—Mini Review—

Mechanism and Control of Mammalian Cortical Granule Exocytosis

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Abstract: *Polyspermy is penetration of the egg cytoplasm by more than a single spermatozoon, and in humans, polyspermy usually results in spontaneous abortion. Following sperm penetration in mammals, cortical granules (CGs), special organelles in eggs, release their contents into the perivitelline space. The CG exudates act on the zona pellucida, causing biochemical and structural changes that result in zona sperm receptor modification and zona hardening, and thus block polyspermic penetration. Significant advances have been made in elucidating signal molecules and signal transduction cascades that play important roles in the CG exocytosis and subsequent polyspermy block. Ca^{2+} oscillation is necessary and sufficient for CG exocytosis as well as for other events of egg activation. The Ca^{2+}-dependent pathways and the proteins involved in membrane fusion may play pivotal roles in the regulation of CG exocytosis. Mammalian oocytes develop their ability to undergo CG exocytosis during maturation. This article reviews the signal molecules and signal transduction cascades involved in CG exocytosis.*

Key words: *Calcium, Cortical granule, Exocytosis, Fertilization, Mammal, Zona pellucida*

Introduction

Polyspermy is penetration of the egg cytoplasm by more than a single spermatozoon. In humans, polyspermy usually results in spontaneous abortion. Births of tetraploid or triploid children have been reported [1, 2], but these polyploidy births result in severe malformations and multiple abnormalities [3, 4]. Therefore, in humans and most other mammals, the primary block to polyspermy is established promptly after fertilization. Although polyspermy is considered an abnormal phenomenon in most mammals and results in developmental failure [5], pig oocytes have a high incidence of polyspermy under physiological conditions [6]. However, pig oocytes present an extraordinary case in which the cytoplasm can remove extra sperm [6], or poly-pronuclear pig eggs can develop to term [7].

Elucidating the mechanism of polyspermy block is very important for reproductive biology. Especially, this is relevant to human *in vitro* fertilization programs in which numerous sperm are used to inseminate an egg. Following sperm penetration in mammals, cortical granules (CGs), special organelles in eggs, release their contents into the perivitelline space, leading to blockade of polyspermy (cortical reaction) [8]. The released contents of the CGs act on the zona pellucida (ZP), causing biochemical and structural changes that result in zona sperm receptor modification and zona hardening, and thus block polyspermic penetration. The incorporation of the sperm membrane into the egg plasma membrane at fertilization also contributes to polyspermy block at the level of the oolemma [6, 9]. While polyspermy is primarily blocked by zona changes in hamster, goat, pig and bovine oocytes, it principally depends on oolemma changes in rabbit oocytes [5]. In mouse, rat, and guinea pig oocytes, both mechanisms are important [5].

In eukaryotic cells, secretory vesicles undergo several ubiquitous steps, such as translocation, docking and priming, before they are ready to fuse with the plasma membrane and release their contents into the extracellular space [10]. This process is called “exocytosis”. Accordingly, CG release is called “CG exocytosis”. Some CG contents are reported to remain during preimplantation development [9], suggesting that it may also play a role in early embryogenesis. Therefore, it is crucial to study the mechanism of CG

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exocytosis for reproductive biology. Significant advances have been made in elucidating signal molecules and signal transducion cascades that play important roles in the cortical reaction and subsequent polyspermy block, and this article reviews the signal molecules/cascades involved in CG exocytosis.

CG Exudates and Polyspermy Block

Mammalian CGs are generally similar in appearance [11–13]. At the ultrastructural level, the size of mammalian CGs ranges from 0.2 to 0.6 μm in diameter [11–13]. The CG contents are uniform in ultrastructural density [12]. However, CGs in some species have been characterized as “light” and “dark” based on differences in electron density [14, 15]. While the difference in ultrastructural density might represent a difference in biochemical composition or different CG maturation stages [13, 16], the biological significance of this difference in density is not yet known.

The mammalian oocyte ZP contains three glycoproteins, ZP1, ZP2, and ZP3 [17]. Of these proteins, ZP3 and ZP2 act as the primary and secondary sperm receptors, respectively, in mouse oocytes. Acrosome-intact sperm binds in a species-specific manner to specific O-linked oligosaccharides located on ZP3. Binding to ZP3 allows sperm to undergo the acrosome reaction. Then, the acrosome-reacted sperm bind to ZP2 on the inner membrane. Consequently, sperm penetrate the ZP and reach the egg plasma membrane [17].

