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Undergo Cortical Granule Exocytosis Following Induced Calcium Oscillations

Allison L. Abbott,* Rafael A. Fissore,† and Tom Ducibella*,‡,[1]

**Department of Anatomy and Cellular Biology, Sackler School of Biomedical Sciences, and ‡Department of Obstetrics and Gynecology, Tufts University School of Medicine and New England Medical Center, Boston, Massachusetts 02111; and †Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01003*

Immature oocytes of many species are incompetent to undergo cortical granule (CG) exocytosis upon fertilization. In mouse eggs, CG exocytosis is dependent primarily on an inositol 1,4,5-trisphosphate (IP_3)-mediated elevation of intracellular calcium ($[Ca^{2+}]_i$). While deficiencies *upstream* of $[Ca^{2+}]_i$ release are known, this study examined whether *downstream* deficiencies also contribute to the incompetence of preovulatory mouse oocytes to release CGs. The experimental strategy was to bypass upstream deficiencies by inducing normal, fertilization-like $[Ca^{2+}]_i$ oscillations in fully grown, germinal vesicle (GV) stage oocytes and determine if the extent of CG exocytosis was restored to levels observed in mature, metaphase II (MII)-stage eggs. Because IP_3 does not stimulate a normal Ca^{2+} response in GV-stage oocytes, three alternate methods were used to induce oscillations: thimerosal treatment, electroporation, and sperm factor injection. Long-lasting oscillations from thimerosal treatment resulted in 64 and 10% mean CG release at the MII and GV stages, respectively ($P < 0.001$). Three electrical pulses induced mean $[Ca^{2+}]_i$ elevations of approximately 730 and 650 nM in MII- and GV-stage oocytes, respectively, and 31% CG release in MII-stage eggs and 9% in GV-stage oocytes ($P < 0.001$). Sperm factor microinjection resulted in 86% CG release in MII-stage eggs, while similarly treated GV-stage oocytes exhibited < 1% CG release ($P < 0.001$). Taken together, these results demonstrate a deficiency downstream of $[Ca^{2+}]_i$ release which is developmentally regulated in the 12 h prior to ovulation.

Key Words: **calcium; egg activation; cortical granule exocytosis; mouse.**

INTRODUCTION

Calcium (Ca^{2+}) is the pivotal regulator of egg activation upon fertilization in a wide array of eggs from different species (Jaffe, 1985). Fertilization of mammalian eggs triggers an initial wave of intracellular calcium ($[Ca^{2+}]_i$) originating from the point of sperm entry and traveling across the egg, followed by repetitive $[Ca^{2+}]_i$ oscillations (Kline and Kline, 1992; Fissore *et al.*, 1992; Miyazaki *et al.*, 1993; Taylor *et al.*, 1993; Whitaker and Swann, 1993). The initiation of these sperm-induced oscillations is primarily inositol 1,4,5-trisphosphate (IP_3) dependent (Miyazaki *et al.*, 1993; Schultz and Kopf, 1995) and is responsible for multiple events of egg activation including cortical granule (CG) exocytosis (Kline and Kline, 1992; Wang *et al.*, 1997a,b,c), establishment of the subsequent zona pellucida (ZP) block to polyspermy (Ducibella *et al.*, 1993; Xu *et al.*, 1994), and meiotic resumption (Kline and Kline, 1992; Whitaker and Swann, 1993).

Fertilization of immature oocytes in a number of species, including sea urchin (Longo, 1978), amphibian (Elinson, 1986), mouse (Ducibella and Buetow, 1994), pig (Wang *et al.*, 1997b), bovine (Wang *et al.*, 1997a), and human (Van Blerkom *et al.*, 1994), however, results in little, if any, CG exocytosis. In the mouse, using quantitative image analysis for CGs, no exocytosis is detected in fertilized, fully grown germinal vesicle (GV)-stage oocytes just 12 h prior to ovulation, whereas, after meiotic maturation, exocytosis is maximal at metaphase II (MII) (Ducibella and Buetow, 1994), the stage at which normal fertilization occurs. Dur-

[1] To whom correspondence should be addressed at the Department of Obstetrics and Gynecology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111. Fax: (617) 636-2917. E-mail: tducibel@opal.tufts.edu.

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ing oocyte maturation when exocytotic competence is acquired, starfish (Chiba *et al.*, 1990), hamster (Fujiwara *et al.*, 1993), mouse (Mehlmann and Kline, 1994; Jones *et al.*, 1995), and bovine (He *et al.*, 1997) immature oocytes also develop competence to release $[Ca^{2+}]_i$ at levels similar to those of mature eggs upon fertilization.

Multiple changes are involved in this maturation-associated development of the egg's $[Ca^{2+}]_i$ release mechanism. A maturation-associated increase in the sensitivity of the egg to IP_3 (reviewed in Fujiwara *et al.*, 1993) results in increased $[Ca^{2+}]_i$ release (Chiba *et al.*, 1990; Mehlmann and Kline, 1994; He *et al.*, 1997) as well as CG exocytosis and extracellular matrix changes (Chiba *et al.*, 1990; Ducibella *et al.*, 1993). Also, there is a 90% increase in the amount of the predominant type-1 isoform of the IP_3 receptor (IP_3R; Mehlmann *et al.*, 1996; Fissore *et al.*, 1999) and a reorganization of the endoplasmic reticulum, resulting in an increase in cortical vesicles (Ducibella *et al.*, 1988), dicarbocyanine dye-stained foci (Mehlmann *et al.*, 1995), and IP_3R type-1-containing foci (Mehlmann *et al.*, 1996). Finally, there is evidence for an increase in intracellular Ca^{2+} stores during meiotic maturation (Tombes *et al.*, 1992). These changes occur less than 12 h before ovulation and culminate in a mature egg competent to release $[Ca^{2+}]_i$ from intracellular stores upon fertilization.

However, it is not known whether these *upstream* deficiencies in the oocyte's $[Ca^{2+}]_i$ release mechanism can completely account for the failure of CG exocytosis in preovulatory oocytes. For example, in the mouse, $[Ca^{2+}]_i$ oscillations upon fertilization of oocytes previously matured *in vitro* to approximately prometaphase I or metaphase I are similar to those observed in MII-stage eggs (Mehlmann and Kline, 1994) but CG exocytosis at this stage is significantly lower than that in MII eggs and occurs in a localized area over the fertilizing sperm head (Ducibella and Buetow, 1994). To test the hypothesis that GV-stage oocytes have additional deficiencies *downstream* of $[Ca^{2+}]_i$ elevation which contribute to the inability to undergo CG exocytosis, we experimentally induced $[Ca^{2+}]_i$ oscillations in both GV-stage oocytes and mature, ovulated MII-stage eggs. Three methods were used to mimic the fertilization-induced $[Ca^{2+}]_i$ elevations that normally occur in MII-stage eggs. CG exocytosis was monitored in response to treatment with thimerosal, electroporation, and sperm factor injection, all of which induce $[Ca^{2+}]_i$ elevations in mouse GV-stage oocytes and/or MII-stage eggs. In addition, $[Ca^{2+}]_i$ levels were monitored. Thimerosal treatment and sperm factor injection induce long-term, fertilization-like oscillations, whereas electroporation can induce an experimentally defined number of $[Ca^{2+}]_i$ elevations. This study demonstrates that GV-stage oocytes are incompetent, relative to MII-stage eggs, to undergo CG exocytosis following each of these three experimental treatments to induce fertilization-like $[Ca^{2+}]_i$ oscillations.

MATERIALS AND METHODS

Collection of Gametes

GV-stage oocytes and ovulated MII-arrested eggs were obtained from 6- to 10-week-old female CF-1 mice (Charles River, Wilmington, MA) following standard gonadotropin injection. For GV-stage oocytes, mice were primed with 5 IU pregnant mare's serum gonadotropin (PMSG) and cumulus-enclosed, fully grown oocytes were collected 44–48 h later by teasing apart ovaries with needles in Earle's balanced salt solution (EBSS) containing 0.3–0.6% bovine serum albumin (BSA), 25 mM Hepes buffer at pH 7.3 (Ducibella and Buetow, 1994). Cumulus cells were removed by repeated pipetting. For MII-stage eggs, mice were primed with 5 IU PMSG and 44–48 h later with human chorionic gonadotropin (hCG); eggs were collected 13–15 h post-hCG injection in the above medium. Cumulus cells were removed by treatment with 0.01% hyaluronidase for 3–5 min at 37°C. To prevent meiotic resumption, isobutyl methylxanthine (IBMX; 200 μM) was added to media used in the collection and treatment of all GV-stage oocytes (Eppig and Downs, 1987) and MII-stage eggs, where noted.

Calcium Imaging

For imaging experiments, oocytes and eggs were collected in TL Hepes (Parrish *et al.*, 1988) containing 10% fetal bovine serum (FBS). Prior to treatment, GV-stage oocytes were kept in M199 medium with 10% FBS and MII-stage eggs were kept in KSOM medium (Ho *et al.*, 1995) with 0.1% BSA. Microinjections were performed in TL Hepes with 2.5% sucrose (w/v) as previously described (Fissore *et al.*, 1995; He *et al.*, 1997). Micromanipulations were performed using Narishige manipulators (Medical Systems, Great Neck, NY) mounted on a Nikon Diaphot microscope (Nikon, Inc., Garden City, NJ). Oocytes and eggs were injected with Fura-2 dextran (Fura-2D, dextran 10 kDa; Molecular Probes, Eugene, OR) which was stored frozen in Ca^{2+}-free phosphate-buffered saline at 1 mM. Approximately 5–10 pl of Fura-2D was injected into the cytoplasm of GV- and MII-stage oocytes by pneumatic pressure (PLI-100 picoinjector; Medical Systems).

Fura-2D fluorescence was monitored as previously described (Fissore *et al.*, 1995; He *et al.*, 1997). Illumination was provided by a 75-W xenon arc lamp and the excitation wavelengths were at 340 and 380 nm. Neutral density filters were used to attenuate the effects of UV light on oocytes. The emitted light was quantified by a photomultiplier tube after passing through a 500-nm barrier filter and the fluorescent signal was averaged for the whole egg. A modified Phoscan 3.0 software program (Nikon, Inc.) controlled the rotation of a filter wheel and a shutter apparatus to alternate wavelengths. $[Ca^{2+}]_i$ was determined using the 340/380 ratio of fluorescence (Grynkiewicz *et al.*, 1985) and R_{min} and R_{max} were calculated as previously described (He *et al.*, 1997). Where spatial imaging was used, Universal Imaging software (Image1/FL; West Rochester, PA) was utilized. Images were captured using an intensifier (Video Scope International Ltd., Sterling, VA) with a SIT66 camera (Dage-MTI, Michigan City, IN).

Thimerosal Treatment

GV-stage oocytes and MII-stage eggs were treated simultaneously, in the same drop of medium, with 200 μM thimerosal in *in vitro* fertilization medium (Hogan *et al.*, 1986) with sodium bicarbonate, 200 μM IBMX, and 0.4% BSA at 37°C and 5% CO_2 for

30 min. Following treatment, oocytes and eggs were transferred to KSOM culture medium containing 200 μM IBMX and 0.1% BSA (Specialty Media, Lavallette, NJ) at 37°C and 5% CO_2 for 2 h.

Electroporation

Oocytes and eggs were transferred from collection medium to KSOM medium prior to treatment. Fewer than 30 oocytes or eggs were transferred to a pulsing dish consisting of two stainless steel wire electrodes 0.5 mm apart attached to a tissue culture dish filled with pulsing medium containing 0.3 M mannitol, 100–200 μM $CaCl_2$, 100–200 μM $MgSO_4$, and 10 mM histidine. Electrical stimulation of 1.2–3.3 kV/cm (60–165 V) and 20–40-μs pulse duration was supplied by a BTX Electro-Cell Manipulator 200 (BTX, Inc., San Diego, CA). Oocytes and eggs were equilibrated in a 1:1 solution of collection medium and pulsing medium before each pulse and remained in pulsing medium for 2 min following each pulse. Three pulses were given, separated by 20 min.

Sperm Cytosolic Fraction Injection

Cytosolic sperm extracts were prepared from boar semen as described in Wu *et al.* (1997, 1998). Briefly, semen samples were washed twice with TL-Hepes medium and the sperm pellet was resuspended in a solution containing 75 mM KCl, 20 mM Hepes, 500 μM EGTA, 10 mM glycerophosphate, 1 mM DTT, 200 μM PMSF, 10 μg/ml pepstatin, and 10 μg/ml leupeptin, pH 7.0. The sperm suspension was sonicated for 15–25 min at 4°C (XL2020; Heat Systems, Inc., Farmingdale, NY). The lysate was then centrifuged twice at 10,000*g* for 45 min at 4°C and the clear supernatant was used as the cytosolic fraction. Ultrafiltration membranes (Centricon; Amicon, Beverly, MA) were used to wash the supernatant (75 mM KCl and 20 mM Hepes, pH 7.0) and concentrate these extracts to 25 mg/ml protein. Aliquots of sperm extracts were frozen at −80°C. A final protein concentration of 2.5 mg/ml in the pipette was used. The total amount of protein injected into an individual egg was 12.5–25 pg.

CG Staining and Quantification

ZPs were removed from GV-stage oocytes and MII-arrested eggs with 0.25% Pronase in EBSS at 37°C. Eggs were fixed in 3% paraformaldehyde and stained with *Lens culinaris* agglutinin (LCA) coupled to biotin (Polysciences, Warrington, PA) and Texas red streptavidin (Gibco BRL, Gaithersburg, MD) according to Cherr *et al.* (1988) as modified by Ducibella *et al.* (1988) and Ducibella and Buetow (1994). The CG density in the cortex was determined by counting LCA-labeled CGs (Ducibella *et al.*, 1990a) in 300 μm^2 of egg cortex and the average CG density for 100 μm^2 for each egg was determined.

Calculation of Cortical Granule Release

For each individual egg, the number of CGs was calculated by multiplying the CG density times the CG-occupied domain area. Percentage CG release in an individual treated oocyte/egg relative to a control group in the same experiment was calculated as follows: $[1 - (N_{treated}/N_{control})] \times 100$, where N represents the individual number of CGs for the treated egg/oocyte or the mean number of CGs for the control. These values for percentage CG release for individual eggs/oocytes were pooled for each group and expressed as mean % CG release.

Assay for ZP2 to ZP2f Conversion

ZPs were isolated and biotinylated as in Moos *et al.* (1994) as modified by Gross *et al.* (1996). Individual biotinylated ZPs were run on a 9% polyacrylamide gel under reducing conditions. The proteins were then electrophoretically transferred onto Immobilon-P (Millipore, Bedford, MA), blocked overnight with 30 mg/L teleostean gelatin in Tris-buffered saline (TBS) with 0.1% Tween 20 at 4°C, and incubated in avidin-biotinylated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 45 min at 4°C. The blots were repeatedly washed for 1–2 h in TBS with 0.1% Tween 20. An ECL kit was used to visualize the proteins (Amersham, Elkhart, IL); the blots were processed according to the manufacturer's instructions and exposed to X-ray film for 10–60 s. Relative amounts of ZP2 and ZP2f protein were quantified with a Millipore Bio-Image System (Millipore) with 2D Analysis software. Percentage ZP2 conversion determined for individual ZP were then pooled and expressed as mean % ZP2 conversion for either control or treated oocytes.

Statistical Comparisons

For treated and control groups, data were expressed as the mean value per group and compared statistically with the paired *t* test using Statview 4.02 software (Abacus Concepts, Berkeley, CA) and the Student unpaired *t* test.

RESULTS

$[Ca^{2+}]_i$ Release and CG Exocytosis in Response to Thimerosal Treatment

The sulfhydryl reagent thimerosal has been demonstrated to induce $[Ca^{2+}]_i$ oscillations in mouse (Carroll and Swann, 1992; Cheek *et al.*, 1993; Kline and Kline, 1994; Mehlmann and Kline, 1994), bovine (Fissore *et al.*, 1995), human (Herbert *et al.*, 1995), and porcine (Macháty *et al.*, 1997) eggs. Thimerosal (200 μM) has been shown to induce similar $[Ca^{2+}]_i$ oscillations in mouse GV-stage oocytes and MII-stage eggs, in the range of 500–900 nM every 5–8 min (Mehlmann and Kline, 1994), which is comparable to that occurring at normal fertilization. In this study, 200 μM thimerosal induced repetitive $[Ca^{2+}]_i$ oscillations in GV-stage oocytes in the range of 260–780 nM, mean peak elevation 511 nM ± 22 (SEM), every 2–4 min (n = 4 oocytes). A representative $[Ca^{2+}]_i$ profile of a thimerosal-treated, GV-stage oocyte is shown in Fig. 1A. $[Ca^{2+}]_i$ monitoring was not performed on MII-stage eggs as it is well known that 100–200 μM thimerosal induces similar $[Ca^{2+}]_i$ elevations (see above) which can be inferred from CG exocytosis following thimerosal treatment determined in this study (data below).

Thimerosal-treated, MII-stage eggs exhibited a reduced CG density (Fig. 1C) relative to untreated controls (Fig. 1B). In contrast, thimerosal-treated, GV-stage oocytes were characterized by a relatively high density of CGs (Fig. 1E) which closely resembled that of untreated controls (Fig. 1D). Indeed, CG quantification demonstrated that relative to controls, thimerosal-treated, MII-stage eggs exhibited

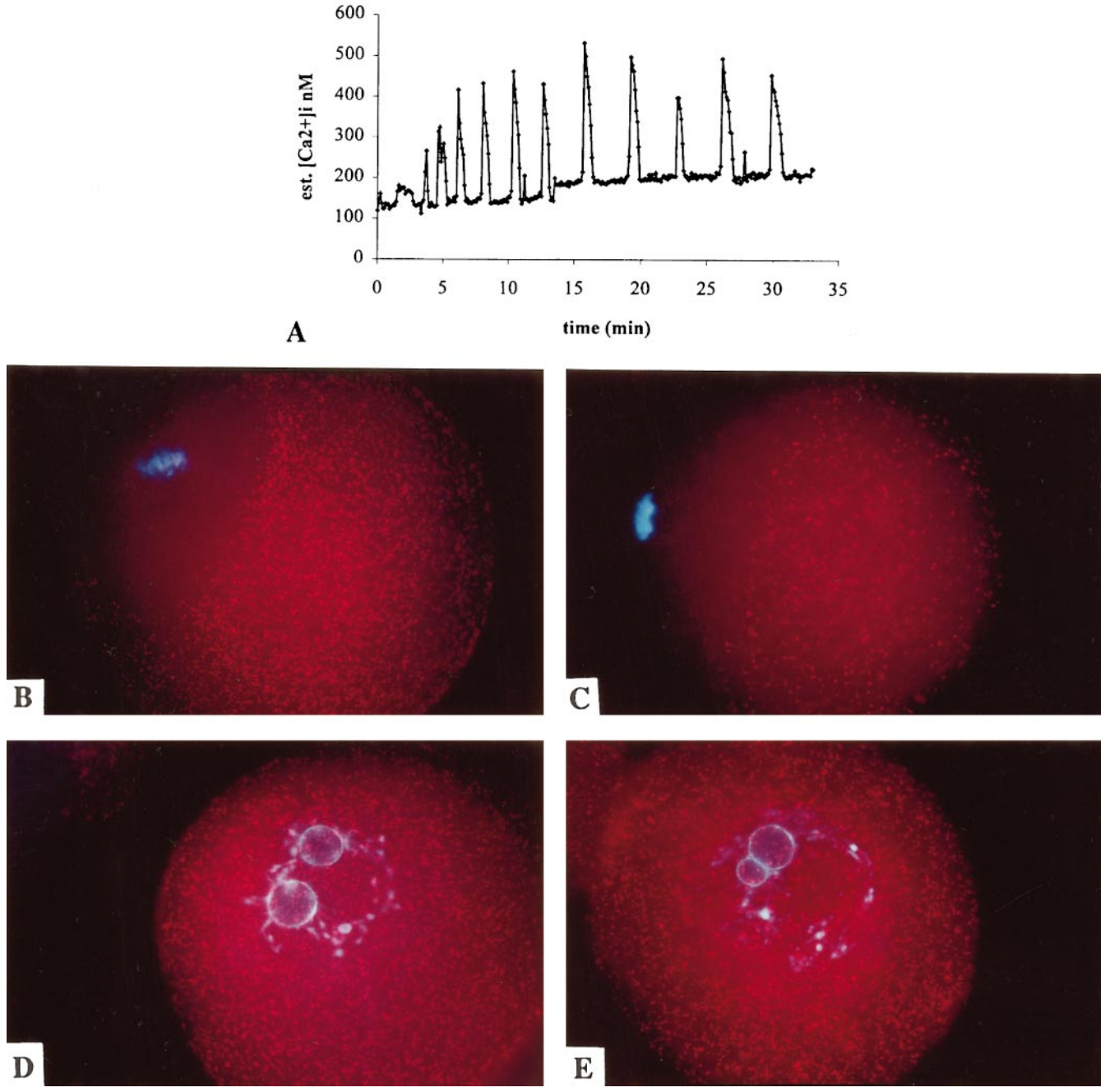


FIG. 1. Effects of thimerosal treatment on MII-stage eggs and GV-stage oocytes. $[Ca^{2+}]_i$ release in a GV-stage oocyte in response to treatment with 200 μM thimerosal for 30 min (A). (B–E) Fluorescent micrographs of eggs or oocytes stained for CGs (red) and chromatin (blue). Untreated, control MII-stage eggs exhibited characteristic high density of CGs, a CG-free domain overlying the spindle, and chromatin aligned in a metaphase configuration (B). Thimerosal-treated, MII-stage eggs were characterized by a reduced density of CGs and chromatin in a slightly more dense, metaphase-like configuration (C). In untreated, GV-stage oocytes, there is the characteristic absence of a CG-free domain, relative high density of CGs, and chromatin at prophase I with the nucleolus staining as a bright rim (D). Thimerosal-treated, GV-stage oocytes had an appearance comparable to untreated oocytes (E). Magnification of images in B–E is ×640.

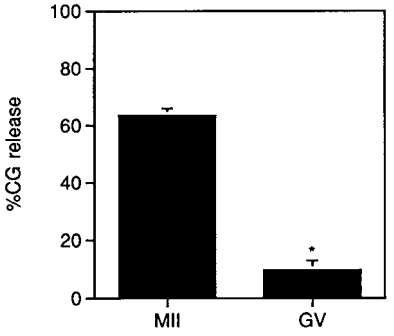


FIG. 2. Thimerosal treatment (200 μM, 30 min) results in greater % CG release in MII-stage eggs than in GV-stage oocytes (* indicates $P < 0.001$). The experiment was performed two times and 19–38 eggs or oocytes were examined for each group. % CG release was calculated as described under Materials and Methods. The data are expressed as means ± SEM.

mean CG release of 64%, whereas thimerosal-treated, GV-stage oocytes exhibited mean CG release of < 10% ($P < 0.001$; Fig. 2).

Zona Modification in Response to Thimerosal Treatment

The low level of CG release detected in GV-stage oocytes following thimerosal treatment was confirmed by the conversion of ZP2 to ZP2f in these treated oocytes (Fig. 3). Control eggs and oocytes had significantly lower ZP2 conversion ($P < 0.001$; Fig. 3). Although < 10% CG release was observed (Fig. 2), the mean % ZP2 conversion was less than, but comparable to, the % ZP2 conversion in thimerosal-treated, MII-stage eggs. This extent of ZP2 conversion after

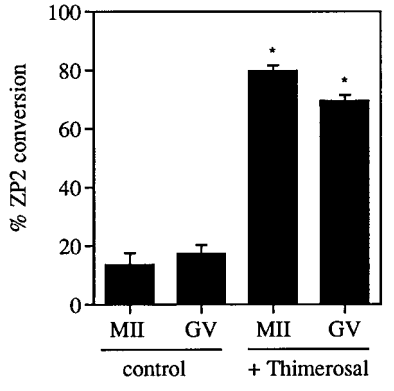


FIG. 3. Thimerosal treatment results in greater conversion of ZP2 to ZP2f in both GV-stage oocytes and MII-stage eggs compared to untreated controls (* indicates $P < 0.001$). The experiment was performed at least twice and 11–28 ZP were analyzed for each group. The data are expressed as means ± SEM.

TABLE 1

Mean $[Ca^{2+}]_i$ Responses upon Electroporation

Group	Baseline (nM)[a]	Peak (nM)[b]	n[c]
MII stage (n = 3)	187 (10)	727 (91)	9
GV stage (n = 3)	159 (14)	652 (56)	9

[a] The mean baseline $[Ca^{2+}]_i$ (SEM).
[b] The mean peak amplitude $[Ca^{2+}]_i$ (SEM).
[c] The number of elevations analyzed.

a low level of CG release is favored by the long duration (2.5 h) in serum-free medium and the smaller perivitelline space of GV stage oocytes than of MII-stage eggs (Ducibella *et al.*, 1990b, 1993).

$[Ca^{2+}]_i$ Release and CG Exocytosis in Response to Electroporation

Although a difference between the competence of GV-stage oocytes and MII-stage eggs to release CGs upon thimerosal treatment was observed, it remained possible that thimerosal treatment inhibited CG release in GV-stage oocytes downstream of $[Ca^{2+}]_i$ elevation. Thus, electroporation was utilized as an alternative technique with a different mode of action to stimulate $[Ca^{2+}]_i$ elevations. Electroporation in Ca^{2+}-containing medium stimulates parthenogenetic activation in eggs of sea urchin (Rossignol *et al.*, 1983), rabbit (Ozil, 1990; Szöllosi and Ozil, 1991), mouse (Vitullo and Ozil, 1992; Ozil and Swann, 1995), and bovine (Collas *et al.*, 1993). Three pulses were given to oocytes and eggs to mimic the early events of fertilization; additional pulses were not given to maintain oocyte viability and minimize nonspecific effects in this experimental system. Using Fura-2 dextran fluorescence, $[Ca^{2+}]_i$ was monitored simultaneously as a GV-stage oocyte or an MII-stage egg was electrically pulsed.

First, electrical pulse parameters were established for MII-stage eggs to create fertilization-like $[Ca^{2+}]_i$ increases. Second, electrical pulse parameters were established such that the $[Ca^{2+}]_i$ profiles for the stimulated GV-stage oocytes were comparable to those for MII-stage eggs. In three MII-stage eggs, electrical stimulation (pulse strength 1.2 kV/cm, pulse duration 20 μs, Ca^{2+} in pulsing medium 100 μM) resulted in a mean peak $[Ca^{2+}]_i$ elevation of 727 ± 91 nM from a baseline of 187 ± 10 nM (Table 1). In three GV-stage oocytes, electrical stimulation (pulse strength 3.3 kV/cm, pulse duration 40 μs, Ca^{2+} in pulsing medium 200 μM) resulted in a mean peak $[Ca^{2+}]_i$ elevation of 652 ± 56 nM from a baseline of 159 ± 14 nM (Table 1). GV-stage oocytes treated with identical pulse parameters as were used with MII-stage eggs exhibited smaller $[Ca^{2+}]_i$ elevations compared to MII-stage eggs and no detectable CG exocytosis.

Following $[Ca^{2+}]_i$ monitoring and electrical stimulation,

CG analysis was performed on the same oocyte or egg for which the $[Ca^{2+}]_i$ profile was known. Figure 4A shows a representative MII-stage egg displaying three $[Ca^{2+}]_i$ elevations corresponding to each time point at which an electrical pulse was given. This egg was characterized by a reduced density of CGs (Fig. 4B) relative to untreated eggs (electroporation controls were similar to thimerosal controls, see Fig. 1B). In contrast, although the GV-stage oocyte shown in Fig. 4D displayed $[Ca^{2+}]_i$ elevations in response to electrical stimulation (Fig. 4C), this oocyte was characterized by a relative high density of CGs (Fig. 4D) with an appearance similar to that of nonpulsed controls (Fig. 1D). Similar results were obtained with a total of three GV-stage oocytes and three MII-stage eggs following identical electroporation treatment and analyses.

After three electrical pulses and under conditions identical to those of the experiments above, groups of MII-stage eggs and GV-stage oocytes displayed significantly different mean % CG releases of 31 and 9%, respectively (Fig. 5). Addition of IBMX in the collection and pulsing medium did not affect CG exocytosis in MII-stage eggs following electroporation (data not shown).

In order to verify that electroporation induced global $[Ca^{2+}]_i$ release similar to that at fertilization, GV-stage oocytes were analyzed by spatial imaging of $[Ca^{2+}]_i$ elevations. Global $[Ca^{2+}]_i$ rise was observed in a normal, spontaneous oscillation (data not shown) as well as upon electrical stimulation (Figs. 4E–4H). Figure 4E shows a representative image of the baseline $[Ca^{2+}]_i$ ($t = 0$). Both Fig. 4F and Fig. 4G show images immediately following the electrical pulse ($t = 0.1$ and 0.4 min, respectively) while Fig. 4H displays a return to baseline $[Ca^{2+}]_i$ level after the pulse ($t = 7.6$ min). This result indicates that the $[Ca^{2+}]_i$ rise is not restricted to one region of the oocyte.

CG Exocytosis in Response to Sperm Factor Injection

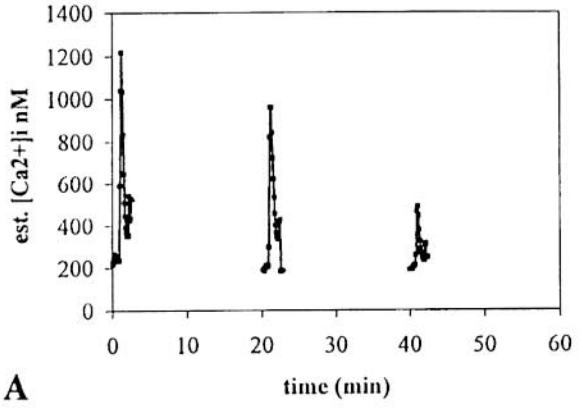
Injection of sperm factor into both mouse GV-stage oocytes and MII-stage eggs results in the stimulation of repetitive $[Ca^{2+}]_i$ oscillations similar to those at fertilization (Wu *et al.*, 1997, 1998). Thus, we determined if sperm factor injection resulted in a similar extent of CG exocytosis at both stages. Injection of sperm factor into MII-stage eggs resulted in a mean CG release of 86% (Fig. 6), whereas $< 1\%$ mean CG release was detected in injected GV-stage oocytes (Fig. 6). These results are consistent with those from thimerosal and electroporation treatments of oocytes and eggs.

DISCUSSION

This study provides evidence that the mechanism to undergo CG exocytosis has one or more deficiencies downstream of $[Ca^{2+}]_i$ elevation in preovulatory, GV-stage mouse oocytes. In previous studies of CG exocytosis, experimental techniques (e.g., fertilization, ionophore, IP_3) did not induce $[Ca^{2+}]_i$ oscillations in GV-stage oocytes that are similar to those of MII-stage mammalian eggs upon normal fertilization. These results gave rise to the idea that the reduced $[Ca^{2+}]_i$ release is responsible for decreased CG exocytosis. The techniques used herein mimic the fertilization-associated oscillatory pattern of $[Ca^{2+}]_i$ elevations and result in similar $[Ca^{2+}]_i$ elevations in GV-stage oocytes and MII-stage eggs. Despite this, GV-stage oocytes exhibited significantly less CG release than MII eggs. We propose that this inability to undergo exocytosis downstream of $[Ca^{2+}]_i$ is due to a maturation-associated change in a factor(s) involved in signaling, translocation, and/or the final steps in secretion.

This demonstration of a deficiency in CG secretion in GV-stage oocytes that lies downstream of $[Ca^{2+}]_i$ elevations is based on the experimental strategy of using three different methods to experimentally induce MII-like $[Ca^{2+}]_i$ oscillations. It is important to note that any one of these methods may have particular limitations, but that it is highly unlikely that three methods would share a common one. For example, if GV-stage oocytes were more sensitive than MII-stage eggs to some oxidative side effect of 200 μM thimerosal, less CG release might result in GV-stage oocytes. On the other hand, electroporation is not likely to have an oxidative side effect, but may create nonphysiological ionic effects via membrane pores. Sperm factor injection would not have the latter two effects, but perhaps may selectively inhibit CG release in GV-stage oocytes by some unknown mechanism. In summary, the combination of nonoverlapping protocols and the ability of each protocol to cause both $[Ca^{2+}]_i$ elevation and CG release in MII eggs strongly supports the concept of a secretory deficiency in GV-stage oocytes.

In the electroporation experiments, the extent of $[Ca^{2+}]_i$ elevation and CG release in MII-stage eggs was dependent upon the pulsing parameters; for example, increasing the pulse amplitude resulted in greater CG release (data not shown). The low level of CG release in MII-stage eggs may be due to an insufficient number or frequency of $[Ca^{2+}]_i$ transients in our experimental system. Multiple studies indicate that CG release begins within 5–10 min of fertilization (Tahara *et al.*, 1996 and references therein), indicating that only a few $[Ca^{2+}]_i$ transients are required to initiate CG exocytosis, though the exact number to initiate and complete exocytosis remains unknown. Electroporation-induced $[Ca^{2+}]_i$ transients in this study were 20 min apart, whereas fertilization (Kline and Kline, 1992; Miyazaki *et al.*, 1993; Whitaker and Swann, 1993), thimerosal treatment (this study; Mehlmann and Kline, 1994), and sperm factor injection (Wu *et al.*, 1997, 1998) all result in $[Ca^{2+}]_i$ elevations < 10 min apart in mouse eggs. In addition, in this study, thimerosal and sperm factor injection resulted in greater CG release than electroporation. The greater number and frequency of oscillations induced by the these methods relative to electroporation may sufficiently activate a Ca^{2+}-dependent enzyme, with properties like Ca^{2+}/


1400
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1000
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est. [Ca2+]i nM
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10
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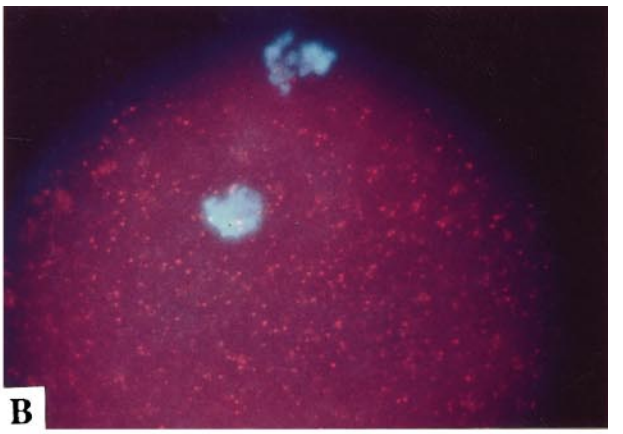
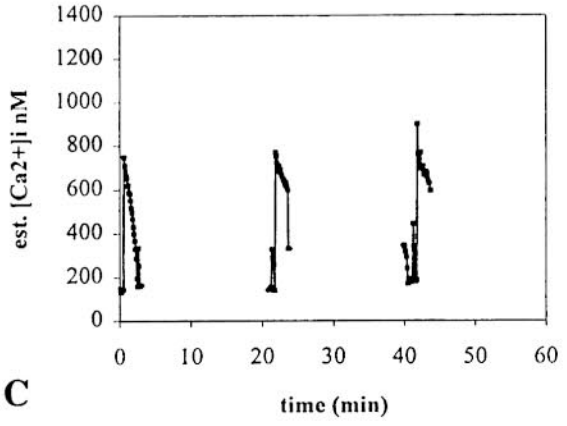
A



B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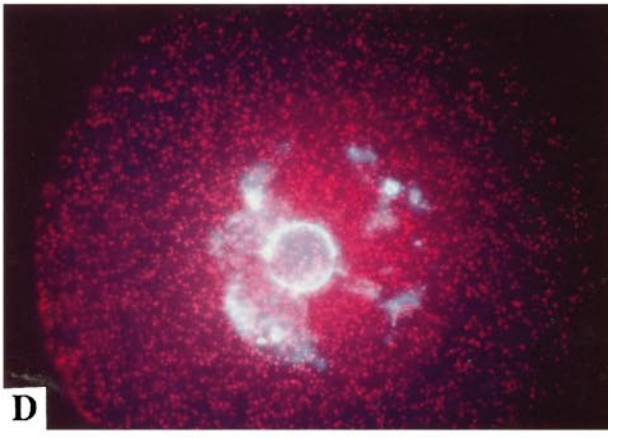
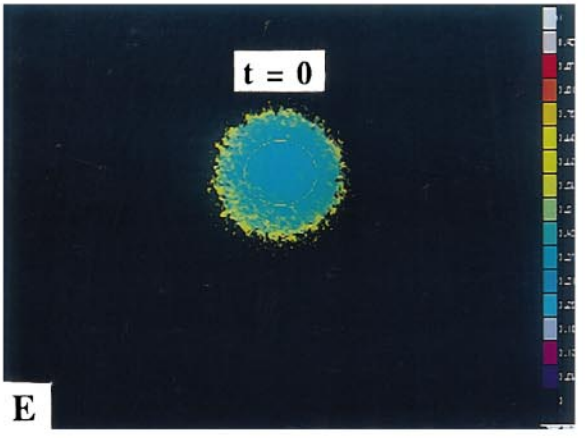
time (min)

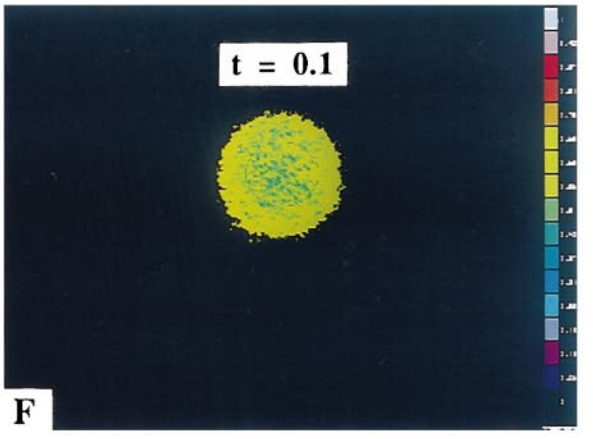
C



D


t = 0

E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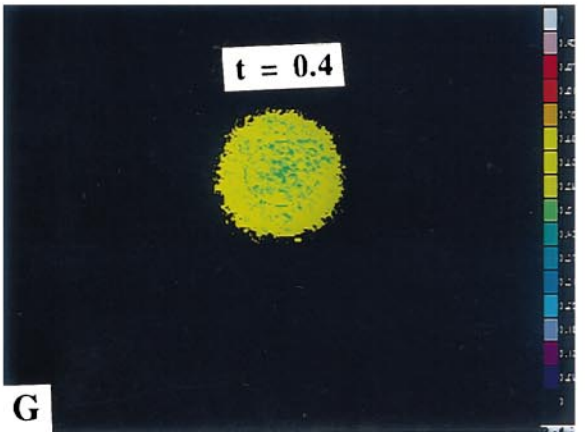
F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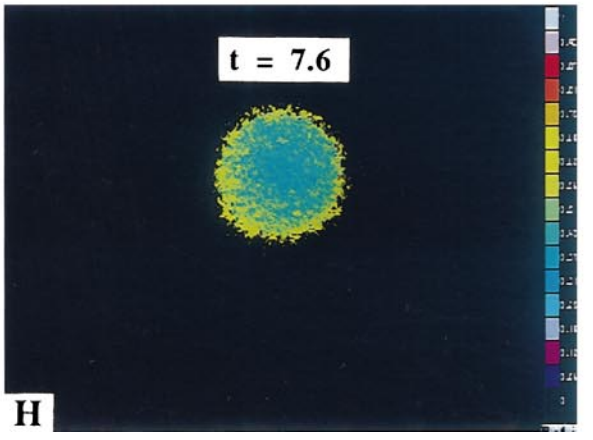
G


t = 7.6

H

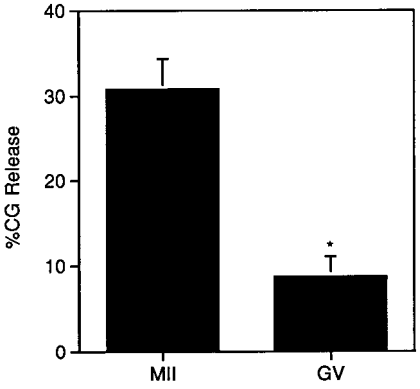


FIG. 5. Electroporation induces significantly greater CG exocytosis in MII-stage eggs than in GV-stage oocytes (* indicates $P < 0.001$). The experiment was performed at least twice and 34–46 eggs/oocytes were analyzed for each group. The data are expressed as means ± SEM.

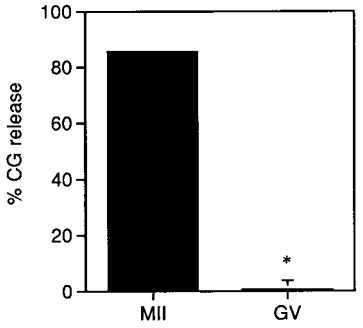


FIG. 6. Injection of sperm factor results in greater % CG release in MII-stage eggs than in GV-stage oocytes (* indicates $P < 0.001$). The experiment was performed two times and 15–34 eggs or oocytes were examined for each group. % CG release was calculated as described under Materials and Methods. The data are expressed as means ± SEM.

calmodulin-dependent kinase II (Dupont and Goldbeter, 1998), thereby resulting in CG exocytosis. It is important to note, however, that despite the low level of CG release in electroporated MII-stage eggs, GV-stage oocytes still exhibited significantly less CG exocytosis following comparable electroporation-induced $[Ca^{2+}]_i$ elevations.

In previous studies with starfish oocytes, high concentrations of IP_3 and polyspermy caused only partial egg envelope elevation, suggesting incomplete CG release and a possible deficiency downstream of $[Ca^{2+}]_i$ (Chiba and Hoshi, 1989; Chiba *et al.*, 1990). The results with mouse oocytes herein are similar in that thimerosal and electroporation treatments induced a small amount (≈10%) of CG exocytosis that was confirmed by ZP2 analysis. It is interesting that *Xenopus laevis* immature oocytes are relatively incompetent to undergo CG exocytosis upon Ca^{2+} injection compared to mature eggs despite being equally competent to support the exocytosis of injected chromaffin granules, suggestive of a deficiency in a CG membrane component(s) to respond to $[Ca^{2+}]_i$ elevation (Scheuner and Holz, 1994). In GV-stage mouse oocytes, although sperm and IP_3 are unable to stimulate CG release, agonists of the PKC pathway (e.g., phorbol ester) can induce partial CG release (Ducibella *et al.*, 1993). However, inhibitors of PKC are unable to prevent fertilization-induced CG exocytosis while they are able to inhibit phorbol ester-induced CG exocytosis (LeFevre and Ducibella, 1997). Therefore, although PKC activation may bypass the observed downstream deficiency, it is not known to what extent its effects are mediated through the normal activation pathway for CG exocytosis.

One hypothesis to account for the incompetence of GV-stage oocytes to undergo CG release in response to induced $[Ca^{2+}]_i$ oscillations is that CGs are unable to translocate to the plasma membrane. Unlike the sea urchin in which CGs are docked at the plasma membrane prior to fertilization (Decker and Kinsey, 1983), the mean distance of CGs in the cortex from the plasma membrane in mouse MII-stage eggs is approximately 0.5 μm and is similar in GV-stage oocytes (Ducibella *et al.*, 1988). Microfilaments are reported to be involved in CG translocation to the cortex in the mouse (Connors *et al.*, 1998) and in CG exocytosis in the eggs of the hamster (DiMaggio *et al.*, 1997), mouse (Tahara *et al.*, 1996), and loach (Ivanekov *et al.*, 1990). In neuronal secretion, a population of secretory granules is tethered to the actin cytoskeleton, their release

FIG. 4. The effects of electroporation on $[Ca^{2+}]_i$ and CG exocytosis. A and B display results from the same MII-stage egg which was monitored for $[Ca^{2+}]_i$ during electrical stimulation with pulses given at approximately 0, 20, and 40 min (A) and then stained for CGs and chromatin (B) as in Fig. 1. Electroporated MII-stage eggs were characterized by a reduced density of CGs. C and D display results from an individual GV-stage oocyte which was likewise monitored for $[Ca^{2+}]_i$ and then stained for CGs and chromatin. Electroporated GV-stage oocytes were characterized by a CG density comparable to untreated controls. In all micrographs, the CG density appears lower toward the perimeter of oocytes and eggs, likely due to the curvature of the cell out of the optical plane of section. E–H display pseudocolor Ca^{2+} imaging of one GV-stage oocyte with a global $[Ca^{2+}]_i$ elevation following an electrical pulse. The $[Ca^{2+}]_i$ scale in these images is arbitrary. $t = 0$ corresponds to a baseline $[Ca^{2+}]_i$ image (E). Times in subsequent panels (F–H) refer to the time following this initial measurement. $[Ca^{2+}]_i$ elevation is observed at the time of the pulse (F) and moments later (G) followed by a return to near baseline level (H). Magnification of images in B and D are ×640 and of images in E–H are ×195. Unlike B and D, the cell in E–H is not compressed.

from which may be regulated by the phosphorylation of synapsin (Greengard *et al.*, 1993; Ceccaldi *et al.*, 1995). Both the release of secretory granules from the actin cytoskeleton and the reorganization of the cortical cytoskeleton, through such Ca^{2+}-dependent actin-depolymerizing proteins as gelsolin and scinderin (Calakos and Scheller, 1996), may be involved in the regulation of secretory granule access to the plasma membrane in somatic cells as well as in mammalian oocytes.

An alternate hypothesis to account for this exocytotic incompetence is that GV-stage oocytes are deficient in a final step of secretion, referred to as docking and fusion. From studies in somatic cells and yeast, a model for the secretory mechanism which involves the interactions between *N*-ethylmaleimide-sensitive fusion proteins (NSF), soluble NSF attachment proteins (SNAP), and SNAP receptors (SNARE) has emerged (reviewed in Südhof, 1995; Calakos and Scheller, 1996; Burgoyne and Morgan, 1998). Complementary sets of SNARE proteins on the vesicle and the target membranes mediate individual membrane fusion events, potentially regulated by rab proteins, kinases, phosphatases, and the Ca^{2+}-sensor candidate protein synaptotagmin (Südhof, 1995). In sea urchins, SNARE proteins thought to be involved in CG exocytosis are detected in the egg cortex (Avery *et al.*, 1997; Conner *et al.*, 1997). In mouse eggs, rabphilin-3A and rab3A are also cortically localized and are suggested to be involved in CG exocytosis because inhibition of rabphilin-3A blocks CG release at a step proposed to lie downstream of $[Ca^{2+}]_i$ elevation (Masumoto *et al.*, 1996, 1998). It is likely that CG exocytosis in mammalian eggs employs a mechanism for secretion similar to that in sea urchin eggs as well as in somatic cells.

Although mechanisms downstream of $[Ca^{2+}]_i$ elevation resulting in exocytosis are beginning to be elucidated in other systems, this remains a poorly understood area of egg activation. Our results demonstrate that this mechanism to respond to elevations in $[Ca^{2+}]_i$ and undergo exocytosis is not functional in mouse GV-stage oocytes compared to MII-stage eggs. The events during oocyte maturation that culminate in a mature, maximally activation-competent egg represent an important developmental problem and one relevant to animal and human-assisted reproductive procedures in which large numbers of immature oocytes are routinely collected.

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