Calcium/Calmodulin-Dependent Protein Kinase II and Calmodulin: Regulators of the Meiotic Spindle in Mouse Eggs

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Elevation of intracellular free calcium causes egg activation by initiating a cascade of interacting signaling pathways that, in unison, act to remodel the cytoplasmic compartment and the nuclear compartment of the egg. We show here that calcium/calmodulin-dependent protein kinase II (CaM kinase II) is tightly associated with the meiotic spindle and that 5 min after egg activation there is a transient, tight association of calmodulin (colocalized with CaM kinase II) on the meiotic spindle. These correlative observations caused us to test whether activation of CaM kinase II mediated the chromosomal transit into an anaphase configuration. We demonstrate that calcium and calmodulin, at physiological levels, along with ATP were capable of driving the spindle (with its associated CaM kinase II) into an anaphase configuration in a permeabilized egg system. The transit into anaphase was dependent on the presence of both calcium and calmodulin and occurred normally when they were present at a ratio of 4 to 1. Peptide and pharmacologic inhibitors of CaM kinase II blocked the transit into anaphase, both in the permeabilized egg system and in living eggs (inhibitors of protein kinase C did not block the transit into anaphase). Using a biochemical approach we confirm that CaM kinase II increases in activity 5 min after egg activation and that a second increase occurs 45 min after activation at the approximate time that the contractile ring of the second polar body is constricting. This corresponds to the approximate time when calmodulin and CaM kinase II colocalize at several points in the activated egg including the region containing midzone microtubules. CaM kinase II appears localized on midzone microtubules as soon as they form and may have a role in specifying the position of the contractile ring of the second polar body.

Key Words: midzone microtubules; contractile ring; cell cycle; cytokinesis.

INTRODUCTION

The conversion of the fertilization-competent egg into the zygote is accompanied by a change in function from one of arrest and maintenance in the egg to one of high metabolic activity as the program of early development unfolds in the zygote. These functional changes are underpinned by numerous structural changes in both the cytoplasmic and the nuclear compartments. The structural alterations needed to mediate conversion of the egg into the zygote depend upon the arrest point in the cell cycle where the egg is fertilization competent. Eggs from different classes of organisms are arrested at different points in the cell cycle:

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Sea urchins are arrested in interphase, ascidians are arrested at meiotic metaphase I, whereas amphibians and mammals are arrested at meiotic metaphase II (Gallicano et al., 1997a). Clearly an egg in interphase, or meiotic metaphase I, must undergo different structural changes at fertilization compared to eggs arrested at meiotic metaphase II. Regardless of the arrest point, the evolutionarily conserved stimulus which releases the egg from cell cycle arrest in all animal species studied to date is a rise in intracellular free calcium ([Ca^{2+}]_i). This central role for [Ca^{2+}]_i as the “switch,” that once turned results in zygotic signal transduction suggests that calcium-dependent enzymes will be involved in many of the changes that accompany the conversion of the egg to the zygote.

Calcium/calmodulin-dependent protein kinase II (CaM kinase II) is a kinase thought to be involved with the exit
CaM Kinase II and the Meiotic Spindle

from M phase in somatic cells (Ohta et al., 1990) and in eggs (Lorca et al., 1991, 1993, 1994; Tombes and Peppers, 1995; Morin et al., 1994; Winston and Maro, 1995). In amphibian eggs CaM kinase II activates the ubiquitin-dependent cyclin degradation pathway as a response to the fertilization-induced elevation in \([\text{Ca}^{2+}]\) (Morin et al., 1994; Lorca et al., 1994; Lindsay et al., 1995) and also acts on c-mos degradation (Lorca et al., 1991, 1993). In mammalian eggs an increase in activity of CaM kinase II can be measured at egg activation (Winston and Maro, 1995); however, little is known about the mechanisms activated by CaM kinase II in mammalian eggs.

Results from several laboratories support a role for the activity of protein kinase C (PKC), a kinase whose conventional isotypes are calcium dependent, as a mediator of several structural changes in the conversion of the fertilization-competent egg into the zygote in amphibians (Bement and Capco, 1989; Grandin and Charbonneau, 1991), mammals (Gallicano et al., 1995, 1997a,b; Moses and Kline, 1995; Ducibella et al., 1993), sea urchins (Olds et al., 1995), annelids (Eckberg and Anderson, 1995), and ascidians (Yokosawa et al., 1989). PKC also has been shown to have a role in the conversion of the cytoplasmic compartment from that of an oocyte to that of a fertilization-competent egg in amphibians (Johnson and Capco, 1997). In eggs of both mammals and amphibians, which are arrested at meiotic metaphase II, PKC acts downstream of the fertilization event.

Moreover, we predicted that direct inhibition of CaM kinase II function would block release of chromosomes from the cell cycle arrest point when the egg was challenged with a rise in \([\text{Ca}^{2+}]\).

The results reported here confirm these predictions. In addition, we have mapped the distribution of calmodulin and CaM kinase II after egg activation and show by detergent extraction that calmodulin forms a tight association with CaM kinase II on the meiotic spindle immediately after egg activation. This tight association with calmodulin disappears, while CaM kinase II remains on the spindle as the chromosomes transit into anaphase II. Further, we show that CaM kinase II becomes localized in the region of the midzone microtubules between anaphase II and telophase II. We provide evidence for a causal role of calmodulin and CaM kinase II in the metaphase-to-anaphase transition using a permeabilized egg system and confirm the activity of the kinase at a biochemical level. Our results provide evidence indicating that CaM kinase II, but not PKC, acts on the chromosomes and meiotic spindle to mediate karyokinesis.

