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Review

The roles of Ca^{2+}, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development

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Abstract

Reviews in *Developmental Biology* have covered the pathways that generate the all-important intracellular calcium (Ca^{2+}) signal at fertilization [Miyazaki, S., Shirakawa, H., Nakada, K., Honda, Y., 1993a. Essential role of the inositol 1,4,5-trisphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. *Dev. Biol.* 158, 62–78; Runft, L., Jaffe, L., Mehlmann, L., 2002. Egg activation at fertilization: where it all begins. *Dev. Biol.* 245, 237–254] and the different temporal responses of Ca^{2+} in many organisms [Stricker, S., 1999. Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* 211, 157–176]. Those reviews raise the importance of identifying how Ca^{2+} causes the events of egg activation (EEA) and to what extent these temporal Ca^{2+} responses encode developmental information. This review covers recent studies that have analyzed how these Ca^{2+} signals are interpreted by specific proteins, and how these proteins regulate various EEA responsible for the onset of development. Many of these proteins are protein kinases (CaMKII, PKC, MPF, MAPK, MLCK) whose activity is directly or indirectly regulated by Ca^{2+}, and whose amount increases during late oocyte maturation. We cover biochemical progress in defining the signaling pathways between Ca^{2+} and the EEA, as well as discuss how oscillatory or multiple Ca^{2+} signals are likely to have specific advantages biochemically and/or developmentally. These emerging concepts are put into historical context, emphasizing that key contributions have come from many organisms. The intricate interdependence of Ca^{2+}, Ca^{2+}-dependent proteins, and the EEA raise many new questions for future investigations that will provide insight into the extent to which fertilization-associated signaling has long-range implications for development. In addition, answers to these questions should be beneficial to establishing parameters of egg quality for human and animal IVF, as well as improving egg activation protocols for somatic cell nuclear transfer to generate stem cells and save endangered species.

Keywords: Fertilization; Egg; Protein kinase; Signaling; Calcium; Cell cycle; Meiosis

Introduction

In recent years, substantial progress has been made in identifying the function, changes in activity, and relative contributions of egg protein kinases (PKs), as well as in beginning to understand how they are regulated by fertilization-associated changes and oscillations in the concentration of the intracellular calcium ions ($[Ca^{2+}]_i$). In addition, the upstream regulators and downstream effectors of these PKs are now under investigation in fertilized eggs. Results of these studies are beginning to fill in gaps in the signaling pathway between the elevation of $[Ca^{2+}]_i$ and events of egg activation (EEA). EEA includes cortical granule (CG) exocytosis, block(s) to polyspermy, cell cycle resumption, and recruitment of maternal mRNAs (Schultz and Kopf, 1995). In addition, given the importance of PK activity and function after fertilization, post-translational protein phosphorylation changes have become increasingly important suspects in "connecting the dots" in the signaling pathway between elevated $[Ca^{2+}]_i$ and the EEA.

Most reviews have focused on the fascinating stories of signaling upstream of the elevation of $[Ca^{2+}]_i$ (Whitaker and

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Swann, 1993; Jones, 1998a; Ciapa and Chiri, 2000; Carroll, 2001; Runft et al., 2002; Williams, 2002; Lee et al., 2006a,b; Malcuit et al., 2006; Miyazaki, 2006; Swann et al., 2006; Townley et al., 2006; Whitaker, 2006). However, relatively little attention has been paid to integrating information about PKs acting downstream of the increase in $[Ca^{2+}]_i$ into a unified picture of their temporal, spatial, and functional (and in some cases, interdependent) roles in initiating development. Oocyte maturation-associated roles of PKs in many organisms are reviewed by Voronina and Wessel (2003).

Many organisms have their own temporal pattern of the elevation of $[Ca^{2+}]_i$ at fertilization (Stricker, 1999; Miyazaki, 2006). The relevance of Ca^{2+} oscillation parameters (Ozil, 1990; Ozil and Huneau, 2001; Ducibella et al., 2002, 2006; Ozil et al., 2005, 2006) raises new questions about PK activity and regulation since many egg PKs are directly or indirectly dependent upon $[Ca^{2+}]_i$. Are Ca^{2+}-dependent PK activities oscillatory? During oscillations, is the protraction of Ca^{2+} signaling and PK activity over a long period of time biochemically advantageous and developmentally relevant? Or is it an insurance policy for egg activation? How is it regulated? Recent studies have begun to shed some light on these questions and raise the prospect that the temporal and spatial behavior of PKs at fertilization has long-range consequences for mammalian development.

The big picture of Ca^{2+}, PKs, and EEA

The increase of $[Ca^{2+}]_i$ at fertilization is considered to be a universal requirement for initiating animal development. As in other cell types, $[Ca^{2+}]_i$ can regulate the activity of proteins using several mechanisms. First, some proteins have dedicated Ca^{2+} binding (C2) domains, which, when occupied, are involved in activating the protein's function. Conventional protein kinase C isoforms (cPKC) represent this group, although activation is also associated with the binding of diacylgylcerol (DAG). Synaptotagmin, a Ca^{2+}-sensitive C2 domain containing protein, regulates exocytosis in many cells, including eggs (Leguia et al., 2006). A second mechanism of Ca^{2+} action is to form a complex with EF hand proteins, such as calmodulin (CaM), which bind to and activate PKs, like Ca^{2+}/CaM-protein kinase II (CaMKII) and myosin light chain kinase (MLCK). Some proteins (e.g., myosin light chains) have their own EF hand domains. A third, indirect mechanism is to regulate PK activity by activating specific phosphatases, several of which are Ca^{2+}-dependent. Finally, the amount of PKs and other proteins, such as "maturation- or M-phase-promoting factor" (MPF) and separase, is indirectly controlled by Ca^{2+}-dependent ubiquitination after fertilization, followed by proteolysis. A sampling of Ca^{2+}-dependent proteins can be found in the sea urchin genome (Roux et al., 2006; Fig. 1, Supplementary Table 2).

As will be discussed in more detail below, these Ca^{2+}-dependent mechanisms regulate egg PK activities, which in turn control the progress of egg activation events. Fig. 1 demonstrates a simple overview of how specific PKs are positioned between the increase in $[Ca^{2+}]_i$ and important EEA. PKC and MAPK (ERK 42/44) activities appear to have targets involved in promoting the normal duration and pattern of the Ca^{2+} oscillations themselves. MAPK and MPF are the gatekeepers of the cell cycle. CaMKII activity promotes cell cycle resumption by stimulating the decrease in MPF, and other functions for CaMKII have been proposed at fertilization based on experimental evidence and its large number of target proteins from studies of many cell types. CaMKII, synaptotagmin, and MLCK have been implicated in CG exocytosis in fertilized eggs.

The effects of changes and oscillations of $[Ca^{2+}]_i$ on these PKs and the mechanisms by which each PK regulates the above EEA are fascinating and emerging stories. Before addressing recent advances in Ca^{2+}-regulated PK function at fertilization, it is important to describe the egg intracellular Ca^{2+} environment that leads to specific predictions about changes in PK activity.

Elevation of egg $[Ca^{2+}]_i$: time, space, and amount are important

Although this review focuses on mammals, it is important to give credit to the historical importance of eggs of other organisms to the entire field of egg activation. Mazia in 1937 reported the increase in $[Ca^{2+}]_i$ in lysates of fertilized sea urchin eggs. Four decades later, using a new Ca^{2+} probe (aequorin) that Shimomura and Johnson (1970) extracted from jellyfish, Ridgway et al. (1977) reported that "Free calcium increases explosively in activating Medaka eggs" (also see Yamomoto, 1954). The fertilization field is indebted to sea urchins, jellyfish, and teleost fish, just as the cell cycle field is to the surf clam (Swenson et al., 1986), starfish (Labbe et al., 1989), and sea urchin (Evans et al., 1983)—where eggs also played a key role. Some highlights of the intertwined history of Ca^{2+} and the cell cycle are presented in the timelines in Fig. 2.

Evidence that intracellular stores of Ca^{2+} were sufficient for egg activation came from (Ca^{2+}) ionophore experiments by Steinhardt et al. (1974). Soon thereafter, a wave of intracellular Ca^{2+} that originated from the point of sperm penetration and appeared to traverse the entire volume of the egg was revealed in several marine organisms (Gilkey et al., 1978; Eisen et al., 1984). Subsequent studies in other species, including many mammals, confirmed the generality of these findings (Stricker, 1999; Runft et al., 2002; Miyazaki, 2006; Whitaker, 2006). The discovery of the Ca^{2+} wave traversing the entire cytoplasm was important since it could help to explain the activation of different events in different locations within such a large cell. For example, CG exocytosis occurs in the cortex, whereas the completion of meiosis takes place under the cortex and at the pole of the mammalian egg usually opposite the site of sperm penetration (Johnson et al., 1975; Talansky et al., 1991), relatively distant from the origin of the Ca^{2+} wave. The spatial and temporal control of the $[Ca^{2+}]_i$ appears to be regulated by Ca^{2+} wave pacemakers in eggs (Sardet et al., 1998; Dumollard et al., 2002).

In the sea urchin, Zucker and Steinhardt in 1979 demonstrated Ca^{2+}-mediated activation of CG exocytosis as workers in this field were cataloguing the EEA that depended upon the

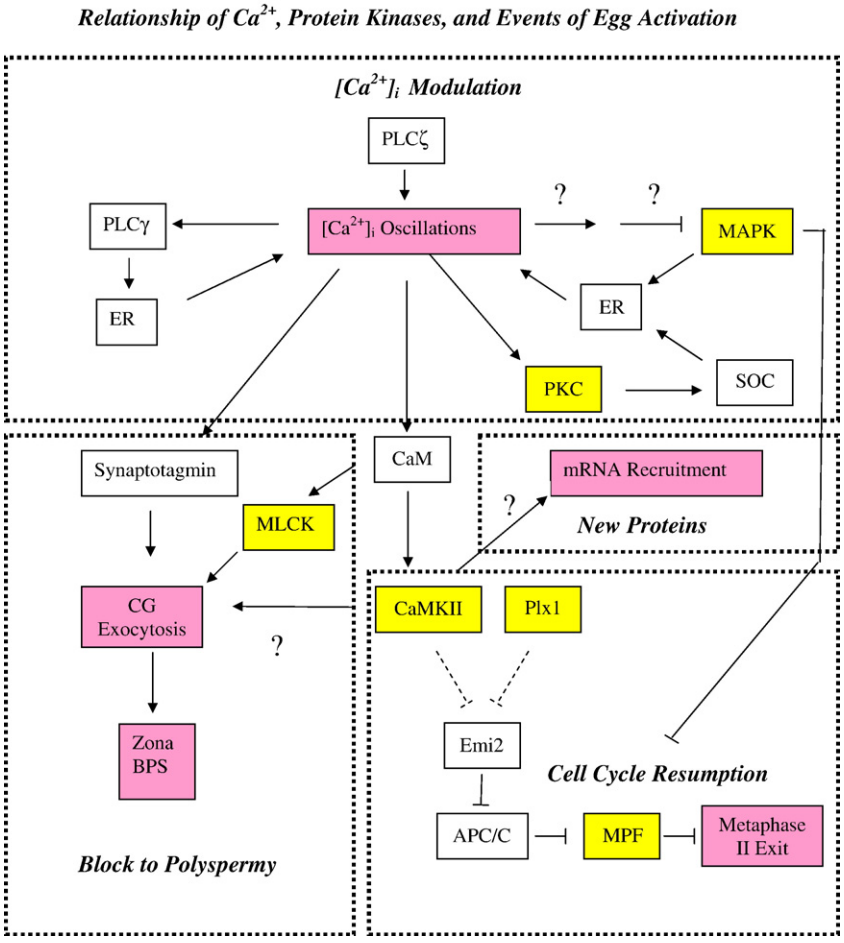

Fig. 1. Simplified model of the relationship of Ca^{2+} signaling to protein kinases (yellow boxes) and the events of egg activation (pink boxes). For some organisms, the box with "$[Ca^{2+}]_i$ oscillations" should be replaced with a box indicating a single rise in $[Ca^{2+}]_i$. In this case, some downstream signaling components may change or have not been investigated. The PLCζ isoform applies to mammalian eggs (Saunders et al., 2007; Miyazaki and Ito, 2006). The isoforms of PKC and other proteins are not shown. Abbreviations: APC/C—cyclosome, BPS—block to polyspermy, CaM—calmodulin, CaMKII—Ca^{2+}/CaM-dependent protein kinase II, CG—cortical granule, ER—endoplasmic reticulum, Emi2—early mitotic inhibitor 2, PLC—phospholipase C, MAPK—MAP kinase, MLCK—myosin light chain kinase, MPF—maturation or M-phase promoting factor, PKC—protein kinase C, SOC—store-operated channels (see text for additional information).

