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Review

Ca^{2+} oscillations in the activation of the egg and development of the embryo in mammals

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Introduction

At fertilization the union of the sperm and egg has two important functions: it brings together their genetic components and it triggers embryonic development. It is often assumed that only the genetic component has an important part to play in further development. In the following review it is argued, on the basis of recent findings, that in disregarding the initial trigger for development, a rise in cytoplasmic Ca^{2+}, an important factor in the control of embryonic development is being ignored.

The review will concentrate on mammalian eggs due to the very characteristic Ca^{2+} changes which constitute the trigger for egg activation, and so embryonic development. Mammalian eggs show low-frequency Ca^{2+} oscillations over a period of several hours. It will be argued that a novel mechanism of Ca^{2+} release is employed by the sperm at fertilization and such Ca^{2+} changes have a role in subsequent development of the embryo in addition to the initial events of egg activation.

For the purposes of this review the female gamete is defined as an 'egg' when it is at a stage that it is normally fertilized, in contrast to an 'oocyte' when it is at an immature stage of maturation and would not normally be fertilized. For all the species discussed here only in the sea urchin is the female gamete correctly termed an egg, having completed all the stages of meiosis.

Fertilization and Ca^{2+}

The release of Ca^{2+} at fertilization was observed directly for the first time in medaka eggs over twenty years ago (Ridgway *et al.*, 1977) and has been observed in all animal eggs examined thus far. A rise in the free cytoplasmic Ca^{2+} concentration within the egg is the endogenous signal for meiotic resumption, indeed all the events of fertilization. The rise in Ca^{2+} is not a peripheral component of the sperm's repertoire. It is the central player since all the events of fertilization are either inhibited by buffering its action (Kline and Kline, 1992) or induced by its microinjection into eggs (Fulton and Whittingham, 1978). Without a rise in Ca^{2+} there is no cortical granule release to prevent polyspermy and there is also no

Abbreviations used in this paper: CSF, cytostatic factor; ER, endoplasmic reticulum; ICM, inner cell mass; IP_3, inositol trisphosphate; MI, first meiotic metaphase; MII, second meiotic metaphase; MPF, maturation promoting factor.

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meiotic resumption and subsequent entry into the embryonic cell cycles.

In medaka (Ridgway *et al.*, 1977), sea urchin (Steinhardt *et al.*, 1977) and frog (Busa and Nuccitelli, 1985), which were among the first species to be studied, the Ca^{2+} signal takes the form of a single wave which passes across the egg from the site of sperm fusion. In mammalian eggs, which are arrested before completing meiosis at metaphase of the second meiotic division (MII), the initial Ca^{2+} signal also takes the form of a wave of Ca^{2+} (Miyazaki *et al.*, 1986). However the signal is more complex because Ca^{2+} oscillations continue at a regular interval following the initial rise and last for several hours (mouse- Cuthbertson and Cobbold, 1985; hamster- Miyazaki *et al.*, 1986; bovine- Fissore *et al.*, 1992; pig- Sun *et al.*, 1992; rabbit- Fissore and Robl 1993; rat- Ben-Yosef *et al.*, 1993; human- Taylor *et al.*, 1993). These oscillations in Ca^{2+} are often of a low frequency with bovine eggs having a particularly long interval between transients -50 min being seen in some eggs (Fissore et al., 1992). In contrast the shortest interval appears that in hamster with an interval of 3 min or slightly longer; the frequency of oscillations increases dramatically as the egg becomes progressively more polyspermic (Miyazaki, 1989). It is interesting to note that the ability of eggs to show Ca^{2+} oscillations in response to sperm fusion is not limited to mammals, since some protostomes show oscillations in addition to the Urochordate ascidian egg and this point will be raised later in the review.

The signal transduction system which links a rise in Ca^{2+} to release from meiosis and cortical granule exocytosis is poorly understood. In most eggs it is maturation promoting factor (MPF) stabilized by the presence of cytostatic factor (CSF) that maintains meiotic arrest (Kubiak *et al.*, 1993; Sagata, 1996). In frog the signal transduction pathway from a Ca^{2+} rise to cell cycle resumption is the most understood, with Ca^{2+} activating calmodulin-dependent protein kinase II known to cause ubiquitinisation of the cyclin component of MPF (Lorca *et al.*, 1993). This ubiquitinisation acts as a molecular tag to cause its degradation. CSF activity is also ablated by a mechanism involving calmodulin-dependent protein kinase II, albeit after a drop in MPF (Lorca *et al.*, 1993). Whether a similar pathway exists in mammals has not been elucidated although parthenogenetic activation of mouse eggs with ethanol can also transiently activate calmodulin-dependent protein kinase II, suggesting it too will be activated during mammalian fertilization (Winston and Maro, 1995).

Mechanism of sperm-induced Ca^{2+} oscillations

In examining the role of Ca^{2+} oscillations in mammalian eggs not only is there a poorly defined signaling pathway for MPF and CSF degradation but also the mechanism by which sperm generates these oscillations is still far from clear. Understanding the mechanism by which sperm induces oscillations should also tell much of their actual role in development. At present there are two main hypotheses. The first proposes a receptor mediated mechanism whereby the sperm binds to an oolemma receptor which activates a phosphatidylinositol-phospholipase C (Foltz and Shilling, 1993). This pathway may be either G-protein coupled or tyrosine kinase linked. Ultimately the first mechanism relies on the conventional second messenger signaling molecule inositol trisphosphate (IP_3), generated by the action of phospholipase C, acting on the IP_3 receptor. The second hypothesis proposes that a sperm factor is released directly into the ooplasm bypassing any membrane association (Swann and Lai, 1997). The main evidence for the second hypothesis is the finding that sperm cytosolic extracts cause Ca^{2+} oscillations when injected into eggs (Swann, 1990, 1994; Homa and Swann, 1994; Stricker, 1997; Wilding et al., 1997; Wu *et al.*, 1997). The response probably involves a novel signaling molecule, a high molecular weight protein, acting on conventional Ca^{2+} releasing channels (Parrington *et al.*, 1996).

