Association between myristoylated alanin-rich C kinase substrate (MARCKS) translocation and cortical granule exocytosis in rat eggs

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Abstract

Cortical granule exocytosis (CGE), following egg activation, is a secretory process that blocks polyspermy and enables successful embryonic development. CGE can be triggered independently by either a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) or activation of protein kinase C (PKC). The present study investigates the signal transduction pathways leading to CGE through activation of PKC or stimulation of a rise in $[Ca^{2+}]_i$. Using Western blot analysis, co-immunoprecipitation and immunohistochemistry, combined with various inhibitors or activators, we investigated the link between myristoylated alanin-rich C kinase substrate (MARCKS) translocation and CGE. We were able to demonstrate translocation of MARCKS from the plasma membrane to the cortex, in fertilized as well as in parthenogenetically activated eggs. MARCKS phosphorylation was demonstrated upon PKC activation, whereas a PKC inhibitor (myrPKC ψ) prevented both MARCKS translocation and CGE in 12-O-tetradecanoyl phorbol-13-acetate (TPA)-activated eggs. We have further shown that upon egg activation the amount of phosphorylated MARCKS (p-MARCKS) and the amount of calmodulin bound to MARCKS were increased. MARCKS translocation in ionomycin activated eggs was also inhibited by the calmodulin inhibitor N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7). These results complement other studies showing MARCKS requirement for exocytosis and imply that upon fertilization, MARCKS translocation is followed by CGE. These findings present a significant contribution to our understanding of CGE in mammalian eggs in particular, as well as cellular exocytosis in general.

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Introduction

Mammalian sperm–egg interaction results in egg activation (Hyslop *et al.* 2004). An increase in intracellular calcium concentration ($[Ca^{2+}]_i$) followed by $[Ca^{2+}]_i$ oscillations, cortical granule exocytosis (CGE) and resumption of the second meiotic division (RMII), are among the earliest events observed following sperm–egg fusion (Raz & Shalgi 1998, Swann & Parrington 1999, Wassarman *et al.* 2001). The calcium transients drive the resumption of the cell cycle by decreasing the activity of both M-phase promoting factor (MPF) and cytostatic factor (Jones 2004). The destruction of MPF, triggered by $[Ca^{2+}]_i$ rise, is mediated by calmodulin (CaM) and by Ca^{2+} /CaM-dependent protein kinase II (CaMKII; Fan *et al.* 2003, Ito *et al.* 2004). Incubation of mouse eggs in the presence of a CaM antagonist prior to *in vitro* insemination, delayed both the fertilization-associated decrease in histone H1 kinase activity and the emission of the second polar body (PBII), but did not block CGE (Xu *et al.* 1996). Other

messengers such as protein kinase C (PKC) and protein tyrosine kinases (PTKs) were suggested as possible inducers of some aspects of egg activation (Kinsey 1997, Raz & Shalgi 1998, Sato *et al.* 2000). We have demonstrated that conventional PKC (cPKC) isoenzymes translocate from the egg's cytosol to the plasma membrane upon PKC activation induced either by phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA), or by 1-oleoyl-2-acetyl glycerol (OAG) which triggers an $[Ca^{2+}]_i$ rise as well. The $[Ca^{2+}]_i$ rise, alone, does not activate PKC α , but OAG induces a more rapid PKC α translocation than TPA, suggesting a synergism between $[Ca^{2+}]_i$ and TPA in accelerating PKC translocation (Eliyahu & Shalgi 2002).

The observation that myristoylated PKC pseudosubstrate (myrPKC ψ), which is a specific PKC inhibitor, inhibited TPA-induced CGE, led to the conclusion that exocytosis can be triggered by two independent pathways: either $[Ca^{2+}]_i$ increase or PKC (Eliyahu & Shalgi 2002).

Recent studies demonstrate that the PKC activity pattern in *Xenopus* eggs, imitates closely the pattern of $[Ca^{2+}]_i$ transients (Larabell *et al.* 2004). In the mouse, cPKC translocates to the egg membrane at fertilization in a pattern that is shaped by the amplitude, duration and frequency of the Ca^{2+} transients (Halet *et al.* 2004).

PKC is known to associate with cytoskeletal elements and/or to phosphorylate them (Inagaki *et al.* 1987). In a previous work we have shown that actin is homogeneously distributed throughout the cytosol of MII eggs and is also localized at the cortex of the egg, mainly above the meiotic spindle (Eliyahu *et al.* 2005). Exposure of eggs to TPA caused a time- dependent depolymerization/reorganization of actin. Drugs that cause polymerization or depolymerization of actin (jasplakinolide and cytochalasin D (CD) respectively) neither induce CGE nor prevent it. However, CD but not jasplakinolide in the presence of TPA, doubled the percentage of eggs undergoing complete CGE as compared with TPA alone (Eliyahu *et al.* 2005). These results suggest that cortical granules are retained at the cortex not solely by actin, but rather by a network of proteins.