$[Ca^{2+}]_i$ (Epel, 1989). It was clear that the elevation of the $[Ca^{2+}]_i$ was one of the earliest events, making it potentially causal for many EEA. With the availability and size of *Xenopus* eggs for studies of MPF and fertilization, lines of research on the cell cycle and Ca^{2+} were bound to intersect. In 1988, Kline demonstrated that intracellular buffering of the $[Ca^{2+}]_i$ with BAPTA in *Xenopus* eggs prevented depolarization, CG exocytosis, and formation of pronuclei (PN); 4 years later his laboratory obtained similar results for CG exocytosis and metaphase II exit in BAPTA-treated, fertilized mouse eggs (Kline and Kline, 1992). Interestingly, sperm chromatin decondensation did not appear to be Ca^{2+}-dependent.

Another chapter in the story of Ca^{2+} and fertilization commenced with evidence that mammalian eggs underwent sperm-induced oscillations in the $[Ca^{2+}]_i$ (Cuthbertson et al., 1981; Miyazaki and Igusa, 1981), while amphibians and sea urchins have a single transient rise in the $[Ca^{2+}]_i$. In mammals, the first Ca^{2+} rise was of longer duration and higher amplitude than subsequent increases, all of which appeared to traverse the egg cytosol (Miyazaki et al., 1993a). This induced a large body of research to determine the upstream causes of these IP_3- and sperm-dependent oscillations of $[Ca^{2+}]_i$ (Swann, 1990; Miyazaki et al., 1993a,b; Kline and Kline, 1994; Saunders et al., 2002; Introduction). Equally important, these periodic increases begged the question of the physiological significance of oscillatory Ca^{2+} signaling, which led to discoveries by the Ozil laboratory that oscillation parameters can influence not only the EEA, but also peri-implantation gene expression and development (Ozil, 1990; Ozil and Huneau, 2001; Ozil et al., 2006).

Mammals proved not to be an isolated case of an unusual temporal profile of $[Ca^{2+}]_i$ over time as other studies demonstrated that eggs of many different organisms exhibit their own Ca^{2+} "signature" at fertilization (Stricker, 1999; Miyazaki, 2006). Of those explored to date, a few have a single, rather symmetrical curve of rising and falling $[Ca^{2+}]_i$ at fertilization. In contrast, many organisms, including worms, mollusks, and mammals, have an oscillatory pattern of $[Ca^{2+}]_i$ over time. In other cases, there is an initial relatively large increase in the amplitude and duration of $[Ca^{2+}]_i$ whose falling phase is longer

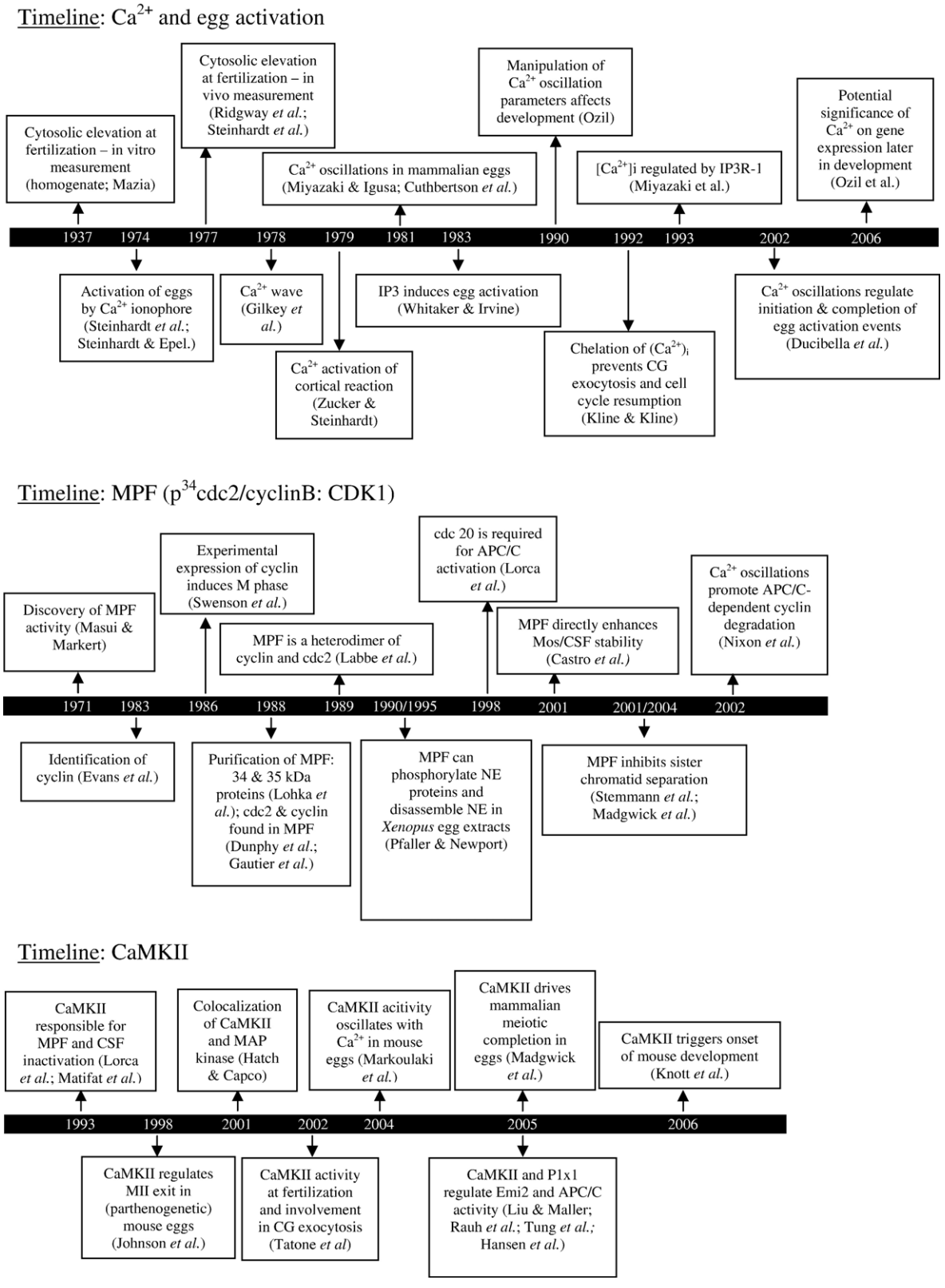

Fig. 2. Parallel timelines of major events in the history of discovery of the importance of Ca^{2+}, MPF (maturation or M-phase promoting factor), and CaMKII (Ca^{2+}/CaM-dependent protein kinase II), all of which regulate cell cycle resumption in eggs arrested in metaphase II of meiosis at the time of fertilization. Due to space limitations, all references could not be included. Undated references (not in the text): Castro et al., 2001; Hatch and Capco, 2001; Lorca et al., 1998; Matifat et al., 1997; Pfaller and Newport, 1995; Steinhardt and Epel, 1974; Steinhardt et al., 1977; Stemmann et al., 2001; Whitaker and Irvine, 1984.

than the rising phase, and, in some of these eggs, minor oscillations follow. Even in many of those eggs with a single symmetrical Ca^{2+} response, oscillations follow quickly during the rapid cleavage divisions. How these profiles came about evolutionarily and whether they have specific benefits for each type of organism remains largely to be determined. There does not appear to be a tight correlation between a type of profile and the stage of meiotic arrest at the time of fertilization (Stricker, 1999). However, there is a relationship between Ca^{2+} oscillation parameters and cell cycle time in mammalian eggs, which has interesting implications for the regulation of PK activity. Before discussing this, it is first relevant to review the PKs that control the egg cell cycle, especially in light of important recent advances in that field.

MPF and CaMKII

Although it had been known that $[Ca^{2+}]_i$ and MPF activity were in some way involved in metaphase regulation of meiosis II in vertebrate eggs (reviewed by Masui, 2000), the detailed connection/pathway between Ca^{2+} and MPF was revealed only recently, as a number of obstacles stood in the way. Although MPF activity was reported in 1971 (Masui and Markert, 1971), purification of its components proved difficult and there was still the matter of MPF regulation via cytostatic factor (CSF) activity, which maintained elevated MPF activity. In the realm of Ca^{2+} research, there were different puzzles. Despite the growing evidence for the universality of Ca^{2+} signaling at fertilization, researchers had to grapple with the failure to detect an increase in $[Ca^{2+}]_i$ during the metaphase to anaphase transition in meiosis I in mouse oocytes (Tombes et al., 1992), and also whether Ca^{2+} was a trigger or modulator for mitotic anaphase (Tombes and Borisy, 1989), despite the fact that these transitions also utilized MPF for M-phase regulation.

Intensive research on cell cycle regulatory proteins in eggs of amphibians and marine organisms, with their large size and/or abundance, assembled major pieces of the MPF and CSF "puzzle", which is still in progress. An early step forward came with the discovery of cyclin, its functional activity, and its heterodimeric partner, the protein kinase CDK1 ($p34^{cdc2}$) (Evans et al., 1983; Swenson et al., 1986; Dunphy et al., 1988; Gautier et al., 1988; Labbe et al., 1989; Lohka et al., 1988). Using *Xenopus* egg extracts in 1993, Lorca et al. provided elegant experimental evidence that the decrease in MPF activity and cyclin destruction were dependent upon the activity of CaMKII rather than PKC (using constitutively active PK constructs). Furthermore, CaMKII activity accounted for the activating effect of Ca^{2+}. Thus, an elevated $[Ca^{2+}]_i$ could stimulate the resumption of the vertebrate egg cycle at fertilization through CaMKII-mediated downregulation of MPF activity. But how did CaMKII activity lead to cyclin destruction?

In vertebrate eggs, the key was to decipher the regulatory controls on the cyclosome (APC/C), the E3 ubiquitin ligase complex that molecularly earmarks cyclin for destruction. As evidence accumulated that the functional status of APC/C could account for cell cycle arrest (reviewed in Tunquist and Maller, 2003; Schmidt et al., 2006; Liu et al., 2007), the APC/C was considered as a type of information processing center for inputs from CSF, MAPK, cell cycle check points, and Ca^{2+}; i.e., the APC/C was the "decider", regarding whether or not the egg would exit meiotic metaphase II arrest to undergo normal development or a meaningless aborted mission of parthenogenesis.

Recently, it was discovered that those proteins most influential and closest to the APC/C were regulated by Ca^{2+}'s old friend, CaMKII, at fertilization. When not faced with fertilization, the APC/C is normally relatively quiescent due to inhibition by a cell cycle regulatory protein. Early mitotic inhibitor 2 (Emi2) (Tung et al., 2005), despite its name, is essential for meiotic but not mitotic cell cycles (Liu et al., 2006). There was also evidence that another PK, Plx1, was involved in egg activation and might act through removing an APC/C inhibitor (Descombes and Nigg, 1998). However, Plx1 was already active in unactivated eggs.

The breakthrough came when three groups reported that APC/C inhibition by Emi2 is lifted in a CaMKII- and Plx1-dependent manner (Liu and Maller, 2005; Rauh et al., 2005; Hansen et al., 2006). Active CaMKII phosphorylated Emi2, creating a docking site for Plx1, which in turn generated a second phosphorylation in Emi2. This second site ear-marked Emi2 for degradation, relieving inhibition on the APC/C. This elegant PK-driven mechanism has similarities to coincidence detector mechanisms that keep major cellular decisions on hold, until several different inputs arrive. Emi2 is made just before metaphase II arrest (Tung et al., 2007) when the egg also develops the ability to undergo normal, maximal elevation of its $[Ca^{2+}]_i$ (Mehlmann and Kline, 1994). CaMKII is activated by the elevation of $[Ca^{2+}]_i$ at fertilization, creating an appropriately phosphorylated target for Plx1.

While these discoveries were made in amphibian eggs, studies of mouse eggs indicated that mammals had not only conserved this pathway but appeared to rely on it heavily since other CSF pathways were not detected (Madgwick and Jones, 2007). Oscillations in $[Ca^{2+}]_i$ promoted APC/C-dependent cyclin B1 degradation (Nixon et al., 2002). By immunofluorescence, CaM and CaMKII were reported to be associated with the metaphase II spindle (Johnson et al., 1998). Antagonists of CaMKII were found to inhibit cell cycle progression in permeabilized (Johnson et al., 1998), parthenogenetically activated (Tatone et al., 1999), and fertilized (Markoulaki et al., 2004) mouse eggs, accompanied by a transient decrease in MPF activity (Tatone et al., 2002). Moreover, in the absence of an elevated $[Ca^{2+}]_i$, expression of constitutively active αCaMKII caused metaphase II exit, cyclin B1 and securin destruction, the decreases in the activities of MPF and MAPK, second polar body extrusion, and formation of female PN (Madgwick et al., 2005; Knott et al., 2006).

