[Frontiers in Bioscience 6, d792-806, July 1, 2001]

CALCIUM AND THE CONTROL OF MAMMALIAN CORTICAL GRANULE EXOCYTOSIS

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TABLE OF CONTENTS

1. Abstract
2. Review of egg activation events at fertilization
2.1. $[Ca^{2+}]_i$ release at fertilization
2.2. Ca^{2+}-induced CG exocytosis
3. Potential Ca^{2+} effectors involved in CG exocytosis
3.1. Calmodulin
3.2. CaMKII and Synapsin I
3.3. PKC
3.4. Synaptotagmin
3.5. Rab 3 and rabphilin-3A
4. Cytoskeletal regulation of CG translocation and exocytosis
5. Kinetics of CG exocytosis
5.1. Kinetics of CG exocytosis in sea urchin eggs
5.2. Kinetics of CG exocytosis in mouse eggs
5.3. Relationship between CG exocytosis and sensitivity to $[Ca^{2+}]_i$
6. Development of secretory competence during oocyte maturation
7. Summary and conclusions
8. Acknowledgements
9. References

1. ABSTRACT

At fertilization, the release of intracellular calcium is necessary and sufficient for most, if not virtually all, of the major events of egg activation that are responsible for the onset of embryonic development. In mammalian eggs, repetitive calcium oscillations stimulate egg activation events through calcium-dependent effectors, such as calmodulin, protein kinases, and specific proteins involved in exocytosis. One of the earliest calcium-dependent events is the exocytosis of cortical granules (CGs), a secretory event resulting in the block to polyspermy and the prevention of triploidy. Emerging studies suggest that CG release in mature eggs is dependent upon calcium-dependent proteins similar to those in somatic cells employed to undergo calcium-regulated exocytosis. In contrast, pre-ovulatory oocytes are incompetent to undergo CG exocytosis due to deficiencies in the ability to release and respond to increases in intracellular calcium. The development of competence to release and respond to calcium is relevant to both animal and human *in vitro* fertilization programs that largely utilize ovarian oocytes not all of which are fully activation competent.

2. REVIEW OF EGG ACTIVATION EVENTS AT FERTILIZATION

The union of egg and sperm at fertilization is the spark that triggers a cascade of events, collectively known as egg activation. Egg activation encompasses mechanisms to both release and respond to intracellular Ca^{2+} ($[Ca^{2+}]_i$) in order to subsequently undergo key events, including cortical granule (CG) exocytosis, cell cycle resumption, and recruitment of maternal mRNAs (figure 1). One of the earliest classes of activation events is CG exocytosis, which results in modifications to the mammalian egg's extracellular matrix, the zona pellucida (ZP) (1), or perivitelline space (2). The contents of thousands of CGs in the mammalian egg cortex are released at fertilization and their structure as well as composition have been reviewed elsewhere (2-5). Based on indirect evidence in mammals and direct evidence in other animals, CGs contain enzymes and other proteins that, upon release, biochemically modify specific egg matrix proteins. Of the three proteins that constitute the mammalian ZP, the ZP-1 protein does not appear to be modified in mice, whereas ZP-2 and ZP-3 are cleaved or glycolytically modified, respectively, to produce a ZP that is no longer compatible

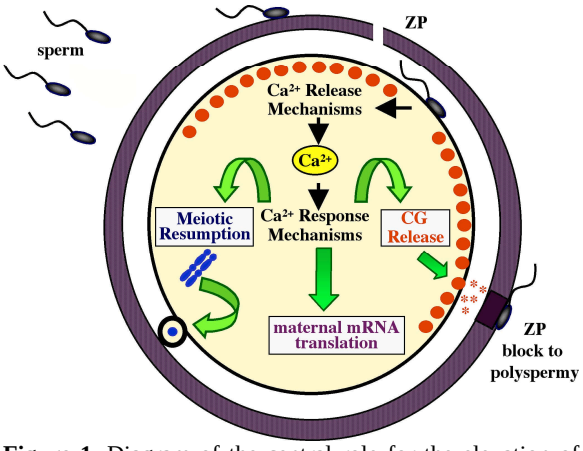

Figure 1. Diagram of the central role for the elevation of intracellular Ca^{2+} in stimulating the major events of mammalian egg activation. Note that both Ca^{2+} release and response mechanisms are required for these events. As discussed in the text, critical components of these two mechanisms develop just prior to ovulation and during meiotic maturation. CG cortical granule; ZP, zona pellucida.

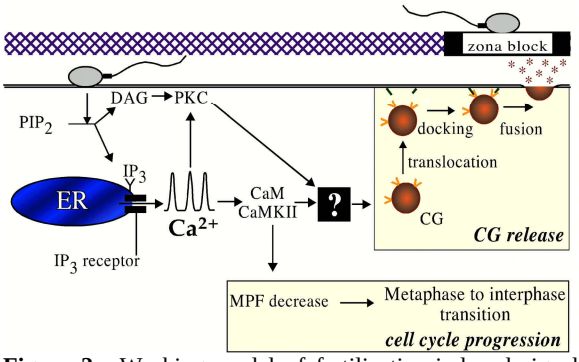

Figure 2. Working model of fertilization-induced signal transduction pathways required for CG secretion and cell cycle progression. While several steps are shown within the box for CG release, steps for cell cycle progression are not shown since they are not the subject of this review. The three Ca^{2+} peaks represent oscillations upon fertilization which normally continue in much greater number for several hours. The question mark indicates evidence for a pathway based on *in vitro* data in other vertebrate eggs or parthenogenetic activation without sperm as well as unknown factors which induce CG exocytosis in response to Ca^{2+} elevation and/or kinase activation. PIP_2, phosphoinositol bisphosphate; DAG, diacylglycerol; PKC, protein kinase C; IP_3, inositol trisphosphate; ER, endoplasmic reticulum; MPF, maturation-promoting factor; CG, cortical granule.

with sperm binding and penetration (1). These extracellular matrix modifications constitute the generation of the ZP block to polyspermy that is relied upon for the primary polyspermy block by many mammals, including humans. The ZP block is crucial for development, because it prevents the lethal condition of triploidy by preventing additional sperm entry into the egg. This is especially relevant to human *in vitro* fertilization in which tens of thousands of sperm are incubated with a single egg.

