rate of rise or frequency of oscillations during this time to reflect this loss of receptors. The level of InsP₃Rs is not reflected in a gradual suppression of Ca²⁺ oscillations and they end abruptly at around the time of pronucleus formation. I can therefore infer that at least 50% of receptors are in excess of requirements and that decreasing InsP₃R levels has little effect on the ability to generate Ca²⁺ transients. Nevertheless, the time at which maximal downregulation is reached coincides with the approximate time that the transients stop after fertilisation, which suggests the possibility of a causal relationship. However, it also correlates with the time of pronucleus formation and entry into interphase which has in fact been shown to be directly related to the cessation of Ca²⁺ transients (Jones *et al.*, 1995a), although more recent evidence has found that the cessation of spiking does not require the formation of a pronucleus (Day *et al.*, 2000). Furthermore, preincubation of MII oocytes in nocodazole for 4 hours stimulates downregulation of InsP₃R expression to 30-40% of control levels, yet has no effect on the pattern of Ca²⁺ oscillations upon subsequent fertilisation (this study), in support of receptor numbers being in excess of requirements. Therefore, it may be that downregulation of InsP₃Rs is a complementary mechanism to ensure that Ca²⁺ signaling ceases once the oocyte enters the first mitotic interphase. Full-blown transients may be achievable when the concentration of InsP₃Rs is above a threshold level. However, a concentration below this threshold level in concert with the embryo being in interphase, may not support transients. It seems reasonable, however, that transition into interphase is the primary factor causing sperm-induced transients to cease since the function of these transients is to induce entry into the first mitosis, hence they are redundant once interphase is reached.

The inability to specifically inhibit fertilisation-induced InsP₃R downregulation in oocytes has prevented me from testing whether there is a causal link between downregulation and the cessation of Ca²⁺ transients. The ability to inhibit downregulation may be useful in future work in order to elucidate the regulation of these transients. However, there is other indirect evidence for an effect of decreased InsP₃R levels on the ability to generate Ca²⁺

transients. Firstly, MII oocytes but not pronucleate stage embryos generate repetitive Ca^{2+} oscillations in response to InsP_3 microinjection (Jones *et al.*, 1995a). It has been shown using InsP_3 injection that after fertilisation (at the pronucleate stage) there is a decrease in both the sensitivity of the InsP_3 -induced Ca^{2+} release mechanism and in the peak Ca^{2+} released (Parrington *et al.*, 1998). This suggests that the InsP_3R itself, and not a decrease in agonist, plays a role in the cessation of Ca^{2+} oscillations after fertilisation. Secondly, the sperm itself is able to induce Ca^{2+} transients during MII but not during interphase after fertilisation (Jones *et al.*, 1995a). However, a decrease in the sensitivity to InsP_3 is also observed in parthenogenetic embryos after activation by Sr^{2+} , ethanol or cycloheximide (Jones and Whittingham, 1996). Since I have demonstrated that parthenogenetic activation, unlike fertilisation, does not stimulate a downregulation of InsP_3Rs , this tells me that this decline in sensitivity occurs independently of a decline in receptor numbers. This suggests that the dominant factor causing the decline in sensitivity is the transition from meiosis (M phase) to interphase. In support of M phase being more sensitive to InsP_3 , it is notable that during mitosis in mouse embryos there is an endogenous increase in Ca^{2+} releasing activity that leads to the generation of Ca^{2+} transients specifically during mitosis (Kono *et al.*, 1996).

The dominant role of the cell cycle in regulating Ca^{2+} release does not preclude a role for the InsP_3R levels in modulating the sensitivity at each stage of the cell cycle. This may be investigated using embryos at the same stage of the cell cycle with different numbers of InsP_3Rs . Preliminary work (for this thesis and other studies) has used this line of investigation to look at the effect of different InsP_3R levels on the sensitivity of Ca^{2+} release, and is discussed below.

Firstly, in the presence of colcemid or nocodazole, fertilisation-induced Ca^{2+} oscillations continue well past the 4-hour point (Jones *et al.*, 1995a; our unpublished results). In this situation the metaphase spindle is disrupted and oocytes remain arrested at MII due to the spindle checkpoint. It is thought that oscillations do not terminate at the normal time due to the oocyte remaining at metaphase of meiosis rather than entering interphase. However, these oscillations become much less frequent as time goes on. Nocodazole has no effect on the ability of sperm to induce downregulation (our unpublished observations) and I have shown that nocodazole itself stimulates downregulation of the InsP_3Rs to 30-40% within 4 hours. Thus, one explanation for the slowing-down of oscillations is that it is due to the parallel decline in receptor numbers. However, the fact that these Ca^{2+} oscillations can continue in the presence of a downregulated level of InsP_3Rs supports the idea that the stage of the cell cycle (MII arrest) is more important in determining the sensitivity of the Ca^{2+} release system.