During mouse oocyte maturation, Emi2 levels appear to increase and Emi2 morpholinos prevent cyclin B1 accumulation and normal metaphase II spindles (Madgwick et al., 2006). Metaphase II eggs undergo parthenogenetic activation after ablation of Emi2 (Shoji et al., 2006). Emi2 degradation is stimulated by Ca^{2+} and precedes that of cyclin B1 (Madgwick et al., 2006; Jones, 2007). These results make it likely that in the

mouse, the release of inhibition on the APC/C also occurs through removal of Emi2 via the activities of CaMKII and Plx1.

Mouse and amphibian eggs appear to diverge somewhat in terms of the pathways implicated in CSF activity (Madgwick and Jones, 2007). Although *Xenopus* utilizes a c-mos pathway-dependent spindle checkpoint, monitored by the Mad and Bub proteins, at least one important PK component of this pathway, rsk90, appears to be missing in mouse eggs (Dumont et al., 2005). Also, the cyclin E/Cdk2 pathway, a component of CSF in amphibians (Tunquist and Maller, 2003), has not been identified in mammalian eggs. Despite the evidence for a c-mos/MAPK pathway constituting a component of CSF activity in mice, its downstream molecular players and impact on the APC/C remain to be identified (see Fan and Sun, 2004; Jones, 2004, 2005; Brunet and Maro, 2005).

In summary, our present understanding is that the primary pathway that signals the resumption of meiosis II is normally driven by the collaboration of least three different PKs. However, these PKs do not work in a linear series typical of a classical signaling cascade. CaMKII and Plx1 act in parallel on Emi2 to relieve inhibition of the APC/C, resulting in enhanced cyclin B destruction and downregulation of the activity of MPF. As discussed below, other PKs provide feedback to enhance the Ca^{2+} signal, indicating that a network of PKs is actually responsible for reactivation of the cell cycle in fertilized vertebrate eggs (see Fig. 1).

PKC

cPKCs (conventional PKCs) appeared to be ideal transducers of second messenger signals at fertilization. The dependence of elevation of $[Ca^{2+}]_i$ on IP_3 and its receptor (Miyazaki et al., 1993a,b; Dupont et al., 1996; Brind et al., 2000; Jellerette et al., 2000; Xu et al., 1994) implied PIP_2 hydrolysis with the resulting generation of DAG. Ca^{2+} and DAG are well-established activators of some isoforms of PKC that allow it to function similar to a coincidence detector. Not unexpectedly, evidence accumulated that PKC played important roles in egg activation, such as CG exocytosis, metaphase II exit, polar body formation, formation of PN, spindle dynamics, and cytoskeletal rearrangements.

As reviewed by Jones (1998b) and Halet (2004), some of these roles should be re-examined due to the reliance on pharmacological activators, parthenogenetic activation, and a variety of different inhibitors, which in some cases generated different results. Moreover, the status of $[Ca^{2+}]_i$ was often not concurrently studied, DAG production was not detected during fertilization in mouse eggs (Halet et al., 2004), PKC isoform status was only recently studied, and PKC enzymatic activity (discussed later) was often not reported. Although there is not sufficient space here for a complete discussion, one fundamental example is the over-reliance on the synthetic DAG, phorbol ester (also called PMA or TPA). There are reports of PMA both augmenting and damping oscillations of $[Ca^{2+}]_i$ in eggs, depending on the mammalian species (see Jones, 1998b). In addition, PMA induces CG exocytosis in a Ca^{2+}-independent manner in pig eggs (Sun et al., 1997) and other events in cells by acting on targets in addition to PKC (Betz et al., 1998; Rhee et al., 2002; Rossner et al., 2004; Byrne et al., 2005). Fertilization-induced CG release is Ca^{2+}-dependent (Kline and Kline, 1992). In the mouse, fertilization-associated CG exocytosis and metaphase II exit are not blocked by the simultaneous use of two permeable PKC antagonists that affect different sites on the kinase (Ducibella and LeFevre, 1997). Thus, egg responses from pharmacological agonists alone do not necessarily tell us what PKC is doing at fertilization (Jones, 1998b; Halet, 2004).

However, several new and interesting functions for PKC are emerging in eggs. In sea urchin eggs, *in vitro* studies have demonstrated that PKC phosphorylates and solubilizes the sperm nuclear lamina in a Ca^{2+}-dependent manner (Collas et al., 1997; Stephens et al., 2002), and PKC is required for the egg respiratory burst after activation (Heinecke and Shapiro, 1992).

There is evidence that mammalian eggs have all three isoform classes of PKC (cPKC: DAG/Ca^{2+}-dependent; nPKC: DAG-dependent; aPKC: DAG/Ca^{2+}-independent) (Gangeswaran and Jones, 1997; Pauken and Capco, 2000; reviewed in Halet, 2004). Furthermore, mouse eggs have a truncated form of PKCδ containing the catalytic region, which may be the form identified as PKM in hamster eggs (Gallicano et al., 1995). Depending on the isoform, enzyme activity is controlled by its own phosphorylation status, membrane targeting, and its two effectors, Ca^{2+} and DAG.

Since studies of PKC function in mammalian eggs have been reviewed (Gallicano and Capco, 1995; Gallicano et al., 1997; Jones, 1998b; Eliyahu et al., 2001; Halet, 2004), they will be only briefly summarized. There is evidence that PKC and PKM are involved in the linearization of cytoskeletal sheets, which may serve as a storage form of intermediate filament protein for early development (Gallicano et al., 1995). Although some studies using pharmacological agonists are consistent with a role for PKC in CG exocytosis in rat eggs (Eliyahu and Shalgi, 2002), further studies are required (see above). Roles for PKC in MARCKS protein phosphorylation and cortical cytoskeleton organization are under investigation in mammalian eggs.

In fertilized mammalian eggs, several hypotheses are emerging for PKC function, primarily based on immunofluorescence studies of PKC isoforms. Different isoforms are localized to different regions of the fertilized mouse egg such as the cortex, spindle, contractile ring of the polar body, and PN (Page-Baluch et al., 2004; Eliyahu and Shalgi, 2002; Fan et al., 2002; Luria et al., 2000; Viveiros et al., 2003). In addition, some change their localization after fertilization. For example, PKCζ/λ and its phosphorylated (active) form are associated with the meiotic spindle and a PKCζ/λ-specific pseudosubstrate inhibitor results in spindle disruption in mature mouse eggs (Page-Baluch et al., 2004). Similar, focal staining of PKCζ/λ and phospho-MARCKS is reported for metaphase II mouse eggs (Michaut et al., 2005).

PKCδ is associated with the egg meiotic spindle and nuclei of early mouse embryos (Viveiros et al., 2003). After fertilization, PKCδ distribution changes from diffused to localized on the spindle even in eggs loaded with a chelator of Ca^{2+} (Tatone et al., 2003). Consistent with this result, PKCδ

is a Ca^{2+}-independent isoform (nPKC). This isoform becomes spindle-associated during metaphase of meiosis I (Viveiros et al., 2003) and regulates the onset of anaphase I in mouse oocytes (Viveiros et al., 2004). These results suggest that these PKC isoforms play a role in spindle integrity and/or function in eggs, and await further analysis. Their Ca^{2+} independence would allow tham to function between Ca^{2+} oscillations and for periods of time longer than a single Ca^{2+} rise. Thus, their temporal pattern of activity may be different from that of cPKCs.

Recent studies provide other interesting roles for PKC isoforms, e.g., in the regulation of fertilization-induced oscillations of $[Ca^{2+}]_i$ and membrane ion channels. cPKCs or their Ca^{2+}/DAG domains that are GFP-tagged rapidly translocate to the plasma during each rise in $[Ca^{2+}]_i$ in fertilized mouse eggs (Halet et al., 2004). Experimental manipulation by PKC overexpression or use of a PKC antagonist resulted in augmentation and inhibition, respectively, of $[Ca^{2+}]_i$ oscillations (Halet et al., 2004; Madgwick et al., 2005). From these studies, a model was proposed in which PKC activity regulates store-operated Ca^{2+} channels in the egg plasma membrane, thereby controlling the duration of $[Ca^{2+}]_i$ oscillations or oscillation parameters. In the fertilized sea urchin egg, a peptide inhibitor of PKC partially inhibited cytoplasmic alkalinization, suggesting a role in regulating the membrane Na^+–H^+ antiporter (Shen and Buck, 1990), but *Xenopus* eggs do not appear to utilize PKC for alkalinization (Grandin and Charbonneau, 1991). However, additional studies are needed to identify the downstream substrate(s) of PKC such that the physiological relevance of this kinase on Ca^{2+} influx can be more precisely understood.

Finally, there is evidence that the PKC substrate, the MARCKS protein, may play important roles in several EEA. First, phosphorylated forms of MARCKS and PKCζ/λ are present at the spindle poles (Page-Baluch et al., 2004) and preferentially located with the centrosome associated with the extrusion of the first polar body, whereas as the MII spindle poles had symmetrical staining (Michaut et al., 2005). Since phosphorylated MARCKS regulates filamentous actin cross-linking in other cells, its other localization in the actin cap of mouse eggs with PKCζ/λ also supports a role for PKC in polar body formation (Michaut et al., 2005). In rat eggs, MARCKS is proposed to be involved in CG exocytosis by modulating cortical actin to facilitate CG translocation to the plasma membrane (Eliyahu et al., 2006; also see Fan et al., 2002). In mouse eggs, however, there is a relatively low signal for phosphorylated MARCKS in the cortex occupied by CGs (Michaut et al., 2005). These studies, as well as others on MLCK, suggest that PKs have important roles in regulating the egg cortical cytoskeleton.

MLCK

The fertilized egg should be a rich resource for investigating Ca^{2+}/CaM-dependent proteins and PKs in light of the activation of CaMKII, and because of the widespread cytosolic elevation of $[Ca^{2+}]_i$ from its wave-like manifestation and CaM's essentially ubiquitous presence in cells including gametes (e.g., Steinhardt and Alderton, 1982; Wilding et al., 1995; Courtot et al., 1999). Other EF hand-dependent proteins are also candidates. Interestingly, the EF hand domains of PLCζ have a critical role in the enzyme's sensitivity to basal levels of $[Ca^{2+}]_i$ and nuclear translocation in mouse eggs (Kouchi et al., 2004, 2005; Kuroda et al., 2006). In addition, CaM mediates cyclic ADP ribose activation of the rynanodine receptor in sea urchin egg microsomes (Lee et al., 1994). Apo-CaM and Ca^{2+}/CaM have effects on IP_3R function, although relatively little is known about their effects in eggs (see Smyth et al., 2002).

Of the Ca^{2+}/CaM-dependent PKs, only CaMKII and MLCK have been investigated in the context of the increase in $[Ca^{2+}]_i$ and specific EEA (to the knowledge of the authors). MLCK is a potentially important regulator of myosin II in nonmuscle cells (Bresnick, 1999). MLCK regulates myosin II by phosphorylating Ser^{19} on the regulatory light chains, stimulating actin-mediated ATPase activity and assembly of myosin II into filaments. Although the only known cellular target of MLCK is myosin II light chain, other PKs can phosphorylate Ser^{19}, including Rho kinase and ZIP kinase (Komatsu and Ikebe, 2004). During events, such as cell division and secretion, Ser^{19} and other sites on myosin II light and heavy chains are phosphorylated by MLCK and other PKs. Although its force-generating function in the contractile ring during cytokinesis is known (Burgess, 2005; Matsumura et al., 2001), the function of myosin II in secretion is not as well understood, although a role in secretory granule translocation is plausible.

After fertilization, myosin II has been reported to participate in cytoskeletal remodeling, CG exocytosis, and cytokinesis, the latter for polar body extrusion and/or cleavage. The peripheral egg cytoplasm undergoes these and many other cytoskeletal alterations (Sardet et al., 2002; Brunet and Maro, 2005). In unfertilized sea urchin eggs, myosin II is localized to the egg cortex and blebbistatin, which antagonizes its ATPase activity, delayed absorption of the fertilization cone (Stack et al., 2006). Consistent with this, microinjection into mouse eggs of a nonphosphorylatable peptide of myosin regulatory light chain blocks sperm incorporation cone disassembly and impedes cell cycle progression (Simerly et al., 1998). These results support a role for PKs in remodeling of the cortical cytoskeleton through effects on myosin II, whose A and B isoforms are differentially localized during meiotic maturation and after fertilization in mouse eggs (Simerly et al., 1998).

