The involvement of protein kinase C and actin filaments in cortical granule exocytosis in the rat

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Abstract

Mammalian sperm–egg fusion results in cortical granule exocytosis (CGE) and resumption of meiosis. Studies of various exocytotic cells suggest that filamentous actin (F-actin) blocks exocytosis by excluding secretory vesicles from the plasma membrane. However, the exact function of these microfilaments, in mammalian egg CGE, is still elusive. In the present study we investigated the role of actin in the process of CGE, and the possible interaction between actin and protein kinase C (PKC), by using coimmunoprecipitation, immunohistochemistry and confocal microscopy. We identified an interaction between actin and the PKC α isoenzyme in non-activated metaphase II (MII) eggs and in eggs activated by phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). F-actin was evenly distributed throughout the egg's cytosol with a marked concentration at the cortex and at the plasma membrane. A decrease in the fluorescence signal of F-actin, which represents its depolymerization/reorganization, was detected upon fertilization and upon parthenogenetic activation. Exposing the eggs to drugs that cause either polymerization or depolymerization of actin (jasplakinolide (JAS) and cytochalasin D (CD) respectively) did not induce or prevent CGE. However, CD, but not JAS, followed by a low dose of TPA doubled the percentage of eggs undergoing complete CGE, as compared with TPA alone. We further demonstrated that myristoylated alanin-rich C kinase substrate (MARCKS), a protein known to cross-link F-actin in other cell types, is expressed in rat eggs and is colocalized with actin. In view of our results, we suggest that the cytoskeletal cortex is not a mere physical barrier that blocks CGE, but rather a dynamic network that can be maneuvered towards allowing CGE by activated actin-associated proteins and/or by activated PKC.

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Introduction

Sperm–egg fusion in mammals results in activation of the egg by triggering a series of biochemical events in a pre-determined developmental program. A very early cellular event observed following sperm–egg fusion is an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) followed by oscillations. Other early events are cortical granule exocytosis (CGE), which leads to modification of the zona pellucida (ZP) and hence to the block of polyspermy and resumption of meiosis (Ben-Yosef & Shalgi 1998, Swann & Parrington 1999, Carroll 2001, Runft *et al.* 2002).

Many biological systems in which Ca^{2+} serves as a second messenger are also regulated by protein kinase C (PKC) through its effect, either positive or negative, on the cellular signal transduction processes (Nishizuka 1988). Various studies have demonstrated expression and even possible involvement of PKC in the egg activation process (Gangeswaran & Jones 1997, Johnson & Capco 1997, Raz *et al.* 1998b, Pauken & Capco 2000, Luria *et al.* 2000, Eliyahu *et al.* 2001). There is evidence that activation of

PKC induces CGE, but there is probably no direct linkage between PKC activation and the exit from metaphase II (MII; Jones 1998, Raz *et al.* 1998a). Studies performed in our laboratory revealed that conventional PKC (cPKC) isoenzymes were activated at fertilization and upon parthenogenetic activation either by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or by 1-oleoyl-2-acetyl-glycerol (OAG), but not by the calcium ionophore ionomycin (Eliyahu & Shalgi 2002). Furthermore, we were able to inhibit CGE by a myristoylated PKC pseudosubstrate (myrPKC Ψ), which is a specific PKC inhibitor, thus suggesting that exocytosis can be triggered independently either by $[Ca^{2+}]_i$ rise or by PKC. In a recent study, it was demonstrated that cPKCs translocate to the egg plasma membrane following a pattern that is shaped by the amplitude, duration and frequency of Ca^{2+} transients (Halet *et al.* 2004).

