Myristoylated alanine-rich C kinase substrate, but not Ca\(^{2+}\)/calmodulin-dependent protein kinase II, is the mediator in cortical granules exocytosis

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Abstract

Sperm–egg fusion induces cortical granules exocytosis (CGE), a process that ensures the block to polyspermy. CGE can be induced independently by either a rise in intracellular calcium concentration or protein kinase C (PKC) activation. We have previously shown that myristoylated alanine-rich C kinase substrate (MARCKS) cross-links filamentous actin (F-actin) and regulates its reorganization. This activity is reduced either by PKC-induced MARCKS phosphorylation (PKC pathway) or by its direct binding to calmodulin (CaM; CaM pathway), both inducing MARCKS translocation, F-actin reorganization, and CGE. Currently, we examine the involvement of Ca\(^{2+}\)/CaM-dependent protein kinase II (CaMKII) and MARCKS in promoting CGE and show that PKC pathway can compensate for lack of Ca\(^{2+}\)/CaM pathway. Microinjecting eggs with either overexpressed protein or complementary RNA of constitutively active αCaMKII triggered resumption of second meiotic division, but induced CGE of an insignificant magnitude compared with CGE induced by wt αCaMKII. Microinjecting eggs with mutant-unphosphorylatable MARCKS reduced the intensity of 12-O-tetradecanoylphorbol 13-acetate or ionomycin-induced CGE by 50%, indicating that phosphorylation of MARCKS by novel and/or conventional PKCs (n/cPKCs) is a pivotal event associated with CGE. Moreover, we were able to demonstrate cPKCs involvement in ionomycin-induced MARCKS translocation and CGE. These results led us to propose that MARCKS, rather than CaMKII, as a key mediator of CGE.

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Introduction

Mammalian oocytes are ovulated while being arrested at the metaphase of the second meiotic division (MII). The fertilizing spermatozoon initiates within the egg a series of biochemical events that lead to rapid mitotic divisions, giving rise to the developing embryo. The transition from MII egg to a developing embryo is brought about by a series of events, generally referred to as ‘egg activation’ (reviewed by Runft et al. 2002, Talmor-Cohen et al. 2002, Jones 2005), but conceptually divided into ‘early’ and ‘late’ events (reviewed by Ducibella et al. 2006). The early events of egg activation commence with a transient rise in intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) followed by Ca\(^{2+}\) oscillations. Shortly thereafter, the activated egg undergoes cortical granules exocytosis (CGE), followed by cell cycle progression. The cortical granules (CGs), residing just beneath the plasma membrane of MII-arrested eggs, secrete their content into the perivitelline space causing alteration of the zona pellucida (ZP) and the plasma membrane, thus establishing a block to polyspermy and ensuring successful egg activation and embryo development (Wassarman et al. 2001, Sun 2003, Gardner & Evans 2006).

It is accepted that the initial rise in [Ca\(^{2+}\)]\(_i\), is both necessary and sufficient for triggering the consecutive events of egg activation (reviewed by Runft et al. 2002). Extensive effort was invested in research directed toward answering the paradigm of how the calcium signal is translated into stimuli that evoke biological processes such as CGE and resumption of the second meiotic division (RMII). Sperm–egg interaction triggers the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP\(_2\); Jones et al. 2000) to inositol 1, 4, 5-trisphosphate (IP\(_3\)) that induces elevation of [Ca\(^{2+}\)]\(_i\), and formation of diacylglycerol (DAG) that activates various members of protein kinase C (PKC). The dichotomous effect of Ca\(^{2+}\) during egg activation was supported by the observation that CGE and RMII require a different number of Ca\(^{2+}\) transients to commence (Ducibella et al. 2002) and a relatively low rise in [Ca\(^{2+}\)]\(_i\), was sufficient for inducing partial CGE, whereas a ‘full scale’ of [Ca\(^{2+}\)]\(_i\) rise was required for the completion of CGE and RMII (Raz et al. 1998a). The difference can be attributed to different
Ca\textsuperscript{2+} requirements or to different downstream effectors participating in each process.

Various studies suggest diverse roles for PKC within the egg (reviewed by Halet 2004): regulation of oocyte maturation (Viveiros et al. 2001), induction of CGE (Eliyahu & Shalgi 2002), modification of the egg actin cytoskeleton (Eliyahu et al. 2005, 2006), and participation in early embryonic development (reviewed by Capco 2001). Both Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent PKC isoforms were identified in rodent eggs (Gangeswaran & Jones 1997, Raz et al. 1998b).

The change in the intracellular distribution of various PKC isoforms, as manifested by its translocation from the cytoplasm to the plasma membrane of the egg, was followed during fertilization (Eliyahu & Shalgi 2002, Halet et al. 2004) as well as during parthenogenetic activation of eggs (Gallicano et al. 1995, Eliyahu & Shalgi 2002). However, it should be noted that activation of PKC by 12-O-tetradecanoylphorbol 13-acetate (TPA) triggered only CGE but had no effect on either RMII or [Ca\textsuperscript{2+}], rise (Raz et al. 1998a), and that Ca\textsuperscript{2+} chelators had almost no effect on PKC activation at fertilization (Tatone et al. 2003). RMII can be triggered only by [Ca\textsuperscript{2+}], rise, whereas CGE can be triggered either by [Ca\textsuperscript{2+}], rise or by PKC activation (Gangeswaran & Jones 1997, Johnson & Capco 1997, Raz et al. 1998b, Pauken & Capco 2000, Eliyahu & Shalgi 2002).