A requirement for MLCK activity in cytokinesis has been reported in both sea urchin and mouse eggs. In the sea urchin, the metaphase–anaphase transition is accompanied by an increase in $[Ca^{2+}]_i$ (Groigno and Whitaker, 1998) and there appear to be two phases of PK-dependent myosin II activity. Inhibitor studies indicated that the early phase required MLCK but not Rho kinase, whereas the later furrowing phase required both PKs, consistent with the idea that different PKs regulate different stages of cytokinesis (Lucero et al., 2006; also see Bement et al., 2005). Cortical contraction is associated with an increase in $[Ca^{2+}]_i$ and Ser^{19} phosphorylation of myosin II (Lucero et al., 2006; Stack et al., 2006). In fertilized mouse eggs, either blebbistatin or the MLCK inhibitor, ML-7, robustly

inhibited the formation of the second polar body (Matson et al., 2006). These inhibitors also prevented spindle rotation, which is required for normal cytokinesis. A Rho kinase inhibitor (Y-27632) had similar effects (Zhong et al., 2005; also see Mabuchi et al., 1993). Unlike the sea urchin egg with a central spindle, vertebrate eggs have a cortical spindle, and rotation to a perpendicular orientation to the egg surface is required for second polar body formation. However, although second polar body formation takes place during the period of Ca^{2+} oscillations in mammalian eggs, the temporal and functional relationship of $[Ca^{2+}]_i$ to polar body cytokinesis requires further investigation (as discussed later).

There is also emerging evidence that MLCK has a role in the development of mouse egg polarity and CG exocytosis, as discussed in more detail in Deng et al. (2005) and Matson et al. (2006). Briefly, CG movements occur during meiotic maturation and fertilization in mammalian eggs and MLCK antagonists inhibit relocation and exocytosis, respectively. MLCK is involved in secretion in other cell types. The Rho-signaling pathway mediates CG translocation during oocyte maturation of sea urchin eggs (Covian-Nares et al., 2004).

In summary, although myosin II is clearly involved in egg cortical cytoskeletal activities, such as cytokinesis, much remains to be done to investigate its role in the EEA. The aforementioned studies raise questions about PK-mediated regulation of myosin's regulatory light chains as well as heavy chains, the identity of PKs involved, which are Ca^{2+}-dependent, and whether the phosphorylation status of other myosin isoforms plays an important role in the EEA (e.g., Weber et al., 2004).

MEK/MAPK

While many studies have characterized the role of the MAPK pathway on oocyte maturation and meiotic arrest at the MII stage, little is known about the function of this pathway on fertilization and the EEA. Because there are many recent reviews in the literature describing the role of the MAPK pathway as a component of the egg's CSF activity (Tunquist and Maller, 2003; Brunet and Maro, 2005; Schmidt et al., 2006; Liu et al., 2007; Madgwick and Jones, 2007), this discussion examines less explored roles, especially the functions that are concerned with egg activation and regulation of Ca^{2+} release during fertilization. Suffice it to say that regardless of whether the MAPK pathway is required for the establishment and/or for the maintenance of the cell cycle arrest prior to fertilization (Madgwick and Jones, 2007), its role in interrupting the meiotic cell cycle so that stage-specific sperm entry is achieved is highly conserved across the animal kingdom. This function of the MAPK pathway is well characterized in species such as the hydrozan jellyfish *Cladonema pacificum* (Kondoh et al., 2006), in some species of starfish (Tachibana et al., 2000) and in the sea urchin (Chiri et al., 1998; Carroll et al., 2000; Kumano et al., 2001), and in eggs of vertebrate species (Tunquist and Maller, 2003; Madgwick and Jones, 2007). Other conserved events of egg activation such as Ca^{2+} release and formation of PN also may be under the control of the MAPK pathway.

In mammals, the first egg activation event thought to be influenced by changes in the levels of MAPK activity is formation of PN. It is well established that inactivation of MPF activity precedes inactivation of MAPK activity and that MPF inactivation is required for the resumption of meiosis and release of the second polar body (Choi et al., 1991; Moos et al., 1995). While the latter events occur within 2 h of fertilization, formation of PN does not occur until 5 to 6 h in the mouse zygote; in this context, it appears that MAPK activity is responsible for delaying formation. Several pieces of experimental evidence support this contention. For example, it was first noted that the expression of a constitutively active form of the kinase responsible for activating MAPK, the dual MEK kinase, simultaneously prevented the fertilization-associated inactivation of MAPK and formation of PN (Moos et al., 1996). Further, okadaic acid (OA)-induced stimulation of MAPK activity in zygotes that already displayed formation of PN rapidly led to disintegration of the pronuclear envelope (Moos et al., 1995), which is consistent with the role of MAPK on nuclear envelope disassembly (Peter et al., 1992). Exposure to OA is likely to result in the activation of several kinases, although it is worth noting that in meiotically incompetent oocytes, OA-induced GVBD occurred in the presence of high MAPK activity but of negligible MPF activity (Chesnel and Eppig, 1995). Lastly, formation of PN is accelerated in zygotes exposed to DMAP, a general kinase inhibitor, and is correlated with a more rapid inactivation of MAPK (Szöllösi et al., 1993; Liu and Yang, 1999). Nonetheless, these results must be weighed against the observation that treatment of eggs with the MEK inhibitor U0126 fails to accelerate formation of PN even though MAPK inactivation is shifted earlier (Phillips et al., 2002). It is worth noting that in the presence of U0126, Mos, the upstream activator of MEK, may still be active (Verlhac et al., 2000a,b), which may account at least in part for the presumed failure of early MAPK inactivation to lead to premature formation of PN.

Several questions remain unanswered regarding the delayed formation of PN and inactivation of MAPK in mammalian zygotes. First, what is the functional need to delay formation of PN in early mammalian zygotes? Perhaps the long cell cycle time evolved to provide time for the egg to reconfigure the sperm chromatin (Morgan et al., 2005); premature formation of PN may result in the incomplete reprogramming of the male genome, which will have catastrophic developmental consequences. Another possibility is that it arises from the need to support persistent $[Ca^{2+}]_i$ oscillations. $[Ca^{2+}]_i$ oscillations are responsible for all events of egg activation, although the completion of certain events requires additional Ca^{2+} stimulation (Ducibella et al., 2002). Because progression into interphase and formation of PN are associated with progressive decline and eventual termination of $[Ca^{2+}]_i$ oscillations (Jones et al., 1995; Day et al., 2000), and given that PLCζ, the putative sperm's Ca^{2+} oscillatory factor, contains a nuclear localization signal and moves there during interphase (Saunders et al., 2002), premature formation of PN may lead to its nuclear sequestration, thereby preventing the delivery of a complete Ca^{2+} activation stimulus. A second question that deserves

further investigation is the molecular pathway that underlies inactivation of MAPK activity during fertilization. In sea urchin eggs, inactivation of MAPK activity has been linked, at least in part, to an increase in phosphatase activity associated with Ca^{2+} release (Kumano et al., 2001). Consistent with this notion, at least two different phosphatases have been described to inactivate MAPK in *Xenopus* oocytes, although their identity remains unknown (Sohaskey and Ferrell, 1999). Interestingly, a MAPK phosphatase also appears to be present in mouse oocytes, and its activity seems to be regulated by Mos (Verlhac et al., 2000a,b), the initiator of the MAPK pathway. Therefore, identification of the molecular mechanism(s) responsible for controlling MAPK activity in eggs/zygotes may deepen our understanding of the mechanism(s) that regulate formation of PN and may provide clues regarding the underpinnings of nuclear reprogramming.

More recent developments point to a regulatory function of the MAPK pathway on $[Ca^{2+}]_i$ release mechanism required for fertilization. As previously mentioned, the persistence of $[Ca^{2+}]_i$ oscillations in mammals is entrained with the cell cycle, i.e., oscillations are consistently observed during the MII stage and they decline as zygotes proceed into interphase (Jones et al., 1995; Day et al., 2000, Jellerette et al., 2004); the underlying mechanism(s) responsible for this association has yet to be resolved. Given that cell cycle progression is regulated by M-phase kinases, it is logical to assume that these kinases are involved in controlling these $[Ca^{2+}]_i$ oscillations. An earlier study, however, using pharmacological inhibitors did not find that abrogation of MPF and MAPK activities caused any obvious effects on the pattern of $[Ca^{2+}]_i$ oscillations (Marangos et al., 2003). Nonetheless, in that study, inhibition of the kinase activities was performed in MII eggs, a stage at which many of the targets could have been already modified. Consistent with this possibility, two recent studies found that oocytes matured in the presence of U0126, a MEK inhibitor that precludes the activation of MAPK, produced $[Ca^{2+}]_i$ responses of low amplitude and of reduced duration in response to fertilization or to injection of PLCζ mRNA (Lee et al., 2006a,b; Matson and Ducibella, 2007). Only one of the studies found that treatment with U0126 compromised the content of the internal Ca^{2+} stores. In addition, inhibition of MAPK activity also prevented the $[Ca^{2+}]_i$ rise associated with nuclear envelope breakdown in sea urchin zygotes (Philipova et al., 2005). Together, these studies demonstrate that during maturation and after fertilization the MAPK pathway and possibly other kinases optimize the oocytes' Ca^{2+} release mechanisms in preparation for fertilization/embryo development.

Two emerging functions of MAPK could account for the aforementioned effects of U0126 on the egg's ability to mount $[Ca^{2+}]_i$ responses. First, several studies demonstrate that MAPK activity is required for the cortical reorganization prior to fertilization. As oocytes mature, they become highly polarized, with a cortical granule free domain and a distinct actin cap overlying the chromatin/spindle, which is near the egg cortex (Longo and Chen, 1985; Maro et al., 1986). The presence of chromatin induces this cortical reorganization near the spindle, although MAPK activity is required for its completion, as injection of chromatin fails to induce cortical reorganization in Mos−/− mice (Deng et al., 2005). Similarly, the translocation of the meiotic spindle to the egg cortex, which occurs immediately prior to the release of the first PB (Wassarman et al., 1976), requires MAPK activity, as is aberrant in the same animal model (Verlhac et al., 2000b). Given this widespread role in cortical reorganization, it is possible that the redistribution of the endoplasmic reticulum (ER) and IP_3R1s that occurs during the later stages of oocyte maturation and that culminates with the formation of ER and IP_3R1 cortical clusters (Kline et al., 1999; Terasaki et al., 2001; Dumollard et al., 2002; FitzHarris et al., 2003, 2007; Stricker, 2006; Boulware and Marchant, 2005), may be regulated, at least in part, by MAPK.

Cortical reorganization of the ER occurs in many species prior to fertilization, and the presence of ER cortical clusters is thought to explain, at least in part, the high susceptibility of eggs to initiate $[Ca^{2+}]_i$ oscillations following sperm entry (Mehlmann et al., 1996; Shiraishi et al., 1995; Kline et al., 1999; Terasaki et al., 2001; FitzHarris et al., 2003; Lee et al., 2006b). It is therefore possible that abrogation of MAPK activity during maturation, as is observed in oocytes treated with U0126, prevents ER and IP_3R1 cluster formation, impairing the ability of eggs to mount $[Ca^{2+}]_i$ oscillations in response to fertilization. A second mechanism by which the MAPK pathway could influence Ca^{2+} release is by directly affecting the function of IP_3R1, the channel thought to mediate the majority of Ca^{2+} release during fertilization (Miyazaki et al., 1993a,b). IP_3R1 shows highly conserved phosphorylation sites for MAPK, and recent reports found using *in vitro* phosphorylation assays that Ser^{436}, which is within one of the consensus sites, is phosphorylated by ERK (Bai et al., 2006; Yang et al., 2006; Lee et al., 2006a,b); ERK also seems to interact with IP_3R1 (Bai et al., 2006). Moreover, an IP_3R1 phosphorylation detected by the MPM-2 antibody in mouse MII eggs was greatly diminished when oocytes were matured in the presence of U0126 (Lee et al., 2006a,b). However, it remains to be demonstrated whether MAPK directly phosphorylates IP_3R1 in eggs under *in vivo* conditions, whether it affects IP_3R1 function in these cells, and whether the decrease in MAPK activity affects IP_3R1-mediated Ca^{2+} release after fertilization.