The strongest evidence against a mechanism based upon IP_3 production is the finding that IP_3 microinjection into mouse (Swann 1994; Jones and Whittingham, 1996), hamster (Swann *et al.*, 1989; Swann 1991, Galione *et al.*, 1994), rabbit (Fissore and Robl, 1993) and bovine eggs (Fissore *et al.*, 1995) always gives high frequency oscillations (one transient every 1-2 min); while those induced by sperm are much lower in frequency (5-20 min). The differences between IP3- and sperm-induced Ca^{2+} transients is detailed in Figure 1. While IP_3 production cannot be ruled out at fertilization, it is difficult to match the experimental data with such a viewpoint. Furthermore microinjection of GTPγS and application of serotonin, which both produce IP_3 by activation of phospholipase Cβ, also induce high frequency oscillations unlike sperm (Miyazaki *et al.*, 1990). It could be argued that microinjection of IP_3 does not mimic the precise, coordinated generation of IP_3 that may occur following egg-sperm fusion and that microinjection is a too simplistic method of mimicking sperm-generated IP_3 release. However this view is hard to marry with the finding that cytosolic extracts of sperm microinjected into eggs mimic the frequency of normal fertilization precisely (Swann, 1990). It is difficult to argue that sperm extract injections, but not those of IP_3, can mimic the spatial aspects of Ca^{2+} release caused by the fertilizing sperm. Furthermore, in hamster eggs IP_3-induced oscillations are quickly damped, that is the IP_3 receptor desensitizes to IP_3 and further application of IP_3 no longer causes Ca^{2+} release (Galione *et al.*, 1994). This does not occur at fertilization since the oscillations are long lasting, so if IP_3 is produced there must be other sensitizing factors involved.

It is the repetitive nature of the Ca^{2+} transients following sperm-egg fusion which govern events associated with fertilization rather then the signaling molecule itself. It could be argued that the exact nature of the signaling molecule may not be too important. In the same way that in studying the mechanism governing lymphocyte proliferation the role of the antigen although primary in recognition is not involved in the downstream lymphocyte proliferation pathway. This scenario is particularly true if the signaling molecule at fertilization does indeed prove to be IP3. The sperm merely generates IP_3 in the egg, which releases Ca^{2+} to then act on meiosis-arresting factors and so causes completion of meiosis. However the one caveat is if the mechanism does not generate IP_3 and a new signaling molecule is involved - its potential multifunctional role may be being overlooked. At present the best candidate for sperm factor protein has been termed 'oscillin' a multimeric protein with a 33 kDa monomeric weight, which shares 53% homology to an *E. Coli* glucosamine-6-phosphate deaminase/isomerase (Parrington *et al.*, 1996). The link between a bacterial enzyme involved in glucosamine metabolism and Ca^{2+} release in mammalian eggs is at present not understood.

Why do mammalian eggs show long lasting Ca^{2+} oscillations?

There must be an underlying reason why mammalian eggs show oscillations and not a single transient as in frog, sea urchin