Subsequent to the onset of CG release, there are two other major classes of egg activation events. Important cell cycle events constitute one class (6). The mature, ovulated mammalian egg is arrested in metaphase II of meiosis. Fertilization stimulates the completion of meiosis, extrusion of the second polar body, formation of pronuclei, and the first mitosis. Prior to egg activation, metaphase II-arrest is maintained by an active complex of the cell cycle regulators, including $p34^{cdc2}$ kinase, cyclin B, and cytostatic factor (6-8). Fertilization induces a Ca^{2+}-dependent inactivation of $p34^{cdc2}$ kinase activity via cyclin B degradation with subsequent resumption of the meiotic cell cycle (9). Changes in the activity of other kinases appear to play important roles in chromosome separation at anaphase II, polar body extrusion, and pronuclear formation (10-12).

A second class of events represents changes in specific proteins required for early preimplantation development. Since the male and female pronuclei are not transcriptionally active, the fertilized egg produces new proteins by translating maternal mRNAs made during oogenesis and by the post-translational modifications of proteins. For the purposes of this article, it is important to stress that both of these classes of egg activation events, like CG exocytosis, are dependent on elevations $[Ca^{2+}]_i$.

2.1. $[Ca^{2+}]_i$ release at fertilization

Fertilization in animals (13) and some plants (14) results in a species-specific pattern of $[Ca^{2+}]_i$ elevations. For example, fertilization in echinoderm eggs is characterized by a single wave of $[Ca^{2+}]_i$ release, while mammalian fertilization is characterized by repetitive $[Ca^{2+}]_i$ oscillations. As depicted in the cartoon in figure 2, in vertebrates (15), these oscillations are dependent primarily upon the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) and the subsequent generation of inositol 1,4,5-trisphosphate (IP_3) (16, 17) and diacylglycerol (DAG) (18). IP_3 acts as a small intracellular second messenger to induce the periodic release of $[Ca^{2+}]_i$ from primary $[Ca^{2+}]_i$ stores in the endoplasmic reticulum (ER) (19-21). This is accomplished by IP_3 binding to its receptor in the ER membrane that acts as a Ca^{2+} gate into the cytosol, opening upon IP_3 binding. The IP_3-induced $[Ca^{2+}]_i$ oscillations begin shortly following fusion of the sperm and egg (22) and continue for hours (23, 24).

A central role for Ca^{2+} as a universal signal for egg activation across nearly all species has long been appreciated (6, 13, 20, 25-28). Upon sperm binding to the egg plasma membrane there is a latent period of a few seconds to a few minutes (29) following which an initial $[Ca^{2+}]_i$ rise is observed. The peak $[Ca^{2+}]_i$ in the mouse egg is in the range of 1 micromolar with subsequent oscillations of a lesser amplitude (20, 23). Multiple elevations are important for the initiation of mammalian development because a single elevation in $[Ca^{2+}]_i$ is not able to elicit the late events of egg activation in newly ovulated eggs (30, 31). In contrast, parthenogenetic activation using electroporation (32) to mimic fertilization-induced $[Ca^{2+}]_i$ oscillations induces the initiation of both early events of egg activation as well as later events of embryonic and early fetal development (33, 34).

2.2. Ca^{2+}-induced CG exocytosis

Both eggs and sperm require an elevation of $[Ca^{2+}]_i$ for the release of the contents of CGs (4, 35) and the acrosome (36), respectively. What is the evidence demonstrating that the rise in $[Ca^{2+}]_i$ is responsible for the exocytosis of CGs in the eggs of many species? One prediction is that the changes in $[Ca^{2+}]_i$ have the appropriate temporal and spatial characteristics; that is, the $[Ca^{2+}]_i$ increase must precede CG release and must take place throughout the entire CG-occupied cortex of this large cell. In the sea urchin, elegant studies by Terasaki demonstrate that the wave of $[Ca^{2+}]_i$ precedes a wave of CG exocytosis by approximately 6 seconds (37). In mammalian eggs, the rise in $[Ca^{2+}]_i$ occurs within 1-4 minutes of the cessation of sperm flagellar motion upon sperm attachment to the egg plasma membrane (38), whereas CG release occurs within the first 5-30 minutes (39-42), shortly following the first $[Ca^{2+}]_i$ transient (43), as described further in section 5 below.

A second prediction is that the $[Ca^{2+}]_i$ increase is necessary and sufficient for CG exocytosis. The inhibition of pathways involved in $[Ca^{2+}]_i$ release results in a block in CG release and/or modifications of the egg's extracellular matrix (44-47). Similar results are obtained with Ca^{2+} chelators that prevent the rise in free $[Ca^{2+}]_i$ when injected into the egg cytoplasm prior to fertilization or egg activation in mammals (23, 31) and sea urchins (48). In vertebrate eggs, $[Ca^{2+}]_i$ is sufficient to promote CG release as shown by Ca^{2+} or IP_3 microinjection, Ca^{2+} electroporation, and treatment with Ca^{2+} ionophore in the absence of sperm (23, 26, 31, 49-52)

3. POTENTIAL Ca^{2+} EFFECTORS INVOLVED IN CG EXOCYTOSIS

How are these $[Ca^{2+}]_i$ oscillations transduced in order to appropriately stimulate the different biological events of egg activation described previously? One general strategy is to investigate Ca^{2+}-dependent proteins that have been studied in more depth in somatic cells and that control similar events, including exocytosis and the cell cycle. Ca^{2+}-dependent proteins, identified in either sea urchin or mouse eggs by Western blot and/or immunofluorescence localization, include protein kinase C (PKC), calmodulin (CaM), Ca^{2+}/CaM-dependent protein kinase II (CaMKII), synaptotagmin, and rabphilin-3A. Their specific functions are under active investigation and some may have multiple roles in egg activation by regulating several events, for example by being positioned relatively 'upstream' in a Ca^{2+} signaling pathway (28, 30, 53). Below, several prominent candidate Ca^{2+} effectors for CG exocytosis will be discussed in light of extensive evidence for their role in secretion in somatic cells as well as their presence in eggs.