Calmodulin (CaM), one of the most common Ca\textsuperscript{2+} transducers, is a regulator of both CGE and RMII (reviewed by Abbott & Ducibella 2001). CaM is present in abundant quantities within eggs and has numerous substrates, of which Ca\textsuperscript{2+}/CaM-dependent protein kinase II (CaMKII) is the most important. CaMKII activity oscillates in synchrony with Ca\textsuperscript{2+} oscillations (Markoulaki et al. 2003, 2004). A Ca\textsuperscript{2+}-mediated stimulation of egg CaMKII activity is required for activation of the anaphase-promoting complex (APC), which in turn is responsible for cyclin B degradation and sister chromatids separation (Madgwich et al. 2005, reviewed by Ducibella et al. 2006). It was hypothesized that CaMKII is required also for CGE and for establishing the block to polyspermy (reviewed by Abbott & Ducibella 2001). Although some reports indicate that CaMKII, like CaM, is localized to sites of exocytosis (Shen et al. 1998, Easom 1999) and is involved in CGE (Tatone et al. 1999, 2002, Markoulaki et al. 2003); a recent report indicated an abnormal CGE in mouse eggs injected with a constitutively active form of CaMKII (CA-CaMKII; Knott et al. 2006).

We have previously suggested that the filamentous actin (F-actin) at the egg cortex is a dynamic network that can be maneuvered toward allowing CGE by activated PKC and its downstream proteins, such as myristoylated alanine-rich C kinase substrate (MARCKS). We and others have demonstrated that MARCKS, a protein known to cross-link F-actin in other cell types and a major PKC substrate, is expressed in rat and mouse eggs (Eliyahu et al. 2005, Michaut et al. 2005 respectively) and is colocalized with actin in non-activated MII eggs (Tsaadon et al. 2006). MARCKS translocates to the egg cortex and dissociates from F-actin in ionomycin-activated eggs, a process that can lead to the breakdown of the actin network, thus allowing CGE to occur. In an earlier study (Eliyahu et al. 2006), we emphasized the link between PKC, actin, CaM, and MARCKS in mediating CGE. We proposed that, as in secretory cells (Danks et al. 1999, Vaaraniemi et al. 1999, Wohlsnland et al. 2000, Arbuzaeva et al. 2002), phosphorylation of the egg’s MARCKS by PKC or interaction of MARCKS with CaM can cause translocation of MARCKS from the plasma membrane to the egg’s cortex and disassembly from the F-actin, thus allowing the CGs to fuse with the plasma membrane and induce CGE. PKC activation promoted MARCKS phosphorylation, whereas a PKC inhibitor (myrPKC\textsubscript{\gamma}) decreased both MARCKS translocation and CGE. We were able to cause translocation of MARCKS and showed an increase in the amount of CaM bound to MARCKS by activating the eggs with ionomycin. MARCKS translocation and CGE were inhibited by the CaM pharmacological inhibitor, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7; Eliyahu et al. 2006). Although biochemical approaches have provided evidence for a link between MARCKS translocation and CGE, and pharmacological inhibitors aided in analyzing the role of MARCKS in the process, one should be wary of data misinterpretation tainted only by pharmacological effects on other cellular targets.

In order to suggest a role of potential mediators in the process of CGE, we employed, in the present study, molecular tools for focusing specifically and directly on CaMKII and MARCKS. We microinjected rat eggs with a complementary RNA (cRNA) of the rat CA-\textalpha CaMKII or with an unphosphorylatable mutant form of MARCKS and examined the ability of the injected eggs to undergo CGE and RMII. The results led us to propose that MARCKS, but not CaMKII, is a key mediator in CGE.

**Results**

**Effect of W7 on MARCKS translocation, CGE and RMII in eggs parthenogenetically activated by OAG**

We have already suggested that CGE is triggered by PKC activation or CaM activation, both pathways acting by causing translocation of the MARCKS protein (Eliyahu et al. 2006). In an attempt to figure out whether these two pathways compensate for one another, we activated, in the present study, both pathways by 1-oleoyl-2-acetylglycerol (OAG), a phorbol ester that causes PKC activation as well as Ca\textsuperscript{2+} elevation (Raz et al. 1998a), in the presence of W7, a CaM inhibitor, and followed translocation of MARCKS, CGE and RMII.

OAG-induced translocation of MARCKS from the plasma membrane (Fig. 1a, A) to the cortex (Fig. 1a, B) of the egg and triggered both CGE and RMII (Fig. 1b, B'). W7 alone...
induced neither MARCKS translocation (Fig. 1a, C'), nor CGE or RMII (Fig. 1b, C'). W7 was able to block OAG-induced RMII (Fig. 1b, D'), but was unable to inhibit either OAG-induced MARCKS translocation (Fig. 1a, D') or CGE (Fig. 1b, D'). CGE was not affected by blocking of the CaM activation pathway, indicating a compensatory pathway, most probably the PKC activation pathway. Further examination of each pathway was performed by employing molecular techniques.

**Involvement of αCaMKII in egg activation**

Accumulating evidence from our previous study implies a direct role for CaM in the process of CGE (Eliyahu et al. 2006). We postulated that CaM, beyond being CaMKII activator, is an active participating link in the pathway leading to CGE by directly binding to MARCKS, thus causing MARCKS translocation. We have hypothesized that CaM, rather than CaMKII, is involved in CGE. In order to contradict the involvement of CaMKII in promoting CGE, we microinjected eggs with a CA-αCaMKII mutant and followed the ability of the eggs to undergo CGE and RMII.