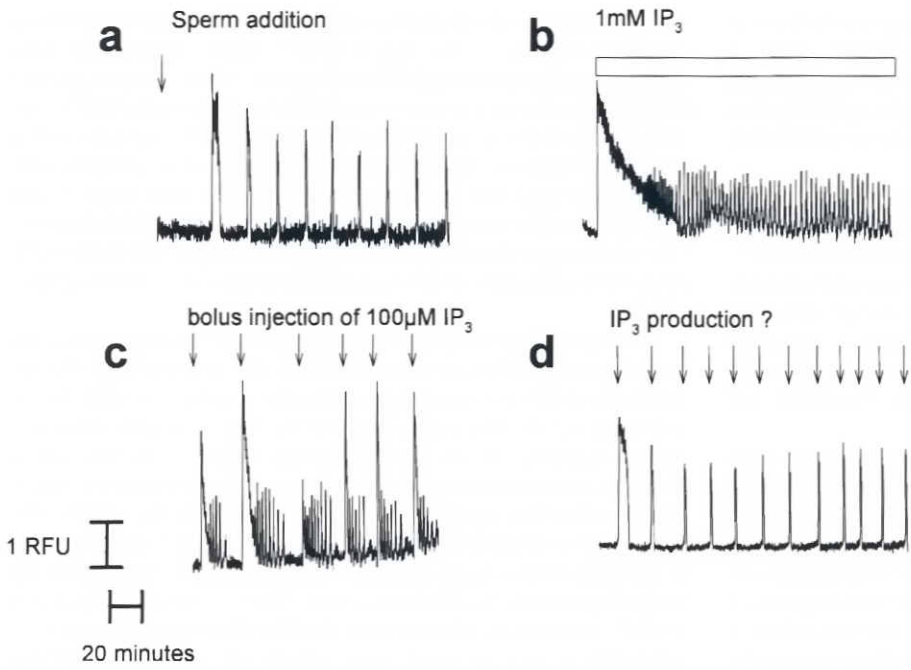

Fig. 1. Changes in intracellular Ca^{2+} in mouse oocytes following sperm or IP_3 addition which show that the frequency of sperm and IP_3 - induced Ca^{2+} oscillations appear very different. **(a)** *Ca^{2+} oscillations induced by sperm, the arrow indicating the time of sperm addition. Note the much higher frequency of oscillations when* **(b)** *IP_3 is allowed to leak gradually from an inserted pipette.* **(c)** *In order to mimic the lower frequency Ca^{2+} oscillations induced by the sperm with IP_3, IP_3 was injected as a bolus into eggs at a final egg concentration of 100μM at the times indicated. Note that each bolus injection induces a series of high frequency oscillations rather than a single transient, which are actually smaller in amplitude to those induced by sperm. If sperm generates IP_3 at fertilization one can only conclude that pulses of IP_3 must be produced at each Ca^{2+} transient as shown in* **(d)**, *but not at a high enough concentration to produce the high frequency oscillations seen in (c). It is difficult to see how this can be so when the Ca^{2+} oscillations induced by sperm are actually larger than those induced by IP_3 (compare a,d with b,c). The x-axis bar represents 20 min and the y-axis bar represents 1 ratio fluorescence unit (RFU) at 420nm/490nm, indo-1 being used to record changes in Ca2+. All figures are represented to the same scale. For explanation of Ca2+ measurement using this system see Jones et al., 1995. For further detail of IP_3 microinjection see Jones and Whittingham, 1996.*

and medaka, independent of the actual mechanism whereby sperm causes Ca^{2+} release (albeit IP3 production or sperm factor release). There are two major differences between mammals and other species with regard to their reproduction which I believe account for the need for oscillations. Both differences are to do with timing and the extended cell cycle in mammals. The first is that mammalian fertilization is internal and as a result of this there is a marked delay between ovulation of eggs, arrested at MII, and fertilization. Medaka and frog have external fertilization and the gametes mix almost instantaneously after being shed. Fertilization therefore occurs very quickly. In the sea urchin mature eggs are stored in gravid females before spawning for extended periods and appear different to most other animal eggs in having completed meiosis. Thus their arrest is in interphase and is caused by an acidification of the cytoplasm so that metabolic activity is greatly decreased (Epel, 1977, 1978; Whitaker and Steinhardt, 1982). The increased length of time between release from the ovary and fertilization in mammals must mean that the mammalian egg is encumbered by a cell-cycle arresting system more replete than non-mammals where the egg is quickly fertilized after release. One would therefore presume that the mammalian sperm contains within it a strong enough signal to necessitate cell cycle resumption. In practice I suggest this signal is a series of Ca^{2+} oscillations which drive exit from meiosis.

The second difference between mammals and non-mammals is the timing between meiotic resumption triggered by the sperm and entry into the embryonic cell cycle at which point the embryo may be regarded as being free-standing, having completed meiosis. This interval is much longer in mammals. For example the time between sperm-egg fusion and formation of pronuclei in the 1-cell embryo of frog is a matter of a few tens of minutes (Newport and Kirschner, 1984) while it is 6-8 h in the mouse (Hogan et al., 1994). In the sea urchin of course the egg has already completed meiosis and the major function of the sperm is to cause an alkalinization of the cytoplasm through a rise in Ca^{2+}. This change in intracellular pH can be induced by a single rise in Ca^{2+} (Whitaker and Steinhardt, 1982).

So why do some non-mammalian species show Ca^{2+} oscillations? In the annelid *Chaetopterus* fertilized at metaphase I (MI) 10 or so oscillations last 7-10 min with the second meiotic division occurring towards the end of this period (Eckberg and Miller, 1995). In the MI-arrested *Nemertean* worm *Cerebratulus*, Ca^{2+} oscillations last 30-120 min, first polar body occurring at about 60 min and the second polar body at 90 min (Stricker, 1996). Also in the same phylum as mammals the deuterostome ascidian egg fertilized at MI shows 12-25 Ca^{2+} oscillations lasting in total 22-25 min, a period that ends with formation of the second polar body (Speksnijder *et al.*, 1989; McDougall and Sardet, 1995). It is therefore clear that eggs arrested at MI such as the protostome *Chaetopterus* and *Cerebratulus*, and deuterostome ascidian show Ca^{2+} oscillations during exit from meiosis analogous to mammalian eggs arrested at MII, albeit usually with a shorter time frame and with a greater frequency. A similar mechanism may underlie this phenomenon, that is the oscillations are ensuring that eggs escape the influence of meiotic-arresting factors. The time at which the egg actually arrests is dependent on the species, but the mechanism the sperm has adopted to relieve arrest (Ca^{2+} release) is the same for all.

So what of the frog? It is arrested at MII and shows only one Ca^{2+} transient. However it enters embryonic interphase within 20 min and therefore has no need of oscillations. The MII-arresting factors are ablated by a single Ca^{2+} transient. It is the speed with which the frog egg exits from meiosis which negates the need of oscillations

in a biological sense. The case is even more pronounced in the sea urchin which has arrested following exit from meiosis. Here a reduction in the cytoplasmic pH probably causes the egg to arrest and the cytoplasmic rise at fertilization induces alkalinization though a Na/H antiporter on the urchin egg plasma membrane (Whitaker and Steinhardt, 1982).

These times may be compared with mammals which show an extended period between fertilization and entry into the first embryonic interphase, 6-8 h in the mouse. A series of oscillations in Ca^{2+} should be preferable to the egg rather than a more general increase in basal levels. This is because an extended rise in Ca^{2+} is known to interfere with many intracellular processes and is therefore regarded as being detrimental or even toxic to cells (Jain and Shohet, 1981; Starke *et al.*, 1986; Bondy, 1989; Grondahl and Langmoen, 1996).