Evidence from several cell types suggests that F-actin is associated with myristoylated alanine-rich C kinase substrate (MARCKS), thus acting as a barrier for excluding the cortical granules from the plasma membrane, and preventing exocytosis (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 (Hartwig *et al.* 1992, Rossi *et al.* 1999). It is suggested that phosphorylation of MARCKS by PKC, or that interaction of MARCKS with CaM, causes its translocation from the plasma membrane to the cytoplasm and its disassembly from the actin filaments, thus allowing the secretory vesicles to fuse with the plasma membrane (Porumb *et al.* 1997, Arbuzova *et al.* 1998, 2002, Danks *et al.* 1999, Vaaranen *et al.* 1999, Wohlsland *et al.* 2000). MARCKS was recently demonstrated to be expressed in rat (Eliyahu *et al.* 2005) and mouse eggs (Michaut *et al.* 2005), and to be colocalized with actin at the plasma membrane (Eliyahu *et al.* 2005).

To further study the role of Ca^{2+} and PKC during egg activation, and to evaluate the significance of MARCKS and CaM during fertilization, we pursued several research avenues, designed to answer the following questions: (1) Does MARCKS translocate during parthenogenetic activation and/or during in vivo fertilization? (2) Is CaM expressed in the rat egg? If so, where is it localized? (3) Does MARCKS associate with CaM during egg activation? (4) Do the inhibitors of PKC or of CaM prevent MARCKS translocation, CGE or RMII? Answering these questions may facilitate the design of a flowchart that depicts various proteins that are involved in egg activation, in general, and in CGE, in particular.

Materials and Methods

Collection of eggs

Wistar-derived rats were housed in air-conditioned, light-controlled rooms, in the animal housing facilities of the Sackler Medical School. Food and water were available *ad libitum*. The study was approved by the Institutional Animal Care and Use Committee.

MI eggs

For induction of ovulation, 25- to 27-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 sacrificed by cervical dislocation 14 h after hCG administration. Cumulus-enclosed MII eggs were isolated from the oviductal 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 vivo inseminated eggs and embryos

hCG-injected female rats were caged overnight with fertile males. Rats were killed 15 h after hCG administration. Egg collection and cumulus cells removal were performed as described above for MII eggs. The eggs were classified according to the various stages of fertilization: sperm binding (SB), fertilization cone (FC) and PBII stages corresponding to 0-15, 15-60 and 60-180 min after sperm attachment respectively. The time after sperm attachment that these stages were observed is deduced from a previous work on in vitro fertilization (Eliyahu & Shalgi 2002).

Parthenogenetic activation

MI-ovulated eggs were parthenogenetically activated by adding three different activators to the incubation medium, all of which are capable of inducing full CGE in rat eggs (Eliyahu & Shalgi 2002).

Ionomycin activation

A 3-min incubation in the presence of 2 μ M calcium ionophore (ionomycin 407950, Calbiochem, San Diego, CA, USA) followed by an additional 0-, 2-, 7- or 17-min incubation in fresh medium lacking the activator. A stock solution of 10 mM ionomycin in DMSO was prepared and kept at 4 °C.

TPA activation

A 5-min incubation in the presence of 30-50 ng/ml TPA (Sigma) followed by an additional 0-, 5- or 15-min incubation in fresh medium lacking the activator. A stock solution of 1 mg/ml TPA in DMSO was prepared and kept at -20 °C.

OAG activation

A 3-min incubation in the presence of 20 µg/ml OAG (Sigma) followed by an additional 0-, 2-, 7- or 17-min incubation in fresh medium lacking the activator. A stock solution of 1 mg/ml OAG in DMSO was prepared and kept at -20°C .

Inhibition of PKC/CaM activity

For inhibiting PKC or CaM, eggs were incubated for 30 min in the presence of either 35 µM myrPKCψ (amino acid 19-27; Biomol, Plymouth Meeting, PA, USA), or 25 µM N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7; Sigma) respectively. Eggs were then activated for 5 min by either ionomycin or TPA at the same concentrations and for the same duration as described in the previous paragraph. Stock solutions of 1 mM myrPKCψ and 5 mM W7 were prepared in distilled, deionized water and stored at -20 or 4°C respectively.

Antibodies

Primary antibodies

Anti-MARCKS goat polyclonal immunoglobulin G (IgG) (N-19, sc-6454; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); MARCKS peptide (N-19; Santa Cruz); anti-phosphorylated-MARCKS rabbit polyclonal IgG (Ser^{159/163}-R, sc-12971-R; Santa Cruz); anti-CaM rabbit polyclonal IgG (FL-149, sc-5537; Santa Cruz); anti-actin rabbit polyclonal IgG (A-5060; Sigma).

Secondary antibodies

Goat anti-rabbit IgG-peroxidase (sc-2004; Santa Cruz); donkey anti-goat IgG-peroxidase (33254; Jackson ImmunoResearch Laboratories, West Grove, PA, USA); donkey anti-goat IgG-Cy-2 (52649; Jackson); donkey anti-rabbit IgG-Cy-2 (51782; Jackson).