3.1. Calmodulin

One of the most common Ca^{2+} transducers in eukaryotic cells is CaM, which is a 17 kDa, EF handed Ca^{2+} binding protein that is highly multifunctional (54). For example, its many intracellular targets include calcineurin, myosin light chain kinase, CaMKII (55), and the type I isoform of the IP_3 receptor (56-59). Because there is evidence that the IP_3 receptor and CaMKII are important in triggering the release of and transducing $[Ca^{2+}]_i$ and that CaM accounts for approximately 0.3% of total protein in mouse eggs (60), it is already clear that CaM is likely an important Ca^{2+} effector at fertilization. However, it is relevant to note that many Ca^{2+}-dependent proteins in cells are not directly controlled by CaM.

Due to its abundance in eggs and numerous important substrates, CaM has been hypothesized to regulate both CG exocytosis and meiotic cell cycle. Of particular interest to these two egg activation events is the presence of CaM at the metaphase II spindle and in the cortex of mouse eggs (12). In fact, cortical localization of CaM is enhanced after $[Ca^{2+}]_i$ elevation (12). In addition, inhibitors of CaM block activation events. CG exocytosis from isolated cortices of sea urchin eggs is inhibited by an anti-CaM antibody, even in the presence of 1mM Ca^{2+} (61). Additionally, a pharmacological inhibitor of CaM, W-7, delays meiotic cell cycle resumption in mouse eggs (31). Although low doses of W-7 do not inhibit fertilization-induced CG exocytosis (31, 42), the results are not conclusive because it has not been possible to use the inhibitor at sufficiently high concentrations due to toxic effects on eggs (31). Thus, further experiments are needed to determine if Ca^{2+}-bound CaM associates with specific effectors required for CG exocytosis.

To understand the mechanism of CaM involvement in CG exocytosis, it is useful to review evidence from somatic cells. There is genetic, biochemical, and pharmacological evidence for a general role for CaM in membrane trafficking events and in regulated exocytosis. In yeast, both pharmacological inhibition of CaM and temperature-sensitive CaM mutations result in defective vacuolar fusion *in vivo* and *in vitro* (62). Likewise, CaM antagonists have been applied to demonstrate a role for CaM in regulated secretion in various cell types, including neurons (63), chromaffin cells (64), and endothelial cells (65), although the role of CaM in chromaffin cells is controversial (66).

In a study of regulated secretion in permeabilized PC12 cells, biochemical fractionation identified CaM as a factor able to stimulate secretion, by promoting the final 'triggering' step of secretion, without additional added cytosolic proteins (67). Because some proteins that are essential for secretion may have a membrane-bound pool, which would not be lost during permeabilization, it is not known if CaM stimulates secretion by acting directly or indirectly on proteins involved in membrane fusion. Interestingly, CaM may regulate secretion in an ATP-independent mechanism, indicating a role for CaM separate from such CaM targets as CaMKII or myosin light chain kinase (67, 68).

In summary, CaM appears to be an important regulator of egg activation events. Although a role for CaM in exocytosis is strengthened by evidence in somatic cells, a clear model for its mechanism of action remains to be elucidated. Deciphering which of CaM's numerous downstream targets are activated at fertilization should continue to be an active area of research in eggs.

3.2. CaMKII and Synapsin I

One important target of CaM is CaMKII, which is also found in all somatic cell types examined (69, 70) and in eggs of many species including sea urchin (71), *X.*

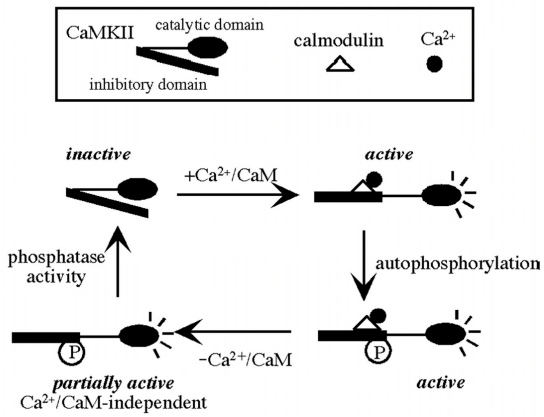

Figure 3. Simple model of the catalytic cycle of CaMKII. Relevant to egg activation is the so-called "memory" phase of the cycle in which the enzyme can maintain activity (via autophosphorylation) even after intracellular Ca^{2+} levels have decreased. This could allow prolonged CaMKII activity during the hours of Ca^{2+} oscillations without continuous elevated cytosolic Ca^{2+} that is generally toxic to all cells. CaM, calmodulin; CaMKII, calcium-calmodulin protein kinase II.

laevis (9), and mouse (12). Like CaM, CaMKII is also a multifunctional Ca^{2+} effector but it is also able to phosphorylate numerous proteins. Of particular interest is its ability to phosphorylate proteins involved in secretion including synaptotagmin, synapsin, synaptophysin, and rabphilin-3A, as well as the IP_3 receptor and calcineurin (69, 70).

Multifunctional kinases like CaMKII can target specific proteins for phosphorylation by localizing to a specific region or structure within the cell. This likely allows for the selective control of a discrete subset of potential targets. In the egg, CaMKII, like CaM, is present at the metaphase II spindle and in the cortex (12, 72). In addition, the enzymatically active form of CaMKII is detected in the egg cortex by immunofluorescence (72). CaMKII localizes to both synaptic vesicles in neurons (73) and to insulin granules in pancreatic beta cells (74). The beta isoform of CaMKII can direct the specific localization of CaMKII oligomers to dendritic spines and cortical areas of neurons through binding F-actin (75). Thus, CaMKII is able to localize to sites of exocytosis. Interestingly, beta CaMKII has recently been found to be predominant isoform in mouse eggs (unpublished data), which suggests that CaMKII in eggs may likewise localize to the actin rich cortex. Finer localization analysis, for example with immunoelectron microscopy, is needed to demonstrate if cortical CaMKII is associated with specific structures such as CGs.

In addition to being appropriately localized to regulate exocytosis, CaMKII has regulatory properties that suggest a role as a critical enzyme in 'decoding' the temporal pattern of repetitive $[Ca^{2+}]_i$ oscillations (figure 3) (69, 70, 76), which are the hallmark of mammalian egg activation. It is of particular interest that after activation by CaM, there is a period of Ca^{2+}-independent CaMKII activity prior to inactivation by phosphatase activity. With this activity, CaMKII is able to maintain activity between rapid Ca^{2+} oscillations in *in vitro* studies (76). Indeed, mathematical modeling supports a role for CaMKII in regulating the timing of the mammalian egg's exit from metaphase II-arrest and the start of the first embryonic mitotic divisions (77).