Given the previous model outlined it is interesting that Ca^{2+} oscillations cease when the egg has escaped from the meiosis-arresting factors, that is by the time pronuclei form in the newly created zygote (Jones *et al.*, 1995). In most biochemical pathways there exists a positive or negative feedback mechanism, for eggs meiosis-arresting factors may influence Ca^{2+} oscillations so that oscillations continue until these factors decline below a threshold level. Eggs can be stopped from entering interphase by microtubule disrupting agents which interfere with the integrity of the metaphase spindle and such eggs remain in meiosis until the agents are washed out (Winston *et al.*, 1995). Eggs prevented from exiting meiosis using the microtubule disrupting agent colcemid and then fertilized continue to oscillate for several hours after normal pronucleus formation would have taken place (Jones *et al.*, 1995). One of the feedback mechanisms could therefore be decondensation of the chromatin which is blocked by colcemid. Another possible candidate could be MAP kinase, a component of CSF, whose decline in activity is associated with pronucleus formation (Moos *et al.*, 1995). Preventing pronucleus formation similarly inhibits the decline in MAP kinase activity.

Parthenogenetic activation

The first definitive function of repetitive Ca^{2+} oscillations induced by sperm has now been discussed - to ensure that the egg escapes from cell cycle arresting factors. But what of parthenogenetic activation, where the stimulus usually induces a monotonic rise in Ca^{2+}. If the egg is capable of completing meiosis with a single transient does this not negate the previous argument?

It has been known for some time that eggs may be parthenogenetically activated since the work of Loeb (1913). Parthenogenetic activation may be mechanical - simply pricking the egg; chemical-ethanol, strontium, Ca^{2+} ionophores; or electrical- depolarization of the egg plasma membrane (reviewed in Whittingham 1980, Whitaker and Steinhardt, 1982; Jaffe 1983). Nearly all methods of parthenogenetic activation rely on raising the intracellular free Ca^{2+} concentration in the ooplasm for their success, in a manner analogous to sperm.

It is clear that non-mammalian species activate more easily than mammalian, for example in the frog mechanical pricking of the egg is all that is required. This is presumably because parthenogenetic activation is a simple procedure in species where sperm induce only one Ca^{2+} transient at fertilization. This signal is easily mimicked by other means.

The success rate of parthenogenetic activation in mammals is related to the age of the egg, freshly ovulated eggs being more difficult to activate than aged (Whittingham, 1980). In most species there exists a window in which the time of normal *in vivo* fertilization takes place, in the mouse this is 14-18 h post hCG; ovulation being around 12 h post hCG. High rates of activation are achieved only when aged eggs are used, that is at time later than when *in vivo* fertilization would have occurred. For example Ca^{2+} microinjection into mouse eggs gives only 16% activation in eggs 13.3 h post hCG while 89% activation at 20 h post hCG (Fulton and Whittingham, 1978).

The age-related success of activation must be due to a decline in the level of the factors responsible for the arrest of egg. Protein synthesis inhibitors cause egg activation if added to eggs for an extended period (Siracusa *et al.*, 1978). This indicates there is a natural turnover of cell cycle arresting factors while the egg is arrested. Most parthenogenetic agents cause a monotonic rise in Ca^{2+}, unlike the repetitive oscillations induced by sperm. For example 7% ethanol treatment of mouse eggs for 7 min is enough to activate mouse eggs, giving a very large Ca^{2+} rise during the time of its addition (Cuthbertson *et al.*, 1981). This single large rise in Ca^{2+}, induced by ethanol and other parthenogenetic agents, is adequate to activate aged eggs where the cell cycle arresting factors have declined since ovulation. Presumably the turnover of arresting factors exceeds their synthesis so net levels decline. The single large Ca^{2+} transient induced by parthenogenetic activation is enough to reduce the factors below the threshold necessary for progression into the embryonic cell cycle. Furthermore the Ca^{2+} ionophore A23187, commonly used to induce parthenogenesis, also causes some inhibition of protein synthesis, which may account to some degree for its effectiveness (Bos-Mikich *et al.*, 1995). Decreasing protein synthesis in combination with a rise in intracellular Ca^{2+} can act synergistically to induce parthenogenesis (Bos-Mikich *et al.*, 1995). The one parthenogenetic agent that is effective in causing high rates of activation in freshly ovulated eggs is Sr^{2+}, because like sperm it causes a series of oscillations and not a monotonic rise (Kline and Kline, 1992), it is therefore 'the exception that proves the rule'.

In contrast, freshly ovulated eggs treated with an agent that causes a monotonic rise in Ca^{2+} show only partial activation. Eggs extrude a polar body, but the chromatids realign on a third meiotic spindle. This has been termed the metaphase III state (Kubiak, 1989). Therefore completion of meiosis has been aborted as the factors which arrest the egg re-emerge. A similar observation was seen in two studies by Collas *et al.* (1993 and 1995) on bovine and rabbit eggs respectively. In these studies pulses of Ca^{2+} were induced in eggs by electrical stimulation and it was found that a single Ca^{2+} transient markedly decreased MPF activity, as judged by a fall in histone H1 kinase activity. However MPF activity rapidly returned and several Ca^{2+} transients were needed to ensure that MPF activity remained low.

It would seem to most readers that a realistic role for Ca^{2+} oscillations is to ensure complete activation. This is needed due to the extended cell cycle and the indeterminate timing of fertilization relative to ovulation in mammals. A system would not have evolved that is 'hit and miss'. Sperm-induced Ca^{2+} oscillations ensure activation of the egg regardless of its post-ovulatory age. It is fortunate on the one hand that experimenters can artificially activate mammalian eggs at high rates by a monotonic Ca^{2+} rise if

they are aged. However on the other hand it is rather confusing for those seeking the mechanism by which sperm induces oscillations when experimenters are confronted by a system that allows activation in some circumstances rather indiscriminately.