PKC is known to be associated with a wide range of cytoskeletal components and/or to phosphorylate them (Inagaki *et al.* 1987). The actin cytoskeleton has long been proposed as the regulatory site of exocytosis. Evidence

from several cell types suggests that a membrane-associated filamentous actin (F-actin) acts as a barrier to exocytosis by excluding secretory vesicles from the plasma membrane (Burgoyne *et al.* 1989, Vitale *et al.* 1991, Trifaro *et al.* 1992) and that its depolymerization is a primary step in the translocation of secretory vesicles to the plasma membrane (Lelkes *et al.* 1986, Sontag *et al.* 1988, Muallem *et al.* 1995). However, other studies have demonstrated polymerization, rather than depolymerization, of actin in some somatic cells undergoing exocytosis (Sha'afi & Molski 1987, Apgar 1991). The presence of a subplasmalemma, actin-based cytoskeleton in eggs is well known (Spudich *et al.* 1988, Bonder *et al.* 1989, Roeder & Gard 1994, Becker & Hart 1996). The microfilaments of this cytoskeleton play a role in many dynamic processes during mammalian egg maturation and fertilization (Sutovsky *et al.* 1997, Connors *et al.* 1998, Terada *et al.* 2000). Processes such as sperm incorporation, CGE, spindle movement and polar body II (PBI) extrusion (Capco *et al.* 1992, Gallicano *et al.* 1992, 1995, Wang *et al.* 2000) are accompanied by reorganization of the actin cytoskeleton (Terada *et al.* 2000).

Various studies investigating the effect of actin-perturbing drugs on CGE have produced inconclusive results. CGE could be inhibited in the eggs of only some vertebrate species and only after treatment with some of the actin-perturbing drugs (Tahara *et al.* 1996, DiMaggio *et al.* 1997, Terada *et al.* 2000, McAvery *et al.* 2002), while other studies showed no inhibition of CGE (Boyle *et al.* 2001, Sun *et al.* 2001). Results regarding the effect of PKC activators on actin filaments are also ambiguous. Activation of PKC by TPA or by diacylglycerol (DAG) caused polymerization of actin, while PKC antagonist inhibited the formation of actin storage (Takashi 1997). However, in other studies, stimulation of PKC by phorbol esters was accompanied by a focal and transient disruption of the cortical F-actin network (Vitale *et al.* 1991, Tchakarov *et al.* 1998, Trifaro *et al.* 2002). These contradictory results reflect the lack of clarity regarding the role of PKC, actin and actin-associated proteins in the regulation of secretory events (Muallem *et al.* 1995).

Evidence from several cell types suggests that F-actin is associated with myristoylated alanine-rich C kinase substrate (MARCKS; Rosen *et al.* 1990, Aderem 1992, Swierczynski & Blackshear 1995, Rossi *et al.* 1999). MARCKS cross-links actin filaments and anchors the actin network to the plasma membrane (Rossi *et al.* 1999). In the present study we investigated the possible interaction between actin and PKC, both of which are known to participate in exocytosis. We examined the hypothesis that the cortical actin filaments serve as a barrier that excludes cortical granules (CGs) from the membrane of MII rat eggs. Changes in the distribution of actin filaments occur as a result of PKC activation during egg activation and enable CGE. Our results demonstrated that CGE cannot be triggered by a simple polymerization/depolymerization of actin filaments but rather PKC activation is required as well.

Materials and Methods

Isolation of eggs

MI-ovulated eggs

For induction of ovulation, 23- to 25-day-old immature Wistar-derived female rats, were injected with 10 IU human chorionic gonadotropin (hCG; Sigma), 48–54 h after administration of 10 IU pregnant mares serum gonadotropin (PMSG; Syncro-part, France). Rats were killed 14 h after the hCG administration. Cumulus-enclosed MII eggs were isolated from the oviducal ampullae into TH medium (Ben-Yosef *et al.* 1995), supplemented with 0.4% BSA (Sigma). Cumulus cells were removed by a brief exposure to 400 IU/ml hyaluronidase (Sigma) in TH medium.

In vivo fertilized eggs

PMSG–hCG-primed immature female rats were caged overnight with fertile males. Rats were killed 15 h after hCG administration. Egg isolation and cumulus cell removal were performed as described above for MII eggs. The eggs were classified according to the various stages of fertilization (Eliyahu & Shalgi 2002): sperm binding (SB), fertilization cone (FC) and PBI stages (0–15, 15–60 and 60–180 min after sperm attachment respectively).