Since CaMKII is particularly suited to respond sensitively to $[Ca^{2+}]_i$ oscillations and is activated upon $[Ca^{2+}]_i$ elevation in eggs (12, 78-79), the hypothesis has recently arisen that CaMKII is required for CG secretion and the block to polyspermy. Current evidence supports a model in which CaMKII is an important regulator of egg activation events, including CG exocytosis and cell cycle resumption. In activated *X. laevis* eggs, peptide inhibitors of CaMKII activity block exit from the metaphase II-arrested state, by preventing its ability to induce cyclin degradation (9). Also, in mouse eggs stimulated with ethanol (79) or Ca^{2+} ionophore (12), the pharmacological inhibitor KN-93 (80) blocks exit from metaphase II-arrest. Furthermore, KN-93 blocks ethanol-induced CG exocytosis while importantly, not affecting the ethanol-induced $[Ca^{2+}]_i$ elevation (79 and see below). However, no evidence to date has shown a physiological role for CaMKII in CG exocytosis at fertilization.

Because this is an emerging area of research in gamete biology, it is appropriate to review the role of CaMKII in other somatic secretory cells where much more is known. Although pharmacological inhibition represents one approach to analyze the role of CaMKII, data interpretation can be complicated by effects on other cellular targets. For example, although KN-93 (80) has been used to examine the role of CaMKII in secretion and blocks secretion in pancreatic islets (54, 81), KN-93 also blocks depolarization-induced Ca^{2+} influx in beta cells (81). Lack of specificity has been reported for an alternate CaMKII inhibitor, KN-62 (81 and references therein). Due to the reliance of regulated exocytosis on elevations in $[Ca^{2+}]_i$, these inhibitors should be used cautiously and care should be taken to discriminate between specific inhibitory effects on CaMKII activity and the additional effects on Ca^{2+} channels, as discussed in Bhatt *et al*. (81).

Peptide inhibitors to CaMKII, including the autoinhibitory domain peptide, CaMKII 281-309 (82) and AIP, the nonphosphorylatable analog of the substrate peptide autocamtide-2 (83) have not been found to have similar effects on other cellular targets. Peptide inhibitors of CaMKII block insulin exocytosis in pancreatic beta cells (81, 84). In mouse eggs, AIP inhibits exit from metaphase II arrest in intact eggs as well as in permeabilized eggs (12).

An alternate approach to determine if CaMKII is required for exocytosis is through the targeted ablation of CaMKII genes. To date, alpha CaMKII is the only isoform for which a knock-out mouse has been generated (85, 86). Additionally, a transgenic mouse line was generated in which an alanine was substituted for the autophosphorylated

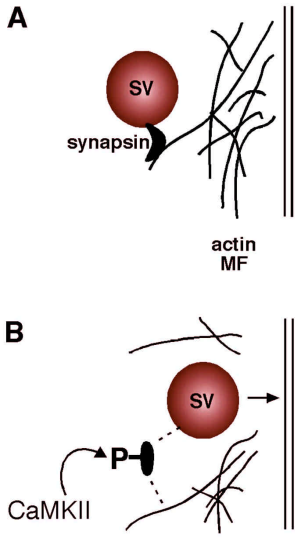

Figure 4. A model for vesicle translocation from a cortical location to the plasma membrane where docking and fusion occur. Egg CGs share a requirement with neurons for vesicle stabilization in the cell cortex and stimulation of translocation upon an increase in intracellular Ca^{2+}. Evidence is discussed in the text in which the protein, synapsin, tethers the vesicle in the resting pool of vesicles, whereas active CaMKII phosphorylates synapsin resulting in the loss of tethering. The mechanism of final translocation is not well understood. SV, synaptic vesicle; MF, microfilaments; CaMKII, calcium-calmodulin protein kinase II.

residue (Thr286) in the alpha CaMKII gene (87). Both lines of mutant mice display defective neurotransmission, specifically in learning and memory (reviewed in 88). In the future, additional knock-outs in beta, gamma, and delta CaMKII as well as cell-specific knockouts of CaMKII isoforms will aid in deciphering CaMKII's action in secretory cells.

Because synapsin I is a neuronal protein target of CaMKII and is involved in secretory vesicle translocation, it may play an important role in regulating CG exocytosis. However, synapsin has not yet been demonstrated in eggs so a role in CG exocytosis remains speculative. From evidence to date, a model has emerged in which CaMKII phosphorylates synapsin I to regulate the translocation of the 'reserve pool' of secretory vesicles to the active zone at the plasma membrane (figure 4; 89). Notably, synapsin I is able to bind to actin and synaptic vesicles when dephosphorylated and exhibits reduced affinity for both of these binding activities upon phosphorylation by CaMKII (89, 90). Mice lacking both synapsin I and synapsin II display a reduced number of synaptic vesicles in the active zone and abnormal synaptic transmission (91). Analysis of CG exocytosis in the eggs of synapsin I and II knockout mice will be useful in testing the hypothesis that CaMKII and synapsin I regulate CG exocytosis.

3.3. PKC

Like CaMKII, PKC may be particularly suited to respond sensitively to $[Ca^{2+}]_i$ oscillations through regulation by $[Ca^{2+}]_i$ and DAG (92). The PKC family of serine/threonine kinases has three classes, distinguished by their requirement for Ca^{2+} and DAG for activation: conventional PKCs that require Ca^{2+} and DAG (cPKC alpha, beta I, beta II, and gamma), novel PKCs that only require DAG (nPKC delta, theta, epsilon, and eta) and atypical PKCs that require neither Ca^{2+} or DAG (aPKC zeta, and iota; 93). The role of PKC in secretion is unclear and several different models have been proposed. These include the regulation by PKC of the release competent vesicle pool size, of plasma membrane Ca^{2+} channels, and the sensitivity of membrane trafficking machinery (94).