Ca^{2+} oscillations following exit from meiosis in fertilized embryos

Thus far a role for Ca^{2+} oscillations in ensuring exit from meiosis in mammalian eggs has been discussed. Yet there is evidence to suggest that Ca^{2+} may be involved in more than just exit from meiosis since in mouse zygotes a Ca^{2+} transient is associated with nuclear envelope breakdown during the first mitotic division (Tombes *et al.*, 1992; Kono *et al.*, 1996). Indeed Ca^{2+} oscillations, similar to those observed at fertilization, but slightly lower in frequency, are seen during the entire period of mitosis (Kono *et al.*, 1996). These changes in intracellular Ca^{2+} are not seen in parthenogenetic mouse embryos. Similar findings have been readily observed in sea urchin embryos, where Ca^{2+} transients are associated with specific events during the period of mitosis such as nuclear envelope breakdown and metaphase-anaphase transition (Poenie *et al.*, 1985; Whitaker and Patel, 1990). This may be a general phenomenon since similar mitotic Ca^{2+} oscillations occur in some mammalian cell lines (Kao *et al.*, 1990).

The fertilized embryo and not the parthenogenetically derived embryo shows mitotic Ca^{2+} transients (Kono *et al.*, 1996). They appear to have an immediate effect on progression through mitosis since BAPTA, a Ca^{2+} chelator, is able to block embryos in mitosis. However BAPTA will also block in mitosis parthenogenetically derived embryos, that do not show global Ca^{2+} transients. It may be that in parthenotes local Ca^{2+} rises are observed that can go undetected by less sensitive recordings. Indeed perinuclear local Ca^{2+} rises are observed in sea urchin parthenotes during entry into the first mitotic division at nuclear envelope breakdown (Wilding *et al.*, 1996). These local releases of Ca^{2+} are seen by confocal microscopy but not in conventional Ca^{2+} recordings which are made usually on whole cells. Local changes may therefore be below the level of detection when the signal is averaged out.

One may tentatively hypothesize that the signal providing the mitotic global Ca^{2+} transients in fertilized embryos and the signal provided by the sperm at fertilization are one and the same. Nuclear transfer experiments reveal this probably to be so. When nuclei of fertilized embryos at the 1- and 2-cell stage are transferred back to an unfertilized MII egg Ca^{2+} transients similar to normal fertilization are always seen (Kono *et al.*, 1995). A similar effect has been shown by cell fusion studies of embryos with MII eggs, where only 1- and 2-cell fertilized embryos and not parthenogenetic embryos are capable of causing activation in the hybrid egg (Zernicka-Goetz *et al.*, 1995), no Ca^{2+} transients occur when nuclei of parthenotes are transferred or cytoplasm from any source (Kono *et al.*, 1995; Fig 2). So this tells us something of further importance that the signal, providing the mitotic oscillations lies either inside the nucleus or is associated with the perinuclear region, which is also transferred in this procedure. Neither location can be ruled out. I favor a perinuclear location merely because this carries with it some endoplasmic reticulum