**Expression of αCaMKII isoform in rat eggs**

The αCaMKII isoform was undetected in mouse eggs, whereas the βCaMKII isoform was predominantly expressed (Abbott et al. 2001). Since we were provided with a rat cDNA encoding the αCaMKII wt isoform, our initial step was to determine whether the αCaMKII isoform is expressed in rat eggs using Western blot analysis. Proteins of either non-activated MII eggs or eggs activated for 20 min by ionomycin (2 mM) were separated on SDS-PAGE (Fig. 2). A single band was detected at ~50 kDa, consistent with the expected molecular mass (MW) of the αCaMKII isoform. The 50 kDa band of ionomycin-activated eggs appeared to be slightly stronger (30%) than the one of non-activated MII eggs (Fig. 2a), maybe due to a better recognition between the antibody and the activated form of the protein. Anti-cdc2 p34 antibody served as a control for standardizing the amount of protein loaded on each lane. Staining intensity of bands indicated a similar amount of proteins in the lysates of non-activated and activated eggs (Fig. 2b). The experiment was repeated three times with similar results.

**Microinjection of αCaMKII protein and cRNA into the eggs**

Once the expression of the αCaMKII isoform in rat eggs was confirmed, the ability of CA-αCaMKII to induce CGE and RMII was examined by microinjecting MII eggs with an αCaMKII-overexpressed protein (0.56 mg/ml) or with rat CA-αCaMKII cRNA (0.5 mg/ml). We monitored the in vitro activity of the CA-overexpressed protein kinase by SignaTECT CaMKII assay system (Promega) and
Figure 2 Expression of αCaMKII in rat eggs. Eggs before or after parthenogenetic activation by ionomycin (2 μM; 20 min) were pooled, lysed and the proteins were separated on SDS-PAGE (300 eggs per lane). The proteins were immunoblotted with either anti-αCaMKII goat polyclonal IgG (a; 1:250) or with anti-cdc2 p34 rabbit polyclonal IgG (b; 1:1000). Secondary antibodies, peroxidase-conjugated donkey anti-goat IgG (1:2500) or goat anti-rabbit IgG (1:5000) respectively, were detected by an ECL detection system. Anti-cdc2 p34 antibody was used as a control for standardizing the amount of protein loaded on each lane. The arrow in (a) points at αCaMKII (50 kDa) and the arrow in (b) points at cdc2 p34 (34 kDa), as calculated from the migration of protein standards with known kDa. The 50 kDa band of ionomycin-activated eggs (lono 20') appeared stronger than that of non-activated MII eggs (MII). At least three independent experiments were performed.

[γ-32P]ATP (data not shown) as well as the ex vivo translation and distribution of the αCaMKII cRNA, 3 h after microinjecting the eggs, in order to ensure that mutated proteins maintain their original intracellular localization (Hatch & Capco 2001). Control, non-injected MII eggs exhibited a very pale staining (Fig. 3B'), reflecting non-specific binding of the anti-FLAG antibody. wt (Fig. 3C') and CA (Fig. 3D') proteins were expressed throughout the egg cytoplasm with a marked concentration at the cortex. The developmental stage of the eggs had no effect on the subcellular distribution of the kinase (Fig. 3C′ and D'). No fluorescence was observed in eggs incubated only in the presence of the second antibody (Fig. 3A').

The effect of CA-αCaMKII on CGE and RMII

We injected MII eggs with wt or CA-overexpressed αCaMKII proteins (0.56 mg/ml) and incubated the eggs for 1.5 h to allow recovery before monitoring RMII (Fig. 4a) and CGE intensity (Table 1). We also injected eggs with αCaMKII cRNAs (0.5 mg/ml), either wt or CA forms, allowed for a 3-h translation period and monitored RMII (Fig. 4b) and the CGE intensity (Table 1). Since CaMKII is known to induce RMII in eggs, the rate of RMII in eggs injected with CA-αCaMKII served as a positive control. We were able to induce RMII in 57% or 69% of the eggs by injecting the overexpressed CA-αCaMKII protein (Fig. 4a) or its cRNA (Fig. 4b) respectively. Injection of either wt αCaMKII protein or wt αCaMKII cRNA into the eggs induced significantly lower rates of RMII (35% and 27% respectively; \(P<0.01\)). Ionomycin or SrCl2 served as additional controls, inducing 86% or 83% RMII respectively (Fig. 4b).

We arbitrarily assigned the value of 1.00 to the CGE intensity of eggs injected with wt protein or wt cRNA. The CGE intensity of treated eggs was calculated relatively by calculating the ratio between the CGE intensity of each experimental group and that of wt-injected eggs. CGE in eggs subjected to ionomycin, SrCl2, or fertilization in vivo served as positive controls (1.92 ± 0.04, 2.53 ± 0.6, 2.57 ± 0.2 respectively; Table 1). Untreated MII eggs exhibited a minimal degree of CGE (0.25 ± 0.12, 0.27 ± 0.11; Table 1). Eggs injected with either PBS, CA-overexpressed protein, or cRNA had CGE intensities (1.05 ± 0.12, 0.93 ± 0.13, or 1.08 ± 0.09 respectively; Table 1) similar to that of wt-injected eggs (1.00; \(P>0.01\)), implying that CaMKII is not involved in CGE. Typical representative micrographs of CGE intensity...
for each treatment group are depicted in Fig. 5: non-injected MII egg (Fig. 5A and A’); wt-, CA-, or PBS-injected eggs (Fig. 5B, B’, C, C’, D, D’ respectively), SrCl₂-activated eggs (Fig. 5E and E’) and in vivo-fertilized eggs (Fig. 5F and F’). We subjected eggs that had already been injected with wt or CA-αCaMKII cRNAs to 2 mM SrCl₂ in order to find out whether the eggs had exhausted their CGE potential. We found (data not shown) an additive effect of SrCl₂, on top of αCaMKII cRNA injection, regarding CGE. As mentioned earlier, CGE can be induced by activated PKC or by its downstream substrates, such as MARCKS. Next, we attempted to examine whether direct interfering with MARCKS will disrupt the ability of the eggs to undergo CGE and RMII.