It is worth noting that MPF activity may also contribute to regulate $[Ca^{2+}]_i$ oscillations at fertilization. For instance, fertilization-associated oscillations in mouse zygotes were abrogated by the addition of roscovitine, a specific MPF inhibitor (Deng and Shen, 2000). While the Ca^{2+} release mechanism(s) affected by the decreased levels of MPF was not discerned in that study, the Ca^{2+} content of the internal stores seemed reduced, suggesting, possibly, an effect of MPF on Ca^{2+} influx and/or in the Ca^{2+}-ATPase pump that regulates Ca^{2+} uptake into the ER. In addition, MPF activity could be modulating the function of IP_3R1, as this channel also contains highly conserved consensus sites for Cdk1/MPF. Two sites, Ser^{421} and Thr^{799}, were found to be to be phosphorylated in IP_3R1 in somatic cells using phospho- and site-specific antibodies (Malathi et al., 2003), although whether this is also the case in eggs remains to be demonstrated. Lastly, a recent study reported that MPF activity is required to maintain $[Ca^{2+}]_i$

oscillations during fertilization in ascidians eggs and it appears that it does so by sustaining IP_3 production (Levasseur et al., 2007). Collectively, research evidence shows that the same M-phase kinases that are involved in the resumption and progression of meiosis during maturation also play critical roles in the regulation of egg activation events after fertilization. Future studies should elucidate the molecules and precise site of action regulated by these kinases.

Oscillatory signaling affects cellular responses

With the discoveries of Ca^{2+} oscillations and Ca^{2+}-regulated PK activities that control important EEA, fundamental signaling and developmental questions arise. Do PK activities change in parallel with $[Ca^{2+}]_i$ (i.e., oscillatory enzyme activity)? If so, does oscillatory PK activity have developmental significance? For example, is development altered if oscillations are replaced by a single continuous $[Ca^{2+}]_i$ rise of the same total (temporal) duration? In animal cloning, how do PKs respond to different parthenogenetic agents with their various effects on $[Ca^{2+}]_i$ and do these responses affect later development? First, some introductory remarks are necessary.

From a historical perspective, perhaps it has not been appreciated that oscillatory signaling has a notable track record in both development and in many adult somatic cells. Thirty years ago, Rapp and Berridge (1977) reported that "Oscillations in calcium-cyclic AMP control loops form the basis of pacemaker activity and other high-frequency biological rhythms" (regarding cardiac pacemaker cells, neurons, insulin secreting β-cells, slime molds, and other cells). With investigations of voltage-sensitive Ca^{2+} channels and better detection methods, Ca^{2+} oscillations were found in many secretory cells, including pituitary gonadotropes, gastric chief cells and chromaffin cells (Fewtrell, 1993), as well as some growth-factor-stimulated cells (Berridge, 1995). It is now appreciated that CG exocytosis in eggs and secretion in somatic cells are likely to have Ca^{2+}-dependent pathways in common (Wessel et al., 2001; Ducibella and Matson, in press). Even the activity of MPF during early cleavage is under the control of oscillatory cyclin synthesis and degradation with each cell cycle.

Furthermore, oscillatory second messenger signaling has been found to generate oscillatory enzymatic responses and to encode information regulating gene expression. In *Dictyostelium*, waves of cAMP generation are followed by oscillations of MAPK (ERK2) activity during aggregation of the slime mold (Maeda et al., 2004). In T lymphocytes, antigen triggering can result in a variety of Ca^{2+} responses (Lewis, 2001), including oscillations, whose characteristics regulate cellular responses. For example, oscillations in $[Ca^{2+}]_i$ reduce the Ca^{2+} threshold for activating transcription factors, increasing signal detection at low stimulation levels, whereas modulation of the oscillation frequency changes the gene expression pattern (Dolmetsch et al., 1998). In neurons and pituitary gonadotropes, the frequency of oscillation in $[Ca^{2+}]_i$ modulates growth/differentiation and transcriptional responses, respectively (Spitzer et al., 2000; Haisenleder et al., 2001). Thus, the number of oscillations may play a role in cell fate determination (Lahav, 2004). Oscillations in $[Ca^{2+}]_i$ drive secretory responses in eggs and pancreatic cells (Kasai and Augustine, 1990; Kline and Kline, 1992; Ducibella et al., 2002, 2006). Receptor-mediated oscillations in $[Ca^{2+}]_i$ are a versatile mechanism for encoding information in the form of oscillation parameters, which regulate cellular and enzymatic responses (De Koninck and Schulman, 1998; Dupont and Goldbeter, 1998; Berridge et al., 2000).

The increasing evidence for oscillatory-encoded information begged the question of whether those fertilized eggs with oscillatory Ca^{2+} responses utilize oscillation parameters to regulate the process of egg activation. Studies of individual EEA indicated that they are sensitive to the amount of Ca^{2+} stimulation (Kline and Kline, 1992; Vitullo and Ozil, 1992; Xu et al., 1996; Lawrence et al., 1998). For example, a dose–response relationship was found between the number of repetitive similar $[Ca^{2+}]_i$ oscillations and exocytosis of egg CGs (Ducibella et al., 2002). The results of Ozil and colleagues (2005, 2006) provide evidence that Ca^{2+} parameters also impact later peri-implantation events and gene expression. If so, it is tempting to speculate that the mechanism may hark back to Ca^{2+}-dependent changes in protein expression and mRNA recruitment after egg activation (Ducibella et al., 2002, 2006). Further investigation is required in eggs, for example, to determine if CaMKII is a positive regulator for mRNA translation as in neurons (Atkins et al., 2004).

When it was found that experimental manipulation of the normal Ca^{2+} oscillation parameters affected the initiation and completion of many EEA (Ducibella et al., 2002; Ozil et al., 2005), this was a clue that egg Ca^{2+}-dependent protein activities could be responding in parallel to the changes in the $[Ca^{2+}]_i$. Consistent with this hypothesis was the following information. First, almost all EEAs are under the control of Ca^{2+}. Second, as the number of oscillations of $[Ca^{2+}]_i$ and total exposure time to elevated $[Ca^{2+}]_i$ were increased in these studies, more EEAs were initiated, incrementally moved forward, and completed (Ducibella et al., 2002, 2006; Ozil et al., 2005; Toth et al., 2006). Third, as already discussed, many EEAs are controlled by PKs, whose activities are under the direct or indirect regulation of Ca^{2+}. Fourth, the activity of at least one PK, MPF, was approximately regulated in a dose–response manner by the total time of elevation of the $[Ca^{2+}]_i$. Consistent with this, cyclin B degradation is regulated by the amplitude and number of oscillations of $[Ca^{2+}]_i$ (Nixon et al., 2002). However, since egg MPF activity is under indirect Ca^{2+} control and downregulated by an elevated $[Ca^{2+}]_i$, it is not an ideal PK to study the relationship of $[Ca^{2+}]_i$ to the activity of those PKs that depend upon $[Ca^{2+}]_i$ for activity. Because MPF activity is under the influence of CaMKII, whose activity is directly regulated by Ca^{2+}/CaM, CaMKII is a better PK to determine more precisely how enzyme activity responds to oscillations of $[Ca^{2+}]_i$ in fertilized eggs.

Oscillatory signaling can regulate the pattern of PK activity at fertilization

Fortunately for developmental biologists, the biochemical relationship of Ca^{2+} and Ca^{2+} oscillations to CaMKII activity

has been given considerable attention. Elegant molecular mechanisms control both the level and duration of CaMKII activity (De Koninck and Schulman, 1998; Hudmon and Schulman, 2002). Regarding the level of activity, Ca^{2+}/CaM binding creates an active subunit ("non-autonomous" activity). More interestingly, after Ca^{2+}/CaM binding of adjacent subunits, trans-autophosphorylation creates a phosphorylated subunit whose activity is now independent of Ca^{2+}/CaM ("autonomous activity"). Sequential trans-autophosphorylation incrementally ramps up the number of active subunits within a single CaMKII multimeric enzyme (Hunter and Schulman, 2005). Thus, the combination of autonomous and non-autonomous modes of activation for 12 subunits provides CaMKII with a potentially wide range of enzymatic activity (rheostat-like), in contrast to an enzyme of few subunits that may be either on or off (switch- or digital-like). In terms of research, monitoring autonomous activity in lysed cells provides an opportunity to measure relative changes in CaMKII activity that had taken place inside the cell (based on the activity of *in vivo* phosphorylated subunits).

Equally interesting is that the phosphorylation status can affect the duration of activity of CaMKII. When phosphorylated on $Thre^{286/287}$, a subunit can maintain activity even after $[Ca^{2+}]_i$ has decreased (see Hudmon and Schulman, 2002). Thus, activity could in theory continue between individual Ca^{2+} oscillatory increases until dephosphorylation or another mechanism downregulates activity. Thus, high-frequency oscillations (<1 per second) of the $[Ca^{2+}]_i$, such as those in neurons, could drive up CaMKII activity without substantial enzymatic decreases between Ca^{2+} peaks (De Koninck and Schulman, 1998). Since $[Ca^{2+}]_i$ is not continuously elevated during oscillations, other Ca^{2+}-dependent proteins without such "autonomous" activity would not be as acutely stimulated, preventing the cell from undergoing the many different Ca^{2+} responses in any cell's repertoire, including cell death. In fact, experimentally induced high-frequency Ca^{2+} oscillations have been reported to cause apoptosis in mouse eggs (Gordo et al., 2000), or to greatly reduce pre-implantation embryo development (Rogers et al., 2004). Conversely, low-frequency oscillations (on the order of seconds or minutes) could result in oscillatory enzyme activity given the increased opportunity for intervening dephosphorylation. The many isoforms and heteromultimeric forms of CaMKII provide additional opportunities for fine-tuning enzyme activity (Bayer et al., 2002).

Thus, the amplitude, frequency, duration, and number of oscillations of $[Ca^{2+}]_i$ can regulate the level and temporal response of enzyme activity, which has been demonstrated for CaMKII *in vitro* (De Koninck and Schulman, 1998). What is relevant to this review is that these characteristics could have implications for development. Oscillation parameters may control the time points and intervals in development in which the enzyme is active and inactive, e.g., if the frequency is low. Oscillatory activity can affect the amount of total enzyme activity over time, a potentially important developmental parameter for events that require either a certain amount of substrate conversion (or downstream cyclin B degradation) or total signaling time to activate a downstream event.

How might the egg utilize oscillatory parameters and information in terms of enzymatic opportunities? Before this question could be addressed, experimental methods had to be developed to manipulate the egg's $[Ca^{2+}]_i$. Since it has been difficult to precisely regulate and monitor most of $[Ca^{2+}]_i$ oscillation parameters in fertilized mammalian eggs (but, see Ozil et al., 2006), most studies to date have used parthenogenetic methods (e.g., Ozil, 1990; Collas et al., 1995). Although $SrCl_2$ causes oscillations, there is considerable variation in amplitude and frequency (Bos-Mikich et al., 1997). With electroporation methods that allow the influx of extracellular Ca^{2+} and that reproducibly regulate $[Ca^{2+}]_i$, the egg's responses have been studied by manipulating oscillation parameters in the absence of sperm (Ozil, 1998; Toth et al., 2006).

Altering the amplitude of $[Ca^{2+}]_i$ has effects on the cell cycle and development. In early electroporation studies, as the extracellular $[Ca^{2+}]$ increased up to point, so did the percent of eggs with PN (to 92%), whereas a lower or higher $[Ca^{2+}]$ resulted in poorer activation responses in the mouse egg (Vitullo and Ozil, 1992). In a subsequent study of rabbit eggs in which $[Ca^{2+}]_i$ was measured, amplitudes similar to those of fertilized mammalian eggs resulted in a higher percentage of eggs with PN than lower amplitudes (Ozil and Huneau, 2001). In that study, repeated low-amplitude pulses that increased $[Ca^{2+}]_i$ incrementally increased the formation of PN, but not to a high level and none of these eggs developed into parthenogenetic fetuses in contrast to fetuses from the higher amplitude treatment.