MATERIALS AND METHODS

Egg Procurement

Superovulation of CD-1 mice was induced by intraperitoneal injection of 5 IU of pregnant mares’ serum gonadotropin (PMSG; No. G04877, Sigma Chemical Corp., St. Louis, MO) followed by 5 IU of human chorionic gonadotropin (hCG; No. CG-10, Sigma Chemical Corp.) 46–48 h later. Female mice were sacrificed 14–16 h after hCG injection followed by removal of each oviduct. Eggs were collected in KSOM medium (Erbach et al., 1994; Ho et al., 1995; 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH2PO4, 0.20 mM MgSO4, 10 mM lactate, 0.20 mM pyruvate, 0.2 mM glucose, 1.0 mM glutamine, 0.01 mM EDTA, 1.71 mM CaCl2·2H2O) modified to contain 4.0 mM NaHCO3 and 20 mM Hepes, pH 7.35 (KSOM-H) by tearing the oviduct with a pair of Dumont No. 5 forceps. Cumulus cells were removed from eggs by brief exposure to 300 μg/ml hyaluronidase (No. H-3506, Sigma Chemical Corp.) followed by washing in KSOM-H. Where not specified, chemicals were purchased from Sigma Chemical Corp.

Experimental Treatments

Ten millimolar ethanol stocks of calcium ionophore A23187 (No. A-1493, Molecular Probes, Eugene, OR) were diluted into KSOM-H to a final concentration of 1 μM, vortexed, and used within 1 min of preparation. Eggs were incubated at 37°C in the ionophore for 2 min, washed three times in KSOM-H, and incubated in KSOM-H at 37°C. For CaM KII and PKC inhibition in living egg and permeabilized egg assays, the following inhibitors were utilized: Membrane-permeant CaM KII inhibitors myristoylated AIP (myr-AIP, 10 μM; No. P-212, BIOMOL, Plymouth Meeting, PA) or KN-93 (10 μM; No. 422708, Calbiochem, La Jolla, CA); and the membrane-permeant PKC inhibitor bisindolylmaleimide (BIM, 100 μM; No. 203290-S, Calbiochem), each diluted in KSOM-H and used within 5 min of preparation. Inhibitors were applied in a 15-min preincubation prior to activation with iono-
phore in living egg assays and prior to permeabilization in permeabilized egg assays. For CaM KII and PKC inhibition after permeabilization, non-membrane-permeant variants of AIP (10 μM, No. P-211, BIOMOL) and PKCδ (100 μM, No. P-150, BIOMOL) were diluted in flushing medium and employed. BIM and PKCδ were used at concentrations reported previously (Galicano et al., 1997b). KN-93 (10 μM) and AIP (10 μM) were used because at those concentrations each was reported to significantly inhibit the activity of CaM KII (Ishida et al., 1995). For CaM KII inhibition in biochemical kinase activity assays, stock KN-93 was added at a final concentration of 10 μM. Lysates were preincubated with inhibitor for 15 min at room temperature prior to the addition of peptide substrate and [γ-32P]ATP.

Confocal Microscopy

Eggs arrested at meiotic metaphase II and activated eggs were examined using scanning laser confocal microscopes after preparation for immunocytochemistry. Both intact and detergent-extracted specimens were analyzed. For the preparation of intact eggs, live specimens were washed briefly in intracellular buffer (ICB; 100 mM KCl, 5 mM MgCl2, 3 mM EGTA, 20 mM Heps, pH 6.8, in H2O), fixed in 2% paraformaldehyde in ICB for 30 min, and then permeabilized by treatment in 2% paraformaldehyde, 0.1% Tween 20 (No. P-1379, Sigma Chemical Corp.) in ICB for 30 min. When specimens were detergent extracted to prepare the cytoskeleton, living eggs were washed briefly in ICB, immersed in the detergent extraction medium (i.e., ICB made 1% with Tween 20 and 200 μg/ml with AEBSF, No. 101500, Calbiochem) for 5 min, and then cytologically fixed in 2% paraformaldehyde in ICB for 30 min. Specimens were then washed three times for 30 min and processed for immunocytochemistry.

Antibodies to the following cellular proteins were used: Anti-calmodulin (No. 05-173, Upstate Biotechnology, Lake Placid, NY); anti-Calmodulin kinase II (made against β and δ subunits of CaM kinase II, No. 06-396, Upstate Biotechnology); and anti-tubulin (No. T-3526, Sigma Chemical Corp.), all at a dilution of 1:100. The following fluorophore-conjugated secondary antibodies were used: FITC-conjugated goat anti-mouse (No. T-3595, Sigma Chemical Corp.) and goat anti-rabbit (No. T-6778, Sigma Chemical Corp.). IgGs diluted 1:100; Cy5-conjugated goat anti-rabbit IgGs (No. PA45004, Amersham, Arlington Heights, IL) diluted 1:250; and ALEXA 568-conjugated goat anti-mouse (Fab2), fragments (diluted 1:500, No. A-11019, Molecular Probes) and goat anti-rabbit IgGs (No. A-11011, Molecular Probes) diluted 1:250. Primary antibodies were diluted in 1% BSA in ICB and applied to eggs overnight at 4°C. Eggs were then washed three times for 30 min and placed in secondary antibody, also diluted in 1% BSA in ICB, for 1 h at room temperature. Finally, eggs were washed three times in ICB for 30 min. To visualize chromosomes in different experiments, incubations in either Hoechst 33342 (10 μg/ml, 5 min incubation) or DAPI (0.5 μg/ml, 15 min incubation) were included in the second-to-last ICB wash. In preliminary experiments we demonstrated that the anti-calmodulin and anti-CaM KII antibodies could bind to their antigens in Western blots (data not shown).