**Involvement of MARCKS in egg activation**

In a previous study, we have used biochemical approaches to provide evidence linking MARCKS translocation to CGE (Eliyahu et al. 2006). In all experiments conducted until now, no direct attempt to mutate or neutralize MARCKS was performed. An accepted method of demonstrating involvement of CaM in CGE via its direct binding with MARCKS would be to try and affect the occurrence of CGE by eliminating the CaM-binding properties of MARCKS. Since the CaM-binding sites are embedded within MARCKS effector domain, performing a mutation designated to hinder the CaM-MARCKS binding is a hard task. As a result, we chose to examine the effect of another direct mutation to the MARCKS protein (m3) on the ability of the eggs to undergo CGE. The three serine residues at the PKC phosphorylation sites (Fig. 6A and A’) were replaced at the m3 mutation to the MARCKS protein (m3) on the ability of the eggs to undergo CGE. The three serine residues at the PKC phosphorylation sites were replaced at the m3 mutation to the MARCKS protein (Fig. 6A and A’).

**Microinjection of MARCKS cRNA into the eggs**

We injected MII eggs with missense (ms), wt, or m3 MARCKS cRNAs, all carrying FLAG tag. Injected eggs were cultured for 3 h to allow protein translation before being monitored for CGE intensity and RMII. We monitored the ex vivo translation of the microinjected wt and m3 MARCKS cRNAs and the distribution of the translated protein and compared it with the intracellular localization of the endogenous protein (Eliyahu et al. 2006). Both wt- and m3-translated proteins were localized mainly on the plasma membrane as well as throughout the cytoplasm (Fig. 6C and D’). Control, non-injected MII eggs and ms cRNA-injected eggs exhibited a pale fluorescence staining, reflecting non-specific binding of the anti-FLAG antibody (Figs 6A’ and 7B’ respectively).

**The effect of m3 MARCKS on CGE**

Eggs microinjected with ms MARCKS cRNA were activated 2.5 h later by 30 ng/ml TPA. CGE intensity of

### Table 1: Cortical granules exocytosis (CGE) fluorescence intensity after microinjection with overexpressed calmodulin-dependent protein kinase II (αCaMKII) proteins or cRNAs.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Eggs (no.)</th>
<th>CGE intensity (M ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Injection of αCaMKII protein (0.56 mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MII</td>
<td>22</td>
<td>0.25 ± 0.12</td>
</tr>
<tr>
<td>Wt</td>
<td>65</td>
<td>1.00</td>
</tr>
<tr>
<td>CA</td>
<td>72</td>
<td>0.93 ± 0.13</td>
</tr>
<tr>
<td>b. Injection of αCaMKII cRNA (0.5 mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MII</td>
<td>20</td>
<td>*0.27 ± 0.11</td>
</tr>
<tr>
<td>PBS</td>
<td>32</td>
<td>1.05 ± 0.12</td>
</tr>
<tr>
<td>Wt</td>
<td>63</td>
<td>1.00</td>
</tr>
<tr>
<td>CA</td>
<td>68</td>
<td>1.08 ± 0.09</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>43</td>
<td>*1.92 ± 0.04</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>24</td>
<td>*2.53 ± 0.6</td>
</tr>
<tr>
<td>Fertilization</td>
<td>22</td>
<td>*2.57 ± 0.2</td>
</tr>
</tbody>
</table>

CGE fluorescence intensity of eggs microinjected with overexpressed, wt, or CA-αCaMKII proteins (a) or cRNAs (b). The eggs were fixed 1.5 or 3-h post-injection respectively, and stained with LCA–biotin (1:100) and Texas Red Streptavidin (1:1000) for CGE labeling. We arbitrarily assigned the value of 1.00 to the CGE intensity of eggs injected with the wt form of either αCaMKII protein or cRNA. The CGE intensity of eggs from other treatment groups was calculated relatively (one-sample t-test; *P < 0.01). At least three independent experiments were performed.
the injected eggs was arbitrarily assigned the value of 1.00 and the CGE intensity of other groups was calculated relatively. Untreated MII eggs exhibited a minimal rate of CGE (0.16 ± 0.06). The rate of CGE intensity in TPA-activated eggs microinjected with m3 MARCKS cRNA was almost half (0.57 ± 0.02) the intensity of TPA-activated eggs microinjected with either ms cRNA (1.00) or wt cRNA (1.01 ± 0.12) or non-injected TPA-activated eggs (0.98 ± 0.64). Typical representative micrographs of the CGE intensity in eggs of various treatment groups are depicted in Fig. 7: non-injected MII eggs (Fig. 7A and A’); non-injected TPA-activated eggs (Fig. 7B and B’) and TPA-activated eggs microinjected with ms, wt, or m3 MARCKS cRNAs (Fig. 7C, C’, D, D’, E and E’ respectively). These results suggest that phosphorylation of MARCKS by PKC is required for CGE to occur. Injections of the various MARCKS cRNA forms did not induce RMII in the eggs.

Two out of the three subfamilies of PKC: conventional PKCs (cPKCs) and novel PKCs (nPKCs) can be activated by TPA (Eliyahu & Shalgi 2002, Quan et al. 2003). In order to determine which subfamily induced MARCKS phosphorylation, thus enabling its translocation and causing CGE, we performed another set of experiments in which MARCKS cRNAs-injected eggs were activated by ionomycin. The CGE intensity of eggs injected with wt MARCKS cRNA was arbitrarily assigned the value of 1.00, whereas the CGE intensity of the other experimental groups was calculated relatively. The CGE intensity induced by ionomycin in eggs injected with m3 MARCKS cRNA was lower (0.58 ± 0.16; P < 0.01) than the one induced by ionomycin in eggs injected with wt MARCKS cRNA (1.00). Non-injected MII eggs had only 0.03 ± 0.02 CGE intensity and non-injected ionomycin-activated eggs had a CGE intensity of 0.65 ± 0.21. These results suggest that, in addition to nPKCs, cPKCs are also participating in the phosphorylation of MARCKS, leading to CGE.

