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The cortical reaction and development of activation competence in mammalian oocytes

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Blocks to polyspermic fertilization are necessary to prevent the incorporation of two sperm nuclei into a zygote's genome, which would result in abnormal development. Many mammalian eggs utilize both an extracellular zona pellucida block to polyspermy and a plasma membrane block. Although little is known about the plasma membrane block in mammals, fertilization results in zona glycoprotein modifications caused by enzymes released by the egg and its cortical granules (CG). This article reviews other recent investigations demonstrating that the oocyte's ability to cause CG release and the block to polyspermy develops near the time of ovulation. The development of normal 'activation competence' is likely to involve preovulatory changes in the oocyte's ability to signal the release of intracellular calcium as well as to respond to this calcium increase, resulting in CG exocytosis. Because normal activation competence appears to have a brief temporal window after oocyte meiotic maturation is resumed and since the oocytes are collected at various stages in assisted reproductive procedures, these studies are relevant to optimizing clinical success.

Key words: activation competence/calcium/cortical granules/in-vitro fertilization/oocyte maturation

Cortical granules

The blocks to polyspermic fertilization (Jaffe and Gould, 1985) are necessary to prevent the incorporation of two sperm nuclei into the zygote's genome that would result in abnormal development. In most mammals, dispermic fertilization is usually prevented by a combination of the fertilized egg's blocks to polyspermy and low sperm number in the Fallopian tube. However, >1% of natural human conceptions (Boue *et al.*, 1975; Golbus, 1981) and ~10% of those from in-vitro fertilization (IVF) (Hammitt *et al.*, 1993) result in triploid embryonic abortions, the majority from dispermic fertilization (Beatty, 1978; Jacobs *et al.*, 1978; Kola and Trounson, 1989). Currently, reproductive technologies (e.g. intrauterine insemination, gamete intra-Fallopian transfer, and IVF) are used to treat infertility, produce needed domestic animals, or help save endangered species. However, much higher sperm concentrations are used than occur in the oviduct during natural conception (in the case of IVF, 100–1000 times higher) and the rate of polyspermy is increased. At the other extreme, only a single spermatozoon is used in intracytoplasmic sperm injection (ICSI) and the normal process of initial spermatozoon–egg interaction appears to be bypassed.

Many mammalian eggs utilize an extracellular zona pellucida (ZP) block to polyspermy and a plasma membrane block, the relative contributions of which vary in different species (Yanagimachi, 1994). In biochemical terms, little is known about the plasma membrane block in mammals (Wolf, 1981) and a possible role for cortical granules (CG) remains unresolved (Yanagimachi, 1994). The membrane depolarization block to polyspermy found in other animal eggs has not been detected in studies of mammalian eggs (Jaffe and Gould, 1985).

Within 10 min of fertilization (Lee *et al.,* 1988; Stewart-Savage and Bavister, 1991; Kline and Stewart-Savage,

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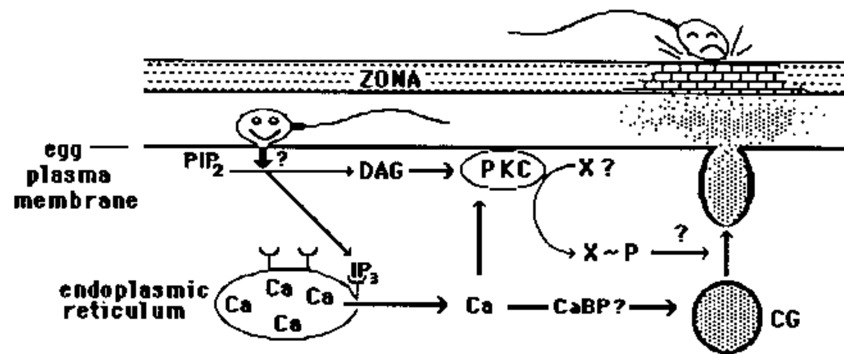

Figure 1. Working model for the mechanism of the cortical reaction in mammalian eggs. The release of egg intracellular Ca continues to be the most important known messenger involved in cortical granule (CG) release, although its downstream targets that ultimately cause exocytosis remain to be firmly established. As described in the text, this model has been compiled from experimental data from many laboratories. See text for abbreviations. PIP_2 = inositol phosphate; DAG = diacylglycerol; PKC = protein kinase C; X = hypothetical protein; IP_3 = inositol 1,4,5-trisphosphate; CaBP = calcium-binding proteins

1994), the cortical reaction releases CG enzymes that change the ZP, resulting in the important extracellular ZP block to polyspermy found in many animals (Wolf, 1981) and humans (Kola and Trounson, 1989). Fertilization-associated biochemical modifications of one or more ZP glycoproteins (designated ZP1, ZP2 and ZP3) have been reviewed extensively in animals (Wassarman, 1990; Bleil, 1991; Dunbar *et al.*, 1991; Liang and Dean, 1993), but much less is known in humans (Shabanowitz and O'Rand, 1988; Ducibella *et al.*, 1995; Moos *et al.*, 1995).