Parthenogenetic activation

MI-ovulated eggs were parthenogenetically activated by two different activators that are capable of inducing full CGE in rat eggs (Raz *et al.* 1998a, Eliyahu & Shalgi 2002). The first activator was TPA (30–100 ng/ml; Sigma); a 5-min incubation in the presence of TPA followed by an additional 5- or 10-min incubation in fresh medium lacking the activator. A stock solution of 1 mg/ml TPA was prepared in dimethylsulfoxide (DMSO) and stored at –20 °C. The second activator was calcium ionophore (2 µM; ionomycin 407950, Calbiochem, San Diego, CA, USA); a 3-min incubation in the presence of ionomycin followed by an additional 17-min incubation in fresh medium lacking the activator. A stock solution of 10 mM ionomycin was prepared in DMSO and stored at 4 °C.

Actin polymerization and depolymerization

MI eggs were incubated for 30 min with the actin-depolymerizing drug cytochalasin D (CD; 10 µg/ml, Sigma), or with the polymerizing drug jasplakinolide/jaspamide (JAS; 1×10^{-7} nM, Molecular Probes, Eugene, OR, USA).

Antibodies and staining agents

Primary antibodies: anti-actin rabbit polyclonal IgG (A-5060, Sigma); anti-cPKC alpha mouse monoclonal IgG (P-16520, Transduction Laboratories, Lexington, KY, USA); anti-MARCKS goat polyclonal IgG (sc-6454, Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibodies:

goat anti-rabbit IgG (sc-2004, Santa Cruz); goat anti-mouse IgG (sc-2005, Santa Cruz); donkey anti-goat IgG (sc-2020, Santa Cruz). Cy-conjugated secondary antibody: donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, PA, USA). Staining agents: Texas Red phalloidin (T-7471, Molecular Probes); Biotinylated lens culinaris agglutinin (LCA)–biotin (B-1045, Vector, Burlingame, CA, USA); Texas Red streptavidin (SA-5006; Vector).

Immunoprecipitation and immunoblotting

An antibody against anti-actin was chemically coupled to protein A sepharose in order to prepare an immobilized antibody affinity reagent for immunoprecipitation (IP buffer), according to Talmor *et al.* (1998). Batches of 1000 eggs, that either had or had not been subjected to activating agents, were lysed in 100 µl IP buffer and kept at -70°C . Upon thawing, the egg lysates were incubated overnight at 4°C with 10 µl immobilized antibody (25% suspension) and then washed by centrifugation with IP buffer and separated by 10% SDS–PAGE under non-reducing conditions. Proteins were transferred onto a nitrocellulose membrane (Amersham) using a wet blotting apparatus (Hoeffer, San Francisco, CA, USA). For immunoblot analysis, blots were blocked with Tris-buffered saline with 5% dry milk and incubated in the presence of various specific primary antibodies. Bound antibodies were recognized by secondary antibodies conjugated to horseradish peroxidase. Detection was performed by an ECL detection system (Pierce, Rockford, IL, USA). Approximate molecular masses were determined by comparison with the migration of prestained protein standards (Amersham).

Immunofluorescence staining and laser-scanning confocal microscopy

Eggs at various developmental stages were fixed for 10 min at room temperature in Dulbecco's phosphate-buffered saline (DPBS) with 3% paraformaldehyde and 0.01% glutaraldehyde. Eggs were then washed in a solution of 3% foetal calf serum (FCS; Biological Industries, Beit-Haemek, Israel) in DPBS (DPBS/FCS). ZPs were removed post-fixation by 0.25% pronase (Sigma) in DPBS/FCS. The ZP-free eggs were washed in DPBS/FCS. For detection of CGE, fixed eggs were labeled by LCA and Texas Red streptavidin (Eliyahu & Shalgi 2002).

For labeling egg proteins, the plasma membrane of ZP-free eggs was permeabilized during 10-min incubation in 0.05% NP-40 in DPBS/FCS solution followed by several rinses in 0.005% NP-40 in DPBS/FCS.