Immunoprecipitation and immunoblotting

We prepared an immobilized antibody affinity reagent for immunoprecipitation (IP), according to Talmor *et al.* (1998). Batches of 500–1000 eggs, that had either been or not been subjected to activating agents, were lysed in 100 µl NP-40 lysis buffer (IP buffer; Talmor *et al.* 1998) and kept at -70°C . Upon thawing, the eggs lysates were incubated overnight at 4°C in the presence of 10 µl of an immobilized antibody (25% suspension) and then washed by centrifugation with IP buffer. Proteins were separated by 10% SDS-PAGE under non-reducing conditions. Proteins were transferred onto a nitrocellulose membrane (Biotrace NT; Gelman, USA) using a wet blotting apparatus (Hoeffer, San Francisco, CA, USA). For immunoblot analysis, blots were blocked with Tris-buffered saline containing 5% dry milk (Talmor *et al.* 1998) and incubated for 18 h at 4°C with either polyclonal antibody to anti

p-MARCKS (1:200; Ser^{159/163}-R, sc-12971-R; Santa Cruz) or anti-actin rabbit polyclonal IgG (1:250; A5060; Sigma) or anti-CaM I rabbit polyclonal IgG (1:200; sc-5537; Santa Cruz) in blocking solution. 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 pre-stained protein standards (Amersham). Densitometric analysis was performed utilizing the Fuji film thermal imaging system (FTI-500; Japan). Quantitation analysis was performed by computerized densitometer analysis (TINA version 2).

Immunofluorescence staining and laser-scanning confocal microscopy

Fixation of eggs

Eggs at various developmental stages were fixed for 10 min at room temperature in 3% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS), supplemented with 0.01% glutaraldehyde, and then washed in a solution of 3% fetal calf serum (Biological Industries, Beit-Haemek, Israel) in DPBS (DPBS/FCS). Zonae pellucidae (ZP) were removed post-fixation by 0.25% pronase (Sigma) prepared in DPBS/FCS and the ZP-free eggs were washed in DPBS/FCS.

Detection of CGE

Fixed eggs were transferred into DPBS supplemented with 1% BSA (fraction V, Sigma), labeled with 5 µg/ml Lens culinaris agglutinin (LCA)–biotin (B-1045; Vector, Burlingame, CA, USA), which binds specifically to cortical granule exudate (Cherr *et al.* 1988, Ducibella *et al.* 1988), washed and labeled with 1 µg/ml Texas Red–streptavidin (SA-5006; Vector). The occurrence of the CGE process was imaged using a laser-scanning confocal microscope. Images were taken at the equatorial plane of the eggs and the percentage of eggs undergoing CGE was calculated. At least three independent experiments (three to four eggs for each group in each experimental day) were performed. The results were statistically analyzed using the Mann–Whitney test.

Permeabilization

The plasma membranes of ZP-free eggs were permeabilized by a 10-min incubation in a solution of 0.05% NP-40 in DPBS/FCS and then washed in 0.005% NP-40 in DPBS/FCS.

Protein labeling

Permeabilized eggs were incubated for 2 h in the presence of anti-MARCKS (1:100). Primary antibodies were detected using a fluorescent-labeled Cy secondary antibody (1:300).

Chromatin labeling and developmental stage assessment

The eggs were incubated for an additional 10 min with 1 µg/ml Hoechst 33342 (Sigma), as a tool for assessing the chromatin stage. RMII was analyzed by monitoring the separation of the chromosomal dyads and the PBII extrusion. The various stages of fertilization were determined by following the sperm and egg chromatin.

Visualization and photography

Cortical granule exudate, MARCKS and DNA labeling were visualized and photographed by a Zeiss confocal laser scanning microscope (CLSM) (LSM 410; 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 a u.v. laser (Coherent Inc. Laser Group, Santa Clara, CA, USA). A × 40 numerical aperture/1.2 planapochromat water immersion lens (Axiovert 135 M, Zeiss) was used for all imaging. Eggs were scanned using the CLSM through the Z-axis to visualize a section at the equatorial plane of the egg.

Assessment of protein translocation

For localization of MARCK and CaM, eggs were scanned using the CLSM Z-axis to visualize sections through their equatorial plane and through the cortex. To allow a better observation of the egg cortex, the eggs, already visualized at the equatorial plane, were manually pressed between the slide and the coverslip and rescanned, thus resulting

in eggs with larger but variable diameters. Since the slides had to be removed from the confocal microscope stage for pressing, and then put back for rescanning, we were unable to locate the exact prescanned eggs. As a result, the images at the equatorial plane and the cortex are not necessarily of the same egg. Confocal micrographs of three to four eggs from each experimental group were densitometrically analyzed.

