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Intracellular calcium in the fertilization and development of mammalian eggs

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Summary

1. Mammalian eggs are arrested at metaphase of their second meiotic division when ovulated and remain arrested until fertilized. The sperm delivers into the egg phospholipase Cζ, which triggers a series of Ca^{2+} spikes lasting several hours. The Ca^{2+} spikes provide the necessary and sufficient trigger for all the events of fertilization, including exit from metaphase II arrest and extrusion of cortical granules that block the entry of other sperm.

2. The oscillatory Ca^{2+} signal switches on calmodulin-dependent protein kinase II (CamKII), which phosphorylates the egg-specific protein Emi2, earmarking it for degradation. To remain metaphase II arrested eggs must maintain high levels of Maturation-Promoting Factor (MPF) activity, a heterodimer of CDK1 and cyclin B1. Emi2 prevents loss of MPF by blocking cyclin B1 degradation, a process which is achieved by inhibiting the activity of the Anaphase-Promoting Complex/Cyclosome. CamKII is not however the primary initiator in the extrusion of cortical granules.

3. Ca^{2+} spiking is also observed in mitosis of one-cell embryos, probably because phospholipase Cζ contains a nuclear localisation signal and so is released into the cytoplasm following nuclear envelope breakdown. The function of these mitotic Ca^{2+} spikes remains obscure, although they are not absolutely required for passage through mitosis.

4. Intriguingly the pattern of Ca^{2+} spikes observed at fertilization have an effect on both pre- and post-implantation development in a manner that is independent of their ability to activate eggs. This suggests that the Ca^{2+} spikes which are set in train at fertilization are having effects on processes initiated in the newly fertilized egg but whose influences are only observed several cell divisions later. The nature of the signals remain little explored but their importance is clear and so warrant further investigation.

Introduction

Ca^{2+} is a ubiquitous intracellular signalling molecule,[1] and intracellular Ca^{2+} (Ca^{2+}_i) changes during signal transduction processes have been widely examined over several decades. Fertilization is one such event where Ca^{2+}_i plays a pivotal role.[2-5] In most species a rise in Ca^{2+}_i is induced by the fertilizing sperm and forms the essential trigger for the egg-embryo transition. This is a remarkable feat of conservation considering that eggs of different species arrest at different points in meiosis, or in some species have completed meiosis.[4] Therefore Ca^{2+}_i is likely having effects on diverse signalling pathways at fertilization in eggs of different species.

Mammalian eggs are ovulated while arrested at metaphase of the second meiotic division (MetII). They remain arrested at this stage until fertilized, and if not degenerate. It makes physiological sense to prevent entry into the embryonic cell cycles without sperm, firstly because development to term of such parthenotes is not possible due to the need to have genes of paternal origin in mammals, and secondly to prevent growth of potentially cancerous cells in the female genital tract.

It has long been known that Ca^{2+}_i is both necessary and sufficient for mammalian fertilization to occur.[6] Thus if Ca^{2+}_i rises are blocked all the events of fertilization are inhibited. The term 'egg activation' is used to describe these events and encompass not only cell cycle resumption out of MetII arrest but also the release of cortical granules (CG), which block the entry of other sperm, and any other events associated with the egg-embryo transition.

This review concentrates on recent developments in our understanding of the signalling pathway used by the Ca^{2+}_i signal from the sperm at fertilization to induce release from egg arrest. It then goes on to examine data supporting the hypothesis that Ca^{2+}_i changes are also important for longer term embryo development, several divisions after its well-established meiotic role. The review hints at some ideas as to how these two temporally separate processes may be connected.

What does calcium do at fertilization?