Eggs were mounted in ICB on a coverslip elevated by wax droplets to prevent compression of eggs. Both the Molecular Dynamics Sarastro 2000 and Leica TCS NT confocal microscopes were used for observation. For the Molecular Dynamics microscope, images of TRITC-labeled specimens were collected using an Argon Laser (514 nm), appropriate filters, and a 60× Plan Apo objective. For the Leica, which permitted simultaneous imaging of DAPI, ALEXA 568, and Cy5 fluorophore-labeled specimens with three lasers (argon UV [363 nm], krypton [568 nm], and Rhodamine [633 nm]) and appropriate filter sets, images were collected using a 63× water objective. All specimens were scanned at 0.5-μm intervals in the z axis. Unless otherwise noted sections were collected through the volume containing the meiotic spindle (i.e., 18–36 optical sections of 0.5 μm).

Permeabilized Assay System

Eggs were permeabilized by immersion in modified ICB (100 mM KCl, 5 mM MgCl2, 5 mM BAPTA, 20 mM Heps, pH 6.8) made 1% with Tween 20 and 1 μg/ml with each of the following protease inhibitors: Aprotinin (No. A-1153, Sigma Chemical Corp.), Bowman-Birk inhibitors (trypsin/chymotrypsin inhibitors, No. T-9777, Sigma Chemical Corp.), chymostatin (No. C-7268, Sigma Chemical Corp.), leupeptin (No. L-2884, Sigma Chemical Corp.), and pepstatin (No. P-4265, Sigma Chemical Corp.). After permeabilization eggs were briefly washed in modified ICB without Tween 20 to remove the detergent. The eggs were then placed into droplets of medium containing the components to be flushed into the specimens. For experiments in which the ratio of calmodulin to free calcium was 1:4, this medium contained 115 mM KCl, 5.75 mM MgCl2, 10 mM diBrBAPTA (tetrasaltsodium, No. 286818-S, Calbiochem), 2.7 mM CaCl2, 0.25 mM calmodulin (No. 208694, Calbiochem), 1 mM ATP, 1 mM phosphocreatine, 50 μg/ml creatine kinase, 1 μg/ml of the protease inhibitors listed above, and 23 mM Heps, pH 6.8. The concentration of free calcium adjusted with a calcium/diBrBAPTA buffer was calculated using WinMMaxChelator Ver. 1.0 (C. Patton, Hopkins Marine Station, Pacific Grove, CA). The ratio of calmodulin to free calcium was adjusted by varying the calmodulin concentration. Kinase inhibitors, when employed, were applied by preincubation for 15 min in the modified ICB medium (lacking Tween 20), and were also included in the complete flushing mixtures during incubation. Permeabilized specimens were incubated for 30 min at 37°C and then cytologically fixed with 2% paraformaldehyde in ICB, washed, and prepared for immunocytochemistry with antibodies or stained with Hoechst or DAPI to detect the configuration of the meiotic spindle or chromosomes, respectively.

CaM Kinase II Activity Assay

CaM kinase II was assayed as follows: For each reaction five eggs arrested at meiotic metaphase II or five activated eggs were washed briefly in PBS and added in 2 μl PBS to a kinase reaction mixture that contained the following components at their final concentrations after the addition of substrate and ATP: 115 mM KCl, 5 mM MgCl2, 5.75 mM diBrBAPTA, 2.1 mM CaCl2, 5.75 mM MgCl2, 6-H2O, 2.2 mM PKI (No. P-203, BIOMOL), 75 μM genistein (No. EI-147, BIOMOL), 10 μM ML-9 (No. EI-268, BIOMOL), 200 μM PKCζ, 240 mM β-glycerophosphate (No. G-6251, Sigma Chemical Corp.), 120 mM para-nitrophenyl phosphate (Sigma 104, Sigma Chemical Corp.), 1 mg/ml of each of the protease inhibitors aprotinin, Bowman-Birk inhibitors (trypsin/chymotrypsin inhibitors), chymostatin, leupeptin, and pepstatin; and 23 mM Heps, pH 6.8. Free calcium was adjusted to 400 nM in the reactions with a calcium/diBrBAPTA buffer. Samples were immediately spun down and flash-frozen by immersion in liquid N2 and remained frozen in liquid N2 or stored at −80°C until the kinase reaction was performed. Sequentially, samples were thawed and 10 μg Autocamtide-2 CaM KII peptide substrate (No. P-101, BIOMOL) and 0.25 μCi [γ-32P]ATP (No.
AA0018, Amersham, Arlington Heights, IL) were added to each tube. Complete reaction mixtures were incubated 30 min at 37°C in a heated microtube mixer (Vortemp 56, Labnet, Woodbridge, NJ). KN-93 (10 μM), a CaM kinase II inhibitor, was added to otherwise identical reaction mixtures for controls at each time point. Control (KN-93) samples were preincubated 15 min at room temperature with gentle agitation prior to the addition of γ²⁵PATP, substrate, and incubation. Reactions were stopped by adding an equal volume of 2× Tricine sample buffer (No. 161-0744, Bio-Rad, Hercules, CA) followed by mixing. Samples were then run on precast Tris-Tricine 16.5% polyacrylamide gels (No. 161-0922, Bio-Rad). Finally, the gels were fixed, dried, and exposed to a phosphor screen for 12 h. Exposed phosphor screens were immediately scanned by a Molecular Dynamics Storm 840 Phosphorimager at 200-μm resolution. Bands corresponding to the molecular weight of Autocamtide-2 were analyzed and band densities were calculated using the Imagequant software package.