Phorbol esters, that mimic DAG as an agonist, have been widely used in order to determine if PKC regulates secretion. However, it is important to additionally determine if PKC is a physiologically relevant agonist of secretion (95). For example, Billiard *et al*. (96) found that although phorbol ester stimulation triggers LH secretion in gonadotropes, pharmacological inhibition of PKC activity only partially blocks physiologically stimulated secretion. Interestingly, a number of phorbol ester-sensitive proteins have been identified including Munc13-1 which interacts with synaptic vesicle proteins and has been proposed to be involved in synaptic vesicle exocytosis (93). Therefore, experiments with phorbol esters should be interpreted cautiously.

These caveats are also relevant to the evidence that PKC activity is required for CG exocytosis in eggs (reviewed in 97, 98). Phorbol esters activate CG secretion in the eggs of several species including frog (99, 100), mouse (101, 102) and pig (103). However, inhibition of PKC activity with several different inhibitors during fertilization does not block CG exocytosis (104). These data indicate that although PKC may act to regulate or enhance secretion, it is not likely to be the primary signaling pathway used at fertilization to trigger CG exocytosis. Because PKCs are present in eggs and are translocated to the cortex of fertilized/activated eggs, a role for PKC in CG exocytosis remains a tenable hypothesis. Multiple isoforms from all three classes have been detected by RT-PCR or western blotting in rat (105) and mouse eggs (106, 107). Gallicano *et al*. (108) demonstrated both PKC translocation to the cell periphery and an increase in PKC activity following fertilization. Future studies identifying PKC targets in mammalian eggs may add considerably to our understanding of egg activation. It is tempting to speculate that the fertilizing sperm may activate several signal transduction pathways that act on the secretory mechanism downstream of $[Ca^{2+}]_i$ release to provide functional redundancy or to finely regulate exocytosis.

3.4. Synaptotagmin

In addition to signal transduction proteins, specific proteins have been demonstrated to mediate membrane fusion events, including neurotransmitter release. Evidence to date supports the SNARE (soluble N-

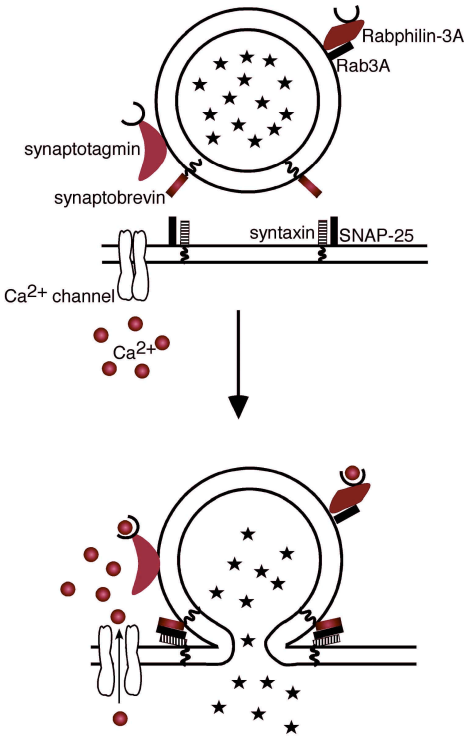

Figure 5. CGs may utilize a secretory mechanism similar to that of synaptic vesicles since similar secretory machinery proteins have been identified in the egg cortex or associated with CGs. The figure is a diagrammatic representation of the process of synaptic vesicle docking and fusion. CGs in eggs share a requirement with neurons for vesicle stabilization in the cell cortex and stimulation of translocation upon an increase in intracellular Ca^{2+}. In the neuron model, note that the stimulus for exocytosis is the influx of extracellular Ca^{2+} via a plasma membrane channel, resulting in Ca^{2+} binding to synaptotagmin. Rabphilin is also thought to bind Ca^{2+}. Some of the prominent protein players of the SNARE hypothesis are included, but many are not illustrated (see reviews, 73, 110-112). The vesicle SNARE, synaptobrevin, associates with the target SNAREs, syntaxin and SNAP-25, to form a trimeric complex involved in membrane fusion and exocytosis of the vesicle contents. In mammalian eggs, the initial large increase in cytosolic Ca^{2+} is primarily from intracellular Ca^{2+} stores in the endoplasmic reticulum, while subsequent oscillations appear to require extracellular Ca^{2+}. Stars indicate vesicle contents.

ethylmaleimide-sensitive factor attachment protein receptor) hypothesis for membrane fusion proposed by Rothman and co-workers (109) and reviewed in (73, 110-112). The molecular mechanisms of the SNARE hypothesis will be only briefly described herein (figure 5). In this model, conserved proteins on the secretory vesicle (v-SNAREs) and on the target membrane, e.g. plasma membrane, (t-SNAREs) interact specifically and govern membrane fusion. There has been extensive work on this mechanism generating genetic, cellular and biochemical evidence in support of this model. For example, specific proteolytic cleavage of syntaxin, SNAP-25, and synaptobrevin by different clostridial toxins blocks neurotransmitter release and synaptic transmission (73). Although this core complex of interacting proteins appears to be universal minimal machinery for docking and fusion, the roles of additional regulatory proteins and putative Ca^{2+}-sensors are still evolving.

A homolog of the candidate Ca^{2+} sensor for synaptic vesicle exocytosis, synaptotagmin, has been identified in sea urchin eggs that localizes near the plasma membrane (113). Additional SNARE members, including synaptobrevin, syntaxin, and SNAP-25 have been detected in sea urchin eggs (113-115) and in mammalian sperm (116), while SNAP-25 has been identified in mouse eggs (117). SNAP-25 is cortically localized in mouse eggs, consistent with the involvement of SNARE members in CG exocytosis (117). Functionally, specific cleavage by botulinum toxin A and tetanus toxin light chain of mouse SNAP-25 (117) and sea urchin synaptobrevin (114), respectively, demonstrates a functional role for these proteins in CG exocytosis.

What it is the model for the action of the leading candidate for the Ca^{2+} sensor, synaptotagmin, in neurotransmitter release? A current working model is that upon $[Ca^{2+}]_i$ elevation, synaptotagmin binds syntaxin and mediates membrane fusion (73). It may also act as a 'clamp' to prevent fusion prior to physiological activation (118). Synaptotagmin is a membrane glycoprotein that binds Ca^{2+}, phospholipids, and other synaptic vesicle-associated or synaptic plasma membrane proteins, including neurexins, AP2, and syntaxin (73). Mice null for synaptotagmin display impaired neurotransmitter release, specifically in the 'fast' component of release, while displaying normal Ca^{2+} independent release (73, 119). Exocytosis is still possible in the absence of synaptotagmin suggesting synaptotagmin is not a required element of the basic fusion machinery.