There are two main events which are triggered directly by the Ca^{2+}_i rise at fertilization: CG release, which is responsible for the block to polyspermy; and initiation of cell cycle resumption out of meiosis from MetII arrest. Before examining these processes in any detail it is first worth commenting on the spatiotemporal aspects of the Ca^{2+}_i signal in mammalian eggs. Some of the first Ca^{2+}_i recordings of eggs were performed on species in which a single Ca^{2+}_i rise was observed to pass across the egg from the site of sperm fusion. Embryonic development quickly ensued. In striking contrast when similar studies were performed on mammalian eggs it was clear that sperm induced a series of Ca^{2+}_i rises (hereafter 'spikes') that lasted several hours.[7-9] Figure 1 illustrates the sperm-induced Ca^{2+} signal in mammalian eggs.

The ability of mammalian eggs to respond to sperm with a long lasting Ca^{2+}_i signal suggested that the long stimulus was required to induce the egg to activate properly.

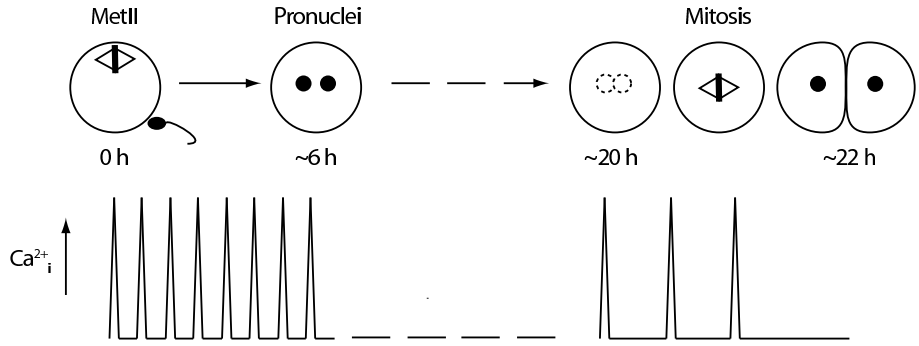

Figure 1. Schematic of Ca^{2+}_i spiking in mammalian eggs. *Met II arrested mammalian eggs show a series of Ca^{2+}_i spikes lasting several hours, until pronuclei form in the 1-cell embryo. Nuclear envelope breakdown at the start of mitosis in the 1-cell embryo is the time at which Ca^{2+}_i spiking is re-initiated. Cessation of spiking is most likely due to nuclear sequestration of PLCζ, which is then released into the cytoplasm during first mitosis.*

This has been borne out by elegant work which has demonstrated that in order to get complete CG release and to induce exit from MetII arrest the egg needs to have experienced multiple Ca^{2+}_i spikes.[10,11] Interestingly fewer spikes are needed to promote CG release than cell cycle resumption[10] but this may make physiological sense if the fertilizing sperm has a more immediate need to block the entry of other sperm than it does to trigger the egg to complete its meiotic division.

The initiator of Ca^{2+}_i release in mammalian eggs is not fully resolved but is a protein delivered into the egg by the sperm. The most likely candidate is a sperm-specific member of the phospholipase C (PLC) family, PLCζ.[12-14] Although other sperm initiating factors have been reported[15-17] they remain to be substantiated and there now appears some consensus that the sperm protein is probably PLCζ acting alone. The generation of a PLCζ knockout mouse would help finally to resolve this issue and is expected within the next few years. Interestingly PLCζ appears to be the initiating factor in mammals and birds,[18] but not in some fish and primitive chordates, suggesting it is a recently evolved PLC member. Regardless of the actual nature of the initiating factor it is clear that the Ca^{2+} spikes at fertilization are driven by inositol trisphosphate production acting on its endoplasmic reticulum receptor ($InsP_3R$).[19-22] PLCζ contains within it a nuclear localisation signal,[14,23,24] which promotes its accumulation in the pronuclei that form at the completion of meiosis II. The nuclear accumulation of PLCζ appears to be the mechanism by which Ca^{2+}_i spikes are terminated[23,25] and accounts for the fact that when pronuclei formation is blocked Ca^{2+}_i spiking continues indefinitely.[26]