CGs contain numerous enzymes that biochemically modify sperm receptors ZP2 and ZP3 [17]. Following fertilization, these ZPs become ZP2f and ZP3f which have lost sperm receptor activity [8–10]. ZP1 protein is not modified [8]. These ZP modifications generate the ZP block that is crucial for the primary polyspermy block in many mammals, including humans. Various studies have examined the contents of mammalian CGs [18, 19]. A few molecules have been directly localized to mammalian CGs using immunocytochemical techniques: ovoperoxidase [20], a 75-kD glycoprotein known as p75 [21, 22], and β-N-acetylglucosaminidase [23]. Additionally, mammalian CGs may also contain heparin binding placental protein [24], a 32-kD protein [25] and several proteinases [16, 26, 27], since these proteins are released from oocytes after CG exocytosis.

Of these proteins, the β-N-acetylglucosaminidase, ovoperoxidase, and proteinases are thought to induce the changes in the ZP that establish the zona block to polyspermy [20, 23, 26, 27]. N-acetylglucosaminidase has been shown to be localized in the CGs of mouse oocytes by immunoelectron microscopy [23]. Following fertilization, CGs release β-N-acetylglucosaminidase, which removes the binding site for sperm β-1, 4-galactosyltransferase (the well characterized sperm receptor for ZP3) so that it can no longer initiate sperm binding [23]. Mouse CGs also contain an ovoperoxidase which hardens the ZP [20]. Proteinases are released from mammalian oocytes after egg activation. The CG proteinase cleaves ZP2 and the proteolyzed form of ZP2 can no longer interact with acrosome-reacted sperm [26]. These modifications of the ZP comprise the ZP block to polyspermy.

In addition to the cortical reaction, an egg plasma membrane block to polyspermy is also known in several species, including humans [31–33]. CG proteinases may also help polyspermy block at the plasma membrane, although the evidence for this is inconsistent [9]. Both the zona block and the plasma membrane block are important in most species, though in the rabbit the plasma membrane block is the primary block to polyspermy [5, 34]. In mouse oocytes, ICSI or artificial activation did not induce the egg plasma membrane to block reinseminated sperm penetration, although CG exocytosis had occurred in these eggs [32, 35]. Sengoku *et al.* also showed in human eggs that pronuclear human oocytes fertilized by sperm were not penetrated by reinseminated sperm [33]. Therefore, these results suggest that the CG contents may not play a role in the establishment of polyspermy block at the plasma membrane. Rather, it has been speculated that sperm membrane incorporation into the oolemma could contribute to the plasma membrane block [31–33].

Some CG conents are reported to remain in the perivitelline space of fertilized hamster, mouse, and human oocytes [9], and to associate with the oocyte surface after fertilization [28–30]. These conents form a new extracellular matrix, called the “cortical granule envelope” [30]. Because the CG envelope persists during preimplantation development until blastocyst hatching [30], it may play a role in early embryogenesis.

Signal Molecules/Cascades Involved in CG Exocytosis

Embryonic development is initiated after the sperm enters the egg and triggers a cascade of events known as “egg activation” [17]. Fertilization results in an increase in intracellular calcium concentration characterized by repetitive Ca^{2+} oscillations [6]. Ca^{2+} oscillation is necessary and sufficient for most of the

major events of egg activation, including CG exocytosis, cell cycle resumption and recruitment of maternal mRNA [17]. Of these events in egg activation, CG exocytosis is one of the earliest calcium-dependent events [9].

The CG exocytosis in mammalian oocytes is induced following fertilization. While intracytoplasmic sperm injection (ICSI) bypasses the physiological cascade of gamete interaction events, CG exocytosis occurs in ICSI [36]. The injection of cytosolic sperm factors into mammalian oocytes has been shown to induce an immediate rise in the free Ca^{2+} concentration, followed by repetitive Ca^{2+} oscillations, which can initiate egg activation events including CG exocytosis [35–39]. Recent studies have demonstrated that a sperm-specific phospholipase C (PLC) (PLCzeta) is the main component of the Ca^{2+} oscillation-inducing activity of sperm [40, 41], which suggests sperm PLCzeta is the sperm cytosolic factor that triggers egg activation during fertilization [42].

Previous studies had demonstrated that the rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) induces the CG exocytosis in eggs for the following reasons. First, the $[Ca^{2+}]_i$ increase always precedes CG exocytosis; in hamster eggs, CG exocytosis occurs shortly after the first $[Ca^{2+}]_i$ transient [43]. Second, the $[Ca^{2+}]_i$ increase has been shown to be necessary and sufficient for CG exocytosis; calcium ionophore A23187 stimulates CG exocytosis in the absence of sperm [44, 45]. In addition, while the microinjection of Ca^{2+} into the egg cytoplasm was shown to be sufficient to promote CG exocytosis [46], the injection of Ca^{2+} chelator BAPTA inhibited Ca^{2+} transients and CG exocytosis induced by sperm [47] or egg activation [48]. Thus, Ca^{2+} oscillation was established as being responsible for the CG exocytosis in eggs.

In somatic cells, Ca^{2+} oscillations are dependent upon the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by PLC, and the subsequent generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [49]. The former induces Ca^{2+} release from intracellular stores through the IP_3 receptor on the endoplasmic reticulum membrane [49], while the latter activates protein kinase C (PKC) [49]. As PLC mRNA and proteins are detected in mouse oocytes [50], mammalian CG exocytosis is probably mediated by activation of the PIP_2 cascade.

In mammals, CG exocytosis is dependent upon signal transduction pathways similar to those employed in somatic cells to carry out calcium-regulated exocytosis [9]. Accumulating evidence suggests that repetitive calcium oscillations stimulate CG exocytosis through Ca^{2+}-dependent effectors [51]. These effectors include IP_3, PKC, calmodulin (CaM), Ca^{2+}/CaM-dependent protein kinase II (CaMKII), Rab3, Rabphilin-3A, Rho, and synaptotagmin. Several Ca^{2+} effectors involved in CG exocytosis will now be reviewed. In addition, the possible involvement of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins will be also discussed.

Potential Signal Effectors Involved in CG Exocytosis

Inositol 1,4,5-trisphosphate (IP_3)

The possible involvement of IP_3 in CG exocytosis was examined by Cran *et al.*, who demonstrated that microinjection of IP_3 into hamster and sheep oocytes induces CG release [52]. Microinjection of IP_3 into mouse oocytes also results in ZP modification [44]. Miyazaki *et al.* demonstrated that IP_3 is essential for the initiation and propagation of sperm-induced Ca^{2+} oscillation by using a function-blocking monoclonal antibody (18A10) to the IP_3 receptor/ Ca^{2+} release channel [53]. Furthermore, monoclonal antibody 18A10 inhibits ZP modification (ZP2-ZP2f conversion) as well as Ca^{2+} release in mouse [54] and human [55] eggs. These results indicate that IP_3, generated by PLC [50], may play a critical role in CG exocytosis.

Protein kinase C (PKC)

Upon fertilization, DAG is generated through the hydrolysis of PIP_2 by PLC [50], and targets PKC. PKC is known to play a important role in sperm acrosomal exocytosis [56]. In mouse oocytes, several PKC isoforms have recently been reported [57]. CG exocytosis in fertilization has been shown to be associated with significant translocation of PKC to the plasma membrane [58, 59]. PKC activation by DAG or phorbol ester induced CG exocytosis and ZP2-ZP2f conversion in mouse, rat and pig eggs [44, 60, 61], suggesting that CG exocytosis is PKC-dependent. However, treatment with some PKC inhibitors failed to block CG exocytosis during fertilization [62]. Future studies should identify the targets of PKC in mammalian eggs, and clarify whether PKC and its effectors contribute to the regulation of CG exocytosis.

Calmodulin (CaM) and calmodulin-dependent kinase II (CaMKII)

CaM is one of the most common Ca^{2+} transducers in somatic cells [63], and is involved in diverse signal

transduction pathways [64]. CaM is considered to play a general role in regulated exocytosis [65]. Thus, it is possible that CaM could play a role in regulating CG exocytosis. However, an inhibitor of CaM, W-7, did not inhibit fertilization-induced CG exocytosis in mouse eggs [48, 66], although it inhibited meiotic cell cycle resumption [48]. Thus, whether CaM is essential for CG exocytosis or not is not known at this point. Further experiments will be needed to verify the contribution of CaM to the regulation of CG exocytosis. CaMKII is one of the important targets of CaM [67], and is found in mouse eggs [68, 69]. In mouse oocytes, an inhibitor of CaMKII, KN-93, markedly inhibited CG exocytosis by ethanol [70, 71], suggesting that CaMKII is an important regulator of CG exocytosis. The activity of CaMKII increases during mouse oocyte maturation [72]. Moreover, Tatone *et al.* showed that CaMKII activity increased transiently 10 min after insemination, then peaked at 1 h and remained elevated 30 min later when most of the oocytes had completed the emission of the second polar body [71]. These results suggest that CaMKII is involved in the initiation of egg activation. However, whether CaMKII is directly involved in CG exocytosis at fertilization is still not certain.

Monomeric GTP-binding proteins

a) Rab 3 and Rabphilin-3A

Accumulating evidence supports a role for monomeric GTP-binding proteins as well as heterotrimeric GTP-binding protein subunits in various steps of the vesicular trafficking pathway [10]. The Rab family of monomeric GTP-binding proteins is a class of proteins that regulates the intracellular traffic of synaptic vesicles [10, 73, 74]. Activated GTP-bound Rabs are able to interact with synaptic vesicles and specific Rab effectors to promote translocation, docking, and fusion with the plasma membrane [73, 74]. Rab3A is involved in Ca^{2+}-dependent exocytosis, particularly in neurotransmitter release [75]. Rabphilin-3A, an effector protein for Rab3A, has a Rab3A binding NH2-terminal domain and a Ca^{2+} binding COOH-terminal domain [76]. In mouse eggs, Rab 3A and Rabphilin-3A are located in the cortical region [77, 78]. When an NH2- or COOH-terminal fragment of recombinant Rabphilin-3A [79] is microinjected into mouse eggs, both fragments block CG exocytosis [77], suggesting that Rabphilin-3A participates functionally in Ca^{2+}-dependent CG exocytosis at fertilization in mouse eggs. Intracellular Ca^{2+} mobilization is thought to regulate Rab3A via Rabphilin-3A [79]. Thus, the Rab-Rabphilin system may play an important role in the signal transduction pathway downstream of sperm-induced intracellular Ca^{2+} mobilization.

b) Rho

Exocytosis is a highly regulated process in which secretory vesicles or granules fuse with the plasma membrane to release their contents. There are several stages in the process of exocytosis (targeting, docking, and fusion of secretory granules), and cortical actin remodeling is believed to regulate exocytosis [80, 81]. Rho GTPases, a family of monomeric GTP-binding proteins, are well known for their pivotal roles in regulating the actin cytoskeleton, cell polarity, gene expression, microtubule dynamics and vesicular trafficking [10]. Thus, Rho GTPases may play an important role in regulated exocytosis through cytoskeletal remodeling. The possible involvement of Rho in CG exocytosis is discussed later under the heading, “Cytoskeletal Regulation of CG Exocytosis”.

SNARE proteins

Exocytotic membrane fusion is now generally accepted to be mediated by members of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) family of proteins [82–85]. The SNARE-mediated process is conserved evolutionally from yeast to humans across different transport events in eukaryotic cells [82–85]. The SNARE proteins include: vesicle-associated membrane protein (VAMP) in the synaptic vesicle membrane, synaptosome-associated protein of 25 kDa (SNAP-25) and syntaxin in the presynaptic plasma membrane [85]. Thus, it is likely that CG exocyotis is also mediated by SNARE proteins, and SNARE proteins have been detected in mammalian eggs [86, 87].

In metaphase II mouse eggs, SNAP-25 mRNA and protein were detected by RT-PCR and immunoblotting analysis, respectively [86]. SNAP-25 is specifically cleaved at a site nine amino acid residues from the COOH-terminal by botulinum neurotoxin A (BoNT/A), resulting in the inhibition of exocytosis in neurons and neuroendocrine cells [88, 89]. When BoNT/A was microinjected into metaphase II mouse oocytes, sperm-induced CG exocytosis was significantly inhibited, and the inhibition was attenuated by co-injection of SNAP-25 [86]. These results suggest that SNAP-25 is functionally involved in the signal transduction pathway downstream of sperm-induced intracellular Ca^{2+} mobilization [86].

Syntaxin was also detected in mouse oocytes at the mRNA and protein levels [87]. In metaphase II oocytes,

syntaxin 4 is located on the plasma membrane and the CG membranes, suggesting that syntaxin 4 co-localizes with CGs and participates in membrane fusion and exocytosis during the cortical reaction. However, whether syntaxin 4 is directly involved in CG exocytosis at fertilization is still not known.

Another member of the SNARE family of proteins is VAMP. The expression of VAMP in mammalian eggs has not yet been reported. In sea urchin eggs, a VAMP homologue is specifically associated with the CG membrane [90]. Interestingly, the sea urchin VAMP homologue resides in the CG membrane before fertilization, whereas it is incorporated into the plasma membrane of the zygote following CG exocytosis [90]. In human sperm, the presence and functional role of the SNARE proteins, including VAMP, were demonstrated in the acrosome reaction [91], which is known as "sperm exocytosis". Thus, the possible involvement of VAMP in mammalian CG exocytosis should be examined.

These results suggest that members of the SNARE family are present in eggs and involved in at least some steps of the regulation of CG exocytosis. Moreover, as the interaction of Rab3A with synaptic SNAREs has been implicated in presynaptic terminals [92], it is possible that the Rab-Rabphilin and the SNARE system may be essential to the physiology of CG exocytosis in a way similar to exocytosis in synaptic cells.

Synaptotagmin has been demonstrated to be a candidate Ca^{2+} sensor for exocytosis [93], although the precise mechanism of Ca^{2+}-sensing by synaptotagmin is still being debated. Synaptotagmin may act as a "clamp" to prevent fusion [93, 94], and upon $[Ca^{2+}]_i$ elevation, synaptotagmin binds syntaxin and mediates membrane fusion [95]. While synaptotagmin has been detected in sea urchin eggs [90], whether synaptotagmin has a role in mammalian CG exocytosis is still unclear.

Analysis of CG Exocytosis

To further clarify the mechanism of CG exocytosis, observations of intact eggs can be used to examine the temporal regulation of CG exocytosis. There are several methods to analyze the dynamics of exocytosis.

First, the distribution of CGs in mammalian eggs can be visualized with lectins, because mammalian CG components are known to be glycosylated. For example, mouse and hamster CGs are labeled by the lectin *Lens culinaris* agglutinin (LCA) [16, 96], which is α-D-mannose-specific, and pig CGs by peanut agglutinin (PNA), which is specific for β-D-galactosyl-(1, 3)-D-N-acetylgalactosamine residues [97]. The time-course of CG breakdown at fertilization has also been estimated by quantifying LCA- or PNA-labeled CGs that remain in the egg cortex after fixation of activated eggs [98]. However, it is not possible with this method to determine the dynamics of exocytosis in single living eggs, because this approach requires fixation of eggs [96] and the distribution of CGs in treated eggs must be compared to that in untreated control ones.

A second method is electrophysiological measurement of membrane capacitance [43, 99], which can reveal an increase in membrane capacitance by addition of CG membrane upon exocytosis. Although continuous measurement enables real-time analysis, membrane capacitance is a measure of the net effect of all exocytotic and endocytotic events (exocytosis increases and endocytosis reduces membrane capacitance). Therefore, when exocytosis and endocytosis are induced simultaneously, the kinetics of total exocytosis are contaminated by temporally overlapping endocytosis.

A third method is real-time observation by fluorescence microscopy, using an impermeable fluorescent membrane probe, such as FM 1-43 [100] and TMA-DPH [66]. These probes, which are highly fluorescent on membrane lipids but not at all in solution, bind to and label only the outer layer of the plasma membrane within a few seconds [101]. Thus, the fluorescence of a whole cell is proportional to its membrane surface area. Exocytosis increases the labeled membrane surface area by fusion of the plasma membrane with membranes of secretory granules, which results in an increase in the total cell fluorescence. On the other hand, endocytosis changes the distribution of the fluorescence but does not affect the total fluorescence intensity of the cell. Thus, the fluorescence method allows us to measure the kinetics of exocytosis, uncontaminated by endocytosis, in single living cells.

With these methods, the time-course of CG exocytosis in mammalian eggs has been examined.

Kinetics of mammalian CG exocytosis

A previous study using LCA-labeling of fixed hamster eggs demonstrated that there was an 85% reduction in the density of CGs by 55 min after insemination, and CG exocytosis was completed 9 min after sperm fusion [98]. In that study, sperm fusion was determined by the Hoechst-transfer method, and the temporal relationship between sperm fusion and CG exocytosis was not determined for the same eggs for which the timing of

sperm fusion with the egg was analyzed.

Continuous electrophysiological measurement showed that membrane capacitance increases immediately after fertilization in hamster eggs (75% of CGs undergo exocytosis within 13 seconds of the first $[Ca^{2+}]_i$ transient) [43]. Although continuous measurement of membrane capacitance enables real-time analysis, the value of membrane capacitance is a measure of the net effect of exocytotic and temporally overlapping endocytotic events. Therefore, observed changes in membrane capacitance do not reflect the direct observation of CG exocytosis.

Fluorescence microscopy of exocytosis in living mouse eggs revealed slower kinetics of release [66]. The increase of fluorescence in the egg started within 5–10 min after sperm attachment, continued at an almost uniform rate, and ceased at 45–60 min. While LCA-labeling requires fixation of eggs [96], this method has significant advantages for studying exocytosis in living eggs.

Although differences have been reported in the time-course of CG exocytosis in the literature, it is likely that mammalian CG exocytosis is a slower process than CG exocytosis in the echinoderm egg. Future studies by live cell imaging with specific CG markers will enable precise analysis of the time-course of CG exocytosis.

Cytoskeletal Regulation of CG Exocytosis

Exocytosis is a phenomenon in which secretory granules fuse with the plasma membrane to release their contents. In the process of exocytosis, actin remodelling takes place to mediate vesicle/granule docking, priming and fusion [80]. In somatic cells, filamentous actin blocks exocytosis by excluding secretory granules from the plasma membrane [102]. Disassembly of cortical actin occurs in response to various stimuli, and allows granules to move to the plasma membrane [102, 103].

The majority of CGs translocate to the cortex during oogenesis, while the rest of CGs translocate during oocyte meiotic maturation [104]. This movement is blocked by the treatment of oocytes with cytochalasin D [104], an inhibitor of microfilament polymerization, suggesting that CGs translocate peripherally in a microfilament-dependent mechanism, although the precise mechanisms by which CGs are translocated remain unknown.

CG exocytosis is also inhibited by agents which disturb the actin cytoskeleton. Treatment with cytochalasin B (an inhibitor of microfilament polymerization) clearly blocked mouse CG exocytosis at fertilization [66, 105]. Jasplakinolide which stabilizes microfilament also prevented CG exocytosis following artificial activation of mouse oocytes [106]. These results suggest that CG exocytosis requires microfilaments. However, disassembly of the actin microfilaments alone does not elicit CG exocytosis [104, 107]. Although the exact role of the actin cytoskeleton is unknown, a recent study suggested that the cytoskeletal cortex is not a mere physical barrier that blocks CG exocytosis, but rather a dynamic network that can allow CG exocytosis by activated actin-associated proteins [107].

In somatic cells, GTP-binding proteins of the Rho family (Rho proteins) regulate the organization of the actin cytoskeleton [108], and are molecular switches that regulate many essential cellular processes, including actin dynamics, gene transcription, cell-cycle progression, cell contraction and cell adhesion [108, 109]. Therefore, Rho proteins are likely to be signaling molecules leading to actin filament-dependent dynamic events at fertilization. Cuellar-Mata *et al.* reported that RhoA localizes around the CGs of sea urchin eggs, and could participate in the Ca^{2+}-regulated exocytosis or actin reorganization that accompany the egg activation [110]. Moore *et al.* demonstrated that *Clostridium botulinum* C3 exoenzyme (C3-CB), a functional inhibitor of Rho, does not affect the modification of the ZP in mouse eggs, whereas C3-CB inhibits emission of the second polar body and cleavage to the two-cell stage [111]. Immunostaining of Rho proteins showed that Rac1 and RhoB are present in the cortical ooplasm, suggesting that the cytoskeletal reorganization regulated by Rho proteins is involved in the events at fertilization [112]. However, whether Rho proteins localize around the mammalian CGs remains to be elucidated. Future studies should elucidate whether Rho proteins and their downstream effectors are involved in the mechanisms that control mammalian CG exocytosis.

Development of Competence for CG Exocytosis during Oocyte Maturation

In mammals, GV stage oocytes do not have the ability to block polyspermy [113]. Preovulatory oocytes don't have the competence to undergo CG exocytosis in response to sperm or artificial stimulation [9, 114]. In mouse oocytes, full competence to undergo CG exocytosis in response to sperm entry develops during oocyte maturation between metaphase I and

metaphase II of meiosis [72, 114]. When IP_3 is microinjected into mouse metaphase II eggs, ZP2 conversion occurs in a manner similar to that observed following fertilization [44]. However, little conversion occurred when IP_3 was injected into GV stage oocytes [44]. This developmental maturation has also been reported in pig and bovine oocytes [45, 114].

What mechanism can regulate this development of competence for CG exocytosis? One possibility could be that the number of CGs is insufficient in the cortex of immature oocytes. In pigs, oocyte maturation is associated with an increase in CGs in the cortex [45]. However, this possibility is unlikely to account for the incompetence of mouse GV oocytes to undergo CG exocytosis, because most CGs in mouse oocytes are already localized in the cortex by the prophase-I stage [113, 115].

Accumulating evidence supports a second possibility, namely, that the incompetence of immmature oocytes to release CGs is due to a lack of or impaired ability to release intracellular Ca^{2+} [9]. Although repetitive Ca^{2+} oscillations are observed in preovulatory GV stage oocytes after fertilization or IP_3 injection, there are fewer oscillations with lower amplitude than in mature metaphase II eggs [44, 116, 117]. Mouse oocytes acquire full competence to release $[Ca^{2+}]_i$ just prior to ovulation [9, 114]. These results suggest that the Ca^{2+} release mechanism is incomplete in GV stage oocytes and develops during maturation [44, 116–118].

Although mouse preovulatory oocytes have morphologically mature CGs with the proper localization, they are not fully competent for exocytosis of CGs in response to Ca^{2+} oscillations in metaphase II eggs [119]. As mentioned before, CG exocytosis in mature oocytes is dependent upon the Ca^{2+} rise and calcium-dependent proteins. For example, CaMKII isoform detected by immunoblotting analysis increased 150% during oocyte maturation [72]. Moreover, the CaMKII activity assay indicated that metaphase II stage eggs have more than 2-fold maximal activity than GV stage oocytes [72], indicating that a maturation-associated increase in CaMKII correlates with the development of competence for CG exocytosis. These results suggest that mechanisms downstream of the Ca^{2+} rise are established during the acquisition of competence for CG exocytosis. However, the precise mechanism of the enhancement of Ca^{2+} sensitivity is still unclear.

Summary

Significant advances have been made in elucidating the machanism of the CG exocytosis and subsequent polyspermy block. As discussed in this review, the Ca^{2+}-dependent signal transduction pathways and the proteins involved in membrane fusion may play pivotal roles in the regulation of CG exocytosis. Mammalian oocytes develop their ability to undergo CG exocytosis during maturation, although the molecular mechanism of the increased Ca^{2+} sensitivity has not yet been determined. As the ability of mammalian eggs to develop competence to respond to Ca^{2+} increase is essential for initiation of early embryonic development, elucidating how mammalian eggs develop competence for CG exocytosis will contribute to the understanding of “egg activation”. Moreover, it is relevant to animal and human assisted reproductive procedures in which immature oocytes are collected. Further research is requisite for elucidating the molecular mechanisms of the development of competence in response to Ca^{2+} increase in mammalian CG exocytosis.

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