The CGE intensity of MARCKS cRNA-injected eggs activated by either TPA or ionomycin imply involvement of nPKCs and/or cPKCs in MARCKS phosphorylation and translocation and hence in CGE in rat eggs. To establish a
direct involvement of cPKCs in the pathway leading to CGE through MARCKS, we quantified the intensity of CGE induced by ionomycin (2 μM) in the presence of myristoylated PKCι (myrPKCι; 35 μM), a specific cPKC pseudosubstrate that served as a PKC inhibitor (Eliyahu & Shalgi 2002), and looked for inhibition of MARCKS translocation. myrPKCι reduced CGE intensity to close to 50% (Fig. 8) and inhibited translocation of MARCKS from the membrane to the cortex in almost 50% of the eggs (data not shown). The CGE intensity in myrPKCι-treated eggs was 0.58 ± 0.03 (P < 0.01), whereas the CGE intensity in ionomycin-activated eggs was arbitrarily assigned the value of 1.00. Untreated MII eggs exhibited a minimal rate of CGE (0.1 ± 0.07). These results support the hypothesis that cPKCs are responsible for almost half of the Ca²⁺-induced CGE, probably by causing MARCKS phosphorylation, on top of the Ca²⁺/CaM-induced MARCKS translocation.

**Discussion**

The block to polyspermy, both at the plasma membrane and at the ZP levels, is imperative for preventing incorporation of more than one sperm into the egg at fertilization, (Sun 2003, Gardner & Evans 2006). Several studies reported that CGE, a major process in preventing polyspermy, can be triggered either by [Ca²⁺], rise or by PKC activation (Gangeswaran & Jones 1997, Johnson & Capco 1997, Raz et al. 1998a, 1998b, Luria et al. 2000, Pauken & Capco 2000, Eliyahu & Shalgi 2002). We have previously demonstrated that upon egg activation, MARCKS could either be phosphorylated by activated PKC or be bound by Ca²⁺-activated CaM (Eliyahu et al. 2005, 2006, Tsaadon et al. 2006). Each pathway can independently cause translocation of MARCKS and its disassembly from the actin filaments, culminating in CGE. In the current study, our first objective was to test whether these two pathways can compensate for one another. We subjected the eggs to OAG that activates both Ca²⁺ and PKC pathways, in the presence of W7, a CaM inhibitor, and followed translocation of MARCKS and its RMII. The observation that W7 inhibited neither MARCKS translocation nor OAG-induced CGE led us to conclude that the PKC pathway can compensate for lack of the Ca²⁺/CaM pathway. There are many potential proteins that may be involved in CGE. Several studies have indicated a role for CaM in promoting CGE in eggs (reviewed by Abbott & Ducibela 2001, Eliyahu et al. 2006). Other studies claim that CaMKII activation is involved in CGE (Markoulaki et al. 2003, Sun 2003, Knott et al. 2006). As mentioned in the Introduction section, CaMKII, like CaM, is localized at the egg cortex, especially at sites of exocytosis (Shen et al. 1998, Easom 1999) and was suggested as a
participant in the signal pathway leading to CGE, on top of its involvement in promoting RMII. Although it is accepted that CGE is triggered by Ca\(^{2+}\) increase, via activation of CaM followed by activation of CaMKII, we could not rule out the possibility that CaM activation leads to a direct interaction with MARCKS, hence to actin reorganization and CGE. The involvement of CaMKII in egg activation events has been the topic of many studies conducted lately. While CaMKII involvement in promoting RMII is well established (Markoulaki et al. 2003, Sun 2003, Madgwich et al. 2005, Ito et al. 2006, Knott et al. 2006), its involvement in CGE is still controversial.

The \(\alpha\)-CaMKII isoform could not be detected in mouse eggs (Abbott et al. 2001) but, in the current study, we could show its expression in rat eggs. In order to determine the role of CaMKII in CGE, we monitored the ability of eggs injected with either protein or cRNA of overexpressed Ca-\(\alpha\)CaMKII to undergo CGE and RMII. Our observations, supported by the work of Knott et al. (2006), led us to suggest that CaMKII is not involved in the signal transduction pathway leading to Ca\(^{2+}\)-induced CGE. Knott et al. (2006) tested the hypothesis that CaMKII is the major integrator of Ca\(^{2+}\)-induced egg activation events, by microinjecting a CA-\(\alpha\)-CaMKII cRNA into mouse eggs. Expression of the mutant cRNA induced a sustained rise in CaMKII activity and triggered cell cycle resumption, though CGE appearance was abnormal. Since they did not report an injection of wt cRNA or any other substance that would serve as a control for isolating the effect of the injection itself, we cannot exclude the possibility that the mechanical process of injection may be the cause for the abnormal CGE. Their results correlate with our observation of a basal CGE intensity detected in all eggs microinjected. In our work, eggs injected with both CA- and wt-overexpressed proteins, as well as those injected with \(\alpha\)-CaMKII cRNAs were unable to undergo CGE, but were able to resume meiosis. Subsequent SrCl\(_2\) activation of the microinjected eggs triggered a maximum rate of CGE, manifesting that the eggs were capable of undergoing CGE. These findings rule out a possible involvement of CaMKII in promoting Ca\(^{2+}\)-induced CGE, but support a major role for CaMKII in promoting RMII, probably by inactivation of both cytostatic factor (CSF) and maturation-promoting factor (MPF; review by Jones 2005). These results can indirectly support our hypothesis that Ca\(^{2+}\)-activated CaM is not only CaMKII activator, but can also act as an active link participating in the pathway leading to CGE, probably by a direct binding to MARCKS and causing MARCKS translocation.