In sea urchin, amphibian and mammalian eggs, CG are cortically localized, 0.1–1 µm diameter, membrane-bound, secretory granules whose contents include enzymes that modify and remove sperm receptors from the extracellular investments of eggs upon fertilization (reviewed by Schuel, 1985; Cran and Esper, 1990; Ducibella, 1991; Hoodbhoy and Talbot, 1994). In the mouse, the fertilization-associated release of CG enzymes changes the ZP: the ZP2 polypeptide is cleaved to $ZP2_f$ (although still held together by intrachain disulphide bonds); ZP3, while apparently not cleaved, undergoes a carbohydrate modification resulting in a loss of sperm-binding activity and an inability to induce the acrosome reaction (Wassarman, 1990; Miller *et al.*, 1993). Studies of factors released by activated eggs suggest that CG contain a trypsin-like enzyme activity (Gwatkin *et al.*, 1973; Wolf and Hamada, 1977), ovoperoxidase activity (Gulyas and Schmell, 1980), a 21–34 × 10^{-3} M_r proteinase that cleaves ZP2 (Moller and Wassarman, 1989), a 75 × 10^{-3} M_r protein of unknown function (Pierce *et al.*, 1990, 1992) and an *N*-acetylglycosaminidase activity that may be responsible for modification of ZP3 (Miller *et al.*, 1993). The 75 × 10^{-3} M_r protein and glycosaminidase have been localized to intact CG. These important studies suggest that mammalian CG contain several proteins with different activities. However, future research remains to establish a complete biochemical inventory of CG contents and how they individually and collectively participate at a molecular level in the blocks to polyspermy.

Mechanism of the cortical reaction

In sea urchin and amphibian eggs (reviewed by Turner and Jaffe, 1989; Nuccitelli, 1991; Bement, 1992), the biochemical pathways involved in activating CG release have been studied and mammalian eggs appear to be similar although not identical to those of marine eggs (reviewed in Miyazaki *et al.*, 1993). Figure 1 should be considered a working model for mammalian CG release. Research on egg activation pathways is proceeding rapidly and, at the time of writing, there are varying amounts of evidence for different parts of this mechanism. Egg activation may be initiated by a sperm receptor-coupled G-protein pathway (Kline *et al.*, 1988; Miyazaki *et al.*, 1990; Williams *et al.*, 1992; Moore *et al.*, 1993), or by spermatozoon–egg fusion followed by the passage of a sperm factor into the egg cytoplasm (Dale *et al.*, 1985; Stice and Robl, 1990; Swann, 1990, 1994), or a combination of several mechanisms. Downstream of these fascinating unresolved early events (Whitaker and Swann, 1993), the elevation of second messengers in the inositol phosphate (PIP_2) cascade leads to the release of intracellular calcium (Clapham, 1995) that is necessary for CG exocytosis.

Inositol 1,4,5-trisphosphate

In sea urchin eggs, fertilization results in a stimulation of PIP_2 turnover and the production of second messengers—inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Ciapa *et al.*, 1992). PIP_2 appears to play a role in the amount of Ca released in activated amphibian eggs

(Larabell and Nuccitelli, 1992). In mammalian eggs, elevated IP_3 causes the release of egg intracellular calcium (Ca) (reviewed in Miyazaki *et al.*, 1993), which is required for CG release. Microinjection of IP_3 causes Ca transients (Miyazaki *et al.*, 1990; Kline and Kline, 1994), CG secretion (Cran *et al.*, 1988) and the ZP2 modification (Kurasawa *et al.*, 1989; Ducibella *et al.*, 1993). Also, an antibody to the IP_3 receptor inhibits sperm-induced Ca transients (Miyazaki *et al.*, 1992) and ZP modifications as well as later events in egg activation (Xu *et al.*, 1994). To the best of our current knowledge, at fertilization, Ca release appears to be mediated principally through IP_3 in mammalian eggs (Miyazaki *et al.*, 1993). Although functional ryanodine receptors (Ayabe *et al.*, 1995) and ryanodine-sensitive Ca stores (Swann, 1992) are present in mouse eggs, ryanodine receptor-mediated Ca release is not required for mouse egg activation (Kline and Kline, 1994; Ayabe *et al.*, 1995).

Calcium

Many studies with marine eggs and isolated cortical preparations from sea urchin eggs (reviewed by Trimmer and Vacquier, 1986) demonstrate that Ca is the most important known positive messenger for CG release. The increase in intracellular free Ca during mammalian fertilization appears to be driven principally by an IP_3-mediated pathway rather than a cADP-ribose, ryanodine receptor mechanism (Miyazaki *et al.*, 1993; Kline and Kline, 1994). Blocking Ca elevation with injected Ca buffers prevents CG release in mouse eggs (Kline and Kline, 1992a). In hamsters (Steinhardt *et al.*, 1974) and mice (Ducibella *et al.*, 1990a,b), the egg's endogenous Ca stores are sufficient to stimulate CG release.

Diacylglycerol

DAG is generated early during sea urchin fertilization (Ciapa and Whitaker, 1986). In amphibian eggs, protein kinase C (PKC) agonists cause egg activation events (Bement and Capco, 1989) and PKC appears to act downstream of Ca (Bement and Capco, 1990). Mouse eggs treated with exogenous phorbol esters or DAG (both protein kinase C agonists) undergo CG release (Ducibella *et al.*, 1993), ZP2 modification (Endo *et al.*, 1987a,b) and other ZP changes (Colonna *et al.*, 1989), all of which are observed after fertilization. Both Ca and DAG may be involved in activating normal CG release because phorbol esters alone do not cause a complete zona reaction (Endo *et al.*, 1987b).

Protein kinase C

Fertilized sea urchin eggs activate phospholipid-dependent PKC (see above), and PKC inhibition down-regulates fertilization responses (Shen and Buck, 1990). In mammalian eggs, Ca or DAG stimulates egg activation and PKC inhibitors down-regulate activation events (Gallicano *et al.*, 1993). In hamster eggs, PKC and its catalytic subunit are involved in cytoplasmic reorganization in activated eggs (Gallicano *et al.*, 1995). In other secretory cells, PKC is involved in granule release (Trifaro *et al.*, 1992; Burgoyne and Morgan, 1993) and may act by phosphorylating a hypothetical protein (X in Figure 1), stimulating exocytosis. There remain several unanswered questions. It is not known whether PKC phosphorylates another kinase, a messenger or a structural protein in the cytoskeleton or secretory granule membrane that is involved in CG release. Equally importantly, it has not been demonstrated in mammalian eggs that fertilization causes PKC-induced CG release. The effects of PKC inhibition on CG release in mammalian eggs undergoing fertilization have not been reported. The critical question is what signalling pathways does the spermatozoon activate at fertilization.