RESULTS

Localization of Calmodulin and CaM Kinase II

The distribution of calmodulin was examined in fertilization-competent eggs arrested at metaphase II and in eggs after activation. Figure 1a shows the typical distribution of calmodulin in an intact, unfertilized egg viewed with a scanning laser confocal microscope. Calmodulin is present in a dispersed pattern throughout the unfertilized egg with an enrichment near the periphery of the egg and around the meiotic spindle. The inset in Fig. 1a shows staining of the same egg with Hoechst to demonstrate chromosome arrest at meiotic metaphase II. At 5, 15, 30, 45, and 55 min (Figs. 1b–1f) after egg activation, the distribution pattern for calmodulin was similar except that the enrichment near the spindle became less intense. The inset in Fig. 1c labeled “control” shows a control for nonspecific binding of the second antibody when no primary antibody was applied. All control images shown were collected in the same experiment as their corresponding labeled sample; the negligible level of staining is typical of all second antibody controls in these experiments. As the activated eggs transit into anaphase and beyond, calmodulin accumulates near the region between the chromosomes (i.e., the region containing midzone microtubules; Margolis and Andreassen, 1993; Cao and Wang, 1996; Fishkind et al., 1996; Wheatley and Wang, 1996). Calmodulin is an abundant protein that functions in many cellular processes and a large proportion of the calmodulin could be floating freely in the soluble fraction of the cell. To test if any calmodulin was tightly associated with cytoskeletal components in the cell, particularly the meiotic spindle, we prepared and analyzed the detergent-resistant cytoskeleton at the same time points employed for intact eggs. Examination of the distribution of calmodulin bound to the detergent-resistant cytoskeleton revealed a distribution pattern very different from that observed in intact specimens. In the unfertilized, detergent-extracted egg, calmodulin was bound to the cytoskeleton at the cortex of the egg and was not associated with the meiotic spindle (Fig. 1g). The insets show the same egg viewed by phase-contrast microscopy (top inset) and after Hoechst staining (bottom inset), demonstrating that the egg's morphology is not altered by detergent extraction. Calmodulin became associated with the detergent-resistant, meiotic spindle within 5 min of egg activation (Fig. 1h) and also remained enriched on the cytoskeleton at the cell periphery. However, by 15 min after egg activation (Fig. 1i) calmodulin was no longer associated with the meiotic spindle and calmodulin remained absent from the detergent-resistant spindle 30 min after activation (Fig. 1j). Such a result might occur if the treatments had induced the spindle to disassemble; however, as a control we show that the spindle could still be detected with anti-tubulin antibodies 15 min postactivation (top inset Fig. 1j; arrowheads indicate the spindle–chromosomes stained with Hoechst are green in this double-labeled image). The top inset shows the chromosomes beginning to transit into anaphase. The bottom inset labeled “control” shows a specimen treated with second antibody but not primary antibody to determine the level of nonspecific staining. Fifty-five minutes after egg activation (Fig. 1k), as the second polar body is undergoing closure of its contractile ring, this stereoscopic view demonstrates calmodulin was enriched on the...
The distribution of CaM kinase II was monitored in intact metaphase II-arrested eggs and in activated eggs at the same time points as calmodulin. In intact specimens CaM kinase II was present throughout the cytoplasm of the egg and was enriched around the meiotic spindle in the metaphase II egg and at each time point after egg activation: 5, 15, 30, 45, and 55 min (Figs. 2a–2f). The bottom insets in Figs. 2a and 2c show the position of the chromosomes; comparison with the CaM kinase II image of the same specimen showed that CaM kinase II was not associated with the chromosomes. The top inset in Fig. 2c labeled “control” shows a specimen treated with a second antibody but no primary antibody. After activation CaM kinase II was present in the region occupied by midzone microtubules (Figs. 2c–2f). This was confirmed by double-labeling for CaM kinase II (red) and for chromosomes (yellow), which demonstrated the presence of CaM kinase II in the region occupied by midzone microtubules (Fig. 2e). CaM kinase II remained near the region occupied by midzone microtubules even as the specimens were completing cytokinesis of the second polar body (Fig. 2f).

Tight association of CaM kinase II with the cytoskeleton was tested by examining the distribution of CaM kinase II after detergent extraction. CaM kinase II was tightly associated with the meiotic spindle in the unfertilized egg (Fig. 2g). The top inset in Fig. 2g shows a phase-contrast optics view and the bottom inset shows a Hoechst view of the same egg demonstrating that detergent extraction did not alter the morphology of the egg as well as showing that CaM kinase II was not associated with the chromosomes on the metaphase plate. CaM kinase II remained associated with the detergent-resistant meiotic spindle 5 and 15 min after egg activation (Figs. 2h and 2i, respectively). The inset in Fig. 2i labeled “control” shows a specimen treated with second antibody when no primary antibody was applied. Figure 2j shows association of CaM kinase II with the detergent-resistant cytoskeleton in the region of the midzone microtubules (arrow). The midzone microtubule region is identified by comparison with the position of DNA in the same egg (bottom inset, Fig. 2j). To demonstrate that CaM kinase II was associated with midzone microtubules, detergent-extracted activated eggs were triple stained for DNA, microtubules, and CaM kinase II. The orange color of the midzone region indicates the colocalization of CaM kinase II (green) and microtubules (red) (top inset, Fig. 2j). The stereoscopic image in Fig. 2k shows an egg 55 min postactivation from a triple labeling experiment where CaM kinase II is labeled green, DNA is labeled purple, and calmodulin is labeled red. Colocalization of calmodulin and CaM kinase II resulted in an orange color. CaM kinase II and calmodulin are colocalized at several points within the specimen (orange areas) including the second polar body and a lesser amount can faintly be detected in the region occupied by midzone microtubules. The cell’s DNA is labeled “D” and midway between the egg and polar body DNA is a ring of CaM kinase II associated with the contractile ring of the second polar body. As the second polar body was emitted, CaM kinase II associated with the inner surface of the contractile ring (Fig. 2k). The “C” indicates the egg cortex.