Results

Distribution of MARCKS during egg activation

The intracellular distribution of MARCKS was examined in fertilized or in parthenogenetically activated eggs. Using a specific anti-MARCKS antibody and confocal microscopy, MARCKS was found to be homogenously distributed throughout the ooplasm and highly concentrated at the plasma membrane of MII eggs, where it could be associated with actin and with the membrane. The labeling intensity at the cytoplasm was weaker than at the plasma membrane (Fig. 1F and J). At the SB stage, MARCKS was observed at the cortical region of the ooplasm, further referred to as 'egg cortex' (Fig. 1G and K). MARCKS translocation during the next hour (PBII stage; Fig. 1H and L) was less prominent.

MARCKS translocated from the egg membrane to the cortex of eggs, parthenogenetically activated (Fig. 2). MARCKS translocation was first detected 5 min after exposure to TPA (Fig. 2B and F) or to ionomycin (Fig. 2B' and F') and reached a maximum at 20 min (Fig. 2D and H, and Fig. 2D' and H' respectively), whereas translocation of

Figure 1 Localization of MARCKS at various stages of *in vivo* fertilization. Eggs were labeled with anti-MARCKS goat polyclonal IgG (1:50) and Hoechst (1 µg/ml). Localization of the antibodies was imaged using a donkey anti-goat IgG Cy secondary antibody (1:300) and CLSM. (A, E and I) Control, second antibody only; (B, F and J) unfertilized egg; (C, G and K) egg at the SB stage; (D, H and L) egg at the PBII stage. (A–D) Light microscopy; (E–L) MARCKS (green) and chromosomal DNA (blue); sperm DNA, yellow arrow; egg DNA, white arrow. (I–L) Cross-section at the equatorial plane of the egg is shown, depicting the cortical area at × 4 magnification. At least three independent experiments were performed (three to four eggs for each group in each experimental day). Each image was taken at the equatorial plane of the egg. Scale bar, 10 µm.

Figure 2 MARCKS translocation following activation by various activators. Sub-cellular localization of MARCKS was visualized after exposing MII eggs to PKC activators: 30 ng/ml TPA or 2 μ M ionomycin for 5 min; or 20 μ g/ml OAG for 3 min. Eggs were fixed at the MII stage (A and E, A' and E', A'' and E''); after a 3–5 min incubation in the presence of an activator (B and F, B' and F', B'' and F''); after a 3–5 min incubation in the presence of an activator followed by an additional incubation in fresh medium lacking the activator (10 or 15 min (C and G, C' and G', D and H, D' and H'); 2 or 12 min (C'' and G'', D'' and H'')). (A–H) TPA-treated eggs; (A'–H') ionomycin-treated eggs; (A''–H'') OAG-treated eggs. Eggs were labeled with anti-MARCKS goat polyclonal IgG (1:50) and Hoechst (1 μ g/ml). Localization of the antibodies was imaged using donkey anti-goat IgG Cy secondary antibody (1:300) and CLSM: MARCKS, green; chromosomes, blue. For localization of MARCKS, eggs were scanned using the CLSM Z-axis to visualize sections through their equatorial plane and through the cortex. To allow a better observation of the egg cortex, the eggs, already visualized at the equatorial plane, were pressed between the slide and the coverslip and scanned. The images at the equatorial plane and the cortex, are not necessarily of the same egg: images at the equatorial plane of the egg (A–D, A'–D', A''–D''); images at the cortex of the egg (E–H, E'–H', E''–H''). At least three independent experiments were performed (three to four eggs for each group in each experimental day). Scale bar, 10 μ m.

MARCKS to the cortex of eggs activated by OAG was first detected 3 min after the onset of the activating stimulus (Fig. 2B'' and F'') and reached a maximum at 15 min (Fig. 2D'' and H''). As expected, resumption of the cell cycle was observed after activation by ionomycin (Fig. 2D') or by OAG (Fig. 2D'') but not by TPA (Fig. 2D).

MARCKS phosphorylation by PKC activation

We used anti-p-MARCKS antibody to determine, by Western blot analysis, whether MARCKS was phosphorylated after PKC activation. Batches of 500 eggs, before or after

parthenogenetic activation by TPA, were lysed and their proteins were separated using SDS-PAGE. As expected (Wu *et al.* 1982, Aderem 1992), p-MARCKS appeared as an 80 kDa band (Fig. 3). The band of p-MARCKS in TPA-activated eggs appeared stronger than in MII eggs (Fig. 3), thus supporting our hypothesis that MARCKS is phosphorylated by PKC during egg activation.