In addition, alterations in the amplitude of $[Ca^{2+}]_i$ are associated with changes in MPF and CaMKII activity. Lower concentrations of IP_3 that reduce the amplitude of $[Ca^{2+}]_i$ do not cause normal egg activation (Kurasawa et al., 1989). Nixon et al. (2002) observed that those fertilized mouse eggs that had a relatively low-amplitude response (<600 nM of $[Ca^{2+}]_i$) also had a lower mean level of cyclin B degradation. This finding leads to the prediction that as $[Ca^{2+}]_i$ increases the activity of CaMKII also increases, leading to increased cyclin B degradation (via mechanisms discussed above). Consistent with this, major increases in the levels of $[Ca^{2+}]_i$ result in further elevations in CaMKII activity in mouse eggs (Markoulaki et al., 2004). However, a thorough study of the response of CaMKII in physiological range of $[Ca^{2+}]_i$ during fertilization has not yet been reported. In this regard, small differences in the mean $[Ca^{2+}]_i$ of pooled eggs after *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have not been associated with corresponding small changes in the mean autonomous activity of CaMKII (Markoulaki et al., 2007). However, the inability to measure non-autonomous activity (Ca^{2+}/CaM binding without autophosphorylation) *in vivo* in eggs leaves room for further studies, e.g., if a CaMKII FRET probe becomes available that responds to both autonomous and non-autonomous activities.

In theory, the duration of each elevation in $[Ca^{2+}]_i$ could also control the total activity and temporal window for CaMKII. In fertilized mouse eggs, the first rise in $[Ca^{2+}]_i$ is longer and of higher amplitude than subsequent rises that tend to be relatively similar to each other. Thus, the first rise is likely to be associated

with a longer period of CaMKII activity. In addition, the mean enzyme activity is still partially elevated 1 min after $[Ca^{2+}]_i$ returns to baseline, suggestive of a short "memory" phase in which autonomous activity *in vivo* persists while $[Ca^{2+}]_i$ has dropped to a basal level (Markoulaki et al., 2004). However, prolonged continuous (non-oscillatory) elevation of $[Ca^{2+}]_i$ for 20 min has a negative effect on CaMKII activity. Although this appears to be due to a large reduction in the amount of activation competent enzyme (Ozil et al., 2005), the mechanism of downregulation requires further investigation. In the fertilized mammalian egg, elevations in $[Ca^{2+}]_i$ continue for several hours or more, and the level of CaMKII activity associated with each rise in $[Ca^{2+}]_i$ continues to be equally stimulated during the same period of time.

Since it does not appear to induce downregulation of CaMKII activity, oscillatory signaling could provide an elegant signaling mechanism to maintain CaMKII sensitivity and activity over a long period of time. The developmental significance of CaMKII is highlighted by evidence that it is a central regulator of many EEA since constitutively active CaMKII in the absence of increased $[Ca^{2+}]_i$ stimulates parthenogenetic early development (Madgwick et al., 2005; Knott et al., 2006). As discussed later, these studies with constitutively active CaMKII and some parthenogenetic activation protocols indicate that non-oscillatory methods can activate mammalian eggs to varying extents, although the developmental competence of these zygotes remains to be carefully evaluated.

The discovery of oscillations in $[Ca^{2+}]_i$ and the fact that some egg PKs are active essentially only when $[Ca^{2+}]_i$ is elevated makes the oscillation frequency a potentially important parameter in regulating the timing of Ca^{2+}-dependent PK activity and EEA, as well as in experimental design. Before discussing the frequency-dependent effects, it is important to note that monitoring Ca^{2+}-dependent PK activity must take into account Ca^{2+} oscillation parameters. Early measurements of CaMKII activity were performed on batches of fertilized mouse eggs, which undergo asynchronous oscillations in the $[Ca^{2+}]_i$. Given their frequency (a 1- to 2-min rise in $[Ca^{2+}]_i$ every 10–20 min), only ~10% of eggs, on average, would have elevated $[Ca^{2+}]_i$ at any one time (sampling) point, resulting in relatively small changes in the mean CaMKII activity over time in these batched eggs. As expected, measurements of CaMKII activity were much larger when eggs were sampled individually but only when $[Ca^{2+}]_i$ was elevated, including a Ca^{2+} reporter probe at low concentration to minimize any effect on enzyme activity (Markoulaki et al., 2003, 2004). If eggs underwent very high frequency, neuronal-like $[Ca^{2+}]_i$ oscillations in which CaMKII activity is maintained between oscillations (see above), the requirements of sampling would not be as restrictive.

The oscillation frequency of $[Ca^{2+}]_i$ is an important parameter of cell cycle regulation in fertilized eggs most likely due to its effect on CaMKII activity. In fertilized mouse eggs, the first large rise in $[Ca^{2+}]_i$ is not sufficient for 2nd polar body formation (Kline and Kline, 1992) or for a major decrease in the level of cyclin B (Nixon et al., 2002), despite a transient CaMKII response (Markoulaki et al., 2003, 2004). Consistent with this, newly ovulated mouse eggs do not undergo a high percentage of formation of PN after ionophore treatment (Abbott et al., 1998), which causes one high-amplitude but limited duration rise in the $[Ca^{2+}]_i$. In addition, multiple $[Ca^{2+}]_i$ rises, each associated with CaMKII activity, are required for sufficient cyclin degradation, as evidenced also by incremental decreases in MPF activity associated with corresponding increases in cell cycle progression (Collas et al., 1995; Ducibella et al., 2002; Nixon et al., 2002).

Some of the most intriguing evidence for the significance of the $[Ca^{2+}]_i$ oscillation frequency comes from observations of egg cycle duration in fertilized eggs of different mammals. Those whose $[Ca^{2+}]_i$ oscillation frequency is relatively rapid develop PN in 5–6 h (e.g., 10–20 min/oscillation; rodents), whereas those that are slower take at least 10–12 h before PN are formed (e.g., 30–45 min/oscillation; domestic animals and primates) (Fissore et al., 1992; Sun et al., 1992; Taylor et al., 1993; Susko-Parrish et al., 1994; Liu and Yang, 1999). Although there are other possible explanations for this correlation, the regulation of cell cycle time by frequency is supported by studies in which experimentally increasing the frequency results in formation of PN ahead of schedule in proportion to the applied frequency (Ozil, 1998). Thus, observations to date suggest that the frequency of oscillations in the $[Ca^{2+}]_i$, CaMKII activity, and most likely the activities of other Ca^{2+}-dependent proteins regulate the rate of progress of various events, such as cell cycle progression and CG exocytosis.

Some non-mammalian eggs resume their cell cycle with a single, prolonged rise in $[Ca^{2+}]_i$ at fertilization (Stricker, 1999). Interestingly, these eggs have very rapid cell cycle times (compared to mammals) and a transient rise in $[Ca^{2+}]_i$ is observed with each early cleavage cycle, which when considered as a whole are oscillations of $[Ca^{2+}]_i$ similar to mammals but shifted into early zygotic development (as discussed in Ducibella et al., 2002, 2006). The prevailing notion is that mammals utilize low-frequency oscillatory signaling to ensure complete activation (Jones, 1998a,b) and set the pace for the EEA in the context of their prolonged cell cycle times. The oscillation frequency of mammalian eggs may be evolutionarily tailored to provide a sufficient duration of time (or, time span in which $[Ca^{2+}]_i$ is periodically elevated) for optimizing EEAs that are involved in later development (Ozil et al., 2006). It should be noted that some species with short meiotic cell cycle times do not have a single $[Ca^{2+}]_i$ rise between fertilization and first cleavage, but have an oscillatory response (Stricker, 1999).

A simplified model of the chain of command from Ca^{2+} to EEA has some features that highlight oscillatory signaling (Fig. 3). Each oscillation of CaMKII activity decreases the cyclin B pool incrementally (Nixon et al., 2002), although some cyclin synthesis continues after egg activation. After cyclin levels decrease sufficiently, MPF activity falls to a level that no longer maintains metaphase II arrest. A transient decrease in MPF is not sufficient for normal cell cycle progression, and the maintenance of a low level of MPF in mammalian eggs appears to be accomplished by continued $[Ca^{2+}]_i$ oscillations. For example, if experimentally imposed oscillations in $[Ca^{2+}]_i$ are

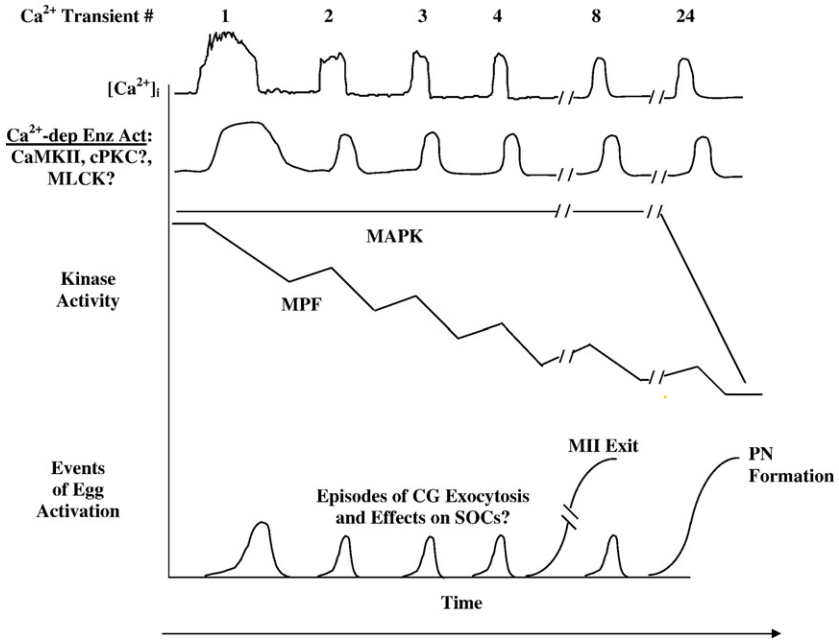

Fig. 3. The relationship of oscillatory Ca^{2+} signaling to the activities of CaMKII, MAPK, and MPF in mammalian eggs. SOC—store-operated Ca^{2+} channels. Abbreviations: CaMKII—Ca^{2+}/CaM-dependent protein kinase II, PLC—phospholipase C, CG—cortical granule, MII—metaphase II of meiosis, MAPK—MAP kinase, MLCK—myosin light chain kinase, MPF—maturation or M-phase promoting factor, PKC—protein kinase C, PN—pronucleus, SOC—store-operated channels (see text for additional information).

terminated prematurely, MPF levels increase again and chromatin remains condensed (e.g., so-called metaphase III state) without progression to the interphase pronuclear stage (Ducibella et al., 2002). In contrast to the decrease in MPF activity, the decrease in MAPK activity, which is also dependent upon $[Ca^{2+}]_i$ and observed in the sea urchin egg (Kumano et al., 2001), does not appear to be incremental and occurs several hours later (Moos et al., 1995; Ducibella et al., 2002). Here again, continued $[Ca^{2+}]_i$ oscillations have been found necessary for downregulation of the activity of this PK, although the mechanism remains to be established.

Like CaMKII, PKC and MLCK are candidates for oscillatory enzyme activity in response to fertilization-induced rises in $[Ca^{2+}]_i$. In amphibian and sea urchin eggs, a single increase in $[Ca^{2+}]_i$ is followed by a MLCK-mediated cortical contractile response and then very rapid mitotic cleavages. This raises the prospect that oscillatory Ca^{2+} signals during early rapid mitotic cleavages in these eggs (reviewed in Whitaker and Larman, 2001) generate oscillatory responses of MLCK that regulate the contractile ring formation and function.

Regarding PKC, immediately following the Ca^{2+} wave, a wave of cortical-associated PKC-GFP fluorescence has been reported in *Xenopus* eggs (Larabell et al., 2004). PKC activity increases by a MARCKS phosphorylation assay by 10 min after insemination in mouse eggs (Tatone et al., 2003). Since most of this activity was reported to be Ca^{2+}-independent, it may be due to PKCδ based on other findings in this and other studies (Viveiros et al., 2001, 2003). After insemination or treatment with PKC activators, various PKC isoforms translocate to regions associated with the egg plasma membrane, meiotic spindle, or pronuclei (Gallicano et al., 1995, 1997; Luria et al., 2000; Eliyahu and Shalgi, 2002; Tatone et al., 2003; Viveiros et al., 2003; Page-Baluch et al., 2004; Halet et al., 2004; Larabell et al., 2004; Zheng et al., 2005). Membrane translocation is associated with activation of cPKC enzyme activity in response to oscillations in $[Ca^{2+}]_i$ in other cell types (Violin et al., 2003).