Two articles supporting our findings have been published lately; Ito et al. (2006) have shown that the CaMKII phosphorylation occurring during egg activation can be suppressed by KN93, a CaMKII inhibitor. They claimed that this phosphorylation is involved in inactivation of p34\(^{\text{Cdc2}}\) kinase and degradation of Mos. In addition, the fact that accumulation and phosphorylation of CaMKII that occurred in eggs undergoing spontaneous activation could be inhibited by KN93 suggests an involvement of CaMKII in spontaneous egg activation (exiting from MII arrest) in rats. We have accumulating data (unpublished) showing that rat eggs undergoing spontaneous activation do not undergo CGE. Gardner et al. (2007) showed that injection of CA-\(\alpha\)-CaMKII cRNA was not sufficient for establishing the plasma membrane block to polyspermy in mouse eggs, though it induced resumption of cell cycle and a moderate CGE. However, treating the eggs with the specific CaMKII inhibitor (myrAIP) led to a decreased block to polyspermy. They concluded that although CaMKII participates in membrane block establishment, other factors are also needed for changing the egg membrane’s receptivity to sperm after fertilization.

Our main focus in the current work was to study the role of Ca\(^{2+}\) and PKC in inducing CGE during egg activation, and attempt to resolve whether MARCKS plays a role in inducing CGE. We have previously suggested that MARCKS may play a role in cortical F-actin disassembly and mediating CGE in rat eggs (Eliyahu et al. 2005, 2006, Tsaadon et al. 2006). We have demonstrated that, in secretory cells, phosphorylation of MARCKS by PKC or its binding to Ca\(^{2+}\)/CaM, causes translocation of MARCKS and disrupts its role as a cross-linker of cortical actin filaments, leading to F-actin disassembly and allowing CGs to fuse with the egg plasma membrane and undergo exocytosis (Danks et al. 1999, Vaaraniemi et al. 1999, Wohlsland et al. 2000, Arbuzova et al. 2002, Eliyahu et al. 2005, 2006, Tsaadon et al. 2006). In the current work, we compared the CGE intensity of TPA-activated eggs injected with either unphosphorylatable MARCKS mutant cRNA, wt MARCKS cRNA, or mS MARCKS cRNA. According to our findings, phosphorylation of the plasma membrane-associated MARCKS protein is essential for exocytosis of the CGs contents, induced by either TPA or ionomycin. The CGE intensity of eggs injected with mutant-unphosphorylatable MARCKS was almost half the intensity of CGE in eggs injected with ms MARCKS cRNA, indicating that phosphorylation of MARCKS by PKC is a pivotal event associated with CGE. The partial CGE in these eggs could be due to the presence of endogenous MARCKS within the eggs that can be phosphorylated by PKC and to the fact that the mutant MARCKS did not lose its CaM-binding ability; thus CGE can be induced by the Ca\(^{2+}\)/CaM pathway in the ionomycin-activated eggs. 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. Michaut et al. (2005) reported the expression of phosphorylated MARCKS (pMARCKS) in mouse eggs, describing pMARCKS as a novel centrosome component that also defines a peripheral subdomain of the cortical actin cap overlaying the egg spindle. They suggested that this localization of pMARCKS implies
its role in the formation of contractile apparatus during PB emission.

PKC isoforms are classified according to their cofactor requirements (Halet 2004). cPKCs are Ca$^{2+}$ and DAG dependent and nPKCs are Ca$^{2+}$ independent but require DAG for activation, whereas atypical PKCs (aPKCs) are neither Ca$^{2+}$ nor DAG dependent. Microinjecting eggs with wt or m3 MARCKS cRNAs prior to activation by ionomycin assisted us in sorting out the PKC isoforms responsible for MARCKS phosphorylation. The reduced CGE intensity of m3 MARCKS cRNA-injected eggs compared with that of wt MARCKS cRNA-injected eggs led us to the conclusion that nPKCs and/or cPKCs are responsible for MARCKS phosphorylation and that cPKCs contribute to the Ca$^{2+}$-induced MARCKS translocation. By exposing ionomycin-activated eggs to PKC$\psi$, a specific cPKC inhibitor, we were able to indicate the involvement of cPKCs in CGE induction via MARCKS translocation. MARCKS translocation from the membrane to the cortex was inhibited in almost 50% of the eggs and the CGE intensity was reduced by close to 50%, as well.

The CGE intensity of TPA or ionomycin-activated eggs injected with m3 MARCKS was reduced by 50%. Matson et al. (2006) reported similar observations, proposing stimulation of the myosin light chain kinase (MYLK2) activity by elevated [Ca$^{2+}$]$_i$ at fertilization of mouse eggs, resulting in activation of the myosin motor involved in CGs translocation. The authors used ML-7, a MYLK2 antagonist, that caused inhibition of PBII formation and reduced CGE intensity by half. According to these results, it is possible that MARCKS acts in concert with MYLK2 in the signaling pathway leading to CGE. CGE is an evolutionary developed mechanism that ensures successful egg activation and embryo development. It is well accepted that a process as important as this will be secured by several pathways and mechanisms. As summarized in the Introduction section, CGE can be triggered by Ca$^{2+}$ increase and/or by PKC activation, whereas RMII can be triggered only by Ca$^{2+}$ increase. Collectively, the above results present direct evidence demonstrating that MARCKS, not CaMKII, is the key regulatory molecule mediating CGE. Figure 9 depicts a flow chart of our view of the possible mechanism of CGE: CGE is mediated by hydrolysis of PIP$_2$, two important second messengers, IP$_3$ and DAG, are generated; the former induces a rise in [Ca$^{2+}$]$_i$ and the latter activates PKC. Two separate pathways, compensating for one another, can bring about MARCKS translocation and lead to CGE. One is the Ca$^{2+}$-independent pathway that involves activation of nPKC, which in turn causes MARCKS phosphorylation via its specific PKC phosphorylation sites. The other is the Ca$^{2+}$-induced pathway that involves activation of Ca$^{2+}$/CaM and its binding to MARCKS, as well as MARCKS phosphorylation by cPKC, both leading to dissociation of MARCKS from F-actin, remodeling of the actin network, and subsequently CGE. **Figure 9** Flow chart of a possible network for CGE. Upon sperm penetration, hydrolysis of PIP$_2$ occurs. Two important second messengers are generated: DAG and IP$_3$. DAG activates nPKCs that cause MARCKS phosphorylation. IP$_3$ induces a rise in [Ca$^{2+}$]$_i$ that causes activation of CaM and cPKCs, both leading to dissociation of MARCKS from F-actin, the former by a direct binding and the latter by phosphorylation. Both Ca$^{2+}$-dependent and Ca$^{2+}$-independent pathways lead to actin reorganization through the dissociation of the MARCKS–F-actin complex, culminating in CGE. We propose that Ca$^{2+}$-activated CaMKII is involved in RMII but not in CGE. Solid arrows (—–) indicate commonly accepted pathways. Dashed arrows (----) indicate pathways demonstrated in our previous and present studies.