Effect of PKC inhibitor on MARCKS translocation

Previously, we have demonstrated that myrPKC ψ , a PKC inhibitor, prevented both PKC translocation and CGE

Figure 3 Phosphorylation of MARCKS after activation by TPA. Eggs, before or after parthenogenetic activation by TPA (50 ng/ml) were pooled, lysed and the proteins were separated on SDS-PAGE (400 eggs per lane). The proteins were immunoblotted with anti p-MARCKS rabbit polyclonal IgG (1:200; A) and anti-actin rabbit polyclonal IgG (1:250; B). Peroxidase-conjugated donkey anti-rabbit IgG secondary antibody was used (1:5000) followed by an ECL detection system. The arrow points to the phosphorylated MARCKS protein at 80 kDa as calculated from the migration of protein standards with known molecular masses. At least three independent experiments were performed.

induced by 30 ng/ml TPA (Eliyahu & Shalgi 2002). To further study the role of PKC in the signal transduction pathways leading to CGE, we stimulated PKC using an experimental design similar to that used in Eliyahu and Shalgi (2002) and followed MARCKS translocation. MII eggs were incubated in the presence of myrPKC ψ , activated by 30 ng/ml TPA in the presence of myrPKC ψ , and then allowed to recover in medium devoid of TPA but containing myrPKC ψ . TPA alone caused a mild translocation of MARCKS (Fig. 4E), which was obliterated by myrPKC ψ (Fig. 4F), thus supporting our hypothesis that PKC activation results in MARCKS translocation. As expected, RMII did not occur in eggs activated by TPA (Fig. 4A–C).

Association of MARCKS with CaM

Binding of MARCKS to CaM can trigger translocation of MARCKS from the plasma membrane to the cytosol. We employed co-immunoprecipitation, and co-immunofluorescence to assess the interplay between MARCKS and CaM.

Expression and localization of CaM

An important initial step toward understanding the relationship between MARCKS and CaM was the study of CaM expression and localization in the egg. As seen in Fig. 5A, CaM appeared as a strong band with an apparent molecular mass of 17 kDa, which is consistent with the expected molecular mass of CaM protein (Hoeftlich & Ikura 2002). The ability of anti-CaM antibody to bind CaM was abolished after 1 h incubation in the presence of 2 μ g/ml CaM peptide (not shown). CaM was evenly distributed throughout the cytosol, was present at the plasma membrane and was highly concentrated at the spindle (Fig. 5B).

Figure 4 Effect of PKC inhibitor on MARCKS translocation. Eggs were fixed at the MII stage (A and D); after a 5-min incubation in the presence of 30 ng/ml TPA followed by an additional 15-min incubation in fresh medium lacking the activator (B and E); after a 30-min incubation in the presence of 35 μ M myrPKC ψ followed by a 5-min incubation in the presence of 30 ng/ml TPA and 35 μ M myrPKC ψ , followed by 15-min incubation in medium containing 35 μ M myrPKC ψ but without TPA (C and F). Eggs were labeled with anti-MARCKS goat polyclonal IgG (1:50) and Hoechst (1 μ g/ml). Localization of the antibodies was imaged using donkey anti-goat IgG (Cy) secondary antibody (1:300) and CLSM. (A–C) Light microscopy and chromosomes; (D–F) MARCKS. Images were taken at the equatorial plane of the egg. At least three independent experiments were performed (three to four eggs for each group in each experimental day). Scale bar, 10 μ m.

Association between MARCKS and CaM during egg activation

A possible interaction between MARCKS and CaM was examined by immunoprecipitating CaM from lysates of 1000 eggs, either at the MII stage or after ionomycin activation, and subjecting the immunoprecipitates to separation by SDS-PAGE under non-reducing conditions. A weak MARCKS band was detected by anti-MARCKS in non-activated MII eggs and a stronger band in activated eggs (Fig. 5C). We performed the following control experiments: (1) immunoprecipitation of IP buffer with the immune complex (Fig. 5C); (2) incubation of the nitrocellulose membrane with any IgG antibody (anti-Src antibody, not shown); (3) incubation of the eggs lysates with any IgG antibody (anti-Src polyclonal antibody) conjugated to protein A sepharose, followed by Western blot analysis using anti-PKC alpha polyclonal antibody (not shown). None of the control groups exhibited MARCKS bands. Our results indicate that, after $[Ca^{2+}]_i$ elevation, CaM interacts with MARCKS, either directly or indirectly.

Effect of CaM inhibitor on MARCKS translocation, CGE and RMII

To determine whether CaM activation induces MARCKS translocation, CGE and RMII, we activated the eggs

Figure 5 Expression and localization of CaM in the egg. (A) Western blot analysis. Samples of 200 MII eggs were pooled, lysed and the proteins were revealed by SDS-PAGE analysis. The proteins were immunoblotted with anti-CaM rabbit polyclonal IgG (1:200). Secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (1:5000) was followed by an ECL detection system. The arrow points to the actin at 17 kDa as calculated from the migration of protein standards with known molecular masses. At least three independent experiments were performed. (B) Immunofluorescence localization. Eggs were fixed at the MII stage and labeled with anti-CaM rabbit polyclonal IgG (1:50) and Hoechst (1 μ g/ml). Localization of the antibodies was imaged using donkey anti-rabbit IgG Cy secondary antibody (1:300) and CLSM. A representative egg is presented. Left panel, light microscopy and chromosomes; right panel, CaM. At least three independent experiments were performed (three to four eggs for each group in each experimental day). Scale bar, 10 μ m. (C) Association of MARCKS with CaM during egg activation. One thousand unfertilized MII eggs or ionomycin (2 μ M)-activated eggs were lysed. The lysis buffer (control) and eggs lysates were immunoprecipitated with anti-CaM rabbit polyclonal IgG ('IP-CaM' on figure). Proteins were resolved by SDS-PAGE analysis and transferred onto nitrocellulose membrane. The blots were probed with anti-MARCKS goat polyclonal IgG (1:50; 'Blot-MARCKS' on figure) and revealed by the ECL detection system. The arrow points to MARCKS at 80 kDa as calculated from the migration of protein standards with known molecular masses. The result of a representative experiment is presented. At least three independent experiments were performed.

by ionomycin in the presence of a CaM inhibitor (W7), and localized MARCKS by immunohistochemistry.