A recent study provides evidence that cPKCα translocates in an oscillatory manner, with each rise in $[Ca^{2+}]_i$ in mouse eggs, which were simultaneously monitored for $[Ca^{2+}]_i$ and EGFP-PKCα location (Halet et al., 2004). The Ca^{2+} binding, C2-domain, of PKC behaved similarly. Interestingly, the extent of translocation was influenced by the amplitude, frequency, and duration of oscillations in the $[Ca^{2+}]_i$. Since only higher amplitude Ca^{2+} spikes (1–3 μM) resulted in detectable translocation, eggs with less robust $[Ca^{2+}]_i$ responses may be at a developmental disadvantage, depending upon the importance of PKC to the EEA (discussed earlier). It is already known that the amplitude of $[Ca^{2+}]_i$ has an effect on cyclin degradation and possibly CaMKII activity, as well.

Studies of the extent to which PKC is activated at fertilization have utilized several different approaches. In the first, the effects of PKC inhibitors on the EEA were investigated with potentially interesting but sometimes conflicting results as mentioned previously. More recently, PKC translocation has been investigated by immunofluorescence using a specific indicator fluorescence probe (Rim-1), GFP/PKC constructs, and antibodies to unphosphorylated and phosphorylated isoforms (the large number of studies are reviewed in Halet, 2004; also see Page-Baluch et al., 2004). Also, the phosphorylation of the PKC substrates, MARCKS protein and myelin basic protein (MBP), have been monitored (Gallicano et al., 1997; Viveiros et

al., 2001; Tatone et al., 2003; Tatone and Carbone, 2006; Michaut et al., 2005; Eliyahu et al., 2006).

Obtaining direct evidence for the specific functions of PKC isoforms in eggs from studies of enzyme activity has been difficult. In part, this is due to the complex regulation of PKC *in vivo*, to three classes of isoforms with different activation requirements, and to whether PKC enzyme activity assays in lysed cells faithfully reflect relative enzyme activity in intact cells (Newton, 2002). For cPKCs, regulation involves phosphorylation by PDK-1, Ca^{2+}-mediated translocation to the plasma membrane, and DAG-mediated conformational change to free the active site. The relative contributions of DAG and $[Ca^{2+}]_i$ to PKC activation in cells have been discussed elsewhere (Oancea and Meyer, 1998; Violin et al., 2003).

Unlike assays for MPF, MAPK, and CaMKII, it is not clear that relative changes in PKC enzyme activity can be as accurately assayed in lysed cells based upon changes in its phosphorylation status that had taken place *in vivo*. For PKC, phosphorylation is an indicator of initial activation but not necessarily enzyme activity, since targeting and a conformational change are also required (see Violin et al., 2003). In biochemical assays, how cell lysates should be handled with respect to $[Ca^{2+}]_i$ and DAG addition brings up the question of their levels in the cell prior to lysis. For these reasons, FRET analysis of PKC activity (Violin et al., 2003) *in vivo* has advantages that should be applied to eggs.

In fertilized mouse eggs, although translocation of cPKCs correlates with oscillations of the $[Ca^{2+}]_i$, DAG production was not detected (Halet et al., 2004). In contrast, phorbol ester (PMA) or ionophore did induce a signal for DAG production in the same study. Addition of exogenous DAG increased the translocation of PKCγ-GFP to the region of the plasma membrane. It has been proposed that cPKCs may be regulated by PIP_2, which increases in fertilized mouse eggs (Halet et al., 2002).

In summary, in fertilized mammalian eggs, several functions have been identified for oscillatory signaling, including setting the pace for the EAA and incremental CG exocytosis, as well as maintenance of low MPF activity and high sensitivity of CaMKII. Other potential functions require further investigation, such as the timing of the decrease in MAPK activity, prevention of apoptosis (Bos-Mikich et al., 1997; Fissore et al., 2002; Rogers et al., 2004), periodic refilling of internal Ca^{2+} stores necessary to maintain oscillations, and regulating the expression of new proteins (not covered herein; Ducibella et al., 2002). These real and potential advantages raise the question of whether of oscillatory Ca^{2+} signaling is required for both normal egg activation and development in mammals and other species.

Is oscillatory signaling necessary?

The fact that all mammalian eggs investigated to date utilize oscillatory Ca^{2+}, CaMKII, and cPKC signaling suggests that there have been adaptive advantages to this mechanism of initiating mammalian development, some of which are discussed above. However, as developmental biologists, we often ask if such a mechanism is necessary for normal development and is it the only way eggs can be activated with high efficiency? These questions are pertinent to experimentally manipulating or stimulating the EEA and development, for example, in the context of somatic cell nuclear transfer (SCNT) used to investigate totipotency, generate embryonic stem cells, and produce animal clones. Animal cloning may become more important for maintaining many endangered species in the future.

It has long been known that parthenogenetic egg activation protocols without oscillatory Ca^{2+} transients stimulate EEA and further development. For example, early studies utilizing an ethanolic medium and replacement of a PN gave rise to apparently normal mice (Surani et al., 1984). From ionophore and other studies, it has been generally found that parthenogenetic agents that cause a single short rise in $[Ca^{2+}]_i$ do not stimulate all EEA in freshly ovulated mouse eggs, and aging eggs for a short time post-ovulation improves activation (Bos-Mikich et al., 1995 and references therein). Older is not necessarily better since mouse eggs aged for 24 h post-ovulation are prone to apoptosis and developmental defects if fertilized (Tarin et al., 1999; Gordo et al., 2000; Fissore et al., 2002). The increased susceptibility to activate in aged mouse eggs is likely due to time/age-dependent decreases in the levels of MPF and MAPK activity in unstimulated eggs, as well as a decrease in the Ca^{2+} requirement to induce the EEA (Xu et al., 1997; Abbott et al., 1998; Vincent et al., 1992; also see Jones and Whittingham, 1996). Lower doses of ionophore or IP_3 were more effective in stimulating EEA in aged eggs than in newly ovulated eggs. Thus, mammalian eggs age rapidly with changes in Ca^{2+} thresholds, which appear to make Ca^{2+} oscillations or prolonged Ca^{2+} treatment no longer required for the EEA. The cause is unknown for these effects of aging, which occur more rapidly in the oviduct than in culture. However, mechanisms that alter PK activity levels appear to be involved due to the decreases in the activities of MPF and MAPK.

$SrCl_2$ is often used to mimic oscillatory Ca^{2+} signaling in parthenogenetic activation procedures in aged rodent eggs, especially for SCNT (Wakayama et al., 1998; Otaegui et al., 1999). Although it can generate a high percentage of diploid parthenogenetic blastocysts, 2–8 h of $SrCl_2$ treatment alters the mean numbers of inner cell mass and trophectoderm cells (and their ratio) compared to embryos from IVF (Bos-Mikich et al., 1997). It is not clear to what extent this is due to differences in binding affinity of Sr^{2+} and Ca^{2+} for Ca^{2+} probes and protein targets. It should be noted that fluorescence probes for Ca^{2+} can report both $[Ca^{2+}]_i$ and the $[Sr^{2+}]_i$ (Tsien, 1999).

Pertinent to this review is whether Sr^{2+} faithfully mimics Ca^{2+}, reproducing the responses of Ca^{2+}-dependent PKs. With $SrCl_2$ treatment, oscillatory increases reported by fluo-3 appear to be lower in amplitude than those from fertilization (Kline and Kline, 1992) (although accurate calibrations are needed of the intracellular rises induced by $SrCl_2$) and may be due to alterations in PKC activity, a positive Ca^{2+} effector (Halet et al., 2004). Although the activities of PKs that directly bind Ca^{2+} or Ca^{2+}/CaM have not been studied in $SrCl_2$-treated mammalian eggs, these eggs exit metaphase II and form second polar bodies,

implying that CaMKII and MLCK have been activated (although other mechanisms have not been ruled out). MPF and MAPK activities are downregulated after $SrCl_2$ injection, rather than extracellular treatment (Okada et al., 2003). In this regard, Sr^{2+} has a higher affinity than Ca^{2+} for the Ca^{2+} activation site on the IP_3R, which is a crucial player in generating oscillations of $[Ca^{2+}]_i$. In addition, Sr^{2+} appears to have a lower affinity for IP_3R inhibitory sites (Marshall and Taylor, 1994). These changes in affinity and enhancement of egg PLC activity (Zang et al., 2005) may explain the longer duration of individual Ca^{2+} oscillatory rises induced by $SrCl_2$ than fertilization, as well as the absence of IP_3R-1 degradation in $SrCl_2$-activated eggs (Brind et al., 2000; Jellerette et al., 2000).

In the absence of oscillatory Ca^{2+} signaling, expression of constitutively active αCaMKII causes the EEA and blastocyst development (above), although CG exocytosis is not normal. These results are subject to many interpretations. Since CaMKII is normally activated by Ca^{2+}/CaM, the importance of $[Ca^{2+}]_i$ is still supported even though it is not elevated. CaMKII may be a master regulator of many EEA. If so, other targets required for the EEA which depend on the $[Ca^{2+}]_i$, but not CaMKII, must not be crucial for early development. For example, some potential Ca^{2+} targets are regulators of $[Ca^{2+}]_i$ oscillations themselves (cPKC; Halet et al., 2004), which are not required for the activity of constitutively active CaMKII. Abnormal CG exocytosis may be explained by the (expected) failure to activate MLCK and synaptotagmin, both of which are Ca^{2+}-dependent. Although it is surprising that so many EEA proceed with constitutively active CaMKII in light of the large number of Ca^{2+}-dependent proteins in all cells, CaMKII has a robust number and wide range of targets.

Identification of all of the downstream targets of CaMKII in eggs and when these targets require phosphorylation are important future objectives. Not only will this information connect the dots between CaMKII and specific EEA (Fig. 4), but also indicate whether CaMKII activity is utilized by the egg throughout the entire period of oscillations of the $[Ca^{2+}]_i$. For example, an event requiring CaMKII activity at 1 or 2 h post-fertilization would provide support for the importance of oscillatory Ca^{2+} and PK activity (recall that prolonged continuous activity of endogenous CaMKII causes downregulation after 20 min). A case in point involves MLCK. There is evidence that MLCK activity is needed for second polar body separation (previously discussed) 2 h after the initial rise in $[Ca^{2+}]_i$ in fertilized mouse eggs, implying a role for an oscillatory increase in $[Ca^{2+}]_i$ at that time. However, parthenogenetically mediated polar body formation from a brief, single elevation of $[Ca^{2+}]_i$ or from constitutively active CaMKII would not have an appropriately timed increase in $[Ca^{2+}]_i$ (although other kinases can also phosphorylate myosin light chain). This paradox remains to be explained.

The activity of the constitutively active CaMKII remains elevated (Madgwick et al., 2005; Knott et al., 2006) and, thus, it is unlikely to oscillate. If so, does oscillatory CaMKII activity in fertilized eggs have no benefit for development (at least through the blastocyst stage)? Is it merely a by-product of oscillatory Ca^{2+} signaling, which prevents downregulation of CaMKII activity and which may have developmental advantages for other reasons? In this regard, parthenogenetic activation (with cycloheximide) without Ca^{2+} or Sr^{2+} affects gene expression patterns (Rogers et al., 2006), as does fertilization with an altered pattern of oscillations of $[Ca^{2+}]_i$ (Ozil et al., 2006).

Although parthenogenetic stimulation and normal fertilization often do not have precisely equivalent effects on the EEA and early development, the egg appears to interpret one aspect of either type of stimulation by a common mechanism, called "summation" (Toth et al., 2006). Given a sufficient amplitude of the $[Ca^{2+}]_i$, the egg appears to readout the length of time that $[Ca^{2+}]_i$ is elevated, whether or not the elevation is continuous (Ozil et al., 2005) or oscillatory (Ducibella et al., 2002; Knott et al., 2006; Toth et al., 2006). In these studies, all of the following were successful in generating a high percentage of mouse blastocytes: a prolonged continuous $[Ca^{2+}]_i$ elevation, or a sufficient number of short oscillatory rises in the $[Ca^{2+}]_i$, or a combination of two different non-overlapping oscillatory protocols, which are insufficient when used individually.

Extending this summation idea to PK signaling at fertilization leads to the hypothesis that PK activity and substrate phosphorylation over time are additive. In theory, the simplest cases to investigate are those eggs with a single, long increase in $[Ca^{2+}]_i$, such as the sea urchin and amphibian. In the sea urchin, ionomycin activation results in a time-dependent increase in myosin light chain phosphorylation, suggestive of cumulative MLCK activity (Lucero et al., 2006; Stack et al., 2006).