For actin labeling eggs were incubated for 2 h in the presence of Texas Red phalloidin (1.1 nM) or with anti-MARCKS (1:100) followed by an incubation in the presence of a fluorescence-labeled Cy secondary antibody (1:300). Chromatin labeling and the fertilization stage of the eggs were assessed by incubating the eggs for 10 min in the presence of 1 µg/ml Hoechst (Sigma). The progression of eggs past the MII stage was analyzed by moni-

toring the separation of the chromosomal dyads. The various stages of fertilization were determined by following the sperm and egg chromatin. The labeling of CG exudates, actin, MARCKS and DNA was visualized and photographed by a Zeiss confocal laser-scanning microscope (CLSM; Oberkochen, Germany). The Zeiss LSM 410 is equipped with a 25 mW krypton–argon laser, a 10 mW helium–neon laser (488, 543 and 633 maximum lines) and an u.v. laser (Coherent Inc. Laser Group, Santa Clara, CA, USA). A $\times 40$ numerical aperture/1.2 planapochromat water immersion lens (Axiovert 135M, Zeiss) was used for all imaging. Eggs were scanned using the CLSM through the Z-axis to perform a section at the equatorial plane of the egg. The stain intensity was measured using the correct mean density values obtained by the LSM software. The fluorescence intensity was expressed relative to values obtained for MII eggs, which were set to 1.0 (arbitrary units).

Results

In the present study we investigated the possible interaction between actin and PKC at the pathway leading to CGE during the process of egg activation.

Expression of actin in the egg

We used Western blot analysis to evaluate the total amount of actin in non-activated MII eggs and in TPA-activated eggs. Egg proteins, before or after parthenogenetic activation of the eggs by TPA, were separated on SDS–PAGE. As shown in Fig. 1, the amount of actin, a 46 kDa protein, was similar in both non-activated MII eggs and in TPA-activated eggs.

Figure 1 Detection of total actin (F- and G-actin) in non-activated MII and TPA-activated eggs. Eggs, before or after parthenogenetic activation by 50 ng/ml TPA, were pooled, lysed and the proteins separated by SDS–PAGE (200 eggs per lane). The proteins were immunoblotted with anti-actin rabbit polyclonal IgG (1:250). Peroxidase-conjugated goat anti-rabbit IgG (1:5000) secondary antibodies were detected by an ECL detection system. A single band was detected at the appropriate molecular mass for actin (46 kDa), as calculated from the migration of protein standards with known molecular masses. At least three independent experiments were performed.

Association between actin and PKC alpha during egg activation by TPA

Coimmunoprecipitation assays were performed to detect the possible interaction between actin and PKC. Lysates of eggs, before or after parthenogenetic activation by TPA, were immunoprecipitated with anti-actin as described in Materials and Methods. The nitrocellulose membrane was incubated in the presence of anti-PKC alpha monoclonal antibody. Our results show an interaction between PKC alpha and actin in non-activated MII eggs and in TPA-activated eggs (Fig. 2). As expected, a single 84 kDa PKC alpha band was detected in non-activated MII eggs whereas TPA-activated eggs exhibited two bands, at 84 and 88 kDa, that may represent the unphosphorylated and the phosphorylated forms of PKC alpha respectively. Several treatments were used as control groups: IP buffer containing only the immune complex; incubation of the nitrocellulose membrane with any IgG antibody (polyclonal anti-Src antibody); incubation of the egg lysate with any antibody (polyclonal anti-Src antibody) conjugated to protein A sepharose. None of the control groups exhibited either an 84 kDa or an 88 kDa band on the nitrocellulose membrane (data not shown).