Ionomycin induced MARCKS translocation from the plasma membrane (Fig. 6D) to the cortex (Fig. 6E) and

Figure 6 Effect of a CaM inhibitor on MARCKS translocation. Eggs fixed at the MII stage (A and D); after a 5-min incubation in the presence of 2 μ M ionomycin followed by an additional 15-min incubation in fresh medium lacking the activator (B and E); after a 30-min incubation in the presence of 25 μ M W7 followed by activation with 2 μ M ionomycin for 5 min in the presence of 25 μ M W7, followed by a 15-min incubation in TH medium that contains 25 μ M W7 without ionomycin (C and F). Eggs were labeled with anti-MARCKS goat polyclonal IgG (1:50) and Hoechst (1 μ g/ml). Light microscopy and chromosomes (blue, A–C); MARCKS (green, D–F). Images were taken at the equatorial plane of the eggs. At least three independent experiments were performed (three to four eggs for each group in each experimental day). Scale bar, 10 μ m.

caused RMII (Fig. 6B). The presence of W7 in the culture medium resulted in inhibition of both MARCKS translocation (Fig. 6F) and RMII (Fig. 6C), possibly indicating the role of CaM activation in triggering both MARCKS translocation and RMII in the eggs.

In order to evaluate quantitatively the role of CaM in CGE and RMII, we studied the capability of eggs, treated with increasing concentrations of W7, to undergo CGE (Fig. 7A and B) and RMII (Fig. 8A and B). MII eggs were incubated in the presence of 2.5–25 μ M W7, activated by ionomycin in the presence of W7, transferred to TH medium that contained 2.5–25 μ M W7 but no ionomycin. Untreated MII eggs served as a negative control for CGE, whereas eggs treated by ionomycin alone served as a positive control (CGE, Fig. 7B; RMII, Fig. 8B). CGE (Fig. 7A) and RMII (Fig. 8A) were inhibited in a dose-dependent manner by W7. Treating eggs with a low concentration of W7 (2.5 μ M) inhibited neither CGE nor RMII (CGE, $P > 0.6$; RMII, $P > 0.6$). At higher concentrations, W7 had a stronger effect on CGE than on RMII (CGE: $P < 0.006$ at 5 μ M, $P < 0.003$ at 10 μ M; RMII: $P > 0.8$ at 5 μ M, $P < 0.036$ at 10 μ M; Mann–Whitney test). Only at 25 μ M W7 were both aspects of egg activation inhibited to the same extent (Figs. 7A and 8A). MII eggs and eggs subjected only to W7 did not undergo CGE (Fig. 7B) or RMII (Fig. 8B). Eggs treated with ionomycin in the presence of W7 presented full inhibition of CGE (Fig. 7B) and of RMII (Fig. 8B), which differed significantly from the positive control eggs (ionomycin activated; CGE,

Figure 7 Effect of a CaM inhibitor on CGE. Eggs, before or after activation by 2 μ M ionomycin in the presence of W7, were fixed and labeled by LCA-avidin (1:500; CGE labeling). LCA-avidin was detected by Texas Red-biotin (1:1000). CGE was imaged using CLSM. The Y-axis presents the percentage of eggs that underwent CGE. Presented values are means $\pm$ s.e. calculated from three to four experiments. (A) Effect of various concentrations of W7. Eggs incubated for 30 min in the presence of 2.5–25 μ M W7, activated for 5 min by 2 μ M ionomycin in the presence of W7, and then transferred for an additional 25 min to TH medium in the presence of W7 alone. Eggs treated with low concentrations of W7 (2.5 μ M) were not significantly different from untreated eggs ($P > 0.6$ at 2.5 μ M), whereas higher concentrations of W7 (5–25 μ M) gave significantly different results ($P < 0.006$, $P < 0.003$ and $P < 0.006$ at 5, 10 and 25 μ M respectively; Mann–Whitney test). (B) Effect of various treatments. Groups of eggs examined: (1) MII stage; (2) after a 30-min incubation in the presence of 25 μ M W7; (3) after 5-min activation by 2 μ M ionomycin followed by 25 min in a fresh medium; (4) after a 30-min incubation in the presence of 25 μ M W7 and then activation by 2 μ M ionomycin for 5 min in the presence of 25 μ M W7, followed by 25-min incubation in fresh medium that contained 25 μ M W7 without ionomycin.