Secondly, a recent study on mouse eggs (Tang *et al.*, 2000) has suggested a general rule that to support Ca^{2+} oscillations stimulated by bovine sperm extract (BSE), the egg or

embryo must be in M-phase but must also have a high level of InsP_3Rs . They compared BSE-induced Ca^{2+} responses in fertilised embryos and parthenogenetic (PG) embryos following activation by ethanol. When the sperm extracts were injected into interphase (G1- and S-phase) 1-cell embryos, neither fertilised nor parthenogenetically-activated embryos showed Ca^{2+} oscillations, suggesting that interphase is insensitive to the BSE stimulation in triggering oscillations. However, the majority of interphase PG embryos could generate a single Ca^{2+} transient, whereas only 7% of interphase zygotes could generate one Ca^{2+} rise with the others not showing any detectable Ca^{2+} increase at all. This suggests that although the effect of interphase dominates, the PG embryos with their full complement of InsP_3Rs (this study) have a more sensitive Ca^{2+} release system than interphase fertilised embryos. In addition, at metaphase of the first mitosis, the majority of PG embryos showed oscillations in response to sperm extract injection while the injected zygotes either showed a single Ca^{2+} rise (<20% of zygotes) or no Ca^{2+} rise at all, again indicating that the level of InsP_3Rs contributes to determining the sensitivity of Ca^{2+} release.

The experimental evidence therefore suggests that the sensitivity of Ca^{2+} release through InsP_3Rs is determined mainly by the stage of the cell cycle but that it is modulated by InsP_3R levels. A change in the stage of the cell cycle could affect the sensitivity of the Ca^{2+} release mechanism in a number of ways. Cell cycle-dependent enzymes could, for example, influence the state of the internal Ca^{2+} stores. One explanation for the termination of oscillations and subsequent decrease in the sensitivity of Ca^{2+} release is a decrease in the size of these internal stores. However, there is in fact a fourfold increase in internally stored Ca^{2+} from GV to fertilised pronucleate stage, including an approximately 25% increase from MII to Pn stage (Tombes *et al.*, 1992). This is therefore not reflective of the decrease in requirement for Ca^{2+} increases from MII to pronucleus formation and entry into interphase. Thus Ca^{2+} store size cannot be the cause of the decrease in the sensitivity of Ca^{2+} release.

Another factor which may influence the sensitivity of Ca^{2+} release is age of the oocyte. As described above, the sensitivity of Ca^{2+} release decreases after fertilisation or parthenogenetic activation and subsequent entry into the first mitotic interphase (Jones *et al.*, 1995a; Jones and Whittingham, 1996). Furthermore, freshly ovulated oocytes are more sensitive to InsP_3 injection than oocytes age-matched for those examined after fertilisation (Jones and Whittingham, 1996; Parrington *et al.*, 1998). This suggests that ageing itself has effects on the sensitivity of Ca^{2+} release mechanisms, which I have shown is independent of any dramatic changes in InsP_3R immunoreactivity. However, the effect of ageing on the sensitivity to InsP_3 is less than that of fertilisation. Age-matched unfertilised oocytes are more sensitive to InsP_3 than Pn stage fertilised embryos, with decreases in both the sensitivity of the Ca^{2+} release mechanism and the peak Ca^{2+} released, after fertilisation (Parrington *et al.*, 1998).

Thus, the decrease in InsP₃Rs at fertilisation may be a physiological adaptation contributing to the cessation of fertilisation-induced Ca²⁺ transients. Alternatively, it may simply reflect the inevitable removal of a functional reserve of receptors, the high level of receptors having performed their role at egg activation. In many somatic cultured cell lines, persistent activation of certain PLC-linked cell surface receptors is known to increase proteolysis of InsP₃Rs and thus lead to InsP₃R downregulation (Bokkala and Joseph, 1997; Wojcikiewicz, 1995; Wojcikiewicz *et al.*, 1994). This is considered an adaptive response which reduces the sensitivity of cellular Ca²⁺ stores to InsP₃ (Bokkala and Joseph, 1997). It is therefore conceivable that the persistent activation of the InsP₃Rs in the oocyte by persistent sperm-generated InsP₃ over the 3-4 hours following fertilisation may lead to an active downregulation of InsP₃R numbers as an adaptive mechanism. This would inhibit Ca²⁺ release and conserve energy since all Ca²⁺ that is released from the internal stores must be pumped back into the stores by the ATP-dependent Ca²⁺ pumps. It would also explain the gradual loss of InsP₃Rs which does not reflect the pattern of Ca²⁺ oscillations. Therefore, although the action of the sperm, that is activation of the egg, depends on the InsP₃R, it may also stimulate its degradation directly as a way of preserving energy once these levels of receptor are no longer required. However, as I have proposed, this stimulation of degradation is just as likely to be a functional adaptation designed to modulate Ca²⁺ oscillations, superimposed upon the dominant effect of the stage of the cell cycle.