3.5. Rab 3 and Rabphilin-3A

In addition to the SNARE proteins, a class of proteins that regulates membrane trafficking events, including Ca^{2+}-regulated exocytosis, is the Rab family of small GTP-binding proteins (reviewed in 120-123). About 40 Rabs have been identified along with a number of Rab effectors, including rabphilin, which has been implicated in exocytosis in mouse eggs (124), mammalian sperm (125, 126), adrenal chromaffin cells (127), neurons (120-122) and PC12 cells (128). Rab 3A and rabphilin-3A localize to the cortex of mouse eggs (124, 129) as well as to synaptic vesicles in neurons (120-122). Functionally, microinjection of peptides corresponding to the conserved effector domain of Rab3 blocks CG exocytosis in sea urchin eggs (130), as do recombinant fragments of rabphilin-3A in mouse eggs (124). Although mice lacking Rab3A are fertile (131), CG exocytosis in these knockout animals has not been specifically examined. It is possible that deficiencies exist in the regulation of CG exocytosis that do not ablate fertility.

A current model for Rab regulation of synaptic vesicle trafficking holds that, when bound to GTP, Rabs are

able to interact with synaptic vesicles and specific Rab effectors to promote translocation and docking to, and possibly fusion with, the active zone at the plasma membrane (120-122). One functional role for Rab3 and rabphilin-3A may be to act to tether a vesicle to the plasma membrane for subsequent fusion (121). Interestingly, rabphilin-3A binds Ca^{2+} and phospholipid, similar to synaptotagmin (120), and contains four phosphorylation sites for CaMKII, the function of which are unknown (132). In addition, rabphilin-3A interacts *in vitro* with alpha-actinin, resulting in cytoskeletal reorganization (133). Because CG translocation is crucial and developmentally regulated (see Section 6 below), the roles of these proteins in CG release needs to be determined.

4. CYTOSKELETAL REGULATION OF CG TRANSLOCATION AND EXOCYTOSIS

One mechanism by which cells regulate exocytosis is through the regulation of the cortical cytoskeleton. For example, upon the stimulation of chromaffin cells, disassembly of cortical actin occurs that is proposed to allow granule movement to the plasma membrane (134, 135). The mechanisms to translocate CGs at fertilization, as well as during oogenesis, remain largely unknown. Although the majority of CGs translocate to the cortex during oogenesis, a small percentage of CGs translocate during oocyte meiotic maturation. Because oocytes mature readily *in vitro*, this subset of CGs provides an experimentally tractable system to examine mechanisms of CG translocation. These CGs were determined to translocate in an actin microfilament-dependent mechanism (136). Additionally, CG release is inhibited by agents which perturb the actin cytoskeleton, including cytochalasin B (42, 137), jasplakinolide (138) and phalloidan (139), although the exact role for microfilaments is unknown. The actin cytoskeleton does not act simply as a barrier to exocytosis, as disassembly of the actin cytoskeleton alone does not trigger CG exocytosis (136). Both the release of secretory vesicles from the actin cytoskeleton as well as the reorganization of the cortical cytoskeleton, through such Ca^{2+}-dependent actin-depolymerizing proteins as gelsolin and scinderin (140), may be involved in the regulation of CG access to the plasma membrane in mammalian eggs.

5. KINETICS OF CG EXOCYTOSIS

In order to understand the mechanism of CG exocytosis, experiments need to be undertaken at the cellular level to understand how individual CGs respond to $[Ca^{2+}]_i$ and fuse with the plasma membrane. Observations of intact activated eggs from different species reveal that they vary in their $[Ca^{2+}]_i$ elevation profile and temporal regulation of CG exocytosis. When these studies are combined with biochemical data on the Ca^{2+} binding affinities of important regulators of exocytosis, the relationship between the $[Ca^{2+}]_i$ concentration and subsequent kinetics of release can provide useful predictions of the molecular mechanisms of the Ca^{2+} response machinery in eggs.

There are several methods to analyze the kinetics of exocytosis that will be briefly summarized. One method is to collect and fix eggs following activation and analyze CG secretion by quantifying CGs that are remaining in the egg cortex. For example, the localization of CGs in mouse eggs can be visualized with *Lens culinaris* agglutinin (141), which can be directly or indirectly conjugated to a fluorophore. Because this method is based on the quantification of intact CGs, CG status in activated or treated eggs must be compared to unactivated controls. However, this approach only provides a 'snapshot' during CG exocytosis and, at very short time intervals after activation, CG quantification includes CGs in the cytoplasm as well as those in the process of exocytosis. The measurement of membrane capacitance is a second method to examine the kinetics of CG exocytosis. Although this offers real time analysis, membrane capacitance is a measure of the net effect of all exocytotic and endocytotic events. Therefore, exocytotic events can be obscured by temporally overlapping endocytosis (142). A third method is real time fluorescence microscopy, using non-specific lipophilic fluorescent indicator probes, such as FM 1-43 and TMA-DPH (available from Molecular Probes, Eugene, OR). These dyes are essentially non-fluorescent in water and become fluorescent upon incorporation into the plasma membrane. Exocytosis results in the presentation of new unlabelled membrane to the dye-filled, aqueous environment, which subsequently becomes labeled such that an increase in fluorescence results from the increase in surface area due to exocytosis. With these techniques, the time course of CG exocytosis in eggs of various species is emerging.