$P < 0.006$; RMII, $P < 0.003$). These results lead us to conclude that CGE, RMII and translocation of MARCKS, are processes that depend on activation of PKC and of CaM.

Discussion

An increase in $[Ca^{2+}]_i$ is observed upon sperm–egg interaction. Several studies reported that CGE can be triggered either by $[Ca^{2+}]_i$ rise, or by PKC activation (Gangeswaran & Jones 1997, Johnson & Capco 1997, Raz *et al.* 1998a, Luria *et al.* 2000, Pauken & Capco 2000, Eliyahu & Shalgi 2002). PKC, as well as CaM, is also able to cause MARCKS translocation in other cell types (Porumb *et al.* 1997, Arbuzova *et al.* 1998, Danks *et al.* 1999, Vaaraniemi *et al.* 1999, Wohnsland *et al.* 2000). We focused on further studying the role of Ca^{2+} and PKC during egg activation and attempting to resolve whether MARCKS and/or CaM play a role in inducing CGE during fertilization of mammalian eggs.

Figure 8 Effect of CaM inhibitor on RMII. Eggs, before or after activation by 2 μ M ionomycin in the presence of W7, were fixed and labeled by Hoechst (1 μ g/ml) and the RMII process was imaged using CLSM. The Y-axis presents the percentage of eggs that underwent RMII. Presented values are means $\pm$ s.e. calculated from three to four experiments. (A) Effect of various concentrations of W7. Eggs incubated for 30 min in the presence of 2.5–25 μ M W7, activated for 5 min by 2 μ M ionomycin in the presence of W7 and then transferred for an additional 25 min to TH medium in the presence of W7 alone. The W7 inhibition was significant only at concentrations of 10 μ M or higher ($P > 0.6$, $P > 0.8$, $P < 0.036$ and $P < 0.003$ at concentrations of 2.5, 5, 10 and 25 μ M respectively; the results were statistically analyzed using the Mann–Whitney test). (B) Effect of different treatments. Groups of eggs examined: (1) MII stage; (2) after a 30-min incubation in the presence of 25 μ M W7; (3) after a 5-min activation by 2 μ M ionomycin, followed by 25 min in a fresh medium; (4) after a 30-min incubation in the presence of 25 μ M W7 and then activation by 2 μ M ionomycin for 5 min in the presence of 25 μ M W7, followed by a 25-min incubation in fresh medium that contained 25 μ M W7 without ionomycin.

MARCKS is highly concentrated at the membrane of MII eggs where it is most probably associated with actin and with the plasma membrane (Eliyahu *et al.* 2005). During the SB stage, MARCKS migrates to the egg's cortex, where it remains during the following PBII stage. Our results imply involvement of MARCKS during early events of egg activation, such as CGE, but they do not exclude the possibility of MARCKS involvement during later events, such as PBII formation. To examine the intracellular signaling pathways leading to CGE, eggs were parthenogenetically activated either by PKC activators or via $[Ca^{2+}]_i$ rise. OAG triggers $[Ca^{2+}]_i$ rise and PKC activation, thus inducing both CGE (Endo *et al.* 1987, Ducibella *et al.* 1993) and RMII, whereas TPA – which acts similar to diacylglycerol (DAG; Nishizuka 1986) – activates PKC and thus induces only CGE (Raz *et al.* 1998a). Ionomycin, which triggers only $[Ca^{2+}]_i$ elevation and does not activate PKC, causes both CGE and RMII (Eliyahu & Shalgi 2002). The present study demonstrated translocation of MARCKS

during parthenogenetic activation by all three activators: TPA, OAG or ionomycin. OAG triggers faster translocation of MARCKS than TPA or ionomycin. Since OAG causes $[Ca^{2+}]_i$ elevation in addition to PKC activation, while TPA does not, and since ionomycin-induced $[Ca^{2+}]_i$ elevation does not cause PKC activation (Eliyahu & Shalgi 2002) we may deduce that the OAG-induced $[Ca^{2+}]_i$ rise combined with PKC activation, is responsible for the accelerated OAG-induced MARCKS translocation. These results complement our previous study which demonstrated acceleration of PKC alpha translocation when triggered by OAG. Both MARCKS and PKC alpha presented similar kinetics of translocation, although in opposite directions: PKC alpha from the cytosol to the plasma membrane (Eliyahu & Shalgi 2002) and MARCKS from the plasma membrane to the cytosol (current study). Taking these results together, including the observations that MARCKS was phosphorylated after PKC activation and that myrPKC ψ inhibited both MARCKS translocation and CGE in TPA-activated eggs, we suggest that TPA induces CGE by activating PKC via phosphorylation and translocation of MARCKS.