Kinase Assays Demonstrate Activation of CaM Kinase II at Egg Activation

The tight association of CaM kinase II with the meiotic spindle and the transient tight association of calmodulin with the spindle suggested that CaM kinase II activity might increase when CaM kinase II and calmodulin are

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**FIG. 2.** Scanning laser confocal images indicating the distribution of CaM kinase II in intact and detergent-extracted metaphase II-arrested eggs and activated eggs. Images are rebuilt from optical sections of 0.5-μm intervals that span the volume through the spindle. (a-f) Intact specimens. (a) Egg arrested at meiotic metaphase II showing CaM kinase II accumulated near spindle. Inset is Hoechst staining of same egg to show chromosomes on metaphase plate. (b) 5 min postactivation. (c) 15 min postactivation; bottom inset shows Hoechst staining in same egg; top inset shows control egg from same experiment with second antibody, but no primary antibody was applied to demonstrate level of nonspecific binding of second antibody. (d) 30 min postactivation. (e) 45 min postactivation CaM kinase II (red) and chromosomes (yellow) double-labeled. (f) 55 min postactivation shows forming second polar body (labeled PBII) and CaM kinase II in region of midzone microtubules as well as in the second polar body. (g-k) Detergent-extracted specimens. (g) Egg arrested at meiotic metaphase II shows CaM kinase II associated with spindle; inset (top, phase-contrast view of same egg; bottom, Hoechst staining of same egg DNA in the first polar body is labeled PBII). (h) 5 min postactivation with CaM kinase II on spindle. (i) 15 min postactivation with CaM kinase II on the spindle. Inset shows control egg from same experiment with second antibody, but no primary antibody was applied to demonstrate level of nonspecific binding of second antibody. (j) 30 min postactivation, CaM kinase II is present in region occupied by midzone microtubules (arrow); bottom inset shows location of DNA. Top inset shows triple-labeled specimen 30 min postactivation—green is CaM kinase II, purple is DNA (arrowheads), red is tubulin, and orange represents the colocalization of CaM kinase II and tubulin. (k) Stereoscopic image of triple-labeled specimen 55 min postactivation—green is CaM kinase II, purple is DNA, red is calmodulin, and orange represents the colocalization of calmodulin and CaM kinase II. PBII, second polar body; C, egg cortex; D, chromosomal DNA between which lie midzone microtubules and the contractile ring (associated with CaM kinase II) of the forming second polar body.
colocalized. We tested this by measuring the activity of the kinase at increasing time intervals after egg activation. To assay for the biochemical activity of CaM kinase II, lysates were made with five unactivated eggs or eggs collected at increasing time intervals after activation. The substrate that we employed to monitor CaM kinase II activity is the specific peptide substrate, Autocamtide-2, which is modified from an autophosphorylation domain of the kinase itself (Hanson et al., 1989). The reaction mixture contained radiolabeled ATP, inhibitors of tyrosine kinase, protein kinase A, myosin light chain kinase, and PKC, as well as protease and phosphatase inhibitors. Comparisons of the amount of CaM kinase II activity in metaphase II eggs and eggs 5, 8, 15, 30, and 45 min after activation are summarized in Fig. 3a, with a representative gel shown in Fig. 3b. CaM kinase II activity elevated within 5 min of egg activation and progressively decreased in activity over the next 30 min. The level of CaM kinase II activity again elevated 45 min after activation; this is the approximate time that the contractile ring of the second polar body is beginning to constrict and that immunocytochemical data shown earlier indicate colocalization of calmodulin and CaM kinase II in the region of the midzone microtubules and at other sites in the specimen (Fig. 2k). As a control, the CaM kinase II inhibitor KN-93 was added to the reaction mixture in parallel experiments and greatly suppressed the level of phosphorylation of the substrate. The lane on the far right is a duplicate time point at 5 min containing KN-93 and electrophoresed on the same gel.

Calcium and Calmodulin Can Induce Release from Meiotic Arrest in a Permeabilized System

The tight association of CaM kinase II with the meiotic spindle throughout all of meiosis and the transient, tight association of calmodulin with the meiotic spindle immediately after activation, coupled with the increased activity of the kinase at times when CaM kinase II and calmodulin colocalized, suggested that these two agents might have a causal role in the release from meiotic metaphase II arrest when the egg is activated. To test this putative causal role, we employed a permeabilized egg system developed by Gallicano et al. (1995). In this system the egg is permeabilized with a weak, nonionic detergent in a physiological buffer that contains a cocktail of protease inhibitors and a calcium chelator to sequester calcium released from internal stores. After permeabilization a variety of defined components can be flushed into the permeabilized eggs including a calcium/BAPTA buffer, ATP, and an ATP regenerating system, as well as inhibitors to specific kinases. In this study we permeabilized eggs at metaphase II and then flushed into the eggs a calcium/BAPTA buffer that adjusted the level of free calcium to 1 μM, an ATP regenerating system, and protease inhibitors. Figure 4a shows an egg 15 min after treatment with flushing media where the ratio of calmodulin to free calcium was 1:4 and the free calcium level was adjusted to 1 μM. Hoechst staining revealed that the chromosomes initiated transit into anaphase. Figure 4b shows an egg 30 min after an identical treatment where the chromosomes, viewed after Hoechst staining, exhibited a telophase configuration. Figure 4c shows a control egg that was permeabilized and incubated for 1 h at 37°C in medium where calcium and calmodulin were omitted. Hoechst staining revealed that the chromosomes remain in the metaphase II configuration. To test whether calcium, in the absence of calmodulin, could cause release from meiotic metaphase II, calmodulin was deleted from the flushing mixture in parallel experiments in which eggs were incubated for 1 h at 37°C. Under these conditions, the chromosomes also remained arrested at meiotic metaphase II.