**Materials and Methods**

**Animals**

Wistar-derived rats were housed in air-conditioned, light-controlled rooms. The study was approved by the Institutional Animal Care and Use Committee.

**Collection of eggs**

**MII-ovulated eggs**

For induction of ovulation, 25- to 27-day-old immature 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; Synco-part; Sanofi, Paris, France). Rats were killed 14 h after hCG administration. Cumulus-enclosed MII eggs were removed from the oviductal ampullae into Toyoada HEPES (TH; Ben-Yosef et al. 1998) medium or into Ca$^{2+}$- and Mg$^{2+}$-free TH (TH$^{-/-}$) medium, both supplemented with 0.4% BSA (fraction V, Sigma; Talmor et al. 1998). Cumulus cells were removed by a brief exposure to 400 IU/ml of hyaluronidase (Sigma).

In vivo-fertilized eggs

PMSG- and hCG-primed immature female rats were allowed to mate and were killed 16 h after hCG administration. Eggs and cumulus cells were treated as described above for MII eggs. Chromosomes were stained with Hoechst 33342 (Sigma) to determine fertilization status.

**Parthenogenetic activation**

MII-ovulated eggs were parthenogenetically activated by four different activators, all of which are capable of inducing CGE in rat eggs (Eliyahu & Shalgi 2002, Tomashov-Matar et al. 2005). MII eggs were incubated in:

1. TH medium for 5 min in the presence of 50 µg/ml 1-oleoyl-2-acyl glycerol (OAG; Sigma), followed by an additional 5-min incubation in TH medium lacking the activator.
Stock solution of 1 mg/ml OAG in dimethylsulfoxide (DMSO) was kept at −20 °C.

2. TH+/− medium for 5 min in the presence of 2 μM calcium ionophore (ionomycin 407950; Calbiochem, San Diego, CA, USA), followed by an additional 45 min incubation in TH medium lacking the activator. Stock solution of 4 mM ionomycin in DMSO was kept at −70 °C.

3. TH+/− medium for 15 min in the presence of 2 mM SrCl2 (Merck), followed by an additional 30-min incubation in TH medium lacking the activator. Freshly prepared 2 mM SrCl2 in TH+/− medium was used.

4. TH medium for 5 min in the presence of 30 ng/ml TPA (Sigma), followed by an additional 15 min incubation in TH medium lacking the activator. Stock solution of 1 mg/ml TPA in DMSO was stored at −20 °C.

Inhibition of CaM and PKC

CaM inhibition

Eggs were incubated for 30 min in the presence of 20 μM W7 (Sigma; Eliyahu et al. 2006) prior to activation by OAG at the same regimen as described in the previous paragraph. Stock solution of 5 mM W7 in double distilled water (DDW) was kept at 4 °C.

PKC inhibition

Eggs were incubated for 5 min in the presence of 35 μM pseudosubstrate (myristoylated PKCδ amino acids 19–27; Biomol, Plymouth, PA, USA; Eliyahu & Shalgi 2002), together with 2 μM ionomycin, followed by an additional 15 min incubation in TH medium containing the inhibitor. Stock solution of 1 mM pseudosubstrate in DDW was kept at −20 °C.

Molecular cloning and sequence analysis

The full-length sequence of rat cDNA encoding αCaMKII wild type (wt) was generously provided by Prof. T Meyer (Stanford University, CA, USA). Human cDNA constructs of MARCKS/80K-L (wt) and mutant (m3) were generously provided by Prof. N Saito (Kobe University, Kobe, Japan). The αCaMKII cDNA was digested with BamHI at the 5′ end and with EcoRI at the 3′ end; the resulting insert of ~1.5 kb was subcloned into several similarly digested plasmid vectors. For protein overexpression, the DNA insert was subcloned into pET-28a plasmid (Novagen, Merck) with an in-frame hexahistidine tag at the 3′ end. For cRNA synthesis, the DNA insert was subcloned into pCMV-Tag 4A plasmid (Novagen, Merck) with an in-frame hexahis-tag at the 3′ end. The resulting insertion of 1.5 kb was subcloned into pET-28a plasmid (Novagen, Merck) with an in-frame FLAG epitope at the 3′ end (Stratagene, La Jolla, CA, USA). Site-directed mutagenesis was performed using the wt αCaMKII clone as a template for making a CA mutant of αCaMKII. The MARCKS cDNAs (wt and m3) were digested with EcoRI at both the 5′ and 3′ ends and were ligated into the same pCMV-Tag 4A plasmid, in the presence of Antarctic phosphatase that catalyzes the removal of 5′ phosphate groups from DNA vector to prevent self-ligation. Both strands of ten independent colonies were sequenced and analyzed for open reading frame by MacVector 6.5 (Oxford Molecular, Oxford, UK).