Calcium-binding proteins

At present, there are a large number of potential calcium-binding proteins (CaBP) (other than PKC) that may be involved in CG exocytosis. These include regulatory molecules, like calmodulin, and structural proteins involved in modulating the cytoskeleton. Like other secretory cells, the mammalian egg cortex contains a layer of filamentous actin (Longo and Chen, 1985) in which mammalian CG are likely to be embedded. The final translocation of CG to the plasma membrane at fertilization may require a modification, shortening, or depolymerization of filaments. In other cell types, these changes are often mediated by a combination of actin-binding proteins and second messengers. At least one of these proteins, gelsolin, acts as an actin-severing protein at ~1 μM Ca (Yin *et al.*, 1990), which is similar to the cytosolic Ca concentration at fertilization. However, gelsolin severing activity is also down-regulated by polyphosphoinositides (Janmey and Stossel, 1987), which makes their analysis at fertilization interesting and challenging.

A role for calmodulin, a Ca-dependent signalling protein, in promoting CG exocytosis is likely from studies of eggs and secretory cells. In sea urchin eggs, antibodies to calmodulin (Steinhardt and Alderton, 1982) and the anti-calmodulin antagonist trifluoroperazine (Baker and Whitaker, 1979), inhibited CG fusion with the plasma membrane in in-vitro studies. In other cells, granule secretion is sensitive to calmodulin inhibitors and antibodies, and calmodulin binds to secretory granule membranes (reviewed in Trifaro *et al.*, 1992).

Development of competence to undergo CG release

In many animals, when immature eggs are fertilized, they fail to undergo CG release and increased sperm penetration of the egg's extracellular coat and/or polyspermy are observed (sea urchins: Longo, 1978; amphibians: Elinson, 1986, mammals: Cherr and Ducibella, 1990). The most outstanding example is in the pig: when human chorionic gonadotrophin was given 2 days early in the oestrous cycle, 92% of the oocytes were polyspermic from in-vivo fertilization of meiotically immature oocytes (Hunter *et al.*, 1976). Both mouse (Ducibella and Buetow, 1994) and human (Van Blerkom *et al.*, 1994) germinal vesicle (GV)-stage oocytes undergo fertilization *in vitro*, but CG release is, respectively, undetectable or severely reduced. Because CG enzymes are responsible for an extracellular block to polyspermy, increased sperm penetration of immature eggs is likely to be due to a failure of normal CG release. The inability of mouse pre-ovulatory oocytes to undergo CG release and establish the ZP block to polyspermy is of increasing interest clinically, because human and domestic animal IVF procedures collect oocytes directly from their follicles prior to ovulation.

The incompetence of fertilized immature oocytes to undergo a normal ZP block could, in theory, be due to an insufficient number of CG, their distance from the plasma membrane, the structure of the oocyte cortex, or an incompetent signalling pathway for CG exocytosis. Before meiotic maturation, CG are absent from the cortex in marsupial (Mate *et al.*, 1992) and pig (Cran and Cheng, 1985) oocytes and during maturation undergo substantial migration into the cortex. In contrast, human CG are present in the cortex of GV and metaphase I stage oocytes, and during in-vitro culture their number may increase (Suzuki *et al.*, 1981; Sathananthan and Trounson, 1982), although this has not been quantified. In quantitative studies, mouse GV-stage oocytes from antral pre-ovulatory follicles have no deficiency in the number or spatial location of CG compared to ovulated metaphase II eggs (Ducibella *et al.*, 1988a,b). The latter studies indicate that CG number and CG distance from the plasma membrane cannot account for the incompetence of full-grown GV-stage mouse oocytes to undergo CG exocytosis.

Because of the central importance of the explosive release of intracellular Ca to CG exocytosis and egg activation in general at fertilization (Jaffe, 1985), maturation-associated regulation of Ca release from cytoplasmic stores is likely to be involved in the development of competence to undergo CG release. Recent evidence indicates that the Ca storehouse is the egg's endoplasmic reticulum (ER). Studies in sea urchin and amphibian oocytes demonstrate that the ER is a continuous network in the cortex, closely associated with CG, and can store as well as release intracellular Ca (see Nuccitelli *et al.*, 1989; Terasaki and Sardet, 1991; Jaffe and Terasaki, 1994; and references therein). Direct and indirect localization studies of the distribution of Ca binding and regulatory proteins are consistent with their localization in the ER. These include the IP_3 receptor, a ryanodine receptor/Ca release channel, calsequestrin and calreticulin (Henson *et al.*, 1989, 1990; McPherson *et al.*, 1992; Kume *et al.*, 1993; Parys *et al.*, 1994; Ayabe *et al.*, 1995; Shiraishi *et al.*, 1995). IP_3 induces Ca release principally from the ER in studies of oocyte cytoplasmic fractions (Han and Nuccitelli, 1990). During meiotic maturation in amphibian (Campanella *et al.*, 1984; Charbonneau and Grey, 1984) and mammalian (Ducibella *et al.*, 1988b; Mehlmann, *et al.*, 1995; Shiraishi *et al.*, 1995) oocytes, the cortical ER appears to increase in quantity or in proximity to CG. Many Ca regulatory proteins (above) also relocate with the ER to the oocyte cortex during maturation. The ER and IP_3 receptor relocation during maturation would provide the optimal arrangement for CG release: the stimulus (spermatozoa), the response machinery (e.g. the ER), and effectors for the zona block (CG) are spatially colocalized. It is noteworthy that relatively little cortical ER is observed in some marine eggs that do not undergo CG release (Luttmer and Longo, 1985). In secretory cells using intracellular Ca as a second messenger, the importance of the spatial organization of stored Ca (and IP_3 receptors because they may colocalize) has been emphasized (Cheek, 1989).