5.1. Kinetics of CG exocytosis in sea urchin eggs

Unlike mammalian CGs, those in sea urchin eggs are docked at the time of fertilization (27, 143, 144). In sea urchins, fertilization results in a single wave of $[Ca^{2+}]_i$ elevation which spreads across the egg from the point of sperm fusion and, after a lag time following the $[Ca^{2+}]_i$ wave of approximately 8-10 seconds (37), CGs undergo exocytosis within seconds (145). This rapid secretion of CGs has been demonstrated by membrane capacitance changes (146) as well as live cell imaging with fluorescent lipophilic probes (37) and differential interference contrast microscopy (37, 147-149). Interestingly, CG exocytosis does not occur in a synchronous wave corresponding directly to the $[Ca^{2+}]_i$ wave, but rather occurs in a stochastic manner with subpopulations of CGs undergoing exocytosis at distinct times following $[Ca^{2+}]_i$ elevation (37, 147, 148). This indicates that within a single egg, individual CGs vary either in their Ca^{2+} sensitivity or in the activity of their fusion machinery. To address this hypothesis, Blank *et al.* challenged isolated sea urchin cortices, consisting of fusion-ready areas of CGs and plasma membrane, with increasing concentrations of Ca^{2+} and determined that subpopulations are heterogeneous in their sensitivity to Ca^{2+} (149). Alternatively, within an intact egg, there may exist local differences in $[Ca^{2+}]_i$ that have not been detected in studies to date.

5.2. Kinetics of CG exocytosis in mouse eggs

Mammalian eggs differ from echinoderm eggs in both their characteristic $[Ca^{2+}]_i$ profile (oscillations vs. single elevation, respectively) and in the kinetics of CG exocytosis (150). Older studies indicate that the ZP block to polyspermy was established by 16 min after stimulation

of an egg (151). Using LCA-labeling of fixed hamster eggs, it was reported that CG exocytosis takes 9 min to complete (41). In this study, however, the timing of sperm fusion with the egg was not determined for the same population of eggs for which the CGs were analyzed. Following membrane capacitance increases immediately after fertilization in hamster eggs, Kline and Stewart-Savage (43) estimated that 75% of CGs undergo exocytosis within 13 seconds of the first $[Ca^{2+}]_i$ transient. This interesting finding however is not based on the direct observation of CGs, but rather on indirect evidence from observed capacitance changes. Fluorescence microscopy of exocytosis in mouse eggs revealed slower kinetics of release, with exocytosis beginning within 5-10 min and continuing for up to an hour (42). This study too does not allow discrimination between CG exocytosis and other exocytotic events. It is important to note that in electron micrographs CGs are the primary population of membrane-bound vesicles. Although there are discrepancies in published data to date, it is clear that, relative to the echinoderm egg, CG exocytosis is a slow process in mammals. This is likely due to the position CGs in mammalian eggs, which are in a non-translocated and non-docked state. CGs in mouse eggs are an average distance of 1-2 microns from the plasma membrane, whereas those of hamsters may be closer (141). Therefore, multiple steps are required for secretion, including the final translocation to the plasma membrane as well as subsequent CG docking and fusion. Alternatively, differences in the $[Ca^{2+}]_i$ profile and $[Ca^{2+}]_i$ buffering properties of eggs may account for the slow release kinetics. Future studies with caged Ca^{2+} and specific CG markers for live cell imaging will allow for precise analysis of the relationship between $[Ca^{2+}]_i$ oscillations and CG exocytosis.

5.3. Relationship between CG exocytosis and sensitivity to $[Ca^{2+}]_i$.

Of all of the events of egg activation studied, the release of CGs appears to be one of the most sensitive to the elevation of $[Ca^{2+}]_i$. CG release and ZP modifications require less stimulation by $[Ca^{2+}]_i$ than cell cycle changes and other events. In response to $[Ca^{2+}]_i$ oscillations in fertilized eggs, CG release occurs before other events, although this does not prove greater sensitivity to $[Ca^{2+}]_i$. However, CG release is likely to require fewer $[Ca^{2+}]_i$ oscillations than other events. For example, microinjection of IP_3, which results in only a small number of $[Ca^{2+}]_i$ oscillations, induces CG release and ZP-2 cleavage without changes in the cell cycle or in the protein synthetic profiles (152). Significant CG release occurs by 15-30 minutes post fertilization (40, 42, 153) in which time 3-4 oscillations occurs (23, 154). Also, CG exocytosis requires 2-10 $[Ca^{2+}]_i$ transients in inhibition studies with BAPTA and fertilized eggs (23). Regarding the other activation events, second polar body extrusion and formation of pronuclei occur at approximately 1 and 4 hours post fertilization corresponding to about 8 and >24 natural oscillations, respectively. Moreover, in fertilized eggs, second polar body extrusion (23) or formation of pronuclei (31) requires a $[Ca^{2+}]_i$ stimulus greater than that required for CG release.

This sensitivity to $[Ca^{2+}]_i$ is not fixed, however, but rather changes dramatically upon extended residence in the oviduct. With increasing time after ovulation, mammalian eggs become more sensitive to agonists of activation *in vitro* or may undergo spontaneous activation *in vivo* (155, 156). In the oviduct, post-ovulatory mouse eggs undergo time-dependent cell cycle and cytoplasmic changes that result in a partially activated state, likely accounting for their time-dependent susceptibility to activate. Spontaneous CG exocytosis is first detected at 4 hours post-ovulation, along with 3% of eggs undergoing anaphase onset (157). By 10 hours post-ovulation, a subset of eggs have released 40-50% of their CGs and at this time, there is a corresponding increase in the extent of ZP modification. In addition to changes in CGs, >60% of eggs were in anaphase and proteins normally synthesized after fertilization were detected. As discussed in Xu *et al*. (157), these time-dependent changes caution against the use of mouse eggs >4 hours post-ovulation in studying the mechanism of normal fertilization and have implications for animal and human *in vitro* fertilization.

Interestingly, these time-dependent parthenogenetic changes are not as pronounced when eggs are collected immediately after ovulation and incubated in culture medium *in vitro*. In contrast to the effect of extended residence of the eggs in the oviduct, *in vitro* culture inhibits CG release and the associated modifications of the ZP, retards cell cycle events associated with completion of the second meiotic reduction, and is not associated with the translation of maternal mRNAs (158). These experiments indicate that residence of mouse eggs in culture medium *in vitro* (which likely takes place under suboptimal conditions) inhibits, rather than accelerates, the spontaneous progression into the interphase-like state, when compared to eggs that reside in the oviduct for increasing periods of time. These results also suggest that for studies focused on *in vitro* fertilization or egg activation that the ovulated eggs should be removed from the oviducts very soon after ovulation. However, the biochemical basis for the difference in these time-dependent changes between eggs aged *in vivo* and *in vitro* is unknown and requires further investigation.