Point mutation and cRNA synthesis

αCaMKII

Threonine at position 286 (T286) was substituted by aspartate, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The sequence of primers used to make this mutation is shown below. The mutated codon is underlined.

Sense: 5′-GCAGAGACGGACAGTGGACCTGGCCTG-3′
Antisense: 5′-CAGGCATCCAGCTCCTGTCGGC-3′

MARCKS

In the mutant that we have received (m3), the three serine residues at the PKC phosphorylation site of MARCKS were replaced with alanine, making the mutant m3 unsusceptible to phosphorylation by PKC. A clone, in which the insertion was ligated backward (will be regarded as missense cRNA; ms) served as another control for injection.

Sequences were verified prior to synthesis of cRNAs. cRNAs were synthesized from linearized pCMV-Tag 4A CaMKII/MARCKS (Ribomax RNA synthesis; Promega) in the presence of 3 mM m7G(5′)ppp(5′)G (NEB; Ipswich, MA, USA), precipitated by isopropanol and resuspended in DEPC-treated water containing 4 U/μl RNasin (Promega).

Protein synthesis

Protein overexpression of wt and CA-αCaMKII subcloned into pET-28a was carried out in BL21 Escherichia coli cells. The expression of the His-tagged protein was induced with isopropyl β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation and lysed in lysis buffer (0.3 mM NaCl, 50 mM Tris (pH 7.6), 1 mM EDTA, 10 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 30 μg/ml aprotinine). Cells lysate was sonicated and clarified by spinning out the insoluble fraction. Clarified lysate was mixed with Ni-NTA His-bind resins. Protein purification was performed on a column, using a Ni-NTA binding kit (Novagen) under denaturing conditions according to the manufacturer’s instructions. The refolded His-tagged protein was then eluted and the main peak pooled. The protein was dialyzed (slide-a-laser; Pierce, Rockford, IL, USA) for purification and its concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories GmbH).

Egg microinjection

The various cRNAs were microinjected into MIIs at a volume of 3–5% of the egg volume, using the FemtoJet microinjector (Eppendorf, Germany). wt or ms cRNAs or PBS served as controls.
**Antibodies**

**Primary antibodies**

Anti-MARCKS goat polyclonal IgG (N-19; sc-6454; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-αCaMKII goat polyclonal IgG (L-15; sc-5391, Santa Cruz Biotechnology), anti-cdc p34 rabbit polyclonal IgG (C-19; sc-954, Santa Cruz Biotechnology), and anti-FLAG mouse monoclonal IgG (F-1804, Sigma).

**Secondary antibodies**

Donkey anti-goat IgG peroxidase (705-035-147; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), goat anti-rabbit IgG peroxidase (111-035-003; Jackson ImmunoResearch Laboratories), donkey anti-goat IgG-Cy-3 (705-165-147; Jackson ImmunoResearch Laboratories), and donkey anti-mouse IgG-Cy-3 (715-165-151; Jackson ImmunoResearch Laboratories).

**Western blot analysis**

Lysis buffer was added to samples of 300 MII eggs collected in 5–10 μl of TH medium (Ben-Yosef et al. 1998). After two cycles of freezing/thawing, the extracts were stored at −70 °C. Proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (BioTrace NT; Gelman Sciences, Ann Arbor, MI, USA), using a wet blotting apparatus (Hoeffer, San Francisco, CA, USA). Blots were blocked by 5% dry milk in TBS (Talmor (Hoeffer, San Francisco, CA, USA). Blots were blocked by 5% dry milk in TBS (Talmor et al. 1998) and incubated overnight at 4 °C in the presence of anti-αCaMKII antibody (1:250) or anti-cdc p34 antibody (1:1000). Bound antibody was recognized by secondary anti-goat IgG antibody (1:2500) or anti-rabbit IgG antibody (1:5000) respectively, conjugated to horseradish peroxidase. Detection was performed by an ECL detection system (Pierce). Approximate molecular masses were determined by comparison with the migration of prestained protein standards (Amersham).

**Immunofluorescence staining**

Eggs were fixed in 3% paraformaldehyde, supplemented with 0.01% glutaraldehyde. ZPs were removed post-fixation by 0.25% pronase (Sigma). The plasma membrane was permeabilized with 0.05% NP-40. Membrane-permeabilized eggs were incubated in the presence of anti-MARCKS (1:50) or anti-FLAG (1:1000) antibodies. Primary antibodies were detected using fluorescent-labeled anti-goat or anti-mouse Cy-3 secondary antibodies (1:250 or 1:1000 respectively).

**CGE quantification and DNA staining**

Fixed eggs were labeled with 5 μg/ml Lens culinaris agglutinin (LCA)–biotin (B-1045; Vector, Burlingame, CA, USA), which binds specifically to CGs exudate (Cherr et al. 1988, Ducibella et al. 1988, Eliyahu & Shalgi 2002), washed, and labeled with 1 μg/ml Texas Red Streptavidin (SA-5006; Vector) for detection of CGE, and with 1 μg/ml Hoechst 33342 (Sigma) for determining cell cycle or fertilization status. Labeled eggs were visualized and photographed with a Zeiss confocal laser scanning microscope (LSM 410 CLSM; Zeiss, Oberkochen, Germany). The intensity of CGE staining was measured in one confocal slice depicting a mean CGE response of each egg. The staining intensity was calculated using the corrected mean density values obtained by the LSM software.

**Statistical analysis**

Data were evaluated by t-test, one-way ANOVA, or Fisher’s exact test. Differences between treatment groups were determined by post hoc test. P<0.01 was considered significant.

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