Although it has been established for some time that Ca^{2+}_i is required to induce cell cycle resumption, the mechanism by which this is achieved has only recently been described. The most downstream target of Ca^{2+}_i is the kinase MPF (Maturation-Promoting Factor or M-Phase Promoting Factor). MPF activity is high in unfertilized eggs and rapidly falls at fertilization.[27,28] MPF is a dimer but its catalytic subunit CDK1 is without activity unless bound to its regulatory partner, which in mammals is cyclin B1. If degradation of cyclin B1 is prevented then eggs remain MetII arrested,[29] even though sperm have fused and Ca^{2+}_i spiking has been initiated. High MPF activity is not just observed during MetII arrest, but is seen in all eukaryotic cells as they pass through mitosis. In mitosis, the decrease in MPF activity at the metaphase-anaphase transition is mediated by the Anaphase-Promoting Complex/Cyclosome (APC/C), a large multimeric protein complex whose activity is essential for exit from mitosis.[30] By the nature of its large multisubunit size APC/C is likely subject to a number of control points in its activity; the most described being the spindle assembly checkpoint (SAC). SAC components are members of the Mad and Bub family as well as MpsI kinase.[31] These act to inhibit APC/C activity before full congression and microtubule attachment of chromosomes on a metaphase spindle. In this way mis-segregation of chromosomes is avoided at anaphase. Although there is evidence that in frog MetII arrest may be achieved by components of the SAC family inhibiting APC/C, in mammals this mechanism appears less important.[32] Recently a novel APC/C inhibitor Early Mitotic Inhibitor I (Emi1) was established as being required to prevent premature APC/C activity as cells enter mitosis.[33,34] A related protein Emi2/Erp1 (Emi1-related protein 1), was then later identified, found to be egg specific, and now seems to be the likely target of Ca^{2+}_i action.[35-38]

Loss of Emi2 at fertilization is achieved through phosphorylation by calmodulin-dependent protein kinase II (CamKII). This fits with the observation that CamKII activity increases at fertilization on a Ca^{2+}_i signal,[39,40] and also constitutively-active CamKII induces cyclin B1 degradation and so cell cycle resumption from MetII arrest.[41] This phosphorylation of Emi2 by CamKII creates a docking site for polo kinase which further phosphorylates Emi2 and this second phosphorylation acts as a trigger for Emi2 loss, through its polyubiquitination by the E3 ligase Skpl-Cullin/F-box protein. This mechanism of degradation thus far has been demonstrated only in frog eggs.[35-38]

However Emi2 appears to be physiologically relevant in mammals for MetII arrest, since when this protein is knocked down in MetII arrested eggs, it induces them to parthenogenetically activate.[42] Furthermore when its synthesis is prevented during oocyte maturation, the oocytes fail to arrest at MetII.[43] Tagging both cyclin B1 and Emi2 with different fluorescent proteins and imaging their degradation simultaneously in the same egg, reveals that Emi2 loss occurs ahead of cyclin B1.[43] Therefore the basic signal transduction pathway which operates at fertilization is likely to be activation of the APC/C through loss in Emi2, stimulated by CamKII phosphorylation. Activation of the APC/C induces degradation of cyclin B1, so MPF falls and oocytes can then resume their second meiotic division.

Interestingly CG release, which occurs ahead of cell cycle resumption, although Ca^{2+} dependent appears not to involve CamKII.[44] Thus a constitutively active mutant of CamKII, although able to induce cell cycle resumption, does not produce the extent of CG release observed with sperm. This suggests that a second, independent signalling pathway is used which is most likely to involve myosin light chain kinase (MLCK), since inhibition of MLCK, blocks CG release.[45]

What does calcium do at first mitosis?

By the time pronuclei form in the 1-cell embryo it is committed to passage through the first cell cycle with S-phase starting shortly after pronuclei are observed. Due to sequestration of PLCζ into the pronuclei, Ca^{2+}_i spiking also stops at around this time.[14,23-26] One would assume that the Ca^{2+}_i spiking has now performed its task and can be disregarded in respect of embryo development. However this is not the case.