In eggs with oscillatory rises in the $[Ca^{2+}]_i$, summation of Ca^{2+}-dependent PK activity appears likely, but it is not necessarily as simple. On one hand, oscillatory CaMKII activity is a simple mechanism to account for the Ca^{2+} oscillation number-dependent decrease in MPF activity and amount of cyclin. However, if phosphatase activity prevailed when $[Ca^{2+}]_i$ decreased between oscillatory rises, the total amount of substrate phosphorylation at the end of each oscillation cycle (peak and subsequent baseline) would be less than immediately after the rise of $[Ca^{2+}]_i$. Unfortunately, little is known about phosphatase activity in relation to regulation of PK activity, phosphatase substrates, and oscillations in $[Ca^{2+}]_i$ in fertilized eggs. However, the CaMKII pathway helps to illustrate what can happen between rises of $[Ca^{2+}]_i$. In mammalian eggs, cyclin synthesis continues after fertilization. If oscillations of the $[Ca^{2+}]_i$ and CaMKII activity are prematurely terminated, cyclin levels appear to increase based on the return of MPF activity to high levels that prevent cell cycle progression (Ducibella et al., 2002). Although perhaps a simplistic prediction, compressing the oscillations into a shorter time period would allow less total cyclin synthesis than during the normal oscillatory period. In this scenario, less total CaMKII activity over time would be required for sufficient cyclin degradation for cell cycle progression. Several observations are consistent with this, although there are caveats (e.g., eggs with a relatively high-frequency pattern of oscillations that is either endogenous or imposed by experimental treatments take a shorter time to develop PN (above)).

Furthermore, since several prominent PKs involved in egg activation are regulated by phosphorylation status, depho-

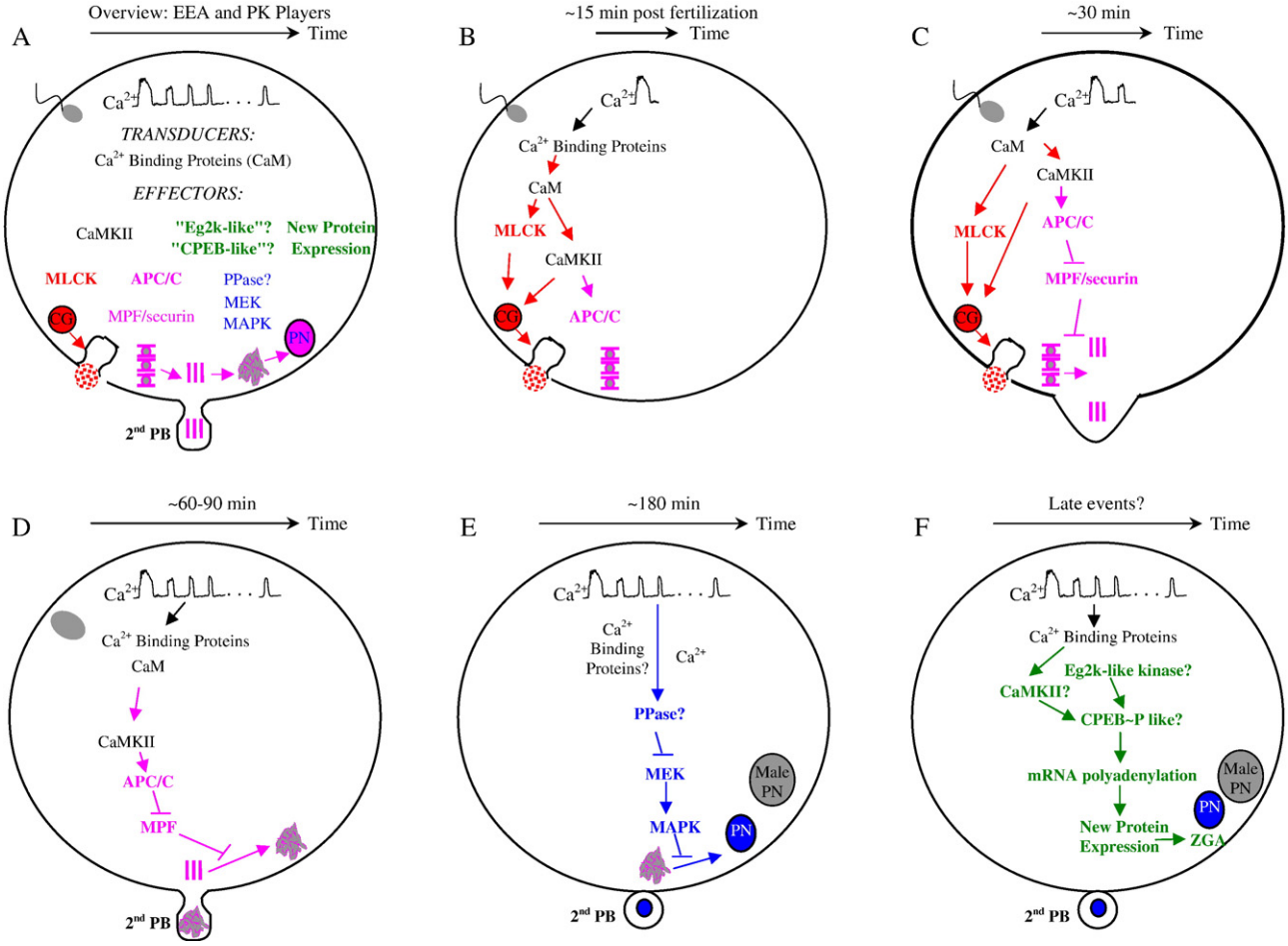

Fig. 4. Model of how signaling pathways change over time in mammalian eggs during oscillatory Ca^{2+} signaling. Some pathways have substantial supporting documentation, such as cell cycle progression (e.g., see Madgwick et al., 2004). Some pathways are highly speculative, such as that for recruitment of mRNAs and zygotic gene activation (ZGA) by a mechanism like that used during egg meiotic maturation, involving an Eg2 kinase and cytoplasmic polyadenylation binding element protein (CPEB) (Mendez and Richter, 2001). Many targets of Ca^{2+} and CaM remain to be investigated. Most abbreviations are identified in the text. Abbreviations: APC/C—cyclosome, CaM—calmodulin, CaMKII—Ca^{2+}/CaM-dependent protein kinase II, CG—cortical granule, Emi2—early mitotic inhibitor 2, MAPK—MAP kinase, MEK—MAPK kinase, MLCK—myosin light chain kinase, MPF—maturation or M-phase promoting factor, PB—polar body, PN—pronucleus, PPase—protein phosphatase (see text for additional information). Times indicated are for the mouse egg.

sphorylation becomes a potentially important mechanism of regulating the amplitude and duration of their activity. In the case of CaMKII, since autonomous CaMKII activity is oscillatory in fertilized mouse eggs and reflects autophosphorylation, oscillatory dephosphorylation of CaMKII is predicted each time $[Ca^{2+}]_i$ returns to a baseline level. Biochemical studies of CaMKII in other cells indicate that dephosphorylation can be achieved by PP1 phosphatase (Strack et al., 1997; Menegon et al., 2002; Bradshaw et al., 2003) or by CaMKII itself (Kim et al., 2001). Little is known in fertilized eggs about the role of phosphatase activities in regulating PK activity, although there is evidence for MAPK phosphatase activity in *Xenopus* eggs (Sohaskey and Ferrell, 1999) and the sea urchin (Kumano et al., 2001). Interestingly, Cdc25C phosphatase activity depends upon CaMKII during mitoses after fertilization in the sea urchin embryo (Patel et al., 1999) and in *Xenopus* (see Walker et al., 1992). Protein phosphatases are found in mouse oocytes (Smith et al., 1998; Swain et al., 2007), changes in PP2A activity have been reported after ethanol activation of mouse eggs (Winston and Maro, 1999), and the effects of okadaic acid on eggs are well known. Thus, cycles of PK and protein phosphatase activities are worth investigating in eggs with oscillatory Ca^{2+}, especially since phosphatases PP2A and PP2B are activated by Ca^{2+} and Ca^{2+}/CaM, respectively.

In summary, although parthenogenetic, non-oscillatory activation procedures stimulate many EEA and some result in offspring (e.g., Dolly, the sheep), normal fertilization induces all EEA and a high percentage normal offspring from newly ovulated eggs rather than aged eggs with altered PK levels and lower developmental potential. Recent studies emphasize the importance of the amplitude and duration of the $[Ca^{2+}]_i$ over time, which are interpreted in terms of summation and which likely have effects through sufficient stimulation of PK activity. Parthenogenetic studies are limited by the absence of both parental imprints. The precise importance of the temporal pattern of $[Ca^{2+}]_i$ for long-range developmental events awaits investigations in which sperm can be engineered to generate

different temporal patterns of $[Ca^{2+}]_i$ in fertilized eggs, or from equivalent types of experiments.

Future directions

In summary, since Mazia's discovery of an elevated $[Ca^{2+}]_i$ in lysates of sea urchin eggs in 1937, studies of many organisms have contributed to our present knowledge of the temporal, spatial, and informational nature of the crucial Ca^{2+} signal that makes the fertilized egg realize its potential as the ultimate or "mother of all" stem cells. Several major conclusions emerge from this review. Ca^{2+}-dependent proteins and PKs play a dominant role in transducing the Ca^{2+} signal into many EEA. Since each of these proteins (CaMKII, cPKC, MLCK, EF hand-dependent, etc.) represents a signaling branch point downstream of Ca^{2+}, the type and complexity of the signaling tree – connecting Ca^{2+} to the EEA (Epel, 1989) – has been greatly clarified. Thus, the phosphorylation status of PK targets regulates not only the cell cycle but also many other EEAs. The amplitude and duration of the Ca^{2+} signal and, by extension, those of specific PK activities control the initiation and completion of the EEA. For species with oscillatory responses, the frequency of signals from Ca^{2+} and PK activity appears to regulate the rate at which the EEAs go to completion. Interestingly, phosphorylation appears to be regulating even the primary signal, itself—the $[Ca^{2+}]_i$. In addition, the hypothesis has emerged that the effects of these PKs have long-range developmental consequences.

This review also raises a myriad of general questions. Many PK targets remain to be identified, and the function of many Ca^{2+}-dependent proteins, different groups of PK, and their PK isoforms require investigation. Does the temporal pattern of the elevation of $[Ca^{2+}]_i$ as well as its amplitude and duration (and frequency, for oscillatory eggs) provide a PK activity response profile that is tailored to optimally activate eggs with different requirements for the initiation of development (e.g., various species are fertilized at different stages of meiosis)? Are species-specific oscillation patterns and changes in $[Ca^{2+}]_i$ regulated by downstream PKs whose activity is directly or indirectly Ca^{2+}-dependent? For example, do oscillations of $[Ca^{2+}]_i$ self-regulate via PK activity feedback? How did the different patterns of $[Ca^{2+}]_i$ response arise during evolution? By analogy with egg meiotic maturation (Mendez and Richter, 2001), does PK signaling regulate maternal mRNA expression, participating in the normal developmental program of early development (see Ducibella et al., 2006)? To what extent will emerging genomic, kinomic, and phosphoproteomic information assist in answering these questions (e.g., Bradham et al., 2006; Roux et al., 2006; Su et al., 2007)? Since the sea urchin kinome has 98% of the subfamilies of PKs as the human (Bradham et al., 2006), it remains a valuable system to study the mechanism of egg activation.

Many specific questions remain as well. Here is merely a sampling. Are phosphatases involved in regulating PK activity changes after fertilization? Which, if any, are regulated by Ca^{2+}? How is MAPK activity downregulated in a Ca^{2+}-dependent manner? Are other isoforms of CaMK or CaMKK involved in the EEA? How prevalent are PK localizations in eggs and how are these achieved for different PK isoforms, such as those for PKC? What key molecular components of the egg's Ca^{2+} releasing machinery, i.e., IP_3R1 and SERCA pumps, are regulated by PKs and by which PKs? In those species with CG translocation at fertilization, do CaMKII, MLCK, PKC, and synaptotagmin act in a co-ordinated manner to de-tether CGs from the cortical cytoskeleton, regulate CG movement to the plasma membrane, modify a dense cortical actin barrier, and regulate fusion, respectively? Does the sperm bring Ca^{2+}-sensitive and PK regulatory factors (in addition to PLCζ) to the site of sperm–egg fusion, assisting in localized initial signal generation?

A robust effort will be needed to address this plethora of questions, and answers will tell us how the ultimate stem cell initiates development, switching from a gamete-based program to a zygotic and embryonic one with maternal inputs (Schultz, 2002; Evsikov et al., 2006; Zeng and Schultz, 2005; Howard-Ashby et al., 2006; Su et al., 2007). In addition, some of these answers are likely to be beneficial to establishing parameters of egg quality for human and animal IVF, as well as improving egg activation protocols for somatic cell nuclear transfer (see Vassena et al., 2007) to generate stem cells or save endangered species.

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