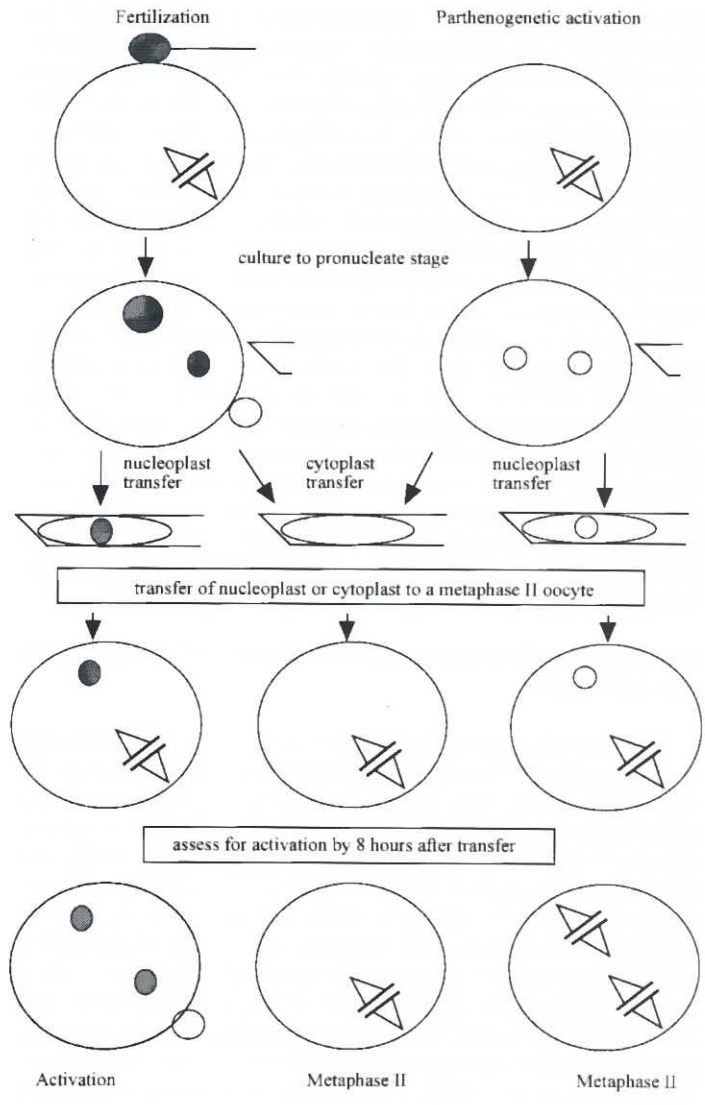

Fig. 2. Nucleoplast and cytoplast transfer experiments of Kono *et al.*, 1995. *The lefthand side of the figure represents nucleoplast and cytoplast transfer carried out on fertilized embryos and the righthand side on parthenogenetically derived embryos. Kono and co-workers transferred either the nucleoplast or cytoplast at the pronucleate stage to a MII egg. In such hybrid eggs they looked for subsequent activation and for Ca^{2+} oscillations. Only in the nucleoplast transfer from a fertilized embryo (shown in grey) did the hybrid show Ca^{2+} oscillations and therefore activate. Note that activation was achieved whether a male of female pronucleus was transferred, hence in the figure both are shown in grey. It was concluded that the Ca^{2+} releasing factor of the sperm resides inside the nucleus or in the perinuclear region and that it could cause Ca^{2+} release when transferred back into a MII oocyte.*

(ER). The ER contains the important Ca^{2+} channels which the sperm-derived Ca^{2+} releasing factor ultimately affects. Therefore if these Ca^{2+} channels have the fertilization derived factors still attached to them then this would explain why oscillations are seen when they are transferred. They have been modified by fertiliza-

tion. Alternatively the Ca^{2+} releasing factor may reside inside the nucleus itself to be released after nuclear envelope breakdown to cause Ca^{2+} release during mitosis. This change in location is similar to many other signaling proteins (Gallant *et al.*, 1995; Crouch and Simson, 1997). It is intriguing to ask the question: does the signaling factor influence the expression of any genes during its time in the interphase nucleus? Maybe this would be done by causing intranuclear Ca^{2+} release at specific times. It is known that intranuclear Ca^{2+} changes do occur in cells and that they can influence gene expression through Ca^{2+}-response elements upstream of certain genes (Hardingham *et al.*, 1997).

The ability of transferred nucleoplasts to cause Ca^{2+} oscillations in recipient MII eggs only works with 1- and 2-cell embryo nuclei, not with those from a 4-cell embryo (Kono *et al.*, 1995). Similarly the fusion of fertilized embryos with MII eggs does not cause egg activation when late 2-cell embryos are used (Zernicka-Goetz *et al.*, 1995). It can only be speculated that the Ca^{2+}-releasing activity residing in the nucleus or perinuclear region has declined to such a low level by the late 2-cell stage or 4-cell stage that it is no longer capable of causing Ca^{2+} release when transferred to a recipient MII egg.

Endogenous change in sensitivity to Ca^{2+} release with the cell cycle

From the above it is clear that Ca^{2+} oscillations in fertilized eggs continue until exit from meiosis has been completed and then are re-initiated during first mitosis. It seems puzzling that in the intervening period of interphase there appears to be no observable Ca^{2+} transients (Jones *et al.*, 1995; Kono *et al.*, 1996). It is tempting to conclude on the basis of the nuclear transfer experiments that the Ca^{2+} releasing factor of sperm is being sequestered into the nucleus and therefore during interphase cannot exert effects on Ca^{2+} levels in the cytoplasm. However if this is true then it does not readily explain why agents such as IP_3 and Sr^{2+}-containing medium which cause oscillations in MII eggs also fail to cause Ca^{2+} oscillations in interphase zygotes (Jones *et al.*, 1995; Kono *et al.*, 1996). The inability of embryos to generate oscillations in response to IP_3 following exit from meiosis is also observed in the ascidian, suggesting a similar cell cycle phenomenon (Albrieux *et al.*, 1997)

The simplest explanation would therefore be that there is an underlying change in sensitivity with the cell cycle. It can only be speculated what is causing this underlying change. The most likely is either some sort of direct modification of the Ca^{2+} channels on the ER responsible for Ca^{2+} release or modification of an accessory factor that binds to these receptors. Calmodulin binds to the ryanodine receptor to affect its ability to release Ca^{2+} in response to cyclic ADP ribose (Lee *et al.*, 1994). There are also binding sites for Ca^{2+}, ATP and other factors on the IP_3 receptor (Bezprozvanny *et al.*, 1991; Bezprozvanny and Ehrlich, 1993; Jayaraman *et al.*, 1996). It is therefore possible that a cell cycle regulated protein may bind these receptors to influence their ability to release Ca^{2+}.

Sr^{2+}- containing medium when added to 1-cell parthenotes in first mitosis induces Ca^{2+} oscillations, in a similar manner to the Ca^{2+} releasing factor of sperm in fertilized 1-cell mitotic embryos (Kono *et al.*, 1996; Bos-Mikich *et al.*, 1997). It has therefore been concluded that to observe Ca^{2+} oscillations in eggs and embryos one needs both an appropriate Ca^{2+} releasing stimulus (for example sperm or Sr^{2+}) and a sensitive Ca^{2+} releasing system (the MII egg or the mitotic one cell embryo). Without the sensitive system (for example in an interphase embryo) oscillations in Ca^{2+} do not occur.

Thimerosal is a thiol modifying reagent which when added to MII eggs causes Ca^{2+} oscillations (Swann, 1991; Cheek *et al.*, 1993). The mechanism of its action remains largely speculative although its ability to cause Ca^{2+} release is not limited to eggs (Bootman *et al.*, 1992). Thimerosal, unlike sperm, IP_3 and Sr^{2+} is able to cause Ca^{2+} oscillations in interphase 1-cell embryos (Jones *et al.*, 1995). It may do this by re-sensitizing the Ca^{2+} channels or channel associated factors through interaction with key thiol containing groups on these proteins. Equivalent changes could well be occurring at fertilization and entry into first mitosis.