It was shown some years ago that a transferred nucleus from a fertilized one- or two- cell embryo had the ability to induce Ca^{2+}_i spiking and so activate an unfertilized MetII egg following fusion of the two.[46,47] However when the fusion experiment was performed with the pronucleus of a parthenote no egg activation from MetII arrest was seen. These observations are now easily explained by the nuclear sequestration of PLCζ into the pronuclei of the fertilized embryo, and its obvious absence from the pronuclei of parthenotes. The fact that PLCζ is stable enough in the pronucleus during the first two cell divisions, also readily explains the observation that Ca^{2+}_i spiking is seen again during the first mitotic division of fertilized embryos but not parthenotes.[48] Presumably during mitosis PLCζ is released into the cytoplasm where it can generate inositol trisphosphate and so Ca^{2+}_i spikes. These spikes however appear non-essential for passage through mitosis because if they are blocked by Ca^{2+} buffers then mitosis proceeds with normal timings.[49] Furthermore parthenotes do not show these spikes and yet readily undergo mitosis, confirming the non-essential nature of Ca^{2+}_i in the mitotic division.

There are however caveats in dismissing the importance of Ca^{2+}_i spikes to the first mitotic division. The first is that raising Ca^{2+} in G2 embryos accelerates entry into mitosis,[49] suggesting that although Ca^{2+} is non-essential it can actually affect passage through this cell cycle transition. In sea urchin embryos Ca^{2+}_i does play an essential role, as shown by the block to anaphase when Ca^{2+}_i changes are buffered.[50] In mammals one could argue that Emi2 should be absent at first mitosis since it is degraded on exit from MetII, and if so passage through mitosis dictated by activation of the APC/C would not be dependent on Ca^{2+}_i as it is during MetII arrest. Emi2 levels have not been assessed during the first mitotic division, however they do increase again following the cessation of Ca^{2+}_i spikes at pronucleus formation,[42,43] making it possible that Emi2 is actually present during the first mitotic division. This stimulates the question of what strategies the embryo uses to degrade Emi2, because presumably this must happen in order for the APC/C to be active during the first mitotic division. So far only a Ca^{2+} dependent mechanism of Emi2 degradation has been described. Future studies are therefore required to assess the way in which the embryo controls Emi2 activity.

What does fertilization-associated calcium do for longer–term development?

Single monotonic rises in Ca^{2+}_i are generally poor activators of mammalian eggs. Eggs may partially complete their second meiotic division, so extrude a polar body, but re-arrest at a new metaphase and stay arrested without the formation of a pronucleus.[51] This is likely due to poor or transient degradation of Emi2, whose activity comes back and inhibits the APC/C allowing cyclin B1 reaccumulation and MPF activity. The general exception to this is the ability with a single Ca^{2+}_i rise to activate eggs that have passed the window of their normal *in vivo* time of fertilization.[52] Here this is best explained by the aged eggs being less able to maintain high MPF levels. Thus the essential nature of the oscillatory Ca^{2+}_i signal may well be to provide a long enough signal in which to ensure the egg escapes meiosis.[53,54] This cannot however be the sole function of Ca^{2+}_i because differing regimens of experimentally-induced Ca^{2+}_i spiking designed to give very high rates of egg activation, do not all induce good quality embryo development.[10,55-60]

In one study eggs were incubated in Sr^{2+}-containing medium to experimentally induce spiking in Ca^{2+}_i for varying periods up to 24 h.[60] Here Sr^{2+} is acting as a Ca^{2+} mimetic on the egg $InsP_3R$, which in terms of channel opening contains both activating and inhibitory Ca^{2+} binding sites. Importantly Sr^{2+} is very much less (600-fold) potent than Ca^{2+} in inhibiting the $InsP_3R$, so effectively sensitizes the receptor.[61] It was shown that eggs incubated for varying times in Sr^{2+}-containing medium to produce high rates of egg activation, go on to form blastocysts with different numbers of inner cell mass cells and trophectodermal cells.[60] The embryos with the least inner cell mass cells, which go on to form all the embryonic structures, were those exposed to Sr^{2+} for the least amount of time. The regime which produced embryos that were