The calcium/calmodulin-induced release from meiotic metaphase II arrest in the permeabilized egg system summarized from replicate experiments is shown in Fig. 5. Figure 5a summarizes the effects of varying the availability of calcium and calmodulin. Seventy percent of the eggs were shown to be released from metaphase arrest when calmodulin and free calcium were present at a 1:4 ratio within 30 min of treatment. In contrast, permeabilized control eggs whose flushing medium did not contain cal-

![Graph](image-url)
modulin or calcium, but had all other buffer components present remained arrested at meiotic metaphase II. Similarly, if calcium was present but no calmodulin was added, the chromosomes remained arrested at meiotic metaphase II. If the transit of chromosomes into an anaphase configuration was dependent on the interaction of calmodulin and calcium, then we predicted that if the ratio of calmodulin to calcium were titrated lower the number of cells exhibiting an anaphase configuration should be reduced. When the ratio of calmodulin to calcium was adjusted to 1:1 or 1:2 in the medium flushed into the permeabilized eggs, the eggs remained arrested at metaphase (Fig. 5b). At a ratio of 1:3 approximately 30% of the eggs exhibited chromosomes that were in an anaphase configuration and at 1:4 approximately 70% of the eggs exhibited an anaphase configuration.

Inhibitors to CaM Kinase II, but Not PKC Block the Release from Metaphase II Arrest in the Permeabilized System and in Living Cells

Inhibition of CaM kinase II greatly reduced the ability of calcium and calmodulin to induce release from arrest at meiotic metaphase II in both the permeabilized system and in living eggs. We employed two different types of inhibitors to CaM kinase II. The first was a peptide inhibitor, referred to as AIP, designed around a highly selective CaM

![Image](https://example.com/image.png)

**FIG. 4.** Chromosomes in permeabilized egg system viewed by epifluorescence microscopy after Hoechst staining. (a) Chromosomes in anaphase configuration 15 min after flushing in calmodulin and calcium (1 μM free calcium) at a 1:4 ratio along with other buffer components. (b) Chromosomes in late anaphase configuration 30 min after flushing in calmodulin and calcium (1 μM free calcium) at a 1:4 ratio along with other buffer components. (c) Control: Chromosomes remain on metaphase plate 60 min after flushing in buffer similar to that in a or b but with calcium and calmodulin omitted. Arrow points to DNA in first polar body. (d) Control: Chromosomes remain on metaphase plate 60 min after flushing in buffer similar to that in a or b but with calmodulin omitted.

![Graph](https://example.com/graph.png)

**FIG. 5.** Quantitation of permeabilized egg experiments in the presence or absence of different effectors of CaM kinase II activity. (a) Effects of altering the presence or absence of calcium and calmodulin in permeabilized egg system. CaM is calmodulin; calcium levels were adjusted with a calcium/diBrBAPTA buffer. Chromosome state was determined after fixation and Hoechst staining by epifluorescence microscopy. Values represent the mean ± SEM from three experiments. (b) Effects of altering the molar ratio of calcium to calmodulin in permeabilized egg system. Calcium levels were adjusted with a calcium/diBrBAPTA buffer to 1 μM free calcium and the concentration of calmodulin was varied to adjust the ratio. Values represent the mean ± SEM from three experiments.
CaM Kinase II autoinhibitory region (amino acid residues RQETVD; Ishida et al., 1995). The second was KN-93, a pharmacological inhibitor of CaM kinase II (Ishida and Fujisawa, 1995). As a control, KN-92 was used. KN-92 is structurally similar to KN-93 but does not inhibit CaM kinase II. For all of these experiments in the permeabilized egg system, the calmodulin to calcium ratio was adjusted to 1:4 in the flushing buffer. In the permeabilized system, 71% of the permeabilized eggs without inhibitors were released from metaphase arrest, whereas in those treated with KN-93 or AIP only 23 or 28% of eggs, respectively, were released from metaphase (Fig. 6a). KN-92, which does not inhibit CaM kinase II, had no inhibitory effect (Fig. 6a). Living eggs were tested with membrane-permeant CaM kinase II inhibitors (Fig. 6b). Living control eggs, untreated with inhibitors and activated with calcium ionophore transited to anaphase in 79% of cases. In contrast, living eggs treated with KN-93 or with myr-AIP exhibited release from metaphase arrest 10 and 24% of the time, respectively, after challenge with calcium ionophore (Fig. 6b). Again, KN-92 did not inhibit transit into anaphase compared to untreated controls (Fig. 6b).