In the current study, we were able to show that, upon egg activation, the amount of p-MARCKS is increased as well as the amount of CaM bound to MARCKS. Recently we have demonstrated that MARCKS is colocalized with actin filaments at the plasma membrane of MII eggs and that actin undergoes reorganization upon egg activation but remains localized at the cortex (Eliyahu *et al.* 2005). In the current study we demonstrated that upon activation by TPA or ionomycin, MARCKS translocates from the egg membrane to the cortex. Thus, egg activation results in morphological dissociation, between the MARCKS and actin. Co-immunoprecipitation could demonstrate the association between MARCKS and actin at the MII stage and the dissociation between the two upon egg activation. Unfortunately it was not possible to precipitate either MARCKS or actin by any of the available commercial antibodies tested.

In a recent study, Michaut *et al.* (2005) were unable to detect an increase in the p-MARCKS immunostaining signal of MII eggs treated with TPA, which is known to activate conventional and novel PKCs. Moreover, p-MARCKS was not detected by immunoblot analysis either before or after egg activation by TPA, which led them to suggest that an atypical PKC isoenzyme was responsible for MARCKS phosphorylation. However, we were able to demonstrate, via immunoblot analysis, the expression of p-MARCKS in MII eggs and an increase in its amount during activation by TPA. The discrepancy between the two studies could be attributed to several differences in experimental methodology: number of eggs used for immunoblotting (500 in our study vs 50 in Michaut *et al.* 2005); use of different antibodies; different species (rat vs mouse) and different protein detection systems (immunoblotting vs immunohistochemistry).

In the current study we demonstrated the expression of CaM, its homogenous distribution throughout the cyto-

plasm, its localization to the meiotic spindle and the interaction between CaM and MARCKS in MII and in ionomycin-activated eggs. The amount of MARCKS bound to CaM increased after parthenogenetic activation, although, in our previous study (Eliyahu *et al.* 2005) we demonstrated a decrease in the total amount of MARCKS in eggs activated by ionomycin or TPA, which was not revealed by immunohistochemistry. There might be several putative explanations for this discrepancy. Firstly, it is possible that the decrease in the amount of MARCKS within any particular egg is undetectable by immunohistochemistry, whereas Western blot analysis – performed on lysates of 500 eggs at a time – is sensitive enough to detect a decrease in the amount of MARCKS. Secondly, although we used the same polyclonal antibody for both assays it is possible that binding of CaM to MARCKS, or phosphorylation of MARCKS by PKC, causes conformational changes that affect MARCKS affinity to the antibody during immunohistochemistry, while the denatured protein retains its affinity to the antibody during Western blot analysis. Our results indicate that CaM interacts with MARCKS, directly or indirectly, after $[Ca^{2+}]_i$ elevation, which raises the possibility that CaM activity is involved in the process of CGE and RMII.

MARCKS translocation from the plasma membrane to the cytosol was inhibited in eggs activated by ionomycin in the presence of W7. This supports our hypothesis that during egg activation, CaM activation is involved in MARCKS translocation, prior to CGE. CGE and RMII were inhibited by W7 in a dose-dependent manner, CGE being more sensitive to the inhibitor concentration than RMII. This difference is in accordance with the suggested segregation of the CGE and RMII pathways, caused by the different sensitivity to $[Ca^{2+}]_i$ of the two processes. It had been shown that a relatively low $[Ca^{2+}]_i$ rise is sufficient for inducing a partial CGE, whereas a higher elevation is required for completion of CGE and induction of RMII (Raz *et al.* 1998a). Xu *et al.* (1996) showed that the use of W7 prior to in vitro insemination delayed the emission of the PBII, but did not block CGE. The difference between the observations of Xu *et al.* and the current results may be derived from the different methods employed. We activated eggs, in the presence of W7, whereas Xu *et al.* (1996) inseminated eggs in culture medium devoid of W7. Electrophysiological studies demonstrated that CGE in hamster eggs commenced as early as 4 s after binding of sperm to the egg membrane (Kline & Stewart-Savage 1994), while RMII was observed 20 min after sperm binding. In view of these facts, the presence of an inhibitor during the activation period is necessary, as even a brief absence of the inhibitor from the culture medium during activation can lead to CGE.

The mechanism of CGE in mammalian eggs is not fully understood. As demonstrated in other cell systems, MARCKS translocation, governed either by PKC phosphorylation or by MARCKS binding to CaM, enabled exocytosis; while inhibition of PKC and of CaM prevented

exocytosis (Vaaranieni *et al.* 1999, Wohnsland *et al.* 2000). In the present study, we have investigated the underlying mechanisms leading to CGE and were able to demonstrate MARCKS translocation in fertilized as well as in parthenogenetically activated eggs. Similar to other cell systems, we have also demonstrated that phosphorylation of MARCKS by PKC, as well as MARCKS binding to CaM, results in translocation of MARCKS from the plasma membrane to the cortex which is followed by release of cortical granule exudate. These results complement other studies showing MARCKS requirement for exocytosis and imply that CGE, RMI and translocation of MARCKS depend on activation of both PKC and CaM. To establish the hypothesis that MARCKS is a mediator in CGE, further experiments such as using MARCKS-specific peptide inhibitors (Rosé *et al.* 2001) should be conducted.

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