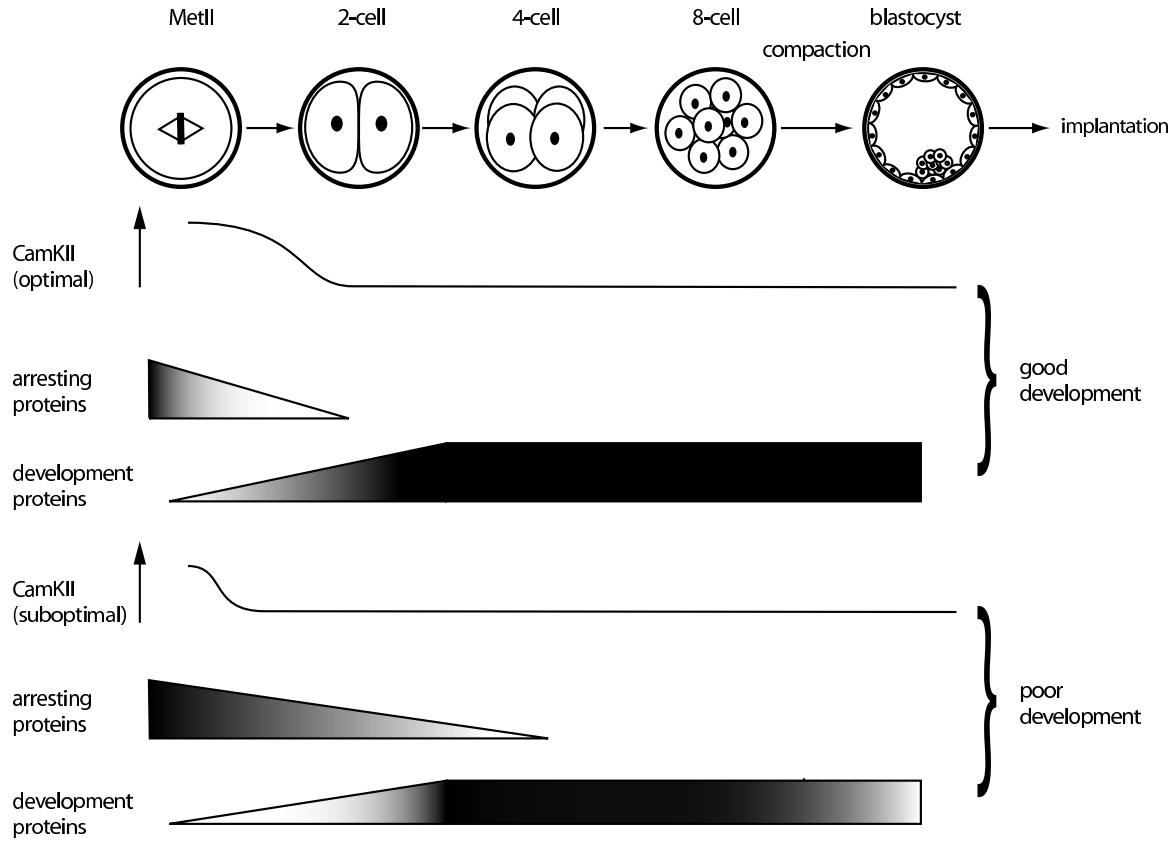

Figure 2. Schematic model of how early events of fertilization can influence embryo development. *Some of the stages of pre-implantation development are depicted leading up to implantation. At fertilization, Ca^{2+}_i spiking switches on CamKII, which has an important role to play in promoting loss, probably through degradation, of a number of proteins responsible for arresting the egg at MetII before fertilization, and also in promoting the synthesis of a number of new embryonic proteins through either transcription or translation. Suboptimal CamKII activation may influence embryo development by hindering both these processes. See text for further details.*