Other than modulating the cessation of sperm-induced Ca²⁺ transients, InsP₃R downregulation may be important for regulating the sensitivity of the Ca²⁺ release system at other stages of embryonic development. I have shown that parthenogenetic embryos appear to develop normally to the blastocyst stage despite having a full complement of InsP₃Rs, but further development of parthenogenetic embryos is limited. To explore the role of InsP₃R downregulation in a fertilised embryo, future work should investigate ways of inhibiting sperm-induced downregulation and monitoring the ability of zygotes to progress through mitotic divisions to form blastocysts and undergo postimplantation development. For example, if degradation is proven to be carried out by the proteasome, expression of an exogenous mutant InsP₃R unable to bind ubiquitin could be used to prevent InsP₃R downregulation in fertilised eggs.

7.3 The role of the InsP₃R in mitosis

The results of my experiments investigating the role of the InsP₃R in mitotic cell cycles clearly showed that ≤10-15% of receptors (compared to MII) are necessary for the formation of the morula (Chapter 6). As discussed in Chapter 6, there is substantial data in the literature to indicate that Ca²⁺ transients are essential regulators of mitotic cell cycles (Kono *et al.*, 1996; Steinhardt and Alderton, 1988; Tombes *et al.*, 1992; Wilding *et al.*,

1996). In view of this, I have proposed that the 10-15% of InsP_3Rs which remain after adenophostin stimulation, are sufficient to allow the generation of small, localised Ca^{2+} transients which drive the cell cycle. Preliminary work has involved imaging changes in cytosolic Ca^{2+} concentration at first mitosis, after depletion of InsP_3Rs using adenophostin. Whereas control fertilised embryos exhibited a detectable Ca^{2+} transient just prior to NEBD, no Ca^{2+} changes were detected in fertilised embryos depleted of InsP_3Rs , despite the timing of NEBD and cleavage to the 2-cell stage being similar in the two groups. The local transients that I propose are therefore not detected when using whole-cell Ca^{2+} measurement techniques. Confocal microscopy may detect and localise such putative transients. In addition, experiments using Ca^{2+} chelators may show that NEBD of first mitosis is blocked at a lower concentration of chelator in embryos with depleted numbers of InsP_3Rs , than in controls. This would be consistent with there being smaller cytosolic Ca^{2+} changes in embryos with depleted InsP_3Rs , so that these changes are suppressed at low chelator concentrations. On the other hand, larger Ca^{2+} changes in control oocytes may not be completely buffered by the same concentration of chelator, leaving a sufficient Ca^{2+} change to drive NEBD. In parallel to these experiments, tracking the localisation of the ER around the time of the NEBD transient may reveal that the ER redistributes to concentrate around the nucleus at this time. This would provide one explanation for how a low level of InsP_3Rs in the zygote may be at a sufficiently high concentration to create a Ca^{2+} gradient and result in a localised transient.

The low proportion of morulae developing to the blastocyst stage under my culture conditions prevented any decisive conclusions being made about the role of the InsP_3R in blastocyst formation, that is formation of the blastocoele, inner cell mass and trophectoderm. It is highly likely that InsP_3 -induced Ca^{2+} release plays a role in the regulation of blastocyst development for the reasons given in Chapter 6. For example, mouse morulae exposed to Ca^{2+} chelators show delayed blastocoele formation (Stachecki and Armant, 1996b), Ca^{2+} release in mouse morulae occurs predominantly through the InsP_3R (Stachecki and Armant, 1996b), and exposure to PLC inhibitors inhibits cavitation, suggesting that PLC-mediated signaling is critical for blastocoele formation (Stachecki and Armant, 1996a). Therefore, further attempts should be made to culture healthy blastocysts and compare the rate of development of those in which InsP_3Rs have been depleted using adenophostin, with that of controls. Indeed, preliminary work of this nature has achieved a higher rate of blastocyst development and has used Hoechst staining to count the number of cells making up the blastocyst in blastocysts cultured from zygotes with depleted InsP_3Rs . There appears to be no significant difference in cell number between these blastocysts and controls (unpublished work). Successful culture of 1-cell embryos to blastocysts may be improved further by transplanting the microinjected 1-cell embryos back into the uterus of the female mouse.

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