Since other investigations have shown that PKC plays an important role in cytoplasmic changes that accompany the conversion of eggs to zygotes, we tested whether the activity of PKC had a role in the release from the meiotic metaphase II arrest. In separate experiments we added two different types of inhibitors of PKC. In the permeabilized assay we used the peptide inhibitor derived from the pseudosubstrate domain of the kinase, PKC8 (amino acids 19–36; House and Kemp, 1987; Hardie, 1988) and in separate experiments we also employed BIM, a pharmacological agent that inhibits PKC by blocking the ATP binding site of the kinase (Muid et al., 1991; Toullec et al., 1991; Kiss et al., 1995). Again the permeabilized egg system was adjusted to a 1:4 calmodulin to calcium ratio in the flushing mixture. Neither of these PKC inhibitors blocked transit into anaphase in the permeabilized system when calmodulin and calcium were present at a 1 to 4 ratio (Fig. 6c). Similar results were obtained when a PKC inhibitor was applied to living eggs and then challenged with calcium ionophore (Fig. 6c).

Since inhibitors of CaM kinase II blocked the ability of calcium and calmodulin to induce release of arrest from meiotic metaphase II, it was possible that addition of CaM kinase II to the permeabilized system would increase the number of eggs that transit into anaphase II. If after the addition of exogenous CaM kinase II there was no increase in the number of eggs that transit into anaphase II, the CaM kinase II tightly bound to the cytoskeleton would be sufficient for anaphase transit. We added purified CaM kinase II (whose kinase ability was confirmed; inset, Fig. 7) to the incubation medium containing permeabilized eggs under the conditions described previously; however, it did not increase the number of eggs whose chromosomes transited into anaphase (Fig. 7). The inability of exogenous CaM kinase II to increase the number of cells that transit into anaphase demonstrates that the amount of CaM kinase II tightly associated with the spindle is sufficient to mediate the transit into anaphase II.

**DISCUSSION**

In this report we show that CaM kinase II is tightly associated with the meiotic spindle, as evidenced by its retention on the detergent-resistant cytoskeleton, in both unfertilized eggs and after egg activation. CaM kinase II also tightly associates with the midzone microtubules that form as the chromosomes transit into anaphase II and remain associated with midzone microtubules even as the second polar body is being emitted. In contrast, while calmodulin is enriched around the meiotic spindle and at later stages around the midzone microtubules region, it is only tightly associated with the spindle, as determined by retention on the detergent-resistant cytoskeleton, 5 min after egg activation and with the midzone microtubules as the second polar body is being emitted. The times during development when calmodulin is tightly associated with the spindle and consequently also associated with CaM kinase II positively correlate with the times when biochemical assays of CaM kinase II activity demonstrate increased kinase activity. These correlative studies suggested that the activation of CaM kinase II could be responsible for the transit in chromosomal organization from metaphase into anaphase at the time of egg activation. To test whether activation of CaM kinase II could cause the transit into anaphase we conducted several additional studies. Using the permeabilized egg system we demonstrated that when in the presence of an ATP regenerating system and a calmodulin to calcium ratio of 1:4, chromosomes in the permeabilized egg would transit into an anaphase, and even a telophase, configuration at the same times that would occur in a living egg activated with calcium ionophore. The CaM kinase II activated by the calcium and calmodulin was already tightly associated with the spindle as demonstrated in the immunolocalization studies. The transit into anaphase was dependent on the ratio of calmodulin to calcium. In addition, in both living eggs activated with ionophore and in the permeabilized egg system incubated with calmodulin and calcium (1:4), inhibitors of CaM kinase II prevented transit into anaphase. Taken together these results suggest a causal role for calcium/calmodulin and CaM kinase II in the metaphase to anaphase transit.

To test whether PKC had a role in the chromosomal transit into anaphase, in separate experiments we applied two structurally different inhibitors of PKC, PKC8 and BIM. Under these conditions the chromosomes transited into an anaphase configuration both in the permeabilized egg system and in living eggs after activation. These results supports our prediction that CaM kinase II, but not PKC, mediates the transit into anaphase for the spindle and for the meiotic chromosomes.

Our biochemical assay experiments differ from those of
Winston and Maro (1995) in that we employed a specific substrate for CaM kinase II, Autocamtide-2, that is derived from the autophosphorylation site on the kinase (Hanson et al., 1989), whereas Winston and Maro used Syntide-2 which can be phosphorylated by other kinases (Colbran et al., 1988; Mochizuki et al., 1993). Moreover, we employed specific inhibitors to CaM kinase II as controls in each of our experiments which allowed detection of phosphorylation due to other kinases. The increase of CaM kinase II activity 5 min after egg activation is confirmed by the observations of Winston and Maro (1995), but we extend their observations by the use of a more extensive time course and a more specific substrate and are able to observe a subsequent increase in CaM kinase II activity 45 min after activation. Our experiments also differ from Inagaki et al. (1997) who reported that an inhibitor of CaM kinase II inhibited formation of the second polar body; however, their study was limited to the application of one pharmacological inhibitor. In addition, Xu et al. (1996) tested a calmodulin inhibitor and reported that it blocked release from arrest at metaphase, yet they did not investigate what role calmodulin played, and their approach also was limited by the application of a single pharmacological inhibitor of calmodulin.