6. DEVELOPMENT OF SECRETORY COMPETENCE DURING OOCYTE MATURATION

Although aging of eggs post-ovulation is associated with increasing sensitivity of Ca^{2+}-dependent events, including CG exocytosis, prior to ovulation oocytes are relatively refractory to agonists of activation, including sperm (159), ionophore (51) and IP_3 (102). The ability of eggs to undergo CG exocytosis arises late in maturation, just prior to fertilization. In the mouse egg, the ability to release CGs over the entire egg cortex develops during oocyte maturation between metaphase I and II, during the 5 hours prior to ovulation (159). In pre-ovulatory mouse oocytes, fertilization results in no detectable CG release, whereas at the metaphase I-stage oocytes, fertilization induces localized CG release over the decondensing sperm head (159). Gradual development of competence to release CGs upon fertilization is also observed during pig (160) and bovine (161) oocyte maturation. Likewise, in the starfish (162, 163) and amphibian (164, 165), competence

to undergo CG exocytosis upon artificial activation by treatment with Ca^{2+}-ionophore or pricking develops late in maturation between the metaphase I and metaphase II stages.

Maturation-associated changes in the $[Ca^{2+}]_i$ release mechanism in oocytes have been demonstrated (reviewed in 166). Deficiencies in $[Ca^{2+}]_i$ release upon fertilization of starfish oocytes that had not completed maturation were first appreciated by Chiba *et al*. (163). In mammals, although fertilization of pre-ovulatory, prophase-I-stage oocytes results in repetitive $[Ca^{2+}]_i$ oscillations, there are fewer oscillations and they are of a lesser amplitude relative to mature metaphase II -stage eggs (167-169). Mouse oocytes attain full competence to release $[Ca^{2+}]_i$ between the metaphase I and metaphase II stages of meiosis. In mouse oocytes, development of competence is associated with a localized release of CGs over the metaphase I chromosomes, but without a ZP-2 modification due to the protective effects of follicular fluid and serum (170). The maturation-associated development of $[Ca^{2+}]_i$ release mechanisms involves changes in mRNA levels (IP_3R isoforms: 171), increases in protein synthesis (IP_3R1: 172, 173), as well as structural changes (ER localization to the cortex: 21, 174-177).

In order to determine the functional significance of deficiencies in mechanisms to elevate $[Ca^{2+}]_i$ in eggs, a full understanding of the mechanisms to respond to $[Ca^{2+}]_i$ is required. Although oocyte maturation is associated with an increase in CGs in the cortex in pig (160) and sea urchin (178, 179), most CGs in mouse oocytes are localized in the cortex by the fully-grown, prophase I-stage (180). However, mouse pre-ovulatory oocytes, despite having morphologically mature CGs in the appropriate localization, are incompetent to exocytose CGs in response to repetitive $[Ca^{2+}]_i$ oscillations that closely mimic those seen at fertilization in metaphase II-stage eggs (49). There is evidence for a similar deficiency in the $[Ca^{2+}]_i$ response mechanism in starfish (163) and *X. laevis* (50). Chiba *et al.* (163) demonstrated that injections of high concentrations of IP_3, which are able to induce robust $[Ca^{2+}]_i$ release, do not result in CG release as assayed by the ability to elevate the fertilization envelope. Interestingly, immature *X. laevis* oocytes are competent to release exogenous secretory granules upon Ca^{2+} injection, but incompetent to release endogenous CGs, suggesting intrinsic deficiencies in the components of CGs (50). The molecular mechanisms for increased Ca^{2+} sensitivity of the secretory machinery in eggs is undetermined and is an exciting area of research which will aid in the understanding of both oocyte maturation and of the development of the secretory mechanism. The ability of a mammalian egg to develop competence to both release and respond to $[Ca^{2+}]_i$ is essential to initiate early embryonic development and is relevant to clinical assisted reproductive procedures in which many immature oocytes are routinely collected.

7. SUMMARY AND CONCLUSIONS

Although the importance of Ca^{2+} as a central regulator of egg activation has long been appreciated, the characterization of $[Ca^{2+}]_i$ response pathways in the egg is still in its infancy. Because Ca^{2+} effector proteins can be involved in multiple events of egg activation, elucidation of the mechanisms to undergo CG exocytosis will likely contribute to the understanding of egg activation as a whole. As highlighted in this review, proteins which control Ca^{2+}-regulated exocytosis are shared among gametes and somatic cells, so the two areas of research will continue to impact each other. We propose that mouse eggs are an excellent system in which to examine conserved mechanisms to undergo Ca^{2+}-regulated exocytosis for the following reasons: 1. unlike in many somatic cells, there is a single population of secretory granules 2. because CGs are within 2 microns of the plasma membrane, the mechanisms to undergo translocation and docking can be studied, and 3. single-cell analysis of CG exocytosis and $[Ca^{2+}]_i$ determination can be routinely performed.

Much more research is required to elucidate the Ca^{2+} response pathway in mammalian eggs, at both the cellular and molecular level. Significant advances have been made in both the Ca^{2+}-dependent signal transduction pathways and in the proteins directly involved in membrane fusion during fertilization, including the identification of SNARE homologs in both eggs and sperm as well as of important regulatory kinases such as CaMKII. The biochemical and cellular changes required for the establishment of the Ca^{2+} response mechanisms are relevant to both animal and human assisted reproductive techniques. For example, in the production of cloned or transgenic domestic animals, pre-ovulatory oocytes are routinely collected and matured *in vitro*. Successful development of Ca^{2+} response mechanisms during oocyte maturation is important for animal cloning as well as for cryopreservation of human pre-ovulatory prophase I arrested oocytes for later maturation, fertilization, and embryo transfer. The development and stability of the secretory mechanism and Ca^{2+} response mechanisms are likely to play an important role in establishing the temporal window for optimal fertilization.

8. ACKNOWLEDGEMENTS

Our research is supported by the grants from the Lalor Foundation (to A.L.A.) and NICHD (HD 24191 to T.D.). We would like to thank Rafael Fissore, Gary Wessel, and Harvey Florman for helpful discussions.

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Key words: Cortical Granule, Exocytosis, Secretion, Calcium, Fertilization, Mammal, Egg, Zona Pellucida, Review

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