Ca^{2+} oscillations drive exit from meiosis and progression through the first mitotic division. It seems possible to bypass both in parthenotes without undue immediate catastrophe. This begs the question: are these oscillations influencing subsequent development?

Evidence against a role for Ca^{2+} oscillations in further development

Parthenogenetically derived embryos do not develop to term because the embryo has not inherited genes from both a paternal and a maternal source (Barton *et al.*, 1984; Barlow, 1995). No manipulation of Ca^{2+} can substitute for genomic shortfalls in parthenotes brought about by imprinting. This is shown by the finding that sperm-like Ca^{2+} oscillations can be induced in MII eggs by replacing the Ca^{2+} in the culture medium with Sr^{2+} (Kline and Kline, 1992) or by providing electrical pulses (Ozil, 1990). For both methods the resulting parthenogenetic embryos do not develop to term. Examining a developmental role for Ca^{2+} in absolute terms is therefore not possible since without both a maternal and paternal contribution there will be no live young

It must therefore be asked whether the Ca^{2+} oscillations seen at fertilization modify any aspect of further development. The effect of Ca^{2+} changes during exit from meiosis and first mitosis must be far acting since it is known that immediate preimplantation development in parthenotes and fertilized embryos is not very different, with a similar time interval between activation and entry into the first S-phase (Abramczuk and Sawicki, 1975) and entry into the first mitotic division (Kaufman, 1983), taking into account the time for sperm penetration. Diploid parthenotes readily progress through the first mitotic division and are seemingly unaffected by a lack of any paternal contribution or Ca^{2+} oscillations that occur in fertilized embryos. Preimplantation development continues to the blastocyst stage in parthenotes but when they are transferred to recipient pseudopregnant females there is a fall off in the implantation rate compared to fertilized blastocysts, for example 50% versus 77% respectively (Kaufman and Gardner, 1974). All parthenotes do not develop past mid-gestation (Kaufman *et al.*, 1977).

Ca^{2+} oscillations and preimplantation development.

So far Ca^{2+} oscillations during exit from meiosis and the first mitotic division have been detailed. As yet mitotic Ca^{2+} transients during the second, third and subsequent mitotic divisions have not been measured directly. They may exist, but in Kono *et al*'s

(1995) experiments the ability of transferred nuclei to cause Ca^{2+} transients ceased at the 4-cell stage. Thus mitotic Ca^{2+} transients, if there, may decrease in amplitude. We need therefore to examine if there is evidence implicating Ca^{2+} changes during exit from meiosis and in 1- and 2-cell embryos with further development.

An indication that Ca^{2+} oscillations during exit from meiosis could effect further development came from the work of Ozil on rabbit eggs (1990). Using an electrical method to provide Ca^{2+} pulses that varied the amplitude of the induced oscillations, he obtained conditions which provided a strong enough stimulus of electrical pulses to give very high rates of activation, i.e. greater than 90%. Eggs treated by these regimens showed no differences between groups in early development over the first few cell cycles. They behaved normally, as one would predict if the role of Ca^{2+} oscillations were merely to complete meiosis and these eggs had done so. However development to the blastocyst was severely compromised in some groups: 33% versus 89% depending on the size of the Ca^{2+} pulses they had received. The eggs exposed to higher amplitude oscillations having the better preimplantation development. Therefore the magnitude of Ca^{2+} oscillations provided to stimulate meiotic resumption were having a marked effect several cell divisions later. The ability of the magnitude of the Ca^{2+} signal (amplitude modulation) to play a role in the signal transduction pathway of Ca^{2+} has recently been reported in B lymphocytes (Dolmetsch *et al.*, 1997). In these cells the amplitude of the Ca^{2+} signal can affect which set of genes are switched on in response to Ca^{2+}, with a low amplitude Ca^{2+} transient switching on the ERK pathway and a much larger Ca^{2+} transient switching on the transcription factors NF-kB and c-Jun. On the basis of the results of Ozil (1990), it is probable that an analogous situation is occurring at fertilization, where the magnitude of the sperm-induced Ca^{2+} oscillations may be affecting the expression of a subset of genes, a process that begins in the 1-cell embryo (Aoki *et al.*, 1997), or the activity of a subset of proteins.

There is further evidence to suggest that changes in intracellular Ca^{2+} early in preimplantation development can have later effects. Low concentrations of ethanol added to 1- or 2-cell mouse embryos for 24h increase the rates of preimplantation development, judged by the rate of blastocyst formation and hatching (Leach et *al.*, 1993). The increased blastocyst formation found by Leach and co-workers using low doses of ethanol was associated with improved development throughout the period of preimplantation suggesting that ethanol was speeding up the rate of cell division. 0.1% ethanol exposed 1-cell embryos had 75.7 ± 21.8 cells at the blastocyst stage while controls had 56.0 ±12.2. This stimulatory effect is not observed when ethanol is added to 4-cell embryos. These effects of ethanol are probably mediated by its ability to raise intracellular Ca^{2+} (Stachecki *et al.*, 1994b). The stimulatory actions of ethanol at low concentrations are probably analogous to the effects of Sr^{2+}-containing medium on intracellular Ca^{2+}. Sr^{2+} induces oscillations when added to 1-cell embryos during the first mitotic division but not during first interphase (Kono *et al.*, 1996). This is due to the cell cycle change in sensitivity already discussed.