CaM kinase II is a multisubunit enzyme that once activated autophosphorylates and reportedly can remain active in this state in the absence of calcium and calmodulin (Hanson and Schulman, 1992; Soderling, 1995). Thus, it is possible that CaM kinase II may first increase in activity after the initial [Ca²⁺]i rise and could remain active even when the calcium level drops. Moreover, mammalian

CaM kinase II in permeabilized egg system inhibit release from arrest. Specimens were viewed 30 min after treatment in an epifluorescent microscope after fixation and Hoechst staining. A23187, live egg controls to demonstrate that the eggs are activation competent. No inhibitor, permeabilized egg without application of inhibitors and with a flushing medium used in Figs. 3a and 3b that causes transit of chromosomes into anaphase. AIP, specific peptide inhibitor of CaM kinase II. KN-93, pharmacological inhibitor of CaM kinase II. KN-92, control structurally similar to KN-93, but does not inhibit CaM Kinase II. (b) Inhibitors of CaM kinase II applied to living eggs block release from arrest at meiotic metaphase II. Specimens were viewed 30 min after treatment in an epifluorescent microscope after fixation and Hoechst staining. A23187, live egg controls without inhibitor demonstrate that eggs are activation competent. myr-AIP (myristoylated AIP) and KN-93 are CaM kinase II inhibitors. KN-92 is a control for KN-93. (c) Inhibitors of PKC applied to permeabilized eggs and living eggs do not block release from arrest at meiotic metaphase II. Specimens were viewed 30 min after treatment in an epifluorescent microscope after fixation and Hoechst staining. A23187, live egg controls without inhibitor demonstrate that eggs are activation competent. PKCψ is a specific peptide inhibitor of PKC. BIM is a specific pharmacological inhibitor of PKC. Values represent the mean ± SEM from three experiments.
CaM Kinase II and the Meiotic Spindle

In somatic cells when the transit into anaphase occurs, the interpolar microtubules dissociate from the centrosome and leave the overlapping assembly ends in the midzone region (Rattner, 1992) as well as bundling the microtubules in the midzone region (Nislow et al., 1992). These midzone microtubules contain an accumulation of spindle-related proteins such as TD-60, CENP-E, and MKLP-1 (Martineau et al., 1995; Wheatley and Wang, 1996) and they are thought to have a role in specifying the location of the contractile ring in cleaving somatic cells (Margolis and Andreassen, 1993; Cao and Wang, 1996; Fishkind et al., 1996; Wheatley and Wang, 1996). Our triple labeling experiment clearly shows that in mammalian eggs CaM kinase II is one of the proteins that accumulates on midzone microtubules, and the association of calmodulin with the CaM kinase II at the time the cytokinetic ring is forming and closing suggests that CaM kinase II activity may play an active role in the cytokinetic process. This result appears to run counter to the classic experiments with echinoderm eggs that indicate that the cytokinesis signal emanates from the spindle asters (Rappaport, 1986, 1991); however, the large size of the echinoderm egg may necessitate different mechanisms of cytokinetic ring specification when the entire embryo must divide (Cao and Wang, 1996). Moreover, the formation of a second polar body also is very different from early cleavage of an entire embryo because the meiotic spindle is closely apposed to the egg plasma membrane and formation of the second polar body is a two-step event requiring first initiation (i.e., an outpocketing of the plasma membrane) followed by contractile ring closure to form a compartment (i.e., the second polar body) containing very little cytoplasm (Marto et al., 1984; Gallicano et al., 1997a,b). Midzone microtubules have been observed previously as second polar bodies in mouse (Verlhac et al., 1996) and rat eggs (Talmor et al., 1998), as well as in cleaving Xenopus blastomeres (Danilchik et al., 1998).

Fertilization is a highly orchestrated process that results in the remodeling of the entire cell. The fertilization-induced rise in $[Ca^{2+}]_i$ triggers a variety of signaling pathways in the cell whose combined actions convert the egg into the zygote. Previously we and others have shown that activation of PKC induces cortical granule exocytosis (Endo et al., 1987; Ducibella et al., 1993), reformation of the Golgi apparatus (Gallicano et al., 1993), and initiates formation of the second polar body (Gallicano et al., 1993, 1995). The differential timing of these events is in part regulated by cleavage of PKC to release its catalytic subunit which is membrane and calcium independent (Gallicano et al., 1995). In this study we illustrate another pathway initiated by calcium, one involving CaM kinase II that acts initially on the meiotic spindle to mediate transit of the chromosomes into anaphase. Subsequently CaM kinase II accumulates in the region of the midzone microtubules and calmodulin again colocalizes with CaM kinase II after the spindle rotates and the contractile ring of the second polar body is constrictioning. Since second polar body formation is a two-step process, first, initiation characterized by an outpocketing of cytoplasm from the egg and, second, constriction characterized by closure of the cytokinetic ring, our results suggest that while PKC is involved in the initiation of the second polar

FIG. 7. Effects of addition of exogenous CaM kinase II to medium flushed into permeabilized eggs, Control, permeabilized egg not exposed to elevated calcium and calmodulin levels. CaM + Ca$^{2+}$, standard flushing medium containing components identical to that used in Figs. 3a and 3b. CaM + Ca$^{2+}$ + CaM KII, flushing medium identical to that in CaM + Ca$^{2+}$ except that exogenous CaM kinase II was added. Values represent the mean ± SEM from three experiments. Inset shows that exogenous CaM kinase II functioned as an active kinase. CaM kinase II, CaM, and Ca$^{2+}$ were present in lanes 1–4, lane 1, no added Autocamtide-2 substrate; lane 2, 5 μg Autocamtide-2 substrate; lane 3, 10 μg Autocamtide-2 substrate; lane 4, 20 μg Autocamtide-2 substrate; lane 5, 10 μg Autocamtide-2 substrate/no added Ca$^{2+}$/no added CaM.
body (Gallicano et al., 1997b), CaM kinase II may act in the second step, that is, the closure of the cytokinetic ring. These observations highlight the temporal and spatial function of a series of kinases that act in concert to restructure the eggs. Mismatching of the spatial position or activity of any of the kinases that participate in these changes would likely disrupt the egg and destroy the cell.

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