most similar in composition to fertilized eggs, were those placed in Sr^{2+} media for 24 h; this period encompassing meiotic exit and first mitosis, both periods in which Ca^{2+}_i spiking is normally observed. More elegant studies have been performed by Jean-Pierre Ozil and his collaborators, using an activation chamber in which eggs can be exposed to a series of artificially induced Ca^{2+}_i spikes of varying amplitude and duration.[62,63] Using such a chamber in which spike frequency and duration are altered it is clear that for protocols which all give high rates of egg activation longer term development is not uniform, with the quality of postimplantation development falling off dramatically for some Ca^{2+}_i spiking regimes.[10,56-59]

At present it is not resolved how Ca^{2+}_i spiking at fertilization, and possibly first mitosis, are having their effects several cell divisions later. It is proposed here that there may be two possibilities (summarized in Figure 2). The first is that a suboptimal Ca^{2+}_i signal fails to stimulate fully the degradation of proteins whose function is essentially to help maintain MetII arrest and whose presence could hinder the cell divisions of the embryo. Given that MetII arrest is so successful, for good physiological reason, and so protracted, it is likely that other signalling pathways are recruited to maintain arrest that are independent of Emi2. One such pathway is likely to be the mos…MAPKinase pathway. In mouse loss of mos, a MAPKinase Kinase Kinase, allows eggs to escape arrest after just a few hours.[64] Furthermore MetII arrest in frog eggs appears to involve more than one signalling pathway.[65] Therefore maintenance of arrest at MetII may involve multiple pathways all of which need to be successfully downregulated for optimal embryonic cell division.

The second possibility is that the Ca^{2+}_i signal needs to switch on the expression of various proteins, whose early expression is needed for good embryo development. This hypothesis is supported by the observations that the number of Ca^{2+}_i spikes experienced by an activating egg can influence the expression of new proteins in the early embryo[10] and that new protein synthesis is required for zygotic genomic activation.[66] In regard of the ability of Ca^{2+}_i to influence protein expression, it is important to note that CamKII can phosphorylate and so switch on cytoplasmic polyadenylation element binding protein (CPEB)[67,68] to stimulate protein expression in hippocampal dendrites through increased mRNA polyadenylation. Given the importance of CPEB in the translational efficiency of

certain mRNA's in oocytes before fertilization[69] it is tempting to speculate that Ca^{2+}_i activated CamKII is also able to affect protein expression through CPEB in eggs. Zygotic transcription begins much earlier than previously thought during the 1-cell embryo stage[70-72] so it remains possible that CamKII is involved in the expression of zygotic proteins. Of course these two possibilities for how Ca^{2+}_i has long-term effects are not mutually exclusive: Ca^{2+}_i could be both involved in stimulating the expression of nascent proteins (e.g. through CPEB) and also in degrading others (e.g. Emi2). Assuming both processes are reliant on Ca^{2+}_i activation of CamKII, then such hypotheses makes the actual dynamics of the signal important, since CamKII activation by Ca^{2+}_i is sensitive to frequency and amplitude.[73,74]

Conclusions

Knowledge of how the oscillatory Ca^{2+}_i signal at fertilization is initiated and the downstream signalling pathways it affects have been elucidated over recent years. The establishment of CamKII as an important transducer of Ca^{2+}_i action in cell cycle resumption opens the possibility of solving the phenomenon of how Ca^{2+}_i can have a much longer term effect on embryo development, several cell divisions later, from its established role in exit from MetII arrest. We may find that optimal embryo development is made possible in eggs by the CamKII-mediated switching on of genes through transcription and/or translation during fertilization combined with the stimulated degradation of proteins required in the protracted cell cycle arrest at MetII.

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