Mitotic Ca^{2+} transients and inner cell mass

Kaufman (1983) has proposed that poor implantation and postimplantation development of parthenotes is due to a deficiency in the number cells forming the inner cell mass (ICM). There is evidence that delaying the timing of implantation for parthenotes, and thereby increasing ICM number, can subsequently improve these parameters. The rate of implantation for such delayed parthenotes can increase to 97% in females with decidua (Kaufman, 1983).

One intriguing question is what influences the division of trophectodermal and ICM cells of the forming blastocyst. Haploid parthenotes have a severely compromised developmental capacity which can be alleviated by preventing second polar body extrusion during activation and thereby making them diploid (Kaufman, 1983). Comparisons have been made between haploid parthenotes, diploid parthenotes and fertilized embryos in the ICM and trophectodermal cell number in resulting blastocysts. It is clear that the ICM content is smaller in parthenotes compared to equivalent fertilized blastocysts, as is the ratio of ICM to trophectodermal cells (Hardy and Handyside, 1996; Mognetti and Sakkas, 1996; Bos-Mikich *et al.*, 1997). It may be that the increased ICM of fertilized embryos gives rise to better preimplantation and postimplantation development and is due to the Ca^{2+} oscillations during exit from meiosis and the first embryonic cell division.

There is a suggestion that Ca^{2+} oscillations during exit from meiosis and first cell division can influence the number of ICM cells in a positive manner (Bos-Mikich *et al., 1997*). In this study ethanol activated parthenotes and fertilized embryos gave the lowest and highest number of ICM cells respectively. Exposing embryos to Sr^{2+}- containing medium during either exit from meiosis or the first cell division acted to increase the number of ICM cells. The mechanism by which this occurs is not known but is presumably due to the ability of Sr^{2+}-containing medium to cause oscillations during this period.

It therefore appears that a rise in intracellular Ca^{2+} during either exit from meiosis and/or during the first mitotic division can affect such parameters as rate of blastocyst hatching, extent of blastocyst formation and inner cell mass content. This rise in Ca^{2+} normally takes the form of oscillations with respect to fertilized embryos. These oscillations may be artificially induced in parthenotes by Sr^{2+}-containing medium or a more general rise above basal Ca^{2+} levels may be induced by ethanol or Ca^{2+} ionophores.

Ca^{2+} changes during late preimplantation development

The review has concentrated on Ca^{2+} changes at or near the time of fertilization and the possible effects such changes can have on later development. There is also evidence to suggest that Ca^{2+} changes later on at the morulae stage can affect development in a positive manner. The induction of a very brief (5 min) Ca^{2+} rise in morulae with either low doses of ethanol or the Ca^{2+} ionophore A23187 can speed up the process of cavitation (Stachecki *et al.*, 1994b). In addition to cavitation Ca^{2+} releasing agents can also increase trophoblast outgrowth and migration when blastocysts are allowed to come into contact with fibronectin coated dishes (Stachecki *et al.*, 1994a). The increase in trophoblast outgrowth *in vitro* would seem to have a beneficial consequence *in vivo* since the implantation rate is doubled (39.4% versus 20.8% in untreated) when such treated blastocysts are transferred to recipient females. In addition there is an increase in the rate of development to term. The beneficial effects of the Ca^{2+} releasing agents can be blocked by prior treatment of the Ca^{2+}

buffer BAPTA (Stachecki and Armant, 1996). It may be that under sub-optimal hormone-deficient *in vitro* culture conditions the application of a Ca^{2+} releasing agent mimics an endogenous Ca^{2+} releasing event or environment.

Summary

Mammalian eggs that are fertilized give an intriguing Ca^{2+} profile, with low frequency oscillations lasting several hours. This review argues that the role of such oscillations is to ensure complete activation - that is, release from meiotic arrest. Like most biochemical pathways there probably exists a feedback mechanism, in this case governed directly or indirectly by cell cycle arresting factors, which control the pattern of oscillations. The feedback mechanism ensures oscillations continue when arrest from meiosis is incomplete, but switches off oscillations when meiosis has been completed. In practice this switching off corresponds to entry into embryonic interphase, thus ensuring that oscillations stop at a time when they are no longer needed and may interfere with other cell events. The Ca^{2+} oscillations reappear during the first embryonic division and may have a role in allowing passage through mitosis. Meiosis- and first mitosis-associated Ca^{2+} transients seem to influence the number of cells in the inner cell mass by an as yet ill defined pathway. Ca^{2+} changes seem also to occur during the morula stage and affect cavitation, hatching and implantation of the blastocyst. How these early and late Ca^{2+} changes are associated remains to be investigated. The role of these Ca^{2+} changes may be a non-genomic complement to the influence of paternally imprinted genes in the developing embryo.

The early changes in Ca^{2+} at fertilization are certainly paternally derived (the Ca^{2+} releasing factor inside the sperm) and it may prove to be the case that so are the later changes in Ca^{2+}. It may not be just idle speculation that these changes in Ca^{2+} forced upon the egg by the sperm are entirely analogous to the conflict theory which explains genomic imprinting (Haig, 1996; Moore and Reik, 1996). The analogy being that the paternal Ca^{2+} releasing factor is a non-genomic factor influencing in a positive manner the developmental potential of the embryo. It will be of considerable interest once the exact nature of the paternal Ca^{2+} releasing factor is known whether it plays a part in any aspect of the development of the embryo. It is known that spontaneous Ca^{2+} oscillations, spontaneous in so much as the inducer of the oscillations is unknown, play a part in governing the differentiation of cell types within the developing embryo (Gu and Spitzer, 1995; Ferrari *et al.*, 1996). It may well prove to be that the sperm provides a factor, capable of inducing long lasting Ca^{2+} oscillations in mammalian eggs, that plays a part in many aspects of embryonic development.

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