Distribution of F-actin during egg activation

The intracellular distribution and organization of F-actin in fertilized or in parthenogenetically activated eggs was examined by immunofluorescence confocal microscopy. F-actin was evenly distributed throughout the cytosol of MII-arrested eggs (Fig. 3A and D) with a marked concentration at the cortex and at the plasma membrane, especially above the metaphase plate (Fig. 4A and E). A decrease in the fluorescence signal of F-actin was detected upon fertilization, at the SB stage, as compared with MII eggs (Fig. 3B and E), the decrease was more pronounced at the FC/PBII stages (Fig. 3C and F). In TPA-activated eggs, we observed a time-dependent decrease of the fluorescence signal (Fig. 4F–H), as compared with untreated MII eggs (Fig. 4E). As expected, eggs treated with TPA remained at the MII stage (Fig. 4B–D).

Figure 2 Association of cPKC alpha with actin upon egg activation by TPA. Eggs (1000 per lane), before (MII) or after (TPA) parthenogenetic activation by 50 ng/ml TPA, were lysed and immunoprecipitated with anti-actin rabbit polyclonal IgG (IP = actin). The proteins were immunoblotted with anti-cPKC alpha monoclonal IgG (1:500). Peroxidase-conjugated goat anti-mouse IgG (1:500) secondary antibody was detected by an ECL detection system. Two bands were detected at 84 and 88 kDa, corresponding to cPKC alpha and phosphorylated cPKC alpha respectively (arrows), as calculated from the migration of protein standards with known molecular masses. At least three independent experiments were performed.

Figure 3 Distribution of F-actin at various stages of *in vivo* fertilization. Fluorescence intensity was calculated as described in Materials and Methods. The values were expressed relative to those obtained for MII eggs, which were set to 1.0 (arbitrary units). Fertilized eggs were fixed and labeled with Texas Red phalloidin (1.1 nM; F-actin labeling) and with Hoechst (1 μg/ml; DNA labeling) and imaged using a laser-scanning confocal microscope. (A and D) Unfertilized egg (MII, relative fluorescence = 1.0). (B and E) Egg at the sperm binding (SB) stage (relative fluorescence = 0.68). (C and F) Egg at the fertilization cone (FC) stage (relative fluorescence = 0.68). Light microscopy (A–C); actin and chromosomes (D–F); sperm DNA (yellow arrow); egg DNA (white arrow). At least three independent experiments (three to four eggs for each group in each experimental day) were performed. Each image was taken at the equatorial plane of the egg. Scale bar, 10 μm.

The temporal decrease observed in the fluorescence intensity of TPA-activated eggs might reflect the depolymerization/reorganization of F-actin.

Effect of actin polymerizing/depolymerizing drugs on the distribution of F-actin and on CGE

To evaluate the role of F-actin in CGE, we incubated eggs in the presence of drugs that induce polymerization or depolymerization of actin (JAS or CD respectively). The distribution of F-actin, before and after incubation in the presence of either drug, was followed (Fig. 5). CD caused aggregation of F-actin within the cytoplasm and a decreased fluorescence intensity, reflecting depolymerization of actin (Fig. 5E). JAS, on the other hand, caused an

Figure 4 Distribution of F-actin during parthenogenetic activation by TPA. Fluorescence intensity was calculated as described in Materials and Methods. The values were expressed relative to those obtained for MII eggs, which were set to 1.0 (arbitrary units). Eggs were fixed at the MII stage (A and E; relative fluorescence = 1.0) or after incubation for 5 (B and F; relative fluorescence = 0.40), 10 (C and G; relative fluorescence = 0.43) or 15 (D and H; relative fluorescence = 0.20) min in the presence of 100 ng/ml TPA. Eggs were labeled with Texas Red phalloidin (1.1 nM; F-actin labeling) and with Hoechst (1 μ g/ml; DNA labeling). Localization of the actin was imaged using a laser-scanning confocal microscope. Light microscopy and chromosomes (A–D); actin (E–H). At least three independent experiments (three to four eggs for each group in each experimental day) were performed. Each image was taken at the equatorial plane of the egg. Scale bar, 10 μ m.

increase in the fluorescence intensity of F-actin throughout the egg, reflecting polymerization of F-actin (Fig. 5F). Eggs treated with either CD or JAS did not progress beyond the MII stage (Fig. 5A–C).