The development of competence to undergo CG release (as well as egg activation) is likely to depend on not only the position of Ca stores and Ca regulatory proteins, but also their concentration and functional status. In mature mammalian eggs, intracellular Ca release is primarily controlled by a mechanism involving IP_3-induced release (Miyazaki *et al.*, 1993; Kline and Kline, 1994; Ayabe *et al.*, 1995; Miyazaki, 1995) as described previously. In theory, this release mechanism could be developmentally gated at many points in the egg's signalling pathway, e.g. the production/elevation of IP_3, the IP_3-responsive Ca-release mechanism and downstream Ca-dependent targets. These categories could be further subdivided. For example, the Ca-release mechanism can be affected by the concentration of cytosolic and luminal (ER) Ca, and the status of the IP_3 receptor (Berridge, 1993; Marshall and Taylor, 1993). Receptor activity, in turn, can be modulated by its phosphorylation state (Supattapone *et al.*, 1988), isoform (Furuichi *et al.*, 1989; Sudhof *et al.*, 1991) and number. A tetramer of four receptors is thought to represent the IP_3-sensitive ER release channel (Wagenknecht *et al.*, 1989; Maeda *et al.*, 1990). Isolation of the IP_3 receptor and modulation of its activity by cytosolic Ca have been reported in amphibian eggs (Parys *et al.*, 1992), whose large

size is advantageous. Investigating maturation-associated changes in this elaborate IP_3 receptor-ER ‘control centre’ for Ca release will serve as an interesting, if initially daunting, challenge. The groundwork has been laid by studies demonstrating that oocytes acquire the ability to undergo full Ca release at the end of maturation (Chiba *et al.*, 1990; Fujiwara *et al.*, 1993; Mehlmann and Kline, 1994).

Mouse oocyte maturation studies indicate that the signalling pathways involved in CG release are not fully developed until metaphase II. Unlike metaphase II eggs, GV-stage oocytes do not undergo CG release in response to Ca ionophore in the absence of extracellular Ca (Ducibella *et al.*, 1990a,c). In this situation, the oocyte must rely on its own intracellular Ca stores that appear to be responsible for the first Ca transient at fertilization. Physiological agonists (spermatozoa or IP_3) do not cause CG release or the ZP block in GV-stage oocytes (Ducibella *et al.*, 1993; Ducibella and Buetow, 1994). In contrast, PKC agonists cause a similar extent of CG release in GV and metaphase II stages, indicating that the final step(s) in exocytosis–CG fusion with the plasma membrane-is not deficient in oocytes before metaphase II (Ducibella *et al.*, 1993). One interpretation of these studies is that there may be a deficiency in the normal process of Ca release that is supported by other investigations. After IP_3 injection or monospermic fertilization, GV-stage oocytes of starfish (Chiba *et al.*, 1990), mice (Mehlmann and Kline, 1994) and hamsters (Fujiwara *et al.*, 1993) do not elevate Ca to the same extent as their mature egg counterparts. Maturation-associated changes in the functional status of the IP_3 receptor have been proposed to account for the development of the ability to elevate Ca to normal intracellular concentrations by metaphase II (Chiba *et al.*, 1990). IP_3-induced regenerative, propagating Ca release, which is important in spreading a wave of Ca release in metaphase II eggs, is not as well developed in GV-stage oocytes (Fujiwara *et al.*, 1993; Mehlmann and Kline, 1994). Also, it has been reported that between the GV and metaphase II stages, there may be a 3- to 4-fold increase in the amount of ionomycin-inducible Ca release (Tombes *et al.*, 1992).

Thus, one working hypothesis is that GV-stage oocytes are deficient in an early Ca elevation step in the CG exocytosis pathway, but not in the proposed late PKC-dependent step (see Figure 1). However, it is not known if all Ca-dependent effectors involved in CG release are present and fully functional. Another (not necessarily competing) hypothesis is that these effectors develop during maturation, perhaps to minimize spontaneous CG release during the long life of oocytes in their follicles, just as capacitation potentiates the spermatozoon’s ability to undergo the acrosome reaction. These studies will require the identification of CaBP involved in CG exocytosis.

During normal meiotic maturation, mouse oocytes acquire the ability to undergo CG exocytosis in three phases (Figure 2A and B): incompetence (GV stage), localized CG release (from GV to metaphase I), and normal global release (metaphase II). Detailed quantitative studies indicate that the percentage of CG loss, location of release, time course of CG release, and sensitivity to thimerosal were all altered in oocytes fertilized before metaphase II (Ducibella and Beutow, 1994). Although thimerosal is thought to increase the sensitivity of the IP_3 receptor, it failed to augment CG release in unfertilized and fertilized metaphase I oocytes at a concentration that augmented CG release in both of these groups of metaphase II eggs. In contrast to the ability to undergo CG release, another important indicator of egg activation, the extent of initial sperm chromatin decondensation, was not affected (Ducibella and Beutow, 1994).

Because some stages of mouse and human fertilized pre-metaphase II oocytes undergo localized CG release in the immediate vicinity of the fertilizing spermatozoon, it appears that a ‘primary’ stimulus for exocytosis is activated in those cases and the oocyte cortex is competent to initiate the process of CG exocytosis. This localized stimulus is likely to be derived from a sperm receptor- or sperm factor-mediated onset of signalling of egg activation (reviewed by Whitaker and Swann, 1993), and possibly from a spermatozoon-mediated sensitization of Ca release (Swann, 1994). However, the spread of stimulation of exocytosis, resulting in global release, does not appear to take place. In sea urchins, CG release is initiated from the point of fertilization, spreads radially (Vacquier and Payne, 1973), and appears to follow the wave of elevated intracellular Ca. Because the Ca wave (reviewed in Nuccitelli, 1991; Miyazaki *et al.*, 1993) and CG release (Ducibella and Buetow, 1994) originate from the locus of fertilization, a wave of Ca-dependent CG exocytosis is also likely to take place in fertilized metaphase II mammalian eggs. Because the first Ca transient in fertilized metaphase I mouse oocytes is similar to that in metaphase II eggs (Mehlmann and Kline, 1994) and localized Ca release has not yet been observed in fertilized hamster oocytes (Fujiwara *et al.*, 1993), it is possible that localized CG exocytosis in metaphase I mouse oocytes is not due to localized Ca release.

Localized CG release can also occur in maturing oocytes in the absence of spermatozoa. During normal meiotic maturation, localized CG release occurs over the metaphase spindle in mouse (Ducibella *et al.*, 1990c; Okada *et al.*, 1993) and hamster oocytes (Okada *et al.*, 1986; Cherr *et al.*, 1988), but has not been detected in oocytes of the pig (Yoshida *et al.*, 1993) and cat (Byers *et al.*, 1992). Is the spindle-associated localized release in rodent oocytes due to a metaphase-associated local release of intracellular Ca?

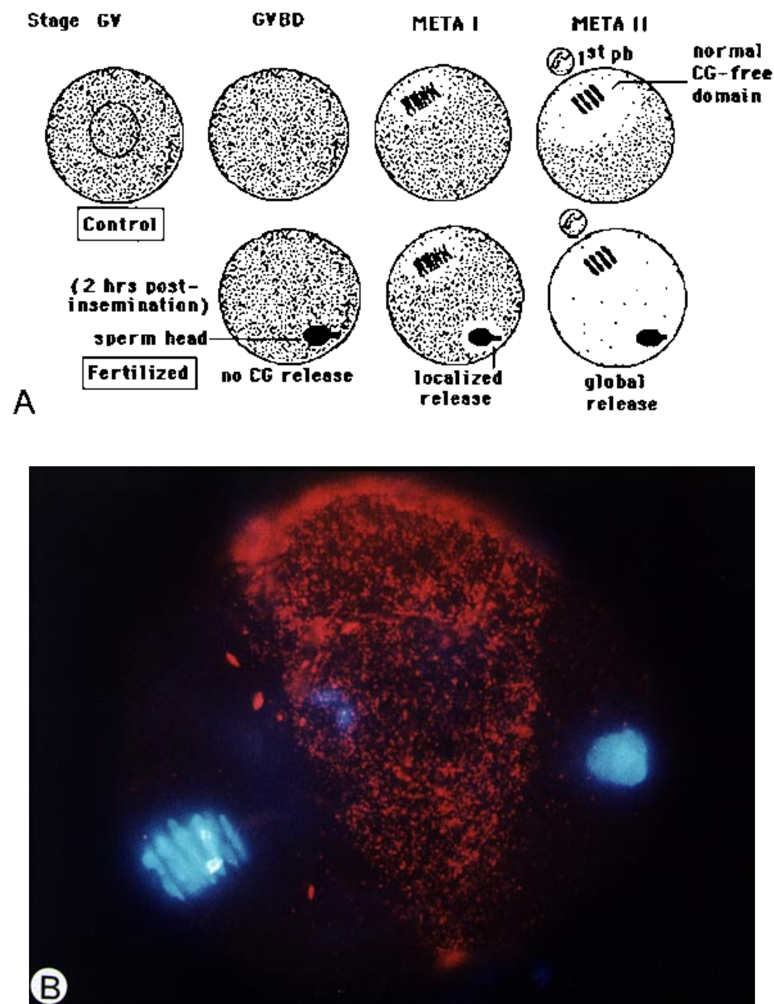

Figure 2. (**A**) The extent of cortical granule (CG) release as a function of oocyte meiotic stage in the mouse is shown. During normal meiotic maturation a CG-free domain appears over the metaphase chromosomes (Control). In oocytes fertilized at the indicated stages of maturation, there is a progression from no quantifiable net CG loss from the cortex at the germinal vesicle stage, to localized release at metaphase (meta) I, and finally to global release at meta II. GVBD = germinal vesicle breakdown; pb = polar body. (**B**) Fluorescence micrograph of localized CG release in a maturing mouse oocyte fertilized at meta I, as depicted in **A** (chromatin is blue, CG are red). Maternal chromosomes are shown on the left and decondensing sperm chromatin on the right, 2 h post-insemination of zona-free oocytes. Note that the CG have been lost over the sperm chromatin, whereas the remaining CG-occupied cortex is still densely populated. The area over the maternal chromosomes has the normal CG-free domain that develops between meta I and II in mouse and hamster eggs. Quantitative studies have demonstrated that neither the sperm- or maternal chromosome-associated CG-free domain can be accounted for by CG redistribution alone (Ducibella *et al.*, 1990c; Ducibella and Beutow, 1994). Multiple exposures were taken at different focal planes. CG staining was achieved with *Lens culinaris* agglutinin–avidin/Texas Red, and chromatin staining with Hoechst 33258/DAPI.

Meiotic maturation requires extracellular Ca, but elevated intracellular Ca was not observed during meiosis I in mouse oocytes (Tombes *et al.*, 1992). Perhaps, there are localized increases in protein kinase activity at the sites of fertilization and the metaphase I spindle that are responsible for localized CG release in some maturing oocytes. Further studies are needed to identify the regulatory and structural molecules involved in CG exocytosis in order to determine whether they are involved in localized CG release as well as normal fertilization-associated exocytosis. Thus, the basis for localized release may remain a mystery until early events at the site of fertilization and maturation-associated changes in the ability to undergo global CG exocytosis are discovered.

Cytoplasmic maturation and human IVF

With the increasing use of human and domestic animal ovulation induction, IVF, and other assisted reproductive technologies, the normal mechanisms that control egg maturation and timing of fertilization are being altered and manipulated externally. For example, in human IVF pro-

grammes ~40% of the egg masses collected contain pre-metaphase II-stage oocytes (Flood *et al.*, 1990; Hammitt *et al.*, 1993). Because of difficulties in staging oocytes within the dense cumulus mass, many clinical groups are likely to be inseminating some oocytes that have not reached metaphase II (Hammitt *et al.*, 1992). If cytoplasmic maturation is not completed before fertilization, egg activation by spermatozoa is not likely to occur normally. In normally maturing human oocytes, it is important to establish when activation competence develops and how long it is maintained. This information will dictate the temporal window in which fertilization (and hence, assisted reproductive procedures) should take place.

In human IVF, many immature oocytes are collected and, even after culture, polyspermy rates are significant (~10%; Hammitt *et al.*, 1993). One prediction from animal studies is that fertilized immature human oocytes will have a higher rate of zona penetration and/or polyspermy. Although polyspermy due to immaturity has been reported in human oocytes (Trounson *et al.*, 1982; Veeck, 1994), carefully controlled studies are not available and are made difficult by the problem of staging oocytes within their dense cumulus. Although meiotically incompetent or delayed GV-stage human oocytes do not become polyspermic, they appear to undergo higher sperm penetration of the zona consistent with their failure to undergo complete CG loss (Van Blerkolm *et al.*, 1994). Delayed fertilization in many mammals also can result in a higher incidence of polyspermy (Austin, 1974). The relative contributions of different causes of polyspermy or multiple sperm penetration of the zona in human IVF have not been rigorously established.

Because precise in-vitro maturation conditions are important (Eppig, 1991) and have not been optimized for human oocytes, a second prediction is that cytoplasmic maturation is likely to be compromised, resulting in decreased clinical success. For example, when mouse GV-stage oocytes underwent meiotic maturation in different culture media and then all were transferred to the same optimized medium for insemination and cleavage, the groups had significantly different percentages of blastocyst development (Van de Sandt *et al.*, 1990; Eppig, 1991). This study indicated the importance of maturation conditions on post-meiotic developmental events. In oocytes from small follicles, acceleration of maturation (*in vivo* via exogenous gonadotrophins or *in vitro* via removal from the follicle) followed by IVF results in a lower percentage of viable embryos compared to embryos from oocytes that developed *in vivo* (Eppig *et al.*, 1992, 1994). In addition, the latter studies demonstrated that accelerated development resulted in apparently normal nuclear (meiotic) maturation, suggesting that normal cytoplasmic maturation had not taken place and is more sensitive to accelerated development. Because eggs from small antral follicles that are stimulated by high concentrations of exogenous gonadotrophins are also collected in human IVF, cytoplasmic maturation and later development may be compromised in some oocytes without necessarily preventing meiotic maturation, fertilization and even the formation of two pronuclei.

In-vitro maturation of human GV-stage oocytes to metaphase II followed by insemination has resulted in reduced fertilization rates and poor clinical success of those eggs that were fertilized (Flood *et al.*, 1990; Hammitt *et al.*, 1993). Possible causes include inadequate culture conditions, inherent oocyte quality and abnormal recruitment. Cytoplasmic immaturity could lead to a failure to develop the signalling pathway mechanisms, informational molecules or nutritional stores that may be required later for normal egg activation or preimplantation development. Just as maturational asynchrony between cumulus–coronal morphology and nuclear maturity of the egg has been observed in stimulated human cycles (Laufer *et al.*, 1984; Hammitt *et al.*, 1993), asynchrony also has been suggested between oocyte nuclear and cytoplasmic maturation, in which the latter appeared to be delayed (Sundstrom and Nilsson, 1988).

In animal IVF, culture conditions play an important role in optimizing fertilization rates by preventing zona hardening. In serum-free medium, the normal maturation-associated loss of CG in mouse oocytes results in ZP biochemical changes, i.e. the conversion of ZP2 to $ZP2_f$ (Ducibella *et al.*, 1990c), leading to a block in sperm penetration (Downs *et al.*, 1986; Choi *et al.*, 1987). Follicular fluid (DeFelici *et al.*, 1985; Kalab *et al.*, 1993), 5% serum (Downs *et al.*, 1986; Choi *et al.*, 1987) and fetuin, a specific protease inhibitor in serum (Schroeder *et al.*, 1990; Kalab *et al.*, 1991, 1993), but not albumin (Downs *et al.*, 1986; Ducibella *et al.*, 1990c) prevent these changes in the mouse ZP. In human eggs, protection from ZP hardening also is likely to be important in maturing oocytes (Ducibella *et al.*, 1990b) because (i) many oocytes are collected before they reach metaphase II, (ii) spontaneous CG loss has been observed in cultured human unfertilized eggs (Rousseau *et al.*, 1977; Tam *et al.*, 1990) and (iii) a biochemical change indicative of ZP hardening and CG loss occurs in some cultured human eggs that fail to fertilize in the absence of serum (Ducibella *et al.*, 1995). Studies are needed of the human ZP during meiotic maturation in the presence or absence of serum, follicular fluid and fetuin.

With regard to routine human IVF, these studies (i) suggest the importance of the stage of the oocyte in timing spermatozoon–egg interaction, (ii) emphasize the need to

devise additional methods for staging and tracking the eggs collected and (iii) support the development of improved methods for in-vitro culture and in-vivo follicular maturation that allow meiotically immature oocytes to complete not only nuclear (meiotic) maturation, but also cytoplasmic maturation with the prevention of ZP hardening. The process of optimizing oocyte quality would be greatly facilitated by biochemical markers of important maturational events, especially those involved later in fertilization or early development. One example is the identification of egg activation and CG exocytosis signalling step(s) that appear to develop between GV breakdown and metaphase II. Just as specific chromatin configurations allow us to track nuclear meiotic progress, a progression of appropriate cytoplasmic markers will provide sequential assessment of cytoplasmic development. These markers would be useful in experimental studies of media optimization for in-vitro maturation of GV-stage oocytes and evaluating new follicular maturation/ovulation induction protocols.

Cytoplasmic response after ICSI

Cytoplasmic maturation is also likely to be important for success in intracytoplasmic sperm injection (ICSI). Although ICSI appears to bypass the initial gamete plasma membrane interactions, the egg cytoplasm still has to respond with a signal(s) to activate a programme for embryonic development and to transform the sperm chromatin into a male pronucleus. Normally, both egg activation (as mentioned previously) and sperm chromatin transformations (Usui and Yanagimachi, 1976; Zirkin *et al.*, 1989; Longo, 1990; Perreault, 1990) require metaphase II mammalian egg cytoplasm, whereas cytoplasm from earlier meiotic stages is not fully competent. Cytoplasmic maturation may be incomplete in ICSI eggs in which the sperm membrane has broken down, but egg activation and complete CG release did not occur (Sousa and Tesarik, 1994).

The process of egg activation in ICSI is of interest for several reasons: (i) ICSI is a new, clinical procedure to treat severe male infertility where routine IVF has failed (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993a,b), (ii) the ICSI procedure appears to bypass important gamete plasma membrane interactions involved in egg activation in unassisted fertilization and (iii) new knowledge about the ICSI activation mechanism may be applied to improve fertilization and pregnancy rates (Edwards and Van Steirteghem, 1993). Although the precise mechanism of egg activation in ICSI has not been demonstrated, an increase in intracellular Ca is probably involved because (i) it has an obligatory role in normal mammalian fertilization (Kline and Kline, 1992a), (ii) Ca oscillations are observed in the human egg after insemination (Taylor *et al.*, 1993), (iii) Ca increases are detected after ICSI (Tesarik *et al.*, 1994; Tesarik and Sousa, 1994) and (iv) eggs that have failed fertilization in ICSI have intact CG (Sousa and Tesarik , 1994), suggestive of a failure of appropriate Ca elevation.

Mammalian fertilization is quickly followed by a series of Ca oscillations. In hamsters, mice, and rabbits (Miyazaki *et al.*, 1993; Fissore and Robl, 1994; Mehlmann and Kline, 1994), following fertilization there is a major Ca transient immediately followed by several hours of regular, periodic transients every 5–10 min, whereas longer intervals have been observed in bovine eggs (Fissore *et al.*, 1992). The amplitude and/or frequency of the initial or first few transient(s) may determine the extent of CG exocytosis (Kline and Kline, 1992a). In the human, studies have been limited by the availability of mature eggs and the use of aged unfertilized eggs. Fertilized human eggs undergo Ca oscillations with a variable (10–35 min) frequency (Taylor *et al.*, 1993). Subzonal insemination (SUZI) of fresh or aged human eggs is followed by a major Ca transient and Ca oscillations (Tesarik and Sousa, 1994). Their frequency (3–15 min) and wave-like propagation from a locus in the egg cortex are similar to those of fertilized animal eggs, and their amplitude decreases with time over 1–3 h.

In contrast, a study of human ICSI reported two widely separate Ca 'episodes'. Human eggs subjected to ICSI are characterized by three phases during the response (Tesarik *et al.*, 1994): an initial Ca increase lasting 2–3 min due to the influx of extracellular Ca associated with injection, followed by a 4–12 h period without detectable responses, and finally, 30–60 min of Ca oscillations with frequencies of between 1 and 5 min. Oscillations are initiated near the cortex, but thereafter their source becomes less focused with time. The initial Ca transient is not due to the interaction of the spermatozoon with the egg cytoplasm because it is detected when either the injection solution without spermatozoa or even a pipette without a lumen (in medium with 1–2 mM extracellular Ca) is used (Tesarik *et al.*, 1994). In this study, this first transient appears to be initiated by exogenous Ca, while the participation of CICR [Ca(exogenous) – induced Ca(endogenous) release] from internal stores has not been rigorously investigated. The injection of 1.8 mM Ca into unfertilized rabbit eggs results in a single Ca transient about one-half the amplitude of that observed upon fertilization (Fissore and Robl, 1994), although the volume of fluid injected in human ICSI may be larger, introducing more Ca. The injection of Ca alone into unfertilized eggs does not cause Ca oscillations typical of those in normal fertilization in animal eggs (Igusa and Miyazaki, 1983; Fissore and Robl, 1994; Kline and Kline, 1994) or those in human ICSI after the long delay (Tesarik

et al., 1994). In any case, little is known about any early effects of this first large Ca transient on the human egg's endogenous Ca and signalling machinery after ICSI. It is entirely possible that this initial Ca elevation is sufficient for egg CG exocytosis, but not for subsequent events like second polar body and pronuclear formation, which are not observed (Tesarik *et al.*, 1994).

The basis for this unusual Ca response in human ICSI may reside in the mechanism controlling intracellular cytosolic egg Ca concentrations and in the question of whether sperm factors are involved in egg activation. The long lag period in which Ca stays low after the initial brief Ca rise is surprising, unless the egg is still 'waiting' for a sperm factor or Ca oscillations have been suppressed temporarily. It is tempting to invoke the former, in which the sperm membranes must break down for a sperm factor(s) to enter the egg cytoplasm. Support for such factors in human ICSI comes from the activity of sperm cytosolic fractions (Dozortsev *et al.*, 1995) and the observation that oscillations fail to appear in sham-injected eggs without spermatozoa in which only the initial Ca increase occurs (Tesarik *et al.*, 1994). By analogy with fertilization events, Ca oscillations should appear shortly after (probably within minutes) the entry of the proposed sperm factor into the egg cytoplasm. The timing of sperm factor release should be indicated by sperm head plasma membrane breakdown. An electron microscopic study in the mouse demonstrates that both the sperm plasma membrane and nuclear envelopes have broken down within 1 h of ICSI (Kimura and Yanagimachi, 1995). Yet, in the human, Ca oscillations do not begin until 4–12 h after ICSI. Interpretation of these data await the identification, after ICSI, of the timing of sperm plasma membrane breakdown in the human egg and of Ca oscillations in the mouse egg. In addition, the identity of the putative sperm factor(s) remains to be established. If a sperm factor participates in egg activation during ICSI (which may or may not be the same sperm factor proposed to be involved in normal fertilization), time may be required to build a sufficient local concentration for activation or to diffuse to the appropriate target, e.g. the cortical ER.

Alternatively, the lag period before Ca oscillations in ICSI may be due to a temporary desensitization of the Ca release mechanism. However, several mechanisms involved in the desensitization of Ca stores do not readily explain the lag period, because the lag is so much longer in duration than that between oscillations after fertilization. The short fertilization-associated interval between normal oscillations is likely to be due to refilling and resensitizing cytoplasmic Ca stores. Refilling involves an ER Ca pump (Kline and Kline, 1992b) with an IP_4-mediated Ca supply (Shirakawa and Miyazaki, 1995); whereas resensitizing is likely to require lowering cytosolic Ca and increasing cisternal ER Ca, both of which are known to sensitize the IP_3 receptor (Marshall and Taylor, 1993; Missiaen *et al.*, 1994; for eggs, see Parys *et al.*, 1992). In order to determine if ICSI has an effect on the sensitivity of Ca stores to undergo release, it would be informative to know if Ca oscillations can be induced shortly after ICSI during the observed lag period. IP_3 injection or refertilization (e.g. SUZI) at various times after ICSI could be used to determine if there are changes in sensitivity to Ca release during the lag period. Although prolonged high cytosolic Ca from the ICSI procedure could inhibit the IP_3 receptor, this is unlikely to explain the long lag because injected cytosolic Ca appears to be rapidly sequestered or pumped out of the cell—Ca concentrations quickly return to baseline in Ca injection studies.

Although one could simplistically interpret ICSI as Ca activation after bypassing spermatozoon–egg membrane interaction, there remain many unanswered questions. Is there any developmental disadvantage to the significant temporal separation of the exogenously induced initial Ca rise (with an expected episode of CG release) and later Ca oscillations? Besides CG release, what events are triggered by the initial, exogenous Ca transient in both the absence and presence of an injected spermatozoon? What initiates the Ca oscillations in ICSI? What is occurring during the Ca lag phase? Do the amplitude and frequency of the Ca oscillations regulate early developmental events, as proposed for mouse and bovine egg activation (Vitullo and Ozil, 1992; Collas *et al.*, 1993), and have these parameters been optimized for clinical success in human ICSI? Does the injection solution play a role in egg activation and, if so, have the solutes been optimized? Why do some eggs that promote sperm plasma membrane breakdown fail to undergo CG release and egg activation (Sousa and Tesarik, 1994)? It has been proposed that some cases of failed egg activation or aberrant early development may be due to abnormal Ca release after fertilization (Homa *et al.*, 1993).

A developmental temporal window for normal egg CG release and activation

The acquisition of competence to undergo normal CG exocytosis and the development of the block(s) to polyspermy are likely to represent a larger, more important and clinically relevant process: development of the ability to undergo normal fertilization and activation of development. Both CG release and other zygotic activation events depend on the release of intracellular Ca, which is regulated by second messengers interacting with cytoplasmic Ca stores. An emerging body of evidence is growing that during the final phase of meiotic maturation, close to the time of ovulation or oocyte retrieval in the case of human

IVF, the oocyte's Ca release mechanism becomes fully competent. Temporal studies on the development of the ability to undergo activation are consistent with the idea that Ca-dependent effectors involved in CG release and cell cycle resumption become functional or change localization during egg maturation and after ovulation. For example, compared to newly formed metaphase II mouse eggs, those several hours after ovulation have a higher percentage or faster rate of pronuclear development after fertilization or artificial activation (Fraser, 1979; Kubiak, 1989) and, within 5–10 h of ovulation, mouse oocytes become increasingly sensitive to Ca injection (Fulton and Whittingham, 1978) and spontaneous activation (Whittingham and Siracusa, 1978). After 10–20 h of ovulation, mammalian egg ageing (Austin, 1974) ensues, including changes in the egg cortex, cortical granules and ability to undergo CG release (reviewed in Ducibella, 1991).

Thus, the mammalian egg cortex and activation machinery appear to be continually changing after meiotic maturation resumes and there is likely to be a temporal window in which normal eggs have the highest likelihood of undergoing a normal cortical reaction and activation of development. This window may entail a shorter time than previously appreciated and is particularly relevant to animal and human IVF in which oocytes and eggs may undergo either unusually rapid recruitment and maturation or prolonged arrest in metaphase II in culture or even intrafollicularly. Although the high percentage of combined fertilization failure and early embryo loss in both ICSI and routine IVF probably has multiple causes, a major causal player is likely to be the cytoplasm, in addition to nuclear and maternal contributions. Characterization of not only the cytoplasmic activation pathways involved in CG exocytosis and cell cycle resumption in mature eggs, but also changes in the activation molecules and machinery during and after oocyte maturation (*in vivo* and *in vitro*) will provide new scientific information with potentially important clinical benefit.

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