Figure 5 Effect of actin-polymerizing/-depolymerizing drugs on the distribution of F-actin. Eggs, before or after 30-min incubation in the presence of either 10 μ g/ml CD or 10^{-7} mM JAS were fixed and labeled with Texas Red phalloidin (1.1 nM; F-actin labeling) and with Hoechst (1 μ g/ml; DNA labeling). Non-treated eggs were fixed at the MII stage (A and D). Treated eggs were fixed after exposure to CD (B and E) or to JAS (C and F). Localization of actin was imaged using a laser-scanning confocal microscope. Light microscopy and chromosomes (A–C); actin (D–F). At least three independent experiments (three to four eggs for each group in each experimental day) were performed. Each image was taken at the equatorial plane of the egg. Scale bar, 10 μ m.

The correlation between PKC activation, F-actin reorganization and CGE was examined by monitoring the capability of eggs, exposed to CD or to JAS, to undergo CGE while PKC was activated by TPA (Fig. 6). MII eggs were incubated in the presence of either CD or JAS and then activated by 30 ng/ml TPA in the presence of either drug. Untreated MII eggs served as a negative control for CGE (Fig. 6A), whereas, eggs exposed only to TPA served as a positive control (Fig. 6D). MII eggs, as well as eggs exposed to either JAS or CD, did not undergo CGE (Fig. 6A–C), nor did they overcome the second metaphase arrest (not shown). As expected, eggs treated with 30 ng/ml TPA, a concentration that triggers only partial CGE (Raz *et al.* 1998a), exhibited moderate fluorescence intensity (Fig. 6D). Addition of CD, but not of JAS, caused enhancement of the CGE fluorescence intensity, indicating that the eggs had undergone complete CGE (JAS, Fig. 6E; CD,

Figure 6 Effect of actin-polymerizing/-depolymerizing drugs on CGE. (A–F) Immunofluorescence confocal microscopy. Eggs, before or after activation by 30 ng/ml TPA in the presence of 10^{-7} mM JAS or 10 μ g/ml CD, were fixed and labeled by LCA–biotin (1:100) followed by Texas Red–streptavidin (1:1000) for CGE detection. Eggs were examined at the MII stage (A); after 30-min incubation in the presence of JAS (B) or CD (C); after 5-min activation by TPA, followed by 25 min in fresh TH medium (D); or after 30-min incubation in the presence of JAS or CD followed by 5-min activation by TPA in the presence of JAS/CD (E and F respectively). The occurrence of the CGE process was imaged using a laser-scanning confocal microscope. At least three independent experiments (three to four eggs for each group in each experimental day) were performed. Each image was taken at the equatorial plane of the egg. Scale bar, 10 μ m. (G) The percentage of eggs undergoing complete CGE under the various treatments. The intensity of the labeling was measured using the corrected mean density values obtained by the LSM image software. The Y-axis represents the percentage of eggs that underwent complete CGE. Values are means $\pm$ S.E.M. TPA in the presence of CD induced significantly higher rates of CGE than TPA alone ($P < 0.005$, two-way ANOVA test). Different letters represent significant differences.

Fig. 6F). There were no significant differences in the ability of eggs to undergo complete CGE between MII-arrested eggs and eggs treated with CD or JAS alone (Fig. 6G, $P > 0.05$, two-way ANOVA test). However, exposing eggs to CD followed by exposure to 30 ng/ml TPA, doubled the percentage of eggs undergoing complete CGE as compared with eggs incubated with TPA alone (88.6 and 46.4% respectively, $P < 0.005$, two-way ANOVA test).

Expression of MARCKS in non-activated MII eggs and in activated eggs

In various cell types, MARCKS, a myristoylated protein, cross-links F-actin and anchors the actin network to the plasma membrane. An important step towards

understanding the role of MARCKS in the process of egg activation was the study of its expression, localization and colocalization with actin. Eggs, before or after parthenogenetic activation, were pooled, lysed and their proteins immunoblotted. As seen in Fig. 7, the egg lysates exhibited a protein band with an apparent molecular mass of 80 kDa, which is consistent with the expected molecular mass for MARCKS protein (Wu *et al.* 1982, Aderem 1992). The protein band of non-activated MII eggs was much stronger than that of TPA- or of ionomycin-activated eggs (Fig. 7). Control eggs were incubated in the presence of anti-MARCKS primary antibody that had previously been exposed for 1 h to a specific MARCKS peptide (2 μ g/ml). No band was observed in this lane (data not shown).

Figure 7 Expression of MARCKS in MII eggs and in eggs activated by TPA or by ionomycin. Eggs, before or after parthenogenetic activation by TPA (50 ng/ml) or by ionomycin (2 μ M), were pooled, lysed and the proteins separated by SDS-PAGE (400 eggs per lane). The proteins were immunoblotted with anti-MARCKS goat polyclonal IgG (1:200). Peroxidase-conjugated donkey anti-goat IgG secondary antibody was used (1:5000) followed by an ECL detection system. The arrow points to MARCKS at $\sim$ 80 kDa as calculated from the migration of protein standards with known molecular masses. At least three independent experiments were performed.

To examine a possible interaction between MARCKS and actin, we double-labeled eggs for the two proteins and examined their colocalization by immunofluorescence confocal microscopy. As already demonstrated, F-actin was homogeneously distributed throughout the cytosol of MII eggs with a marked localization at the membrane and cortex (Fig. 8C). MARCKS was highly concentrated at the membrane of MII eggs (Fig. 8B). A distinct colocalization between MARCKS and actin was noted at the plasma membrane (Fig. 8D). Our results demonstrate, for the first time, expression and localization of MARCKS protein and its colocalization with actin in the rat egg.

Discussion

The initial signal of egg activation, shortly after sperm-egg interaction, is an increase in $[Ca^{2+}]_i$. PKC is also an active participant in some aspects of egg activation, as it is in other cell types (Kline & Kline 1992, Ducibella *et al.* 1993, Raz & Shalgi 1998, Eliyahu *et al.* 2002). The expression of various PKC isoenzymes and their capability to be activated in eggs have already been demonstrated (Gangeswaran & Jones 1997, Johnson & Capco 1997, Raz *et al.* 1998b, Luria *et al.* 2000, Pauken & Capco 2000, Halet *et al.* 2004). In a recent study we showed that CGE can be triggered either by a $[Ca^{2+}]_i$ rise or by PKC (Eliyahu & Shalgi 2002). Since microfilaments play a role in many dynamic events during mammalian egg maturation and fertilization, and since PKC activation causes reorganization of actin leading to exocytosis in somatic cells (Lelkes *et al.* 1986, Sontag *et al.* 1988, Capco *et al.* 1992, Gallicano *et al.* 1992, Muallem *et al.* 1995, Takashi 1997, Wang *et al.* 2000), we chose to study the involvement of both actin and PKC in CGE. In the current work, we examined the affect of TPA, as a parthenogenetic activator, on the amount and distribution of total actin within the egg and on its interaction with PKC alpha.

Figure 8 Immunofluorescence colocalization of MARCKS and F-actin. Eggs were fixed at the MII stage and labeled with anti-MARCKS goat polyclonal IgG (1:50; MARCKS labeling; green), Texas Red phalloidin (1.1 nM; actin labeling; red) and Hoechst (1 μ g/ml; DNA labeling; blue). Control eggs were labeled with the primary antibody, which had been previously incubated for 1 h with 2 μ g/ml of the specific MARCKS peptide. Localization of the antibodies was imaged using a secondary donkey anti-goat IgG Cy antibody (1:300) and a laser-scanning confocal microscope. Light microscopy (A); MARCKS (B); F-actin (C); colocalization between the MARCKS and F-actin (D; yellow). At least three independent experiments (three to four eggs for each group in each experimental day) were performed. Each image was taken at the equatorial plane of the egg. Scale bar, 10 μ m.

The amount of actin in both non-activated MII eggs and in TPA-activated eggs was similar. This finding is not surprising as most cells have large actin storage ready for an immediate response to intracellular events (Welch *et al.* 1997). A coimmunoprecipitation assay was used to identify a possible, direct or indirect, interaction between PKC and actin. A band of PKC alpha, an isoenzyme known to participate in exocytosis in other cell types (Benussi *et al.* 1998) was detected in lysates of non-activated MII eggs. Lysates of activated eggs exhibited two bands of PKC alpha, which probably represent the unphosphorylated and the phosphorylated forms of the isoenzyme as described by Bornancin & Parker (1997). cPKC isoenzymes are known to undergo autophosphorylation during activation. The interaction observed between actin and the phosphorylated and unphosphorylated forms of PKC implies an involvement of both proteins in the egg activation process.

Non-activated MII eggs demonstrated a homogenous distribution of F-actin throughout the cytosol as well as a specific localization at the egg membrane, mainly above the meiotic spindle. Activation of the eggs by sperm or by TPA caused depolymerization/reorganization of the F-actin, as manifested by a decrease in the intensity of the fluorescence signal. The most significant changes observed took place in the cytosol and not at the plasma

membrane. We attribute it to the strong actin signal detected at the plasma membrane of non-activated MII eggs, which can mask minor fluorescence changes. Another possibility is that the actin-binding proteins that anchor the F-actin to the plasma membrane render the membranous actin more stable than the cytosolic actin. However, since the total amount of actin appeared similar in Western blots of both activated and non-activated eggs, we can safely assume that there is a change in the ratio of G-actin to F-actin.

TPA induced polymerization of actin in Tubifex eggs while PKC antagonists inhibited the polymerization (Takashii 1997). Depolymerization of actin by CD enhances CGE in zebrafish eggs, while polymerization of actin by phalloidin inhibits CGE (Becker & Hart 1999). They conclude that F-actin acts as a mechanical barrier to exocytosis by excluding CG vesicles from the plasma membrane and that this barrier has to be removed prior to membrane fusion. To study the effect of PKC activation on actin reorganization eggs were treated with actin-polymerizing and -depolymerizing drugs. As expected, CD caused depolymerization of actin while JAS caused polymerization. In an attempt to follow the interplay between F-actin reorganization, PKC activation and CGE, eggs were treated with either CD or JAS with or without a further exposure to TPA. Although CD or JAS alone did not induce CGE, CD but not JAS increased the intensity of the CGE fluorescence signal caused by TPA. The mechanism by which CGs are anchored to the egg cortex is unclear. The observation that CD did not cause spontaneous CGE indicates that the reorganization of actin is not adequate to allow CGE, thus implying that the egg cytoskeletal cortex is not a simple barrier that blocks constitutive exocytosis, as suggested for other cells. A more likely possibility is that actin-associated proteins and/or PKC need to be activated in order to cause CGE. We suggest that these proteins are activated in response to parthenogenetic activation by TPA, and they, in turn, could cause the remodeling of the cortical microfilamentous actin that is ultimately required for CGE.

Evidence from several cell types suggests that MARCKS cross-links F-actin and anchors the actin network to the plasma membrane (Rosen *et al.* 1990, Aderem 1992, Swierczynski & Blackshear 1995, Rossi *et al.* 1999). An important initial step towards understanding the role of MARCKS during egg activation was the study of its expression, localization and colocalization with actin. We have shown that MARCKS is present in abundance in MII eggs but that levels decrease upon egg activation, as depicted by the decreased band intensity in Western blot analysis. We should bear in mind that the decrease in band intensity could also reflect a decrease in the affinity of the antibody to the protein, caused by phosphorylation of MARCKS. Immunohistochemistry and confocal scanning microscopy enabled us to demonstrate MARCKS localization at the plasma membrane of non-activated MII eggs and its colocalization with F-actin. In view of the

mentioned data, we assume that MARCKS is associated with F-actin and we suggest the hypothesis that, for CGE to occur, PKC has to phosphorylate an actin-bound protein